Fe− and Co−Bleomycins Bound to Site Specific and Nonspecific DNA Decamers: Comparative Binding and Reactivity of Their Metal Centers†

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ABSTRACT: Co− and Fe−bleomycins (Blms) have been reacted with DNA_a, d(GGAA**GC**TTCC)$_2$, containing a specific site for cleavage, and DNA_b, d(GGAA**AT**TTCC)$_2$, a closely related nonspecific 10-mer, to survey whether features of structure and reactivity of these adducts vary systematically as a function of the base sequence of the DNA oligomer. The ESR spectrum of NO−Fe(II)BlmDNA_a is rhombically perturbed in comparison with that of NO−Fe(II)BlmDNA_b, which is nearly identical to the spectrum of NO−Fe(II)Blm. The ESR spectrum of Fe(III)BlmDNA_a in phosphate buffer is low-spin; that of Fe(III)BlmDNA_b is high-spin as seen with Fe(III)Blm alone. According to absorbance spectroscopy, O_2−Fe(II)BlmDNA_a is stabilized in comparison with the DNA_b adduct. Similar stabilization of O_2−Co(II)Blm bound to DNA_a but not to DNA_b was also observed by ESR spectroscopy. HO_2^-−Co(III)Blm A_2 binds in slow exchange on the NMR time scale to DNA_a at its 5′-G-pyrimidine-3′ site of cleavage. In contrast, fluorescence and NMR spectroscopy demonstrate that most of HO_2^-−Co(III)Blm A_2 binds stoichiometrically in fast exchange to DNA_b. The reactions of Fe(III)BlmDNA_a and Fe(III)BlmDNA_b with ascorbate and O_2 reveal that the latter becomes activated and cleaves its 10-mer, producing base propenals, at a faster initial rate. Thus, in two series of metallobleomycins, (A) NO−Fe(II)Blm, O_2−Fe(II)Blm, Fe(III)Blm in phosphate buffer, and HO_2^-−Fe(III)Blm and (B) O_2−Co(II)Blm and HO_2^-−Co(III)Blm, the metal domain of each species interacts differently with DNA depending upon its base sequence.

Bleomycin is a natural product which is useful in the treatment of human cancer. The structure contains both a metal and a DNA binding domain which are tethered through a small oligopeptide unit (Figure 1). As an iron complex, FeBlm[1] becomes activated in the form of HO_2^-−Fe(III)Blm to carry out single- and double-strand DNA breakage and base release (Petering *et al.*, 1990, 1996; Stubbe & Kozarich, 1987). Double-strand scission is poorly repaired and is thought to be responsible for at least part of the toxicity of the drug (Byrnes & Petering, 1994a,b). The reactions which damage DNA are site specific, showing a strong preference for 5′-G-pyrimidine-3′ dinucleotide sites (Steighner & Povirk, 1990). Both backbone cleavage and base release are initiated by reaction of HO_2^-−Fe(III)Blm or another undefined species derived from it with the C4′ hydrogen of the deoxyribose moiety bearing the pyrimidine base (Wu *et al.*, 1985).

Structural insight into the chemistry of these DNA-damaging reactions has been slow in appearing. Information about the conformations of free or DNA-bound forms of FeBlm has not yet been obtained. However, studies have shown that the oxidation of Co(II)Blm by dioxygen proceeds by the same pathway as that of Fe(II)Blm and produces analogous products, HO_2^-−Co(III)Blm (Form I) and Co(III)-Blm (Form II) (Fulmer & Petering, 1994; Xu *et al.*, 1992a,b). Conveniently, both cobalt species are diamagnetic and the former is stable in the absence or presence of DNA so that NMR studies can be done on them and their DNA adducts. These comparable properties have lent strength to the hypotheses holding that analogous species of Fe- and CoBlm have similar or identical structures and that such CoBlm structures may serve as models for FeBlms.

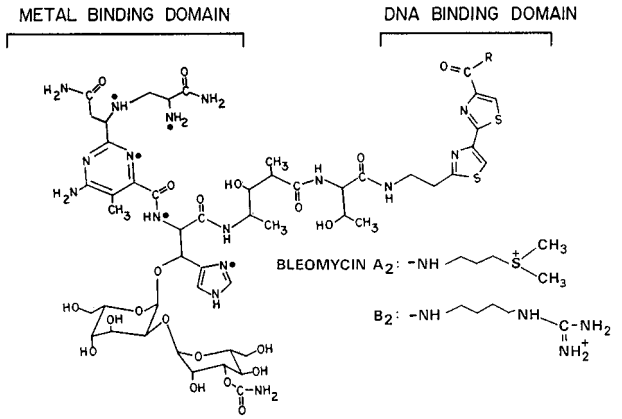

FIGURE 1: Covalent structure of bleomycin.

The solution structure of HO_2^-−Co(III)Blm A_2 is consistent with five-coordinate binding of the cobalt ion to the nitrogen atoms highlighted with dots in Figure 1 (Xu *et al.*, 1994; Wu *et al.*, 1996a). In addition, besides the conformational structure imposed on the metal domain by Co(III),

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[1] Abbreviations: Blm, bleomycin; Blm A_2, major component of the clinical mixture, blenoxane (see Figure 1); Form I, HO_2^-−Co(III)Blm; Form II, Co(III)Blm; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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the linker region is tightly folded against the metal domain and the bithiazole is folded back over the sixth coordination site which bears the peroxide. In the recently published studies on the structure of Form I bound to DNA oligomers containing both 5′-GC-3′ and 5′-GT-3′ cleavage sites, it was shown that the metal domain−linker structure remained intact upon binding to DNA (Mao *et al.*, 1996; Wu *et al.*, 1996b). Furthermore, the pyrimidinyl unit of the drug hydrogen bonds to guanine in the minor groove, forming a pyrimidine (drug)-guanine-cytosine base triple that supplies the specific recognition interaction between the drug and the guanine base (Wu *et al.*, 1996b). The bithiazole partially intercalates between the base pairs containing cytosine and the next base along the sequence in the 3′ direction (Mao *et al.*, 1996; Wu *et al.*, 1996b). In the process, the peroxide becomes localized in proximity to the C4′ hydrogen of the deoxyribose linked to cytosine, where DNA cleavage is initiated by HO_2^-−Fe(III)Blm (Mao *et al.*, 1996; Petering *et al.*, 1996; Wu *et al.*, 1996b).

This structural information provides a partial rationale for the site specificity of binding of Form I to DNA, as well as for its analog HO_2^-−Fe(III)Blm, if the cobalt and iron species adopt the same conformations when associating with DNA. Similarly, it begins to provide a detailed hypothesis for site specific cleavage of DNA; namely, selective binding to 5′-G-pyrimidine-3′ sites localizes the drug and positions the metal domain favorably at the site to initiate the DNA-damaging reactions of the drug (Mao *et al.*, 1996; Petering *et al.*, 1996; Wu *et al.*, 1996b).

Structural studies of Form I−DNA adducts clearly demonstrate that both the DNA and the metal domains interact with DNA. Other studies have also provided a strong indication that the metal domain of various metallobleomycins associates with DNA. Thus, the ESR signal of NO−Fe(II)Blm changes as the nitrosyl complex is titrated with calf thymus DNA (Antholine & Petering, 1979; Kennedy *et al.*, 1995). Similarly, the high-spin ESR signal of Fe(III)-Blm in phosphate buffer becomes low-spin in the presence of calf thymus DNA (Albertini & Garnier-Suillerot, 1984; Li *et al.*, 1997). Finally, the ESR signal of O_2−Co(II)Blm bound to oriented fibers of salmon sperm DNA reveals that dioxygen and, by inference, the metal domain are rigorously oriented with respect to the axis of the DNA helix (Chikira *et al.*, 1989).

Initially, the titrations of NO−Fe(II)Blm and Fe(III)Blm in phosphate buffer with DNA, showing the perturbations in ESR spectra, were thought to indicate simple binding phenomena, in which the two ESR spectra in each titration represented free and DNA-bound drug. However, recent fluorescence studies of the binding of the bithiazole moiety of Fe(III)Blm to calf thymus DNA in phosphate buffer showed that much of the drug remained high-spin when the bithiazole was stoichiometrically bound to the polymer (Li *et al.*, 1997). Thus, it has been hypothesized that the metal domain can adopt two conformations while bound to DNA that are dependent on the base pair to drug ratio (Kennedy *et al.*, 1995).

It is in this context that we decided to survey metal domain properties of Fe- and CoBlm species in the presence of two DNA 10-mers, one containing a specific site of binding, d(GGAA**GC**TTCC)$_2$ (DNA_a), and a related one in which 5′-AT-3′ was substituted for 5′-GC-3′, d(GGAA**AT**TTCC)$_2$ (DNA_b). The results support the hypothesis that, when these metallobleomycin species bind to DNA, the metal domain can adopt two different conformations, which depend on whether a specific site of binding is present. They also add support to the hypothesis that corresponding Fe- and CoBlm species have similar structures. Finally, they suggest that both of these different conformations play key roles in determining site specificity of drug binding to and cleavage of DNA.

METHODS

Materials. Blenoxane, the clinical mixture of bleomycin, largely made up of Blm A_2 and B_2, was a gift of Bristol Myers Co. The two DNA 10-mers were made as previously described (Mao *et al.*, 1996). Chemicals used in various experiments were reagent grade or of the highest purity available.

Binding of NO−Fe(II)Blm to DNA. ESR spectroscopy was used to examine the interaction of NO−Fe(II)Blm with DNA. Samples were prepared within an anaerobic chamber and placed into an anaerobic cell equipped with a gas chromatographic septum and a detachable 4 mm ESR tube. The reaction mixtures contained 0.10 mM Fe(II)Blm with or without 0.10 mM DNA_a or DNA_b in 20 mM phosphate buffer at pH 7.4. Once the samples were prepared, the anaerobic cell was removed from the chamber. The stock NO solution (*ca.* 2 mM) was prepared by first purging H_2O with argon and then with NO. It was added to the contents of the anaerobic cell through the septum with a Hamilton gas-tight syringe. Once excess NO had been removed by evacuation, the ESR tube was sealed and removed from the anaerobic cell. ESR spectra were collected at 77 K with a Varian E112 Century Series spectrometer.

Binding of O_2−Fe(II)Blm and O_2−Co(II)Blm to DNA. The reactions of Fe(II)Blm or Co(II)Blm, DNA_a or DNA_b, and O_2 were observed spectrophotometrically using the buffer as the reference sample. All starting reaction mixtures consisted of 0.31 mM oligonucleotide, Blm, and Fe^{2+} in 20 mM phosphate buffer at pH 7.4 and 25 °C.

ESR spectra of the same concentrations of Co(II)Blm, DNA_a or DNA_b, and O_2 as used above were collected on the Varian E112 Century Series spectrometer at 77 K. The computer program VIKING was employed to collect and average repeated scans of each sample. GRAPHER digitized each spectrum, and QUATTRO PRO for Windows organized and graphed the data.

Interaction of Fe(III)Blm with DNA. Reaction mixtures of 0.10 mM Fe(III)Blm and 0.10 mM DNA_a or DNA_b in 20 mM phosphate buffer at pH 7.4 and 25 °C were examined by ESR spectroscopy at 77 K by the same procedures as described for O_2−Co(II)Blm.

Binding of HO_2^-−Co(III)Blm A_2 to DNA Examined with Fluorescence and NMR Experiments. In order to obtain equilibrium constants for the binding of Form I to DNA_a and DNA_b, fluorescence spectroscopy was used to follow the interaction of Form I with each oligomer. The resulting data were analyzed according to eq 1, as previously described, to obtain binding constants (K) and binding site size, n (number of drug molecules per 10-mer) (Chien *et al.*, 1977; Mao *et al.*, 1996).

$$1/[HO_2^-\text{−Co(III)BlmDNA}] = 1/(nK[DNA_o][HO_2^-\text{−Co(III)Blm}]) + 1/(n[DNA_o]) \quad (1)$$

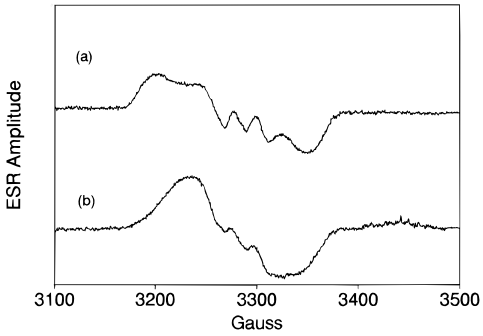

FIGURE 2: ESR spectra of Fe(II)Blm−NO in the presence of DNA_a (a) and DNA_b (b) under anaerobic conditions. The reaction mixture contained 0.10 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ added to 0.10 mM Blm with 0.10 mM DNA_a or DNA_b, respectively, in 20 mM phosphate buffer at pH 7.4. Nitric oxide (0.10 mM) from a buffer solution saturated with NO initiated the reactions. Samples were kept at 25 °C for an interval and then frozen in liquid dinitrogen for ESR measurement.

In this equation, $[DNA_o]$ represents the total concentration of DNA base pairs.

One-dimensional ^1H NMR spectra of Form I bound to DNA_a and DNA_b were obtained as previously described (Mao *et al.*, 1996). One:one ratios of HO_2^-−Co(III)Blm A_2 and DNA_a or DNA_b at a concentration of 2.0 mM were mixed in 20 mM phosphate buffer at pH 7.4 and 25 °C.

Reaction of Fe(III)Blm and Ascorbate with DNA. Fe(III)-Blm (0.1 mM) and 0.1 mM DNA_a or DNA_b were mixed in 20 mM phosphate buffer at pH 7.4 and 25 °C. Reactions were initiated with the addition of 0.25 or 1 mM ascorbate. At various times, aliquots of the reaction mixture were removed and either examined by ESR spectroscopy under the same conditions as with NO−Fe(II)Blm or treated as previously described to analyze for base propenal formation through the formation of the malondialdehyde−thiobarbituric acid adduct (Fulmer & Petering, 1994).

RESULTS

Binding of NO−Fe(II)Blm to DNA. DNA_a or DNA_b was mixed with Blm, Fe^{2+}, and NO under anaerobic conditions to achieve 1:1 ratios of 10-mer:drug. The ESR spectrum of each adduct was recorded at 77 K as shown in Figure 2. The spectrum of NO−Fe(II)BlmDNA_b in Figure 2b was nearly identical to that of NO−Fe(II)Blm in the absence of the oligomer or that of the major contributor to the ESR spectrum of NO−Fe(II)BlmDNA at small ratios of calf thymus DNA to drug (Antholine & Petering, 1979; Kennedy *et al.*, 1995). In contrast, the nitrosyl complex bound to DNA_a displayed an ESR spectrum that was rhombically perturbed in comparison with the other spectrum (Figure 2a). It is identical to that observed when NO−Fe(II)Blm is bound to calf thymus DNA at base pair:drug ratios of greater than 30. These results suggest that the nitrosyl complex can adopt at least two conformations with respect to DNA, one in the presence of nonspecific sequences and the other when specific cleavage sites are present. The first has little effect on the axial binding site of NO in the metal domain; the other conformation does perturb the NO site, presumably through metal domain−DNA interactions.

Binding of O_2−Fe(II)Blm and O_2−Co(II)Blm to DNA. A previous study showed that O_2−Fe(II)BlmDNA became increasingly stable as the base pair:drug ratio increased and was stable for hours at a ratio of 30−50:1 (Fulmer & Petering, 1994). The dioxygen adduct is characterized by an absorbance spectrum with a peak at 385 nm. After this absorbance band was searched for in aerobic mixtures of DNA_a or DNA_b plus Blm and Fe^{2+}, Figure 3 reveals that O_2−Fe(II)Blm could be detected in the presence of DNA_a. At the ratio of 30 base pairs or 3 oligomers per FeBlm molecule, O_2−Fe(II)Blm gradually underwent complete oxidation−reduction over the course of 15 min to yield Fe(III)Blm with its twin absorbance bands at 365 and 385 nm (Figure 3). DNA_b was unable to provide any stability to Fe(II)Blm in the presence of O_2 so that only HO_2^-−Fe(III)Blm and Fe(III)Blm species were observed over the same time period.

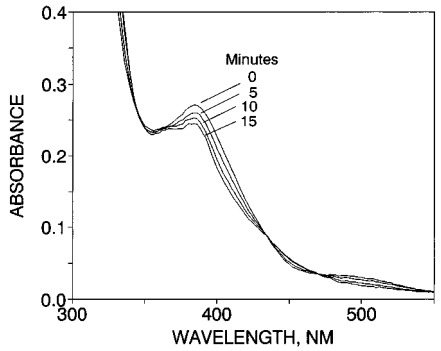

FIGURE 3: UV−vis spectra of Fe(II)Blm oxidation in the presence of DNA_a. The reaction mixture contained 0.313 mM DNA_a and 0.104 mM Blm and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, in 20 mM phosphate buffer at pH 7.4 and 25 °C.

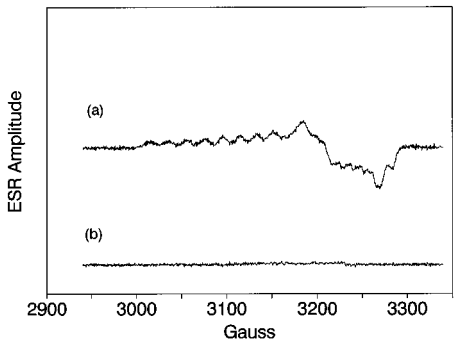

FIGURE 4: ESR spectra of aerobic Co(II)Blm in the presence of DNA_a (a) or DNA_b (b). $CoCl_2$ (0.313 mM) was added to 0.313 mM Blm with 0.313 mM DNA_a or DNA_b, respectively, in 20 mM phosphate buffer at pH 7.4. Samples were kept at 25 °C for an interval and then frozen in liquid dinitrogen for ESR measurement.

A similar experiment was performed, substituting Co^{2+} for Fe^{2+}. The formation of O_2−Co(II)Blm was detected by absorbance and ESR spectroscopy (Figure 4). As with O_2−Fe(II)Blm, DNA_a stabilized O_2−Co(II)Blm as detected by the presence of an absorbance maximum at 468 nm and the characteristic ESR spectrum of this species that has been previously reported (Figure 4a) (Xu *et al.*, 1992b; Chikira, 1989). The ESR spectrum remained at full intensity even after incubation of the sample at room temperature for 4 days. In the presence of DNA_b, the complex oxidized to a diamagnetic species within 15 min (Figure 4b).

These experiments show that both of the dioxygenated species are stabilized preferentially by the site specific 10-mer. That this effect was more dramatic with O_2−Co(II)-Blm than with O_2−Fe(II)Blm is consistent with their relative stability when bound to a range of concentrations of calf thymus DNA (Xu *et al.*, 1992b; Fulmer & Petering, 1994).

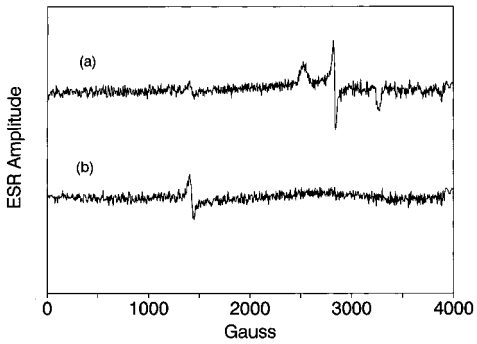

FIGURE 5: ESR spectra of aerobic Fe(III)Blm in the presence of DNA_a (a) or DNA_b (b). $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (0.10 mM) was added to 0.10 mM Blm with 0.10 mM DNA_a or DNA_b, respectively, in 20 mM phosphate buffer at pH 7.4. Samples were kept at 25 °C for an interval and then frozen in liquid dinitrogen for ESR measurement.

Binding of Fe(III)Blm to DNA. Fe(III)Blm forms an adduct with phosphate which converts the largely low-spin ESR signal of the iron center into a $g = 4.3$ high-spin signal (Albertini & Garnier-Suillerot, 1984; Li *et al.*, 1997). Upon addition of DNA, the low-spin signal was restored, indicative of the displacement of phosphate from the Fe(III)Blm structure. When Fe(III)Blm in phosphate buffer was reacted with DNA_a, its high-spin ESR signal was converted to the low-spin signal previously observed (Figure 5a). No such conversion was detected when DNA_b replaced DNA_a (Figure 5b).

Fe(III)Blm in HEPES buffer is largely low-spin (Li *et al.*, 1997). According to a previous ESR study, when Fe(III)Blm in this buffer was added to a 10:1 ratio of base pair:drug, a small shift in the low-field g value from 2.41 to 2.43 was observed (Albertini & Garnier-Suillerot, 1984). A comparison of the ESR spectra of Fe(III)Blm bound to DNA_a and DNA_b showed that the lowest-field g value was displaced from 2.42 observed with free Fe(III)Blm to 2.43 in the presence of DNA_a but was unperturbed by DNA_b.

Both of these results are consistent with the interpretation which holds that the metal domain of Fe(III)Blm binds to the oligomer containing the specific site differently than it does to the 10-mer which has no preferential binding site. Furthermore, they suggest that a specific association of the metal domain with the GC site in DNA_a is responsible for the change in the spin state of Fe(III)Blm in phosphate buffer.

Reaction of Fe(III)Blm and Ascorbate with DNA. The aerobic reaction of Fe(III)Blm with ascorbate in the presence of DNA_a or DNA_b was examined in two ways. First, the capacity of 1 mM ascorbate and ambient dioxygen to activate Fe(III)Blm to HO_2^-—Fe(III)Blm in the presence of either 10-mer in 20 mM phosphate buffer was determined by ESR spectroscopy. After the reactions were started, samples were frozen at 30 or 60 s for analysis. As seen in Figure 6, the activated species ($g = 2.26$, 2.18, and 1.94) was readily detected with either DNA_a or DNA_b. In addition, the low-spin form of Fe(III)BlmDNA_a could be seen as shown by the resonance at $g = 2.43$ (Figure 6C). The spectrum of the activated species in each case was identical to that in the absence of DNA. As shown in Figure 6A,B, the concentration of HO_2^-—Fe(III)BlmDNA_b declined rapidly in comparison with that of HO_2^-—Fe(III)BlmDNA_a and was much smaller than that of the DNA_a adduct at 60 s.

Second, an experiment was done to measure the time-dependent extent of strand scission of DNA_a and DNA_b by

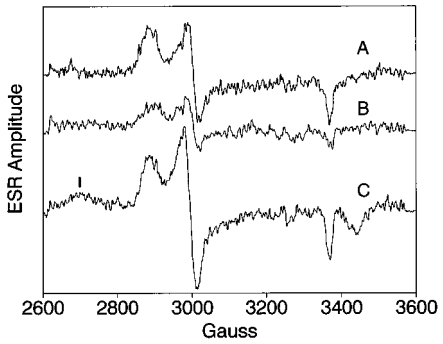

FIGURE 6: ESR spectra of HO_2^-—Fe(III)Blm bound to DNA 10-mers formed by reaction of Fe(III)Blm with ascorbate and O_2: (A) DNA_b at 30 s of reaction, (B) DNA_b at 60 s of reaction, and (C) DNA_a at 60 s of reaction. Conditions were as follows: 0.1 mM Fe(III)Blm, 0.1 mM 10-mer, and 1 mM ascorbate. The g value at 2.43 is noted by a bar on spectrum C.

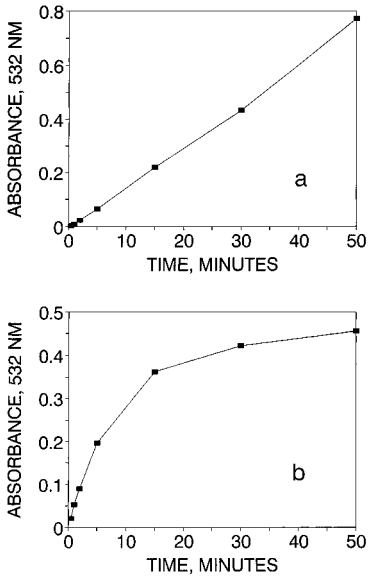

FIGURE 7: Rate of base propenal formation upon reaction of Fe(III)Blm and ascorbate with DNA_a (a) and DNA_b (b). DNA_a or DNA_b (0.10 mM) was reacted with 0.1 mM Fe(III)Blm and 0.25 mM ascorbate in 20 mM phosphate buffer at pH 7.4 and 25 °C.

Fe(III)Blm in the presence of O_2 and a small excess of ascorbate which limited the reaction. Using a 2.5:1 ascorbate:Fe(III)Blm ratio, the formation of base propenal was monitored over time (Figure 7). The reaction involving DNA_a was linear over 50 min, causing 30% of the 10-mer molecules to be damaged in this time period. Unexpectedly, the initial rate of base propenal formation from DNA_b was significantly faster, consistent with the ESR results described above. The maximal extent of cleavage was achieved in 30 min, leveling off at 30% of the oligomer in the reaction mixture. Like other examples of reactions between metallobleomycin species and DNA_a and DNA_b, the properties of strand scission of the two oligomers differ.

Binding of HO_2^-—Co(III)Blm A_2 (Form I) to DNA. Equilibrium binding of Form I to DNA_a and DNA_b has been examined in fluorescence titration experiments. According to the analysis of these titrations, Form I binds to DNA_a with an equilibrium constant of $(3.9 \pm 0.3) \times 10^6$ and a binding site size n of 0.99 ± 0.03 drug molecule/10-mer (Figure 8). The constants for the association of Form I with DNA_b are $(1.9 \pm 0.4) \times 10^6$ and 0.99 ± 0.02 drug molecule/10-mer.

The interaction of Form I with calf thymus DNA and oligomers containing 5′-G-pyrimidine-3′ sequences has been

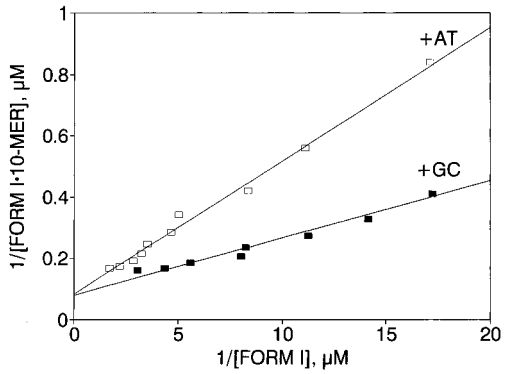

FIGURE 8: Graphical analysis of the fluorescence titrations of HO_2^-−Co(III)Blm A_2 with DNA_a and DNA_b: (■) DNA_a and (□) DNA_b. Conditions were as follows: 12.5 μM DNA_a or DNA_b plus Form I in 20 mM HEPES buffer at pH 7.4 and 25 °C.

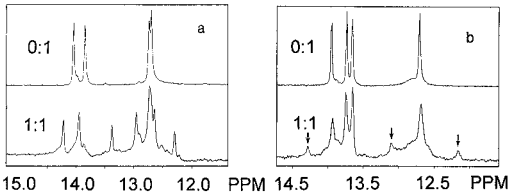

FIGURE 9: ^{1}H NMR spectrum of the DNA imino region of DNA_a (a) or DNA_b (b) bound to HO_2^-−Co(III)Blm A_2. DNA_a or DNA_b (2.0 mM) was reacted with 2.0 mM Form I in 20 mM phosphate buffer and 0.1 M KCl at pH 7.4 and 25 °C. The resonances with arrows are derived from a minor component.

examined by NMR spectroscopy. It has been characterized previously as a slow exchange process, in which the binding of the drug lifts the degeneracy of the DNA protons (Xu *et al.*, 1994; Wu *et al.*, 1996b; Mao *et al.*, 1996). During the one-dimensional titration of DNA_a with HO_2^-−Co(III)Blm A_2, this is conveniently observed by the splitting of the degenerate DNA imino protons (Figure 9a).

The titration of DNA_b with Form I at 25 °C resulted in broadening of the imino proton peaks of the free oligomer without an observable shift in their chemical shifts. The two bithiazole protons were shifted significantly upfield, but their precise chemical shifts have not yet been determined (data not shown). Also, a hint of a new set of imino proton peaks appeared, shown by the arrows in Figure 9b, that may represent a minor, slow exchange adduct between Form I and DNA_b.

According to the results of the fluorescence titrations described in Figure 8, all detectable Form I existed as an adduct with DNA_b in the NMR experiment of Figure 9. Evidently, the majority of Form I interacted with DNA_b in fast exchange on the NMR time scale but did not localize at a particular site as with DNA_a. As with the other examples, HO_2^-−Co(III)Blm A_2 binds in two different ways to DNA depending upon the nature of the nucleotide sequence.

DISCUSSION

For a number of years, it has been evident that the presence of DNA can alter properties of the metal centers of various metallobleomycins. Initially, it was shown that, upon titration of NO−Fe(II)Blm with calf thymus DNA, the ESR spectrum of the free nitrosyl adduct became more rhombic (Antholine & Petering, 1979; Kennedy *et al.*, 1995). Then, it was observed that high-spin Fe(III)Blm in phosphate buffer or at pH 4 is converted to its low-spin form upon titration with calf thymus DNA (Albertini & Garnier-Suillerot, 1984). Furthermore, upon interaction with DNA, high-spin N_3^-− and CN^-−Fe(III)Blm lose their exogenous ligands (Albertini & Garnier-Suillerot, 1984; Li *et al.*, 1997). Finally, it has been established that the dioxygen ligand of O_2−Co(II)Blm that is bound to oriented DNA fibers is rotationally constrained to a plane perpendicular to the helix axis (Chikira *et al.*, 1989).

These results indicate that the metal domains of each of these species can interact significantly with natural DNA samples. Moreover, it has also been established that adducts of Fe(III)Blm with phosphate or cyanide ligands form stoichiometrically with DNA and then, while bound to the polymer, undergo conversion to low-spin Fe(III)Blm with dissociation of either ligand as the ratio of DNA base pairs to drug is increased or as a function of time, respectively (Li *et al.*, 1997). Similarly, considering the range of association constants measured for various metallobleomycins, 5×10^4−10^6, it can also be assumed that NO−Fe(II)Blm stoichiometrically binds to DNA through its bithiazole tail under the conditions of the titration noted above (Petering *et al.*, 1990). If that is correct, then DNA-bound NO−Fe(II)Blm undergoes a change in metal domain environment as the DNA:drug ratio increases (Kennedy *et al.*, 1995). These results with native DNA samples make it clear that metallobleomycins can adopt at least two conformations when associated with DNA, one in which the metal center behaves much as it does in the absence of DNA and the other in which properties of the metal domain are perturbed by the polymer (Kennedy *et al.*, 1995).

Recent NMR studies of the interaction of HO_2^-−Co(III)-Blm A_2 with DNA 10-mers, containing 5′-G-pyrimidine-3′ sites of cleavage have demonstrated in more detail how the metal domain of a metallobleomycin can interact specifically with DNA base pair sequences (Mao *et al.*, 1996; Wu *et al.*, 1996b). In particular, it has been observed that the Co(III) center becomes oriented in the minor groove through hydrogen bonding interactions between the pyrimidine ring nitrogen (N3) and the amino group and the guanine 2-amino group and its N3 nitrogen (Wu *et al.*, 1996b). In the same structure, extensive hydrogen bond interactions between the peroxide group and groups on the drug and DNA may help orient it toward the C4′ hydrogen with which HO_2^-−Fe(III)Blm reacts to start DNA strand cleavage (Wu *et al.*, 1996b).

In this context, the present results demonstrate that the metal domain properties of a number of Fe- and CoBlm species consistently vary depending on whether they are reacted with a nonspecific or a specific DNA 10-mer. This is well-illustrated with NO−Fe(II)Blm. The ESR spectrum of the nitrosyl complex bound to DNA_b was almost identical to that of NO−Fe(II)Blm in the absence of DNA and to that of the major contributor to the ESR spectrum of the adduct when bound to calf thymus DNA at a ratio of base pairs to drug of 10:1 (Figure 2) (Antholine & Petering, 1979; Kennedy *et al.*, 1995). In contrast, the ESR spectrum of NO−Fe(II)Blm bound to DNA_a has the same rhombic appearance as that of this adduct bound to calf thymus DNA at a ratio of 30 base pairs to 1 drug molecule (Antholine & Petering, 1979; Kennedy *et al.*, 1995). On the basis of the binding affinity of a variety of metallobleomycins for DNA, it is hypothesized that NO−Fe(II)Blm is fully bound to both DNA_a and DNA_b (Mao *et al.*, 1996; Petering *et al.*, 1990).

Thus, the different ESR spectra of the metal domain in the two drug−DNA complexes occur while NO−Fe(II)Blm is bound to these oligomers. In this context, these experiments clarify what occurs in the titration of NO−Fe(II)Blm with calf thymus DNA. The drug progressively moves from predominantly nonspecific locations to specific sites as the base pair:drug ratio increases. These results indicate that the metal domain adopts different equilibrium structures when NO−Fe(II)Blm associates with the two DNA 10-mers. One of them, which is observed in the presence of the nonspecific 10-mer, only marginally perturbs the NO group from its environment in the absence of DNA. The other detected with DNA_a clearly places the metal center in a new chemical environment. We hypothesize that the metal domain−DNA interactions, which are responsible for specific metal domain binding in this case, are similar to those which characterize the complex of HO_2^-−Co(III)Blm with DNA_a oligomers containing a specific, 5′-GC-3′ binding site, namely, two hydrogen bonds between guanine and the drug pyrimidinyl residue (Wu *et al.*, 1996b).

The different behavior of NO−Fe(II)Blm when bound to DNA_a and DNA_b can be directly extended to dioxygenated Fe(II)- and Co(II)Blm. It is evident from the results of Figures 3−5 that DNA_a significantly stabilizes both dioxygen species in comparison with DNA_b. Earlier, the progressive stabilization of the O_2−Fe(II)Blm and O_2−Co(II)Blm, seen as the base pair:drug ratio increased, was simply attributed to the increase in the average distance between drug molecules bound along the DNA molecule (Fulmer & Petering, 1994; Xu *et al.*, 1992b). As the base pair:drug ratio increased, the rate at which two molecules of dioxygenated Fe(II)- or Co(II)Blm could react with one another would decrease. One can now enlarge this hypothesis; it is suggested that as the base pair:dioxygen adduct ratio becomes larger, individual drug molecules preferentially bind to specific sites of cleavage. This interaction between specific binding sequences and the metal domain contributes thermodynamic and/or kinetic stabilization that retards bimolecular reaction with other drug molecules.

According to this hypothesis, at small ratios of DNA to drug, much of the drug binds to nonspecific sequences such as DNA_b and rapidly undergoes oxidation−reduction as in reaction 2. At larger ratios, the relatively few drug molecules per unit of DNA localize at specific sites such as the 5′-GC-3′ sequence within DNA_a because of added thermodynamic stabilization from specific DNA−metal domain interactions (reaction 3, in which M is Fe or Co).

smaller base pair:drug ratio

$$2O_2\text{−M(II)BlmDNA}_b \rightarrow HO_2^-\text{−M(III)BlmDNA}_b + \text{M(III)BlmDNA}_b + O_2 \quad (2)$$

larger base pair:drug ratio

$$O_2\text{−M(II)BlmDNA}_b + \text{DNA}_a \rightarrow O_2\text{−M(II)BlmDNA}_a + \text{DNA}_b \quad (3)$$

The present results showing long-term stability of O_2−Co(II)BlmDNA_a are consistent with the previous demonstration that O_2−Co(II)Blm is enormously stable when bound to calf thymus DNA at ratios of at least 20 base pairs to 1 drug molecule (Xu *et al.*, 1992b). Presumably, this stabilization is related to the rigorous organization of the dioxygen unit in this complex that is observed when O_2−Co(II)Blm is bound to oriented fibers of salmon sperm DNA fibers at a ratio of 50 base pairs to 1 drug molecule (Chikira *et al.*, 1989). According to that work, the dioxygen molecule is confined to a plane approximately perpendicular to the DNA helix axis. By inference, the entire metal domain was likewise constrained such that the plane of the metal binding site was also approximately perpendicular to the helix axis as described for HO_2^-−Co(III)Blm bound to a site specific DNA 10-mer (Wu *et al.*, 1996b). We hypothesize that this observed constraint on metal domain conformation results from the same guanine−pyrimidinyl (drug) hydrogen bonding thought to specify the interaction of HO_2^-−Co(III)Blm A_2 with guanine at DNA cleavage sites (Wu *et al.*, 1996b).

The model for differential binding of O_2−Co(II)Blm to DNA_a and DNA_b can also be applied to the interaction of HO_2^-−Co(III)Blm A_2 with each of these 10-mers. In contrast to the localized, slow exchange binding of Form I to the 5′-GC-3′ site in DNA_a, it associates with DNA_b largely in a fast exchange mode. Because it is stoichiometrically bound to DNA_b under the conditions of the NMR experiments as discussed below, this fast exchange behavior must result from its rapid movement between nonspecific sites of binding. Consistent with this interpretation is the fact that, with minor exception, most of the bound Form I did not break the degeneracy of the imino protons and did not cause observable changes in their chemical shifts (Figure 9b). Nevertheless, the upfield shift in the two bithiazole protons of Form I suggests that binding of the drug does involve intercalation of the bithiazole moiety into the DNA structure (Wüthrich, 1986). According to these results and interpretation, drug molecules are highly mobile along nonspecific stretches of DNA sequences.

Additional detail can be ascribed to the binding of Form I to DNA. It was observed that HO_2^-−Co(III)Blm associates with each oligomer with large equilibrium constants (4×10^6 for DNA_a and 2×10^6 for DNA_b) (Figure 8). Initially, the similarity in binding constants for Form I to DNA_a and DNA_b was surprising, considering that Form I localizes at the 5′-GC-3′ site in DNA_a. Nevertheless, one can begin to rationalize the two similar equilibrium constants by observing that the calculation (eq 1) assumes that each drug molecule binds equally to all possible binding sites in each oligomer. Whereas that assumption approximates the situation in the Form I−DNA_b adduct according to the analysis of its NMR spectrum in Figure 9b, it does not represent the case for Form I−DNA_a. Specifically, there are about 14 sites in DNA_b involving the central nucleotides on either strand which contribute substantially to the binding of Form I (Mao *et al.*, 1996; Wu *et al.*, 1996b). In contrast, there are only two sites, the 5′-GC-3′ sequence on either strand, which participate in the association of Form I with DNA_a. Calculating site binding constants for the interaction of Form I with each 10-mer on the basis of these numbers of sites produces values of 2×10^6 and 1.4×10^5 for DNA_a and DNA_b, respectively.

In light of these revised constants, the larger differences in site binding constants becomes qualitatively comparable with the observed specificity of Form I for 5′-G-pyrimidine-3′ sites. They are also in harmony with the NMR result showing that most of Form I moves rapidly between sites along the length of DNA_b but is confined to the 5′-GC-3′ site in DNA_a (Mao *et al.*, 1996). Thus, from the NMR and

fluorescence data, one can hypothesize that the rate of transfer from nonspecific to specific sites is relatively large and is thermodynamically favorable.

This differential kinetic and equilibrium behavior must relate to the fact that specific DNA−metal domain interactions are established in the presence of DNA_a which are largely absent from the nonspecific DNA_b sequence. These include at least the two hydrogen bonds made between G5 of DNA_a and the pyrimidinyl moiety of the drug (Wu *et al.*, 1996b). Nevertheless, the presence of a minor slow exchange component in the one-dimensional NMR spectrum of Form I−DNA_b suggests that a drug−DNA structure, perhaps similar to that of Form I−DNA_a, can form between Form I and DNA_b.

The ESR spectrum of Fe(III)BlmDNA in phosphate buffer and the rate and extent of base propenal formation during its reaction with ascorbate and O_2 are also dependent on the nature of the DNA 10-mer (Figure 5). Fe(III)Blm in phosphate buffer, which forms a phosphate−Fe(III)Blm adduct that is high-spin, binds to DNA_a with an association constant of at least 10^5 M^{-1}, according to fluorescence titrations that will be presented elsewhere, becoming low-spin in the process (Figure 7). It remains high-spin when associated with DNA_b.

The titration of the phosphate adduct with calf thymus DNA is accompanied by the same high- to low-spin transition while the drug is bound to the polymer that was observed with the two 10-mers (Li *et al.*, 1997). Thus, like NO−Fe(II)Blm, there is direct evidence that the metal domain of this adduct species can adopt two conformations with respect to DNA while the rest of the molecule is bound to the polymer. Furthermore, it is evident that the low-spin ESR signal of DNA-bound Fe(III)Blm represents the drug bound to specific sites of cleavage. This conclusion was fortified by the ESR spectral observation that the lowest-field *g* value of Fe(III)Blm was perturbed by DNA_a but not by DNA_b.

The difference in behavior of Fe(III)Blm with the two 10-mers is correlated with the differential capacity of Fe(III)-Blm in aerobic phosphate buffer to cleave DNA_a or DNA_b in the presence of ascorbate and O_2 (Figure 7). Using a small excess of ascorbate with respect to Fe(III)Blm to cause limited reduction of Fe(II)Blm and to reduce O_2−Fe(II)Blm to HO_2^-−Fe(III)Blm, cleavage of DNA_a proceeded slowly over a 30 min period (Figure 7 and reactions 4−6).

$$\text{Fe(III)BlmDNA}_a + {}^1/_2\text{Asc} \rightarrow \text{Fe(II)BlmDNA}_a + {}^1/_2\text{Asc(oxidized)} \quad (4)$$

$$\text{Fe(II)BlmDNA}_a + O_2 \rightarrow O_2\text{−Fe(II)BlmDNA}_a \quad (5)$$

$$O_2\text{−Fe(II)BlmDNA}_a + {}^1/_2\text{Asc} + H^+ \rightarrow HO_2^-\text{−Fe(III)BlmDNA}_a \quad (6)$$

Under the same conditions, base propenals were initially produced more rapidly when Fe(III)Blm was bound to DNA_b. Consistent with these different rates of DNA strand cleavage were time-dependent differences in the concentrations of HO_2^-−Fe(III)Blm present with DNA_a and DNA_b (Figure 6). Although reaction 6 may participate in the activation process of Fe(III)BlmDNA_b, Fe(II)BlmDNA_b also reacts very rapidly in the presence of O_2 to produce Fe(III) species which can only be generated in a bimolecular process (Figure 3):

$$\text{Fe(II)Blm(DNA}_b) + O_2 \rightarrow O_2\text{−Fe(II)Blm(DNA}_b) \quad (7)$$

$$2O_2\text{−Fe(II)Blm(DNA}_b) + H^+ \rightarrow HO_2^-\text{−Fe(III)Blm(DNA}_b) + \text{Fe(III)Blm(DNA}_b) + O_2 \quad (8)$$

Thus, once Fe(III)BlmDNA_b was reduced to Fe(II)BlmDNA_b, a pathway for rapid reaction existed which led to strand scission and base propenal formation.

Differences in the rates of base propenal formation have also been seen in reactions of Fe(III)Blm, ascorbate, and O_2 with calf thymus DNA at base pair:drug ratios of 25:1 and 7.5:1.[2] The larger ratio behaves like the reaction involving DNA_a and indicates that FeBlm was localized on specific sites of binding or cleavage; the latter resembles the reaction with DNA_b and implies that many nonspecific sites of calf thymus DNA were initially populated by the drug.

It was surprising to find that strand cleavage could occur rapidly at nonspecific DNA sites (Figure 7). Possibly, a species of HO_2^-−Fe(III)BlmDNA_b, analogous to the slow exchange component of Form I−DNA_b, carried out strand cleavage. Apparently, the high specificity of reaction of FeBlm bound to natural DNA for 5′-G-pyrimidine-3′ sites was not due to the inability of HO_2^-−Fe(III)Blm to react efficiently with other sites. Starting from Fe(III)Blm, ascorbate, and O_2, it is hypothesized that site specificity of reaction results from the ability of Fe(III)Blm, O_2−Fe(II)-Blm, and HO_2^-−Fe(III)Blm to move rapidly among nonspecific sequences and, thus, to quickly search the linear domain of DNA for specific sites. There, they localize because of specific metal domain−DNA interactions as described above (Mao *et al.*, 1996; Petering *et al.*, 1996; Wu *et al.*, 1996b). However, there is both facile activation of Fe(III)Blm and reaction of HO_2^-−Fe(III)Blm with nonspecific DNA when no specific sites are present, which produces base propenals as observed in site specific damage. Thus, it appears that the metal domain of the activated species can adopt similar conformations with respect to specific and nonspecific sites. This places the reactive peroxo group in proper orientation to the C4′ hydrogen where the cleavage reactions begin. Whereas this probably occurs quantitatively during equilibrium binding of HO_2^-−Fe(III)Blm to 5′-G-pyrimidine-3′ sites, at nonspecific sites, this interaction might be represented by a small equilibrium conformer as seen in Figure 9 or by a transition state in the initial reaction at the C4′ hydrogen.

This paper has compared properties of binding and reaction of two related series of complexes with DNA 10-mers containing specific and nonspecific sites of binding: (A) NO−Fe(II)Blm, O_2−Fe(II)Blm, Fe(III)Blm, and HO_2^-−Fe(III)Blm and (B) O_2−Co(II)Blm and HO_2−Co(III)Blm A_2. With each complex, the observed behavior with the two 10-mers was distinctly different, implying that different metal domain interactions with the central 5′-GC-3′ (DNA_a) and 5′-AT-3′ (DNA_b) sequences were involved. That the particular metal domain−DNA interactions responsible for these divergent properties are the same for O_2−Fe(II)Blm and O_2−Co(II)Blm seems likely, because both complexes exhibited qualitatively similar behavior with DNA_a and DNA_b. That they control the structure and reactivity of all of the iron

[2] W. Li and D. H. Petering, to be submitted for publication.

and cobalt complexes bound to DNA_a and DNA_b is also plausible, since, in each case, the observed properties for the free metallobleomycin species were strongly perturbed only in adducts involving DNA_a, the 10-mer containing the specific binding sites. If this argument is correct, the ongoing structural analysis of DNA adducts of HO_2^-−Co(III)Blm A_2 and related cobalt complexes will be transferable to corresponding adducts of FeBlm species with DNA.

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REFERENCES

Albertini, J. P., & Garnier-Suillerot, A. (1984) *Biochemistry 23*, 47−53.
Antholine, W. E., & Petering, D. H. (1979) *Biochem. Biophys. Res. Commun. 91*, 528−533.
Byrnes, R. W., & Petering, D. H. (1994a) *Radiat. Res. 134*, 343−348.
Byrnes, R. W., & Petering, D. H. (1994b) *Radiat. Res. 137*, 162−170.
Chien, M., Grollman, A. P., & Horwitz, S. B. (1977) *J. Biol. Chem. 256*, 11636−11644.
Chikira, M., Antholine, W. E., & Petering, D. H. (1989) *J. Biol. Chem. 264*, 21478−21480.
Fulmer, P., & Petering, D. H. (1994) *Biochemistry 33*, 5319−5327.
Kennedy, M. C., Antholine, W. E., Li, W., Mao, Q., & Petering, D. H. (1995) *Inorg. Chim. Acta 240*, 535−540.
Li, W., Antholine, W. E., & Petering, D. H. (1997) (submitted for publication).
Mao, Q., Fulmer, P., Li, W., DeRose, E., & Petering, D. H. (1996) *J. Biol. Chem. 271*, 6185−6191.
Petering, D. H., Byrnes, R. W., & Antholine, W. E. (1990) *Chem.-Biol. Interact. 73*, 133−182.
Petering, D. H., Mao, Q., Li, W., DeRose, E., & Antholine, W. E. (1996) in *Metal Ions in Biological Systems* (Sigel, S., & Sigel, H., Eds.) Vol. 33, pp 619−947, Marcel Dekker, Inc., New York.
Steighner, R. W., & Povirk, L. F. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8350−8354.
Stubbe, J., & Kozarich, J. (1987) *Chem. Rev. 87*, 1107−1136.
Wu, J. C., Kozarich, J. W., & Stubbe, J. (1985) *Biochemistry 24*, 7562−7568.
Wu, W., Vanderwall, D. E., Lui, S. M., Tang, X.-J., Turner, C. J., Kozarich, J. W., & Stubbe, J. (1996a) *J. Am. Chem. Soc. 118*, 1268−1280.
Wu, W., Vanderwall, D. E., Turner, C. J., Kozarich, J. W., & Stubbe, J. (1996b) *J. Am. Chem. Soc. 118*, 1281−1294.
Wüthrich, K. (1986) in *NMR of Proteins and Nucleic Acids*, p 269, John Wiley and Sons, New York.
Xu, R. X., Antholine, W. E., & Petering, D. H. (1992a) *J. Biol. Chem. 267*, 944−949.
Xu, R. X., Antholine, W. E., & Petering, D. H. (1992b) *J. Biol. Chem. 267*, 950−955.
Xu, R. X., Nettesheim, D., Otvos, J. D., & Petering, D. H. (1994) *Biochemistry 33*, 907−916.

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