Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Vallee, B. L., & Wacker, W. E. C. (1970) *Proteins (2nd Ed.) 5*, 1–192.
Vallee, B. L., & Falchuk, K. H. (1981) *Philos. Trans. R. Soc. London, Ser. B 294*, 185–197.
Vallee, B. L., & Falchuk, K. H. (1983) *Biological Aspects of Metals and Metal Related Diseases* (Arkar, B., Ed.) p 1, Raven Press, New York.
Wacker, W. E. C. (1962) *Biochemistry 1*, 858–865.
Watson, D. K., & Mandrianakis, E. N. (1982) *Biochemistry 21*, 249–256.

Iron–Bleomycin–Deoxyribonucleic Acid System. Evidence of Deoxyribonucleic Acid Interaction with the α-Amino Group of the β-Aminoalanine Moiety†

J. P. Albertini and A. Garnier-Suillerot*

ABSTRACT: The Fe(III) complex of bleomycin (BLM) is, at pH 4, in the high-spin form. At pH 7, the coordination of the α-amino group of the β-aminoalanine moiety of BLM converts it to a low-spin species: BLM·Fe(III)·αNH_2. The conversion of the high-spin species to the low-spin one can also take place at pH 4 (i) by addition of ligands L such as N_3^-, $S_2O_3^{2-}$, and SCN^- or (ii) through interaction with DNA. Moreover, the addition, at pH 7, of DNA to BLM·Fe(III) that has been previously complexed with one of these ligands L displaces this latter from its position. These results suggest that (i) the ligand L occupies the same site of coordination as the α-amino group and (ii) an interaction occurs between the β-aminoalanine moiety of BLM and DNA that lowers the pK_d of the α-amino group, promoting its coordination to iron.

Bleomycins (BLM) are a family of glycopeptide antibiotics clinically prescribed for the treatment of selected neoplastic diseases (Carter, 1978). The BLM have both deoxyribonucleic acid (DNA) interacting and metal-binding sites, and the biological activity is related to this bifunctionality. BLM induces a degradation of DNA in a reaction that has been shown to depend, in vitro, on the presence of ferrous ion and molecular oxygen (Sausville et al., 1976, 1978; Takita et al., 1978). BLM is capable of binding Fe(II) to yield an air-sensitive complex Fe(II)·BLM. It has been postulated that the oxidation of this complex by dioxygen yields Fe(III)·BLM and a radical that is responsible for DNA damage.

Up to now, the X-ray crystallographic analysis of Fe(II)·BLM and Fe(III)·BLM has not been reported; nevertheless, the structure of a copper complex of BLM was investigated by X-ray crystallographic analysis of a biosynthetic intermediate of BLM (P3A) (Itaka et al., 1978). It has thus been demonstrated that the secondary amine nitrogen, pyrimidine ring nitrogen, deprotonated peptide nitrogen of histidine residue, and histidine imidazole nitrogen coordinate as the basal planar donor and the α-amino nitrogen of β-aminoalanine coordinates as the axial donor; the metal site has fundamentally a square-pyramidal structure with four chelate rings of 5–5–5–6 ring members. Spectroscopic evidence taken together with titration results establishes the amino–pyrimidine–imidazole–sugar region of BLM as important for binding Fe(II) and Fe(III) [Dabrowiak et al. (1980) and references cited therein], but the detailed geometry and stereochemistry of the iron-binding site remain to be elucidated. Nevertheless a structure has been proposed for iron complexes of BLM involving the same coordination geometry as P3A·Cu(II), except for the carbamoyl group at the sixth coordination site (Itaka et al., 1978; Dabrowiak et al., 1978; Umezawa & Tomohisa, 1978). It should be emphasized that this is a proposed structure and that on grounds of nuclear magnetic resonance (NMR) measurements, other suggestions have been made (Oppenheimer et al., 1979a,b).

Sugiura has focused attention on the role of the α-amino nitrogen of the β-aminoalanine portion of BLM, showing that this ligand is necessary for effective molecular oxygen binding and efficient oxygen reduction by the iron complex of BLM (Sugiura, 1979, 1980). It has then been suggested that the molecular oxygen binds in a position trans to the α-amino nitrogen of the β-aminoalanine portion.

The BLM·Fe(III) complex obtained at pH 7 is in the low-spin form; a decrease of the pH down to 4 converts it in a high-spin species (Burger et al., 1979). The complex of Fe(III) with depyruvamide bleomycin (dep-BLM)—a derivative of BLM lacking the β-aminoalanine amide portion—does not exhibit this pH dependency of the spin state, being in the high-spin state even at pH 7 (Sugiura, 1980). It has thus been suggested that the spin-state change of the BLM-Fe(III) complex between pH 4 and 7 takes place through coordination of the α-amino nitrogen of the β-aminoalanine moieties (Burger et al., 1979; Sugiura, 1980).

In this paper, we report experiments showing that at pH 4 the high-spin BLM·Fe(III) species can be converted to a low-spin species (i) by addition of ligands L such as N_3^-, $S_2O_3^{2-}$, and SCN^- or (ii) by addition of DNA. On the other hand, the addition at pH 7 of DNA to BLM·Fe(III) that has been previously complexed with one of the above-cited ligands L displaces in fact this latter from its position. These results suggest that (i) the same position can be occupied either by the α-amino nitrogen or by a ligand L and (ii) an interaction occurs between the β-aminoalanine portion of BLM and DNA, which lowers the pK_d of the α-amino group promoting its

† From the Laboratoire de Chimie Bioinorganique, U.E.R. de Santé, Médecine et Biologie Humaine, Université Paris-Nord, 93012 Bobigny, France, and the Laboratoire Associé au C.N.R.S. No. 71, Département de Recherches Physiques, Université Pierre et Marie Curie, 75230 Paris Cedex 05, France. *Received March 31, 1983; revised manuscript received August 3, 1983.*

0006-2960/84/0423-0047$01.50/0

coordination to Fe(III). These findings should help to provide a better understanding of the molecular mechanism of this drug. This investigation was done essentially by absorption, circular dichroism (CD), and electron paramagnetic resonance (EPR) measurements.

Materials and Methods

Purified BLM A_2, which contains (3-aminopropyl)dimethylsulfonium [$-NH(CH_2)_3S^+(CH_3)_2$] at the terminal amine, and dep-BLM A_2 were kindly provided by the Laboratoire Roger Bellon. Standard Fe(II) solutions were prepared from reagent-grade $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$. Calf thymus DNA purchased from Sigma Chemical Co. All other reagents were of the highest quality available, and deionized distilled water was used throughout the experiments. Samples of Fe(III)·BLM were prepared by two different methods. (i) The first was directly by the stoichiometric addition of Fe(II) salt to the drug in aqueous solution in the open air. To obtain the low-spin species, this reaction was performed in Hepes [*N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid] buffer, 0.05 M, pH 7.4; to obtain the high-spin species, the reaction was performed either at pH 7 and then the pH was lowered to 4 or directly at pH 4. In this second case, the formation of the complex is slow, and one must wait several hours to ensure complete reaction. (ii) The second was by the stoichiometric addition of the antibiotics to the Fe(III) salt in aqueous solution either at pH 7.4 or at pH 4 as described above. The dep-BLM·Fe(III) complex was prepared by this method. In every case the spin state of the complex was checked by EPR spectroscopy. BLM·Fe(III) can be kept several days in Hepes buffer, pH 7.4, as well as in acetate solution, pH 4, without modification of their spectroscopic characteristics. It should be emphasized that in all the experiments described the results do not depend on the method of synthesis of BLM·Fe(III).

Absorption spectra were recorded on a Cary 219 spectrophotometer, CD spectra were recorded on a Jobin Yvon dichrograph Model Mark V, and EPR spectra were recorded on a Varian CSE 109 spectrophotometer at −180 °C. Potentiometric measurements were obtained with a Tacussel pH meter, Model Isis 20000, at 25 °C under a nitrogen atmosphere, with a Metrohm EA 147 glass electrode.

Results

BLM·Fe(III) Complex as a Function of pH. It is now well documented that at pH 4 the BLM·Fe(III) complex is in the high-spin form; an increase of the pH up to 5.5 converts it to the low-spin species (Burger et al., 1979; Dabrowiak, 1980). We monitored the conversion of the high-spin form to the low-spin form via CD studies. The CD spectra recorded at various pH values between 4 and 5.5 exhibit isodichroic points at 475, 380, 305, and 240 nm. The CD spectra of the high-spin and low-spin forms are shown in Figure 1.

The potentiometric titration of BLM·Fe(III) was performed in 0.1 M $NaClO_4$ with NaOH, 0.1 M, in the pH range 3.5–7. The number of protons $\bar{n}$ released per bleomycin has been plotted as a function of pH in Figure 1 (insert) ($\bar{n}$ has been calculated classically as $[Na^+]_{added} - [OH^-]/[BLM \cdot Fe(III)]$) (Bjerrum, 1941). As can be seen, one proton is released between pH 4 and 5.5 when the conversion of the high-spin to the low-spin form takes place. This occurs with a pK of 4.6. This result is in good agreement with the finding of Burger et al. (1979) suggesting the dissociation of one proton, with a pK of 4.3, during the spin-state change. In the following, we will note BLM·Fe(III)·αNH_2 as the complex in the low-spin form with the α-amino nitrogen coordinated to

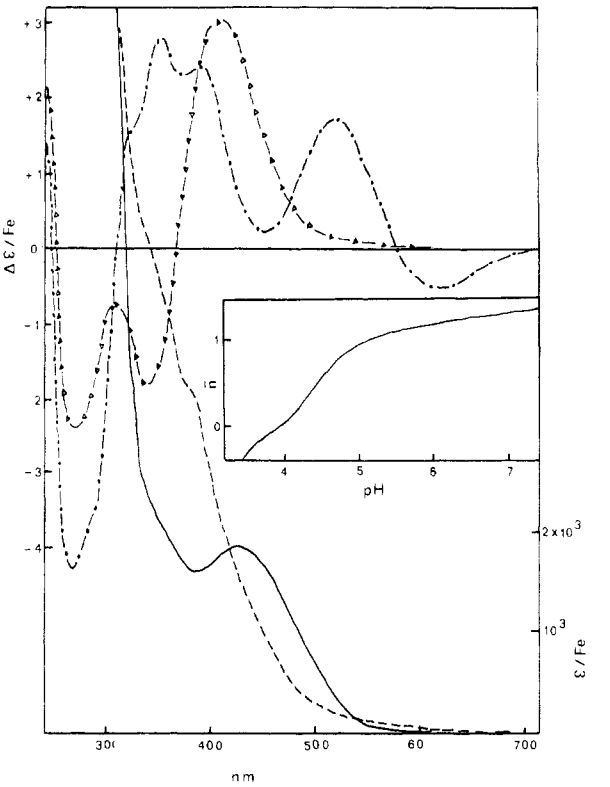

FIGURE 1: Absorption and CD spectra of the low-spin and high-spin BLM·Fe(III) complexes: (upper curves) CD spectra of the low-spin (●) and high-spin (▲) species; (lower curves) absorption spectra of the low-spin (---) and high-spin (—) species. (Insert) Potentiometric titration of the BLM·Fe(III) complex. $\bar{n}$ is the number of protons released. BLM·Fe(III) was 1.3×10^{-3} M in $NaClO_4$, 0.1 M.

Fe(III) and BLM·Fe(III) as the complex in the high-spin form.

The constant of association K_1 of the α-amino nitrogen to BLM·Fe(III) can be evaluated from the following equilibrium:

$$\underset{\text{high spin}}{\text{BLM}\cdot\text{Fe(III)}} + \text{“}\alpha NH_2\text{”} \overset{K_1}{\rightleftharpoons} \underset{\text{low spin}}{\text{BLM}\cdot\text{Fe(III)}\cdot\alpha NH_2} \qquad (1)$$

where “αNH_2” stands for the α-amino nitrogen of the β-aminoalanine portion of BLM. The concentrations of low-spin and high-spin species have been evaluated from CD and potentiometric titration data. A pK_d of deprotonation of the α-amino nitrogen of 7.5 (Solaiman et al., 1980; this work) has been used to calculate the concentration of “αNH_2”. A value of order of 10^6 is obtained for K_1 where

$$K_1 = [\text{BLM}\cdot\text{Fe(III)}\cdot\alpha NH_2]/([\text{BLM}\cdot\text{Fe(III)}][\text{“}\alpha NH_2\text{”}])$$

BLM·Fe(III) and L (N_3^-, SCN^-, and $S_2O_3^{2-}$). The addition of small aliquots of a solution of NaN_3 to a solution of the high-spin BLM·Fe(III) at pH 4 gives rise to the formation of a low-spin species ($g_z = 2.544$, $g_y = 2.222$, $g_x = 1.831$). The absorption spectrum is characterized by the appearance of a band at 500 nm, and the CD spectra exhibit isodichroic points. In a typical experiment, the BLM·Fe(III) concentration was taken equal to 5×10^{-4} M, and at a molar ratio of $[N_3^-]/[\text{Fe(III)}]$ equal to 3, the evolution of the different spectra levels off. The absorption and CD spectra of the species thus obtained are shown in Figure 2. The strong absorption band in the visible is assigned to a charge-transfer transition $N_3^- \rightarrow$ Fe(III). This first experiment demonstrates the binding of N_3^-.

In the second experiment, the pH of the solution at a $[N_3^-]/[\text{Fe(III)}]$ molar ratio of 3 was raised to 7. This gives rise to the release of the N_3^- ligand as it is attested by the decrease of the absorption band at 500 nm. The CD spectrum

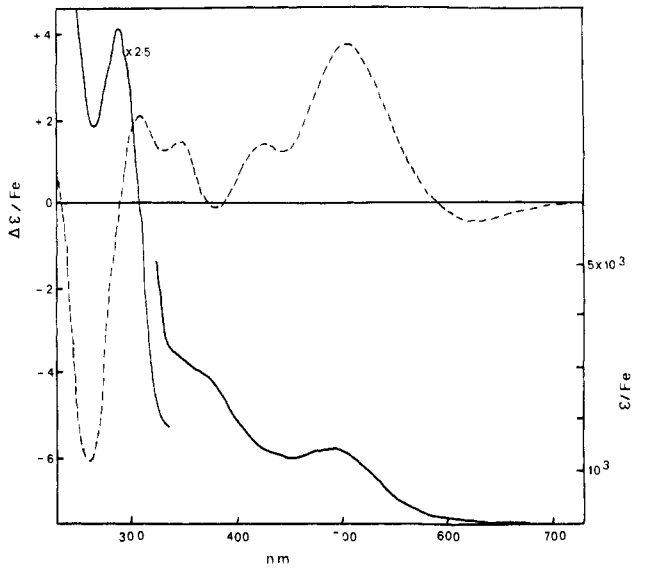

FIGURE 2: BLM·Fe(III)·N_3^- complex absorption (—) and CD (---) spectra. Experimental conditions: N_3^- at 1.5×10^{-3} M was added to BLM·Fe(III), 5×10^{-4} M, at pH 4.

evolves to that of the low-spin BLM·Fe(III)·αNH_2 species with the presence of isodichroic points at 415 and 325 nm, as well as the EPR spectrum. This process is reversible: the BLM·Fe(III)·N_3^- complex is recovered by decreasing the pH to 4. This second experiment suggests that the α-amino nitrogen supersedes N_3^-.

In a third experiment, an excess of N_3^- was added to the BLM·Fe(III)·αNH_2 complex at pH 7. At a molar ratio of $[N_3^-]/[Fe]$ equal to 160, the absorption spectrum characteristic of the BLM·Fe(III)·N_3^- complex is completely recovered; the CD spectra exhibit the same isodichroic points as in the second experiment. This suggests that N_3^- has superseded αNH_2.

From the foregoing results, we can infer a competition between the α-amino nitrogen and N_3^- for the same position; this can be summarized by the following equilibria:

at pH 4 and $0 \leq [N_3^-]/[Fe] \leq 3$

$$\underset{\text{high spin}}{\text{BLM·Fe(III)}} + N_3^- \rightleftarrows \underset{\text{low spin}}{\text{BLM·Fe(III)·}N_3^-} \qquad (2)$$

at $4 < \text{pH} < 7$ and $[N_3^-]/[Fe] = 3$

$$\underset{\text{low spin}}{\text{BLM·Fe(III)·}N_3^-} \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} \underset{\text{low spin}}{\text{BLM·Fe(III)·}\alpha NH_2} \qquad (3)$$

at pH 7 and $0 \leq [N_3^-]/[Fe] \leq 160$

$$\underset{\text{low spin}}{\text{BLM·Fe(III)·}\alpha NH_2} + N_3^- \rightleftarrows \underset{\text{low spin}}{\text{BLM·Fe(III)·}N_3^-} \qquad (4)$$

Taking into account the equilibria (Sillen & Martell, 1964)

$$\text{“}\alpha NH_3^+\text{”} \rightleftarrows \text{“}\alpha NH_2\text{”} + H^+ \qquad pK_d = 7.5 \qquad (5)$$

and

$$HN_3 \rightleftarrows N_3^- + H^+ \qquad pK_d = 4.7 \qquad (6)$$

one evaluates 1.5×10^6 as the value of the stability constant of BLM·Fe(III)·N_3^-, defined as

$$K_2 = [\text{BLM·Fe(III)·}N_3^-]/([\text{BLM·Fe(III)}][N_3^-])$$

In the case of Fe(III) and N_3^-, the value of the first stability constant is about 2×10^5 (Sillen & Martell, 1964).

The same kind of experiments were performed with SCN^- and $S_2O_3^{2-}$, and similar results were obtained. In the case of

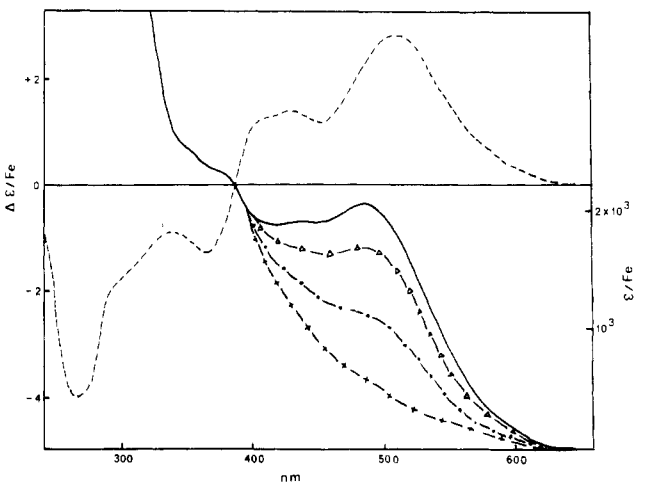

FIGURE 3: BLM·Fe(III)·SCN^- complex absorption (—) and CD (---) spectra. Experimental conditions: SCN^- at 3×10^{-2} M was added to BLM·Fe(III), 5×10^{-4} M, at pH 4. The lower panel shows the absorption spectra of the solution at pH 4.5 (▲), 5.1 (●), and 5.8 (×).

SCN^-, its addition to a BLM·Fe(III) solution at pH 4 gives rise to the formation of a low-spin species ($g_z = 2.609$, $g_y = 2.225$, $g_x = 1.773$). At a BLM·Fe(III) concentration of 5×10^{-4} M, a molar ratio of $[SCN^-]/[Fe(III)]$ equal to about 650 is necessary to ensure the complete formation of the complex. Its absorption and CD spectra are shown in Figure 3; the two strong absorption bands at 490 and 440 nm can be assigned to charge-transfer transitions of $SCN^- \rightarrow Fe(III)$. An increase of the pH from 4 to 6 gives rise to the substitution of SCN^- by the α-amino nitrogen of BLM; this is attested by the decrease of the visible absorption band (Figure 3), the CD spectra which exhibit isodichroic points at 410 and 310 nm, and the EPR spectra. A stricking feature of the absorption spectra is the absolute invariance of the two bands at 370 and 390 nm during the process (Figure 3). The addition of an excess of SCN^- to the BLM·Fe(III)·αNH_2 complex at 7 gives rise to the back-formation of some SCN^- complex, but it cannot be completely recovered: for instance, at an initial concentration of BLM·Fe(III)·αNH_2 and SCN^- of 5×10^{-4} and 2 M, respectively, only about 2×10^{-4} M BLM·Fe(III)·SCN^- complex is formed.

Here again, a competition between the α-amino nitrogen and SCN^- for the same position is involved. If one takes into account the equilibria

at pH 4 and $0 \leq [SCN^-]/[Fe] \leq 650$

$$\underset{\text{high spin}}{\text{BLM·Fe(III)}} + SCN^- \rightleftarrows \underset{\text{low spin}}{\text{BLM·Fe(III)·}SCN^-} \qquad (7)$$

at $4 < \text{pH} < 7$ and $[SCN^-]/[Fe] = 650$

$$\underset{\text{low spin}}{\text{BLM·Fe(III)·}SCN^-} \underset{H^+}{\overset{OH^-}{\rightleftharpoons}} \underset{\text{low spin}}{\text{BLM·Fe(III)·}\alpha NH_2} \qquad (8)$$

at pH 7 and $0 \leq [SCN^-]/[Fe] \leq a$ (a higher than 2)

$$\underset{\text{low spin}}{\text{BLM·Fe(III)·}\alpha NH_2} + SCN^- \rightleftarrows \underset{\text{low spin}}{\text{BLM·Fe(III)·}SCN^-} \qquad (9)$$

and (Sillen & Martell, 1964)

$$HSCN \rightleftarrows SCN^- + H^+ \qquad pK_d = 0.8 \qquad (10)$$

as well as equilibrium 5, the value of the stability constant of BLM·Fe(III)·SCN^- defined as

$$K_3 = [\text{BLM·Fe(III)·}SCN^-]/([\text{BLM·Fe(III)}][SCN^-])$$

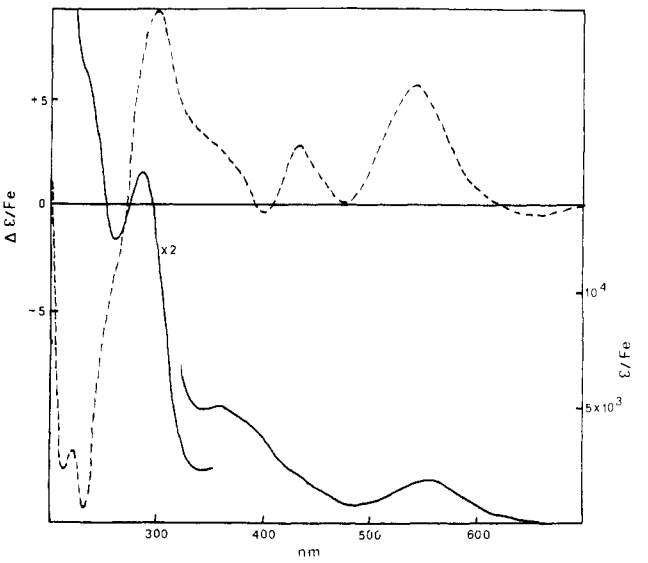

FIGURE 4: BLM·Fe(III)·$S_2O_3^{2-}$ complex absorption (—) and CD (---) spectra. Experimental conditions: $S_2O_3^{2-}$ at 3.5×10^{-3} M was added to BLM·Fe(III), 5×10^{-4} M, at pH 4.

is of the order of 600. In the case of Fe(III) and SCN^-, the value of the first stability constant is of about 200 (Sillen & Martell, 1964).

The system BLM·Fe(III)·$S_2O_3^{2-}$ exhibits the same behavior as the two above-cited systems; i.e., at pH 4, a low-spin species ($g_z = 2.394$, $g_y = 2.225$, $g_x = 1.890$) is formed by addition of $S_2O_3^{2-}$ to BLM·Fe(III). The formation of the complex is complete at a molar ratio $[S_2O_3^{2-}]/[Fe(III)]$ equal to 7; its absorption and CD spectra are shown in Figure 4. As previously, the strong band at 550 nm in the absorption spectrum can be assigned to the charge-transfer transition of $S_2O_3^{2-} \rightarrow$ Fe(III): in that case, this is confirmed by Raman resonance spectral data (J. P. Albertini et al., unpublished results). Here again, a superseding of $S_2O_3^{2-}$ by the α-amino nitrogen is obtained by increasing the pH to 6: the absorption band at 550 nm disappears, and the CD spectra exhibit isodichroic points at 500, 460, and 430 nm. This process is pH reversible. The addition, at pH 7, of an excess of thiosulfate, i.e., a molar ratio of $[S_2O_3^{2-}]/[Fe(III)]$ of about 12, gives rise to the back-formation of BLM·Fe(III)·$S_2O_3^{2-}$. Thus for this ligand, equilibria analogous to (2), (3), and (4) can be postulated. All the spectroscopic data are gathered in Table I.

BLM·Fe(III)·αNH_2 and DNA (pH 7.4). The addition of DNA to BLM·Fe(III)·αNH_2 (300 μM) in Hepes buffer, 0.05 M, pH 7.4, gives rise to some modification of the CD spectrum of the complex. As it can be seen in Figure 5, a slight increase of the positive band at 510 nm is observed, but the most characteristic feature is a net increase of the positive peak at 390 nm; the variation of $\Delta\epsilon_{390}$ as a function of the molar ratio [nucleotide]/[Fe] has been plotted in Figure 5 (insert). Assuming that the modification of the peak at 390 nm is due to the binding of the complex to DNA, we determined from this curve the concentration of bound complex to DNA ($\Delta\epsilon_{390} = 3.6$) and of free complex ($\Delta\epsilon_{390} = 2.5$). The binding data analyzed by Scatchard plots (Scatchard, 1949) give an apparent equilibrium constant of 3.5×10^5 M^{-1}. One molecule of complex binds for every 2.6 bases pair in DNA. This compares with the results obtained by Povirk et al. (1981), Roy et al. (1981), and us (Albertini & Garnier-Suillerot, 1982) in the cases of complexes of BLM with Fe(III), Cu(II), and Co(III), respectively. This result strongly suggests that the modification of the CD band at 390 nm can be used as an indication of the binding of the BLM·Fe(III)·αNH_2 complex

Table I: Absorption and CD Data for BLM·Fe(III)·L Complexes

	absorption		CD	
complex	λ (nm)	ϵ (M^{-1} s^{-1})[a]	λ (nm)	$\Delta\epsilon$ (M^{-1} cm^{-1})[b]
BLM·Fe(III), pH 4	420	1800	410	3.2
	350	2000 (sh)	340	−1.8
			275	−2.3
			240	4.0
			218	−3.1
BLM·Fe(III)·αNH_2, pH 7			620	−0.6
			515	1.8
	380	2300	390	2.5
	360	2500	350	2.8
			325	1.6
	290	21500	295	−3.2
			275	−4.6
			235	4.5
BLM·Fe(III)·N_3^-			620	−0.5
	490	1500	505	3.9
			425	1.5
			380	−0.2
	360	3100	345	1.5
			315	2.0
	290	23000	260	−6.0
BLM·Fe(III)·SCN^-	490	2100	510	2.7
	430	1900	430	1.4
	380	2330	405	1.2 (sh)
	360	2500	370	−1.3
			310	−1.7 (sh)
			270	−4.0
BLM·Fe(III)·$S_2O_3^{2-}$			655	−0.5
	550	2000	544	5.5
	440	2000 (sh)	436	2.6
	396	4200	403	−0.5
	364	5200	360	2.5 (sh)
	290	26000	304	8.9
			265	−3.0
	240	32000	235	−14.0
			215	−11.0

[a] ϵ, molar absorption coefficient. [b] $\Delta\epsilon$, molar circular dichroism coefficient.

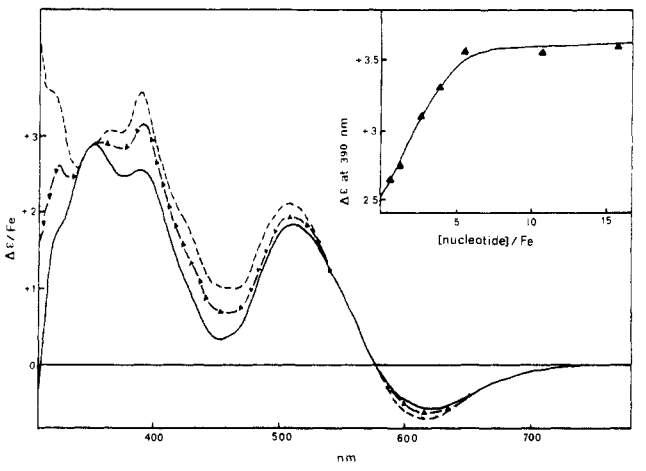

FIGURE 5: CD spectra of BLM·Fe(II)·αNH_2 complex in the presence of DNA. Experimental conditions: 3×10^{-4} M BLM·Fe(III)·αNH_2 in 0.05 M Hepes buffer, pH 7.4; [nucleotide]/[Fe] = 0 (—), 2.7 (Δ), and 11 (---). (Insert) Variation of ϵ at 390 nm as a function of the [nucleotide]/[Fe] molar ratio.

to DNA. It should be noticed that we have not observed concomitant modification of the CD spectrum of DNA in the 260-nm region. This result is at variance with that obtained in the case of the DNA–BLM–Co(III) system (Albertini & Garnier-Suillerot, 1982). A slight modification of the EPR spectrum of the complex is also observed by addition of DNA: an increase of the g_z value from 2.40 to 2.43 with almost no

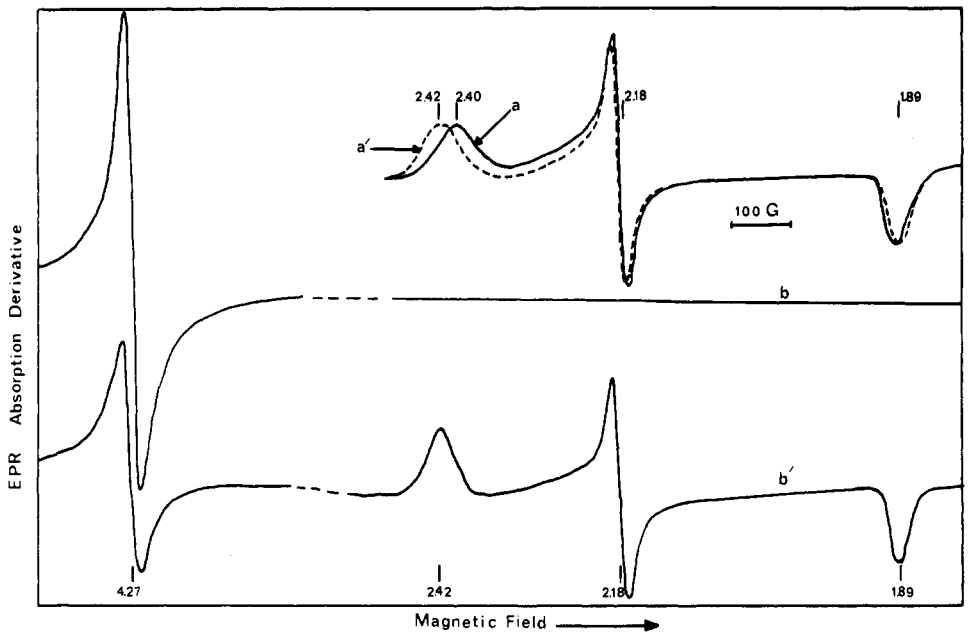

FIGURE 6: EPR of BLM·Fe(III) complexes in the presence or absence of DNA. Experimental conditions: (upper curves) 220 μM BLM·Fe(III) in 0.05 M Hepes buffer, pH 7.4, in the absence (curve a) and presence (curve a′) of DNA at [nucleotide]/[Fe] = 20; (lower curves) 200 μM BLM·Fe(III) in 0.05 M acetate solution, pH 4, in the absence (curve b) and presence (curve b′) of DNA at [nucleotide]/[Fe] = 20. g values are indicated.

modification of g_y and g_x (Figure 6).

BLM·Fe(III) and DNA at pH 4. BLM·Fe(III) can be kept in sodium acetate, pH 4, for several days without any damage. The addition of DNA to BLM·Fe(III) converts some percentage of this high-spin form to a low-spin form; this percentage depends predominantly on two parameters: (i) [nucleotide]/[Fe] molar ratio and (ii) the time elapsed after the addition of DNA to the complex.

Figure 6 shows the EPR spectrum of BLM·Fe(III) in the absence (curve b) and in the presence (curve b′) of DNA at a [nucleotide]/[Fe] molar ratio equal to 15. Since the evolution of the system as a function of time is very fast, the samples were frozen to liquid nitrogen temperature within the 30 s following the addition of DNA. In Figure 7, the percentage of low-spin form and of high-spin form ($\Delta t \simeq 30$ s) has been plotted as a function of the [nucleotide]/[Fe] molar ratio. As can be seen in Figure 6, the great similarity between the spectrum of BLM·Fe(III)·αNH_2 at pH 7 and the low-spin form of BLM·Fe(III) in the presence of DNA is striking, and this strongly suggests that DNA promotes the coordination of the α-amino nitrogen to Fe(III) according to the equilibrium at pH 4

$$\underset{\text{high spin}}{\text{BLM·Fe(III)}} + \text{DNA} \rightleftarrows \underset{\text{low spin}}{\text{BLM·Fe(III)·}\alpha NH_2\text{–DNA}} \qquad (11)$$

As time elapses, the two EPR signal disappear, and the percentages of low-spin and high-spin form present as a function of time have been plotted in Figure 7 (insert). Most probably, due to the presence of the phosphate group of DNA, Fe(III) dissociates from BLM and is not detected by EPR spectroscopy due to its fast relaxation. This compares with the instability of BLM·Fe(III), which has been observed in sodium phosphate buffer (Burger et al., 1979, 1981; Melnik et al., 1981).

dep-BLM·Fe(III) and DNA. The dep-BLM·Fe(III) complex is in the high-spin state at pH 4 as well as at pH 7. The addition of DNA to this complex either at pH 7 or at pH 4 does not give rise to a conversion of high-spin form to the low-spin form. This experiment allows one to rule out the

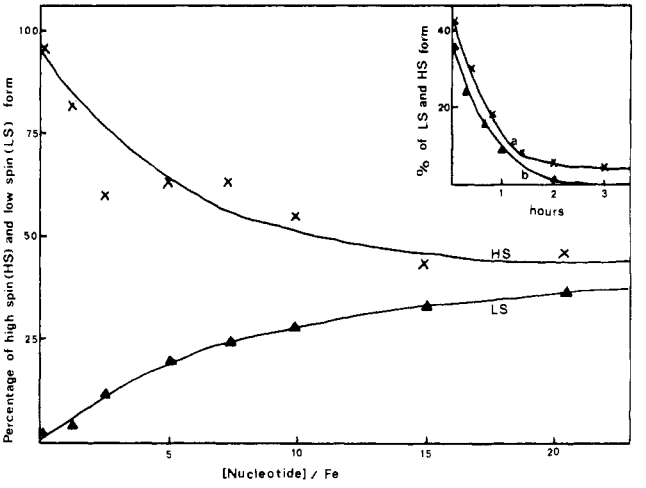

FIGURE 7: BLM·Fe(III)–DNA system at pH 4. The percentage of high-spin (HS) and low-spin (LS) species, determined from the EPR data, has been plotted as a function of the [nucleotide]/[Fe] molar ratio. Experimental conditions: 3×10^{-4} M BLM·Fe(III) in acetate solution, 0.05 M, at pH 4. The time elapsed after the addition of DNA and the freezing of the solution to liquid nitrogen temperature is 30 s. (Insert) Variation of the percentage of high-spin (curve a) and low-spin (curve b) species as a function of time at [nucleotide]/[Fe] = 15 and 25 °C.

eventuality that, in the case of the BLM·Fe(III) complex, this conversion could have been due to a direct binding of DNA to iron.

Titration of BLM–DNA System. One of the conclusions that can be drawn from the foregoing data is that DNA promotes the coordination of the α-amino group to BLM·Fe(III). The simplest hypothesis to explain this observation is that through interaction with DNA, the pK_d of deprotonation of the α-amino group has been lowered.

To test this hypothesis, titrations of DNA and BLM–DNA, in NaCl, 0.2 M, were respectively performed in the pH range 4–10. The solutions were titrated with NaOH, 0.1 M, or HCl, 0.1 M. The number $\bar{n}$ of protons released per BLM was calculated as reported above.

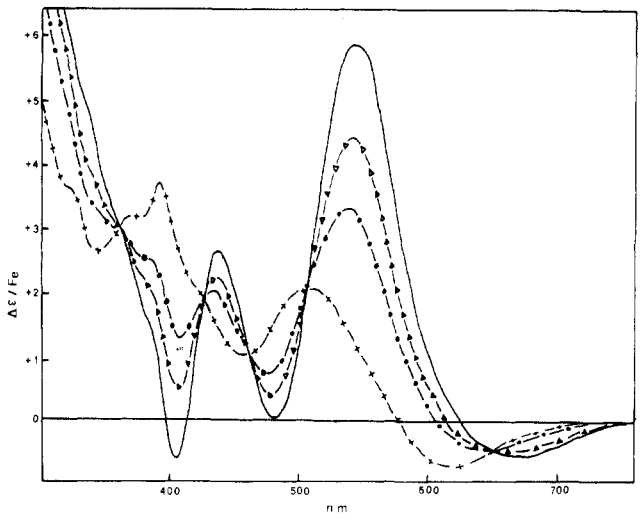

FIGURE 8: CD spectra of BLM·Fe(III)·$S_2O_3^{2-}$ in the presence of DNA. Experimental conditions: 5×10^{-4} M BLM·Fe(III) and 6.3×10^{-3} M $S_2O_3^{2-}$ in 0.05 M Hepes buffer, pH 7.4; [nucleotide] = 0 (—), 10^{-3} (△), 2×10^{-3} (●), and 8×10^{-3} M (×).

In the case of the DNA–BLM system, the protons released by DNA have been taken into account (about 0.2 proton per nucleotide are released between pH 4 and 10) and substracted from the data obtained. In that condition, one proton per BLM is released between pH 5.5 and 9. A pK_d of about 7 was thus determined.

BLM·Fe(III)·L and DNA at pH 7.4. As we have reported above, the addition, in Hepes buffer, pH 7.4, of $S_2O_3^{2-}$ to the BLM·Fe(III)·αNH_2 complex at a [$S_2O_3^{2-}$]/[Fe] molar ratio of about 12 ensures the complete formation of BLM·Fe(III)·$S_2O_3^{2-}$. The subsequent addition of increasing amount of DNA gives rise to the decrease of the absorption band at 550 nm, indicating the release of $S_2O_3^{2-}$. the CD spectrum is strongly modified as well; as can be seen in Figure 8, the spectrum of BLM·Fe(III)·$S_2O_3^{2-}$ evolves to that of DNA-bound BLM·Fe(III)·αNH_2 with the presence of isodichroic points at 650, 505, 460, 425, and 360 nm; at a [nucleotide]/[Fe] molar ratio of about 16, the spectrum of DNA-bound BLM·Fe(III)·αNH_2 is completely recovered. The superseding of $S_2O_3^{2-}$ by the α-amino nitrogen has thus been possible through interaction of the complex with DNA. If one assumes that the free complex is BLM·Fe(III)·$S_2O_3^{2-}$, the concentrations of DNA-bound complex and free complex have been determined from the value of $\Delta\epsilon$ at 540 nm. The binding data analyzed by Scatchard plots give an apparent equilibrium constant of 1.2×10^4 M^{-1}. One molecule of complex binds for every 2.8 base pairs in DNA. The binding constant obtained here is lower than that obtained when BLM·Fe(III)·αNH_2 binds to DNA in the absence of $S_2O_3^{2-}$. This can be accounted for by the fact that in that case $S_2O_3^{2-}$ has to be displaced from its position.

The same phenomenon is observed when DNA is added to BLM·Fe(III)·N_3^- complex, i.e., a decrease of the absorption band at 490 nm due to the release of N_3^- and the evolution of the CD spectrum to that of DNA-bound BLM·Fe(III)·αNH_2 (Figure 9).

These experiments can then be summarized by the equilibrium at pH 7

$$\underset{\text{low spin}}{\text{BLM·Fe(III)·L}} + \text{DNA} \rightleftarrows \underset{\text{low spin}}{\text{BLM·Fe(III)·}\alpha\text{NH}_2\text{–DNA}} + \text{L} \quad (12)$$

where L stands for either N_3^-, $S_2O_3^{2-}$, or SCN^-.

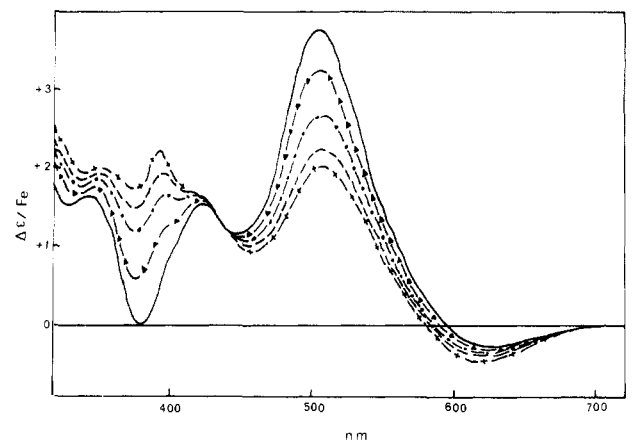

FIGURE 9: CD spectra of BLM·Fe(III)·N_3^- in the presence of DNA. Experimental conditions: 5×10^{-4} M BLM·Fe(III) and 0.11 M N_3^- in 0.05 M Hepes buffer, pH 7.4; [nucleotide] = 0 (—), 0.5×10^{-3} (△), 10^{-3} (●), 1.5×10^{-3} (---), and 2×10^{-3} M (×).

Discussion

The present study has two important implications regarding binding of ligands to iron–bleomycin complexes. Sugiura recently reported that the ferric bleomycin complex can bind a host of ligands much in the same way as seen with ferric homoglobin (Sugiura, 1980; Sugiura et al., 1982). On the other hand, ferrous bleomycin complex can also bind ligands such as CO, NO, and O_2 (Sugiura, 1982; Antholine & Petering, 1979; Sausville et al., 1978; Burger et al., 1979; Albertini et al., 1982). It has been suggested (Sugiura, 1979, 1980) that all these ligands coordinate the iron in a position trans to the α-amino nitrogen of the β-aminoalanine moiety (Sugiura, 1979, 1980). This hypothesis appears quite reasonable in the case of the BLM·Fe(II) complex with O_2 and oxygen antagonists since it has been shown that the dep-BLM is unable to activate molecular oxygen (Sugiura, 1979). A quite different situation is found when the BLM·Fe(III) complex binds L ligands such as those used in this work, i.e., N_3^-, SCN^-, and $S_2O_3^{2-}$. The first interesting result drawn from our experiments is that there is, in fact, a competition between the L ligand and the α-amino nitrogen for the same position.

The second result that emerges from this study is the possibility, through interaction with DNA, to partially convert the high-spin BLM·Fe(III) complex to the low-spin form at pH 4. From the closer similarity between the EPR of BLM·Fe(III)·αNH_2 at pH 7 and that of the BLM·Fe(III) complex in the presence of DNA at pH 4 (Figure 6, curves a and b′), we can infer that the α-amino nitrogen is still bound to the metal in the latter. Yet, coordination of the α-amino group at low pH should take place only if its pK_d has been lowered. Indeed, our titration data of BLM in the presence of DNA shows that this is the case. In addition, taking into account that at pH 4 DNA (at [nucleotide]/[BLM] $\simeq$ 15) converts about 35% of BLM·Fe(III) complex from the high-spin form to the low-spin one (Figure 7) and using the value of 10^6 for the formation constant K_1 of BLM·Fe(III)·αNH_2 (eq 1), we calculated a value of 6.8 for the pK_d of α-amino nitrogen in the presence of DNA, which compares with the value obtained by direct titration. The conclusion can thus be drawn that, in the presence of DNA, the pK_d of the α-amino group is lowered from 7.5 to about 6.8–7.0. Indeed, the interaction of the α-amino group with DNA is not unexpected since Huang et al. (1980) have reported two types of DNA-induced fluorescence quenching of BLM: a nonionic type of quenching probably resulting from the partial intercalation of BLM bithiazole rings into DNA molecules and an ionic type

of quenching involving the C-terminal amine and the β-aminoalanine moiety. In particular, they have shown that a methylsulfonylation of the α-amino group brings about some modification in the ionic type of quenching. In our experiments, therefore, coordination of the α-amino nitrogen at pH 4 can be accounted for by the decrease of its pK_d owing to interaction with DNA. The same explanation holds for the superseding of L by α-NH_2 when DNA is added to a BLM·Fe(III)·L complex at pH 7: a lowering of the pK_d of the α-amino group should increase its ability to compete with L.

The lowering of the pK_d of the α-amino group through interaction with DNA could now help to explain the behavior of the oxygenated complex of BLM·Fe(II) in the presence of DNA. In fact, two opposite behaviors have been observed for this complex in the presence of DNA depending on the molar ratio [nucleotide]/[BLM]. (i) At a low molar ratio of [nucleotide]/[BLM] of about 10, Povirk showed that the rate of oxidation of BLM·Fe(II) is influenced by DNA, the time constant of the kinetics of oxidation of the complex decreasing from $\tau = 35 \pm 1$ s in the absence of DNA to 0.56 ± 0.02 s at a [nucleotide]/[BLM] $\simeq 10$ (Povirk, 1979). (ii) At a high molar ratio of [nucleotide]/[BLM] ≥ 60, we have shown that the oxygenated complex of BLM·Fe(II) is largely stabilized, the time constant of the kinetic of oxidation being then of about 1 h (Albertini et al., 1982). These two observations are absolutely not conflicting and can be accounted for by two different effects. Thus, as we have stated above, when DNA is present to bind most of the BLM·Fe(II) complex, the interaction increases the proton lability of the α-amino group, and its σ-donating ability should then decrease—in so far as the pK of amines can be taken as a measure of their σ-bond strength (Carter et al., 1974; Collman et al., 1983)—giving rise to a destabilization of the oxygenated species; this can account for the data of Povirk (1979). At a molar ratio of [nucleotide]/[BLM) ≥ 60, this effect is still present but is canceled out by a second one that is most probably the building up of a protective pocket around the complex like globin does around the heme in hemoglobin.

Acknowledgments

We are indebted to Dr. J. Desbordes from the Laboratoire Roger Bellon, who kindly supplied us with bleomycin A_2 working standard, and to B. Maurin for technical assistance in the EPR measurements.

References

Albertini, J. P., & Garnier-Suillerot, A. (1982) *Biochemistry 21*, 6777–6782.

Albertini, J. P., Garnier-Suillerot, A., & Tosi, L. (1982) *Biochem. Biophys. Res. Commun. 104*, 557–563.

Antholine, W. E., & Petering, D. H. (1979) *Biochem. Biophys. Res. Commun. 91*, 528–533.

Bjerrum, J. (1941) in *Metal Amine Formation in Aqueous Solution*, Haase, Copenhagen.

Burger, R. M., Peisach, J., Blumberg, W. E., & Horwitz, S. B. (1979) *J. Biol. Chem. 254*, 10906–10912.

Burger, R. M., Peisach, J., & Horwitz, S. B. (1981) *J. Biol. Chem. 256*, 11636–11644.

Carter, M. J., Rillema, D. P., & Basolo, F. (1974) *J. Am. Chem. Soc. 96*, 392–400.

Carter, S. K. (1978) in *Bleomycin Status and New Developments* (Carter, S. K., Crooke, S. T., & Umezawa, H., Eds.) Academic Press, New York.

Collman, J. P., Brauman, J. I., Doxsee, K. M., Sessler, J. L., Morris, R. M., & Gibson, Q. H. (1983) *Inorg. Chem. 22*, 1427–1432.

Dabrowiak, J. C., Greenaway, F. T., Iongo, W. E., Husen, M. V., & Crooke, S. T. (1978) *Biochim. Biophys. Acta 517*, 517–526.

Dabrowiak, J. C., Greenaway, F. T., Santillo, F. S., Crooke, S. T., & Essery, J. M. (1980) *ACS Symp. Ser. No. 140*, 237–247.

Huang, C. H., Galvan, L., & Crooke, S. T. (1980) *Biochemistry 19*, 1761–1767.

Itaka, Y., Nakamura, H., Nakatani, T., Muraoka, Y., Fuju, A., Takita, T., & Umezawa, H. (1978) *J. Antibiot. 31*, 1070–1072.

Melnik, D. L., Horwitz, S. R., & Peisach, J. (1981) *Biochemistry 20*, 5327–5331.

Oppenheimer, N. J., Rodriguez, L. O., & Hecht, S. M. (1979a) *Biochemistry 18*, 3439–3445.

Oppenheimer, N. J., Rodriguez, L. O., & Hecht, S. M. (1979b) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5616–6220.

Povirk, L. F. (1979) *Biochemistry 18*, 3989–3995.

Ray, S. N., Orr, G. A., Brewer, F., & Horwitz, S. B. (1981) *Cancer Res. 41*, 4471–4477.

Sausville, E. A., Peisach, J., & Horwitz, S. B. (1976) *Biochem. Biophys. Res. Commun. 73*, 814–822.

Sausville, E. A., Stein, R. W., Peisach, J., & Horwitz, S. B. (1978) *Biochemistry 17*, 2746–2754.

Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.

Sillen, L. G., & Martell, A. E. (1964) in *Stability Constants of Metal-Ion Complexes*, The Chemical Society, Burlington House, London.

Solaiman, D., Rao, E. A., Antholine, W., & Petering, D. H. (1980) *J. Inorg. Biochem. 12*, 201–220.

Sugiura, Y. (1979) *Biochem. Biophys. Res. Commun. 88*, 913–918.

Sugiura, Y. (1980) *J. Am. Chem. Soc. 102*, 5208–5215.

Sugiura, Y., Suziki, T., Kawabe, H., Tanaka, H., & Watanabe, K. (1982) *Biochim. Biophys. Acta 716*, 38–44.

Takita, T., Muraoka, Y., Nakatani, T., Fuju, A., Itaka, Y., & Umezawa, H. (1978) *J. Antibiot. 31*, 1073–1077.

Umezawa, H., & Takita, T. (1980) *Struct. Bonding (Berlin) 40*, 73–99.