Vol. 110, No. 3, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 10, 1983 Pages 827-836

Mössbauer Studies of Iron-Bleomycin Complexes

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Received December 20, 1982

The Mössbauer technique was used to investigate the metal ligand bond in Fe(III) and Fe(II) oxygenated bleomycin complexes. Distinct differences in the Mössbauer spectra were observed for these two types of complexes. In the case of the oxidized Fe(II) bleomycin complex a single pH dependent high spin paramagnetic site is observed. The spectra for Fe(III) bleomycin also revealed this high spin paramagnetic site, however, an additional pH dependent site was observed. Mössbauer spectra revealed that this second site converted from a high spin ferric in 1 molar HCl to a high spin ferrous state above pH 0.5. These two sites are suggested to arise from the Fe(III) bleomycin complex existing in at least two distinct conformations. It is additionaly postulated that the ferrous site arises from an electron transfer between the bithiazole moiety in bleomycin to the bound ferric ion.

Bleomycins are water soluble glycopeptides which induce various antitumor activities in animal experiments and are clinically useful in the chemotherapy of certain human tumors. The Fe(II)-bleomycin complex is postulated to be active through reduction of molecular oxygen to a

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0006-291X/83/030827-10$01.50/0

more reactive species resulting in cleavage of a susceptible DNA bond (1). Though the mechanism of DNA breakage by bleomycin is not clearly defined, the in vitro DNA cleaving activity requires the presence of both oxygen and Fe(II).

EPR studies of the Fe(III) bleomycin complex (2) show that Fe(III) forms both a high and low spin complex with bleomycin that are in a pH-dependent equilibrium. At pH 2.4 EPR and optical spectra indicate no interaction between Fe(III) and bleomycin while at pH 3.7 a high spin complex forms and at pH 7 the complex is low spin. In addition the EPR spectra reveal no difference in the iron state whether it be in the Fe(III) form or oxidized from Fe(II). A recent abstract (3) reports Mössbauer studies on the Fe(III) bleomycin A_2 complex. Though there was no mention of the pH, Mössbauer spectra revealed a high spin Fe(III), a low spin Fe(III), and a high spin Fe(II).

This work is an examination, employing the Mössbauer technique, of the electronic and molecular structure of the iron (Fe(III) and Fe(II)) environment in bleomycin as a function of pH. Mössbauer spectroscopy has the unique property that it can detect both the presence of iron with any electronic configuration (including diamagnetic iron) and the chemical state of the iron being observed. In contrast a technique such as ESR, commonly used to investigate the iron environment, is restricted to those paramagnetic iron sites having half integer spins thereby eliminating iron sites with integer spins (unless special techniques are employed).

Present Mössbauer studies have revealed distinct and interesting interactions in the iron bleomycin complexes. In the case of the Fe(II) bleomycin O_2 complex a single pH dependent high spin paramagnetic site is observed. Fe(III) bleomycin also revealed this high spin site, however an additional pH dependent site was also observed. This site converts from a high spin ferric to a high spin ferrous state. It was also observed that the bleomycin iron complex

remains intact at high hydrogen concentration (1M) suggesting an extremely stable complex.

EXPERIMENTAL PROCEDURES

MATERIALS: Bleomycin sulfate which contained approximately 60% bleomycin A_2, 30% bleomycin B_2, and 10% other bleomycins, a mixture similar to that used in the ESR studies (2), was obtained from Aldrich Chemical Company. Solutions were prepared in a 20 mM sodium phosphate buffer. A molecular weight of 1550 was assumed. The pH of the solutions was adjusted, at room temperature, with NaOH and/or HCl and measured with a Radiometer PHM64 pH meter. After setting the pH, the optical spectra were recorded between 300 and 500 nm and compared with literature results. The samples were brought to 77 K within ten minutes following final pH adjustment. The iron was enriched with ^{57}Fe and in the form of a chloride salt. All experiments were carried out under aerobic conditions by purging the solutions with oxygen.

METHODS: Mössbauer experiments were performed in the transmission geometry mode using a 30 mCi cobalt source in a Pd matrix at room temperature, with the absorber at liquid nitrogen temperature. The bleomycin iron complex was contained in a Delrin sample holder which was mounted to the cold finger of a continuous flow cryostat. (Lake Shore Cryotronics, Westerville, Ohio).

Analysis of the Mössbauer spectra was performed using standard least mean squares fitting procedures assuming Lorentzian line shapes. The output of the computer program (4) (line positions, line width and intensities) was used to determine isomer shifts and the effective magnetic field and quadrupole splittings.

RESULTS

An iron powder sample was used for calibration of the instrument. The effective instrumental line broadening, including linewidth source and geometrical effects, was at most 10%. Figure 1a-d are the

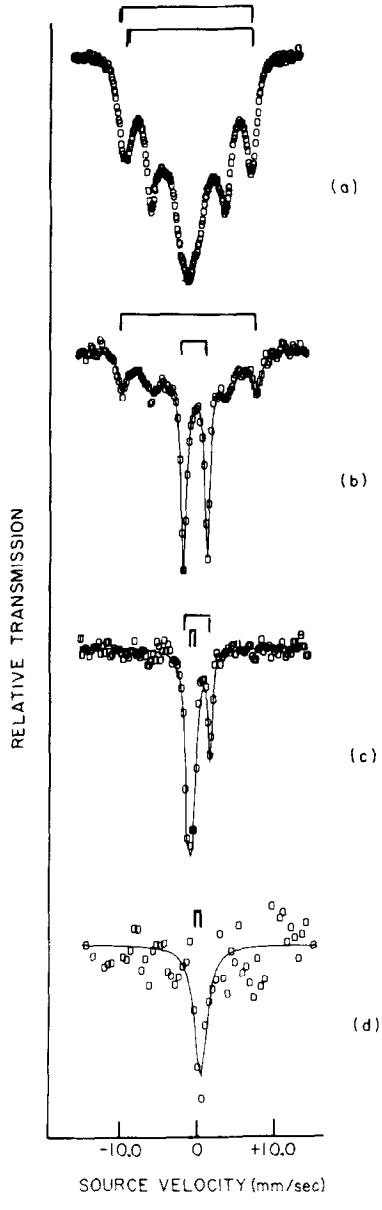


Figure 1. Mössbauer transmission spectra of BLM (Fe^{3+}) at 80 K in zero applied magnetic field and for pH values < 1/2 [1M hydrogen ion concentration], 1/2, 3.5, and 7 for (a), (b), (c) and (d) respectively; the dots give the experimental results and the solid curves are best fits based on Lorentzian line shapes. The pH 7 spectra has been presented though not analyzed due to the low statistics.

spectra of the Fe(III)·bleomycin sample at a temperature of 80 K and various pH values. At a 1M hydrogen ion concentration (Fig. 1a) bleomycin gives a typical paramagnetic high spin Mössbauer spectrum similar to Ferrichrome A at 2.5 K (5). To check this assignment the temperature was increased to 110 K where an increased broadening of the lines and smearing of the spectrum (typical of paramagnetic behavior) occurred. Fig. 1a reveals a superposition of at least two slightly different hyperfine patterns as revealed by the differences in linewidths between the first and sixth line. The first line can be resolved into two lines and there are hints of a substructure around the central lines. Stick diagrams above this spectrum indicate the positions of the outermost resonance lines. An analysis of the spectrum in terms of static hyperfine parameters yields an effective magnetic field H = 540 kG, a quadrupole spliting of ΔE_q = 0.022 mm/s and an isomer shift with respect to iron of 0.446 mm/s for site I and H = 535 kG, ΔE_q = 0.020 and isomer shift of 0.443 mm/s for site II. The above parameters, for both sites, are indicative of a high spin Fe(III) state (Fe^{3+}, S = 5/2). At pH = 0.5, spectra from two different sites were clearly observed as shown in Fig. 1b. One of the paramagnetic sites (I) remains and still gives a split spectrum. The second site changes its character: quadrupole doublet with large splitting ΔE_q = 3.046 mm/s, and an isomer shift of 1.265 mm/s, indicative of a high spin ferrous state (Fe^{2+}, S = 2) (6).

At pH 3.5 (Fig. 1c), three peaks are observed. Two correspond to the quadrupole doublet of site II (Fe^{2+}, S = 2) and the third, revealed at higher resolution by line fitting (Fig. 2a), to be part of another quadrupole doublet with a splitting ΔE_q = 0.730 mm/s, and isomer shift 0.332 mm/s (Fe^{3+}, S = 5/2). Site I is indicative of a high spin ferric state whose environment is different from that at lower pH, given the changes in quadrupole splitting (ΔE_q) and isomer shift (Table I).

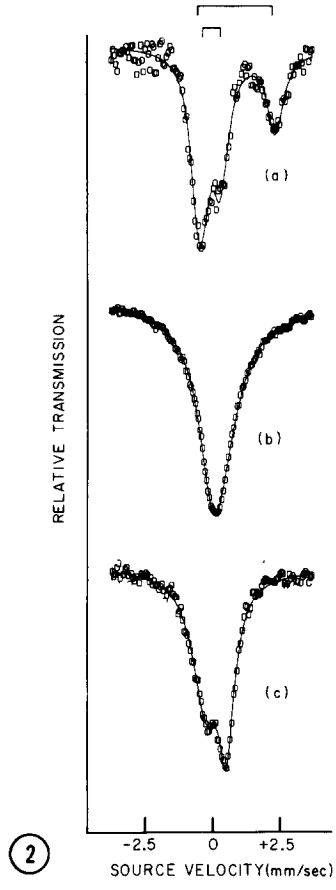
TABLE 1. HYPERFINE PARAMETERS: Effective Magnetic Field (Heff), Quadrupole Splitting (ΔE_q) and Isomer Shift (δ(Fe)), with respect to iron, of Blm (Fe^{3+}) for Different pH Values.

pH	site	Heff (kG)	ΔE_q (mm/sec)	δ (Fe)[c] (mm/sec)
a	I	540 ± 2.5	0.022	0.446
a	II	535 ± 2.5	0.020	0.443
1/2	I	540 ± 5.0	0.022	0.446
1/2	II	--------[b]	3.046	1.265
3.5	I	--------[b]	0.730	0.332
3.5	II	--------[b]	3.046	1.265

[a] 1M hydrogen ion concentration
[b] No observed magnetic splitting
[c] Standard deviation ±0.008

All of the hyperfine parameters obtained for Fe(III)·bleomycin are listed in Table I. To check whether these spectra correspond to anything other than a bleomycin site we ran blank experiments (no bleomycin added) and obtained the spectra shown in Fig. 2b, and 2c. Fig. 2b shows the spectrum of ferric chloride in buffered solution and Fig. 2c of powdered crystals of $FeCl_3$, both at 80 K. A comparison of Figures 1 and 2 clearly demonstrates that the iron in the solutions was influenced by bleomycin.

The Mössbauer spectra of Fe^{2+}. BLM·O_2 as a function of pH, are shown in Fig. 3. For the three pH values (Fig. 3a, b and c) the spectra show a single paramagnetic site (I') similar to site I in the pH 0.5 spectrum of Fe(III)·bleomycin. There are some slight differences in the parameters as can be seen by comparing Table I with Table II. Site I' in general shows slower relaxation rates, indicative perhaps of greater separation between the individual Kramers doublets, for each value of pH, as compared with site I. More importantly, however, the counterpart of site II for Fe(III)·bleomycin,



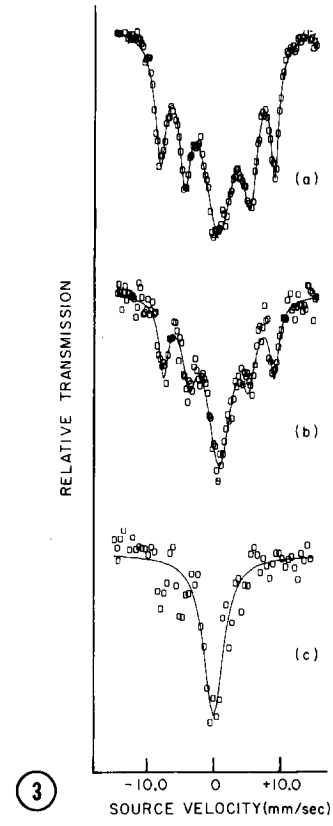


Figure 2. Higher velocity resolution transmission spectra of (a) BLM (Fe^{3+}) at pH 3.5, (b) $FeCl_3$ in buffer, (c) and $FeCl_3$ powdered crystals; all at 80 K.

Figure 3. Mössbauer transmission spectra of BLM (Fe^{2+})·O_2 at 80 K in zero applied magnetic field and for pH values <1/2 [1M hydrogen ion concentration], 1/2, 3.5 for (a), (b) and (c) respectively. The dots give the experimental results and the solid curves are best fits to the data based on Lorentzian line shapes.

characterized by the large quadrupole doublet, is absent in the spectra of Fe(II)·Blm·O_2.

DISCUSSION

These Mössbauer spectra of the iron-bleomycin complex reveal a number of interesting features. First is the persistence of metal complex formation at extremely low values of pH as seen from a

TABLE II. HYPERFINE PARAMETERS: Effective Magnetic Field (H_{eff}), Quadrupole Splitting (ΔEq) and Isomer Shift ($\delta(Fe)$) with respect to iron, of Blm $(Fe^{2+})\cdot O_2$ for Different pH Values.

pH	Site	H_{eff} (kG)	ΔE_q (mm/sec)	$\delta(Fe)^c$ (mm/sec)
a	I'	546	0.037	0.411
1/2	I'	536	0.108	0.485
3.5	I'	---[b]	----[b]	0.041

[a] 1M hydrogen ion concentration
[b] No observed magnetic splitting
[c] standard deviation ±0.008

comparison of Figures 1 and 3 with Figure 2. From potentiometric titration data (7) it has been established that the imidazole and the amino group of the α-amino carboximide moiety bind the metal ion with a subsequent disappearance of their pK_a's. Additionally, proton NMR studies (8) suggest a kinetically unfavorable interconversion from the complexed to uncomplexed forms at low pH ($\leq$ 2.5). It is interesting to observe such a stable ion-ligand complex in acidic solutions. Surprisingly very little thermochemical data exists to compliment this point.

Secondly, and probably most importantly, we have observed two distinct binding sites for the Fe(III)· bleomycin complex. Since the present study has been carried out on a mixture of bleomycins (60% A_2, 30% B_2, and 10% other bleomycins) the possibility exists that the two sites can be attributed to binding with the A_2 and B_2 forms. However, if this were the case why is only a single site observed in the oxidized Fe(II) complex ? This existence of two sites has also been observed in Mössbauer and NMR studies (3,9) of the bleomycin A_2 form and therefore it would appear likely that the Fe(III)· bleomycin A_2 complex exists in at least two distinct conformations. Thus, our Mössbauer studies reveal that either the A_2 and B_2 forms are degenerate in their spectra or B_2 does not

bind iron. This discrepancy requires further studies on the seperate forms of bleomycin.

One of the two sites which we observe agrees well with an earlier EPR study (2) in that a ferric high spin state around pH 3.5 was detected. However, the second site, which must be involved in a redox type of reaction, is unique since the iron is in a Fe^{2+}, S = 2 state. Such a site has been confirmed elsewhere (3). Since this redox reaction is a pH dependent process (Table 1) a possibility is that at a given pH the bleomycin undergoes a conformational change in which electron transfer from the bleomycin to Fe(III) can occur. NMR studies (9) at pH 7 show that two types of Fe(III)• bleomycin complexes coexist and that in one of the complexes there is possible coordination of the bithiazole group to the metal. Since thiazole's are nucleophilic it is plausible that electron sharing or transfer from thiazole to Fe(III) can occur. It is well established that complexes of bleomycin with Fe(II) are cytotoxic so it is possible that bleomycin offers more to the mechanism than just intercalating to DNA thereby bringing the Fe(II) close to a deoxyribose moiety. For example when Fe(II) is oxidized to Fe(III), in vivo, the bleomycin could reduce the Fe(III) back to its ferrous state and the whole cycle repeat with the Fe(II)•bleomycin complex acting as a catalyst.

Finally, the Mössbauer spectra reveal distinct differences in the iron environment between the Fe(III) and oxidized Fe(II) bleomycin complexes (Tables 1 and 2); an observation contrary to a previous EPR and optical study (2). Such differences between the two iron forms have also been reported from fluorescence quenching studies of bleomycin iron complexes (10).

ACKNOWLEDGMENTS: We would like to thank Dr. Jack Lebowitz of the University of Alabama for suggesting the problem and Dr. R. L. Berger of the Laboratory of Technical Development for constant support and helpful discussions.

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