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Undesirable Reactions in Bleomycin Biomimetic Catalysis Systems Caused by Iodosobenzene

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The study of the catalytic mechanisms of enzymes, especially heme proteins, has often been approached and facilitated by the use of analogues to mimic enzyme catalysis. For example, the heme protein cytochrome P-450 functions with a flavoprotein reductase, NADPH, and molecular oxygen in the hydroxylation and epoxidation of certain substrates.[1] The catalytic oxygenation of small molecules has been achieved in metalloporphyrin models for this system by using peroxides, peroxy acids, or iodosobenzene as surrogate activators.[2]

The oxidations catalyzed by heme proteins and model systems may implicate a common active iron-oxygen complex formed by the reaction of various oxidants with the iron porphyrin sites. In horseradish peroxidase, and by analogy in cytochrome P-450, the hypervalent iron species responsible for the catalysis is often formulated as an iron-oxene complex at an oxidation level formally equivalent to ferryl iron bound to an oxygen atom containing a full octet of electrons.[1] The complex in peroxidases[3,4] and in catalase, called compound I, contains an additional oxidizing equivalent in a porphyrin π cation radical. For ferric tetraphenylporphyrin model systems, a species analogous to the enzymic oxidant compound I is produced from the ferric porphyrin and iodosobenzene.[5,6] A description of the activation and oxygen-related chemistry of these models as they relate to cytochrome P-450, horseradish peroxidase, and catalase remains under active investigation.

Metal complexes of the antitumor drug bleomycin have also been reported to achieve chemical transformations similar to those catalyzed by the heme proteins cytochrome P-450, horseradish peroxidase, and chloroperoxidase. These reactions include hydroxylation, epoxidation, and oxidative N-demethylation, which are accomplished upon activation of ferric bleomycin with peroxides, with iodosobenzene, or with other oxidants.[7-9] It has been suggested that the activation of ferric bleomycin by iodosobenzene and by peroxide are analogous and that there are mechanistic similarities between the iron-drug complex and cytochrome P-450 in small molecule oxidations.[9,10] Ferric bleomycin reacts with hydrogen peroxide to give a species termed "activated bleomycin" that has unique EPR and optical spectroscopic characteristics. The same species is produced by the aerobic activation of Fe(II)-bleomycin.[11,12] Activated bleomycin is the kinetically competent form of the drug that cleaves DNA. Though the redox state of activated bleomycin is formally equivalent to compound I (activated bleomycin requires two reducing equivalents for discharge to ferric bleomycin[13]) its iron-oxygen moiety is different from a ferryl-oxene species as found in horseradish peroxidase compound I[3,4] in that it contains low-spin ferric iron and at least one iron-bound oxygen atom.[11,14] No direct demonstration has ever been made that iodosobenzene generates this same species or any other hypervalent iron species from Fe(III)-bleomycin nor does iodosobenzene activate the drug[15] for the DNA cleavage characteristic of hydrogen peroxide-activated Fe(III)-bleomycin.[11]

One problem associated with the oxidizing conditions used in experiments with porphyrin model systems and in heme proteins is the rapid decomposition of the porphyrin macrocycle upon treatment with oxidants such as H_2O_2 or iodosobenzene.[1,16,17] Model catalysis experiments with high turnovers of the activated metal have made use of substituted porphyrins especially resistant to oxidative damage by iodosobenzene.[5,6,17-20] The similar reports of catalysis by ferric bleomycin with iodosobenzene have not addressed the possible breakdown of the metal-drug complex upon treatment with this reagent nor has any spectroscopic information been published about this system.

In our investigation of the spectroscopic properties of the putative complex formed upon treatment of ferric bleomycin with iodosobenzene, we found a rapid destruction of the drug. No EPR evidence for an activated metal oxidant was found. The iron binding capacity of the molecule is compromised, and the bithiazole moiety is altered by iodosobenzene. These observations make it difficult to equate the chemistry occurring with iodosobenzene and Fe(III)-bleomycin to that reported for isolated and characterized compound I analogues,[5,6] especially since reaction conditions appearing in the literature involve long incubations with large molar excesses of iodosobenzene.[10] The fact that iodosobenzene can achieve the epoxidation of alkenes with $Fe(ClO_4)_3$, Zn(II)-bleomycin,[21] Cu(II) complexes[22] including Cu(II)-bleomycin,[10] or simple metal triflates[23] raises the question of the true nature of the catalytic oxidant in experiments with Fe(III)-bleomycin, especially since the binding of Fe(III) by the drug is not very strong.[24] In this communication we address the issue of the integrity of iron-bleomycin under various conditions and conclude that the drug is quickly modified by iodosobenzene, even in the absence of Fe(III).

Experimental Section

Bleomycin sulfate (BLENOXANE) was generously supplied by Bristol Drugs. Bleomycin A_2 was isolated by preparative thin-layer chromatography (TLC) on silica plates in 0.6 M ammonium acetate/0.04 M ammonium hydroxide, in 50% methanol.[25] Bleomycin A_2 was eluted from the silica in 50% methanol/water. Ammonium oxalate was added to remove the calcium dissolved from the silica binder, and the drug was

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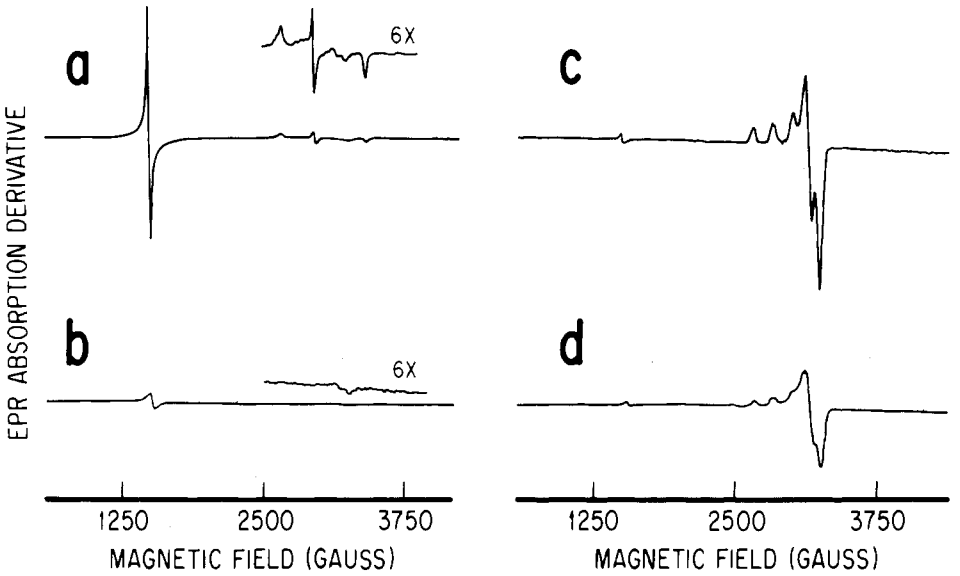

Figure 1. EPR spectra of Fe(III)–bleomycin A_2 in 4:1 methanol–H_2O: (a) 0.7 mM Fe(III)–bleomycin A_2, no additions; (b) 0.7 mM Fe(III)–bleomycin A_2 after treatment with excess iodosobenzene (23 mM); (c) sample from part a, after addition of 0.84 mM Cu(II); (d) sample from part b after addition of 0.84 mM Cu(II). The signal at 1500 G (g = 4.3) is due to high-spin Fe(III)–bleomycin A_2 and the signal at higher field (g = 2.45, 2.18, 1.89) is due to the low-spin complex.[8] The signal in part c is due to Cu(II)–bleomycin A_2 ($g_{\parallel}$ = 2.22, $g_{\perp}$ = 2.06).[22] EPR conditions: microwave frequency, 9.078 GHz; microwave power, 5.0 mW; modulation amplitude, 2.5 G.

desalted over a gel permeation column. The peak fractions were pooled and were applied to a cation-exchange column (Mono S, Pharmacia) in 10 mM ammonium acetate. The drug was eluted in a linear gradient of ammonium acetate. The pooled bleomycin A_2 fractions were lyophilized to remove ammonium acetate. The final material chromatographed as a single spot on TLC (R_f = 0.45) in the ammonium acetate/ammonium hydroxide solution in methanol, described above. The concentration of bleomycin A_2 was determined optically (ϵ_{292} = 15.1 mM^{-1} cm^{-1}).[24]

EPR spectra were recorded on a Varian E-112 spectrometer equipped with a Varian NMR gaussmeter. Optical spectra were recorded on a modified Cary 14 spectrophotometer (Aviv Associates, Lakewood, NJ). Fluorescence spectra were recorded on a Perkin–Elmer fluorescence spectrophotometer, Model MPF-3L.

Iodosobenzene was prepared from (diacetoxyiodo)benzene according to a published procedure[26] and was used within 3 weeks of preparation. The solvent used in all sample preparations was (4:1) methanol/H_2O, unless otherwise stated. Iron bleomycin was usually prepared in H_2O by combination of ferric ammonium sulfate with a small excess of bleomycin A_2, followed by a brief incubation.

Samples used for EPR experiments at 77 K contained 0.8 mM bleomycin A_2 and 0.7 mM Fe(III), and were incubated for 15 min with 23 mM iodosobenzene. Cupric sulfate (0.84 mM) was added after recording the initial EPR spectrum, and a second EPR spectrum of the sample was recorded after overnight incubation with Cu(II).

Another EPR experiment was performed at 8 K and low microwave power (10–50 μW) to check for the appearance of a signal near g = 2 that might arise from a species analogous to compound I of HRP (HRP = horseradish peroxidase): the easily saturated signal arising from the coupled S = 1, S = $^1/_2$ Fe(IV)–porphyrin radical system has been described and is observed at temperatures below 30 K.[27] Excess iodosobenzene in a minimum of methanol was added to 0.7 mM Fe(III)–bleomycin in a 4:1 methanol–water mixture after equilibration of both solutions at −15 °C. The sample was immediately frozen in liquid nitrogen and was examined by EPR spectroscopy. A second addition of iodosobenzene was made subsequent to defrosting, and the sample was reexamined.

Samples for optical experiments contained 0.44 mM bleomycin A_2 and 0.38 mM Fe(III) with 0.6 mM iodosobenzene added in two 0.5-mg portions. The sample was centrifuged before recording the spectrum, which was corrected for the small absorbance due to Fe(III) plus iodosobenzene, without bleomycin A_2.

The change in fluorescence of bleomycin A_2 upon treatment with iodosobenzene was monitored in a sample prepared by dilution to 3 mL of a 25-μL incubation mixture that contained 0.9 mM bleomycin A_2, 1.1 mM Fe(III), and 29 mM iodosobenzene. The reagents were mixed and incubated for 15 min at room temperature before dilution. Iodosobenzene or Fe(III) plus iodosobenzene did not contribute to the observed fluorescence. Also, no change in the fluorescence of bleomycin A_2 was observed when it was added to a preincubated mixture of Fe(III) plus iodosobenzene.

Thin-layer chromatography of bleomycin A_2 after treatment with iodosobenzene was performed on Silica 60 plates in the methanol–acetate buffer used for the isolation of bleomycin A_2.

Results and Discussion

The sensitivity of bleomycin A_2 to iodosobenzene was demonstrated in several experiments. Fe(III)–bleomycin A_2 in methanol–water exhibits a high-spin iron EPR spectrum (g = 4.3) with a small contribution due to low-spin Fe(III)–bleomycin (g = 2.45, 2.18, 1.89).[11] Both the high- and low-spin iron EPR signals decrease after a brief incubation with excess iodosobenzene, as shown in Figure 1, with only a trace of the high-spin iron signal remaining. No evidence for a signal characteristic of activated bleomycin (g = 2.26, 2.17, 1.94)[8] was found in a similar sample frozen 30 s after addition of iodosobenzene to Fe(III)–bleomycin A_2: this sample exhibited 50% of the g = 4.3 signal intensity found before addition of iodosobenzene (not shown). Another experiment demonstrated that the broad g = 4.3 signal due to high-spin Fe(III) in methanol–water without bleomycin A_2 is also nearly abolished subsequent to addition of iodosobenzene (not shown).

The EPR experiment performed at 8 K revealed no signal near g = 2 other than that due to the trace of Cu(II)–bleomycin,[28] which is ubiquitous.

To test the integrity of the drug molecule after treatment of Fe(III)–bleomycin A_2 with iodosobenzene, Cu(II), which binds more strongly to bleomycin than does Fe(III),[19] was used to elicit the EPR signal of Cu(II)–bleomycin A_2 (Figure 1, right panel).[28] Some Cu(II)–drug complex was formed, though the EPR characteristics of this complex were slightly different from those of bona fide Cu(II)–bleomycin A_2 in an untreated sample. The EPR signal intensity due to the Cu(II)–drug signal in the treated sample was greatly reduced compared to the intensity of the Cu(II)–bleomycin A_2 signal elicited upon addition of 1.2 equiv of Cu(II) to an untreated sample of Fe(III)–bleomycin A_2.

The EPR results demonstrate that (1) iodosobenzene abolishes the EPR signal of both Fe(III)–bleomycin and Fe(III) in methanol–water and (2) the bleomycin A_2 molecule is modified by iodosobenzene. Iodosobenzene in methanol water mixtures is

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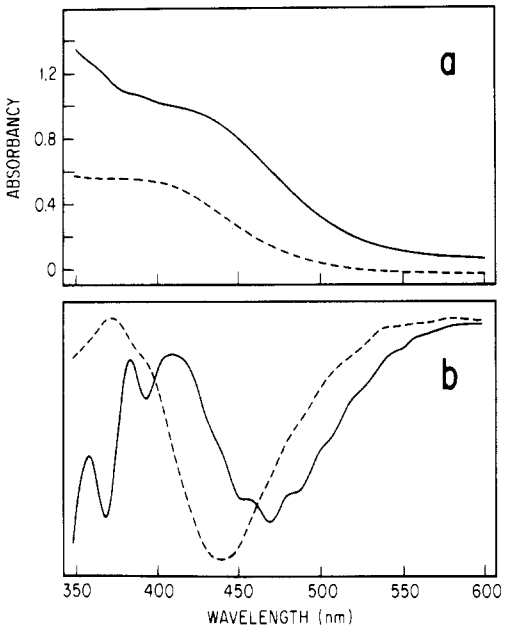

Figure 2. (a) Optical spectra of Fe(III)–bleomycin A_2: solid line, 0.38 mM Fe(III)–bleomycin A_2; dotted line, same sample after addition of iodosobenzene. (b) Derivative functions of the spectra in part a: solid line, Fe(III)–bleomycin A_2; dotted line, same sample after addition of iodosobenzene.

polymeric,[29] and the loss of EPR signal could reflect the binding of iron to the polymer. The results at 8 K suggest that iodosobenzene does not activate Fe(III)–bleomycin to a complex similar to compound I.

Optical Spectrophotometry. The addition of iodosobenzene to Fe(III)–bleomycin A_2 causes a color change from the orange-yellow characteristic of the ferric drug complex to a stable pale yellow. Optical spectra of samples before and after treatment with iodosobenzene are shown in Figure 2. A derivative function of the optical spectrum of the mixture clearly shows the disappearance of features found in the spectrum of the starting metal complex. The persistent yellow color in the treated sample arises from an unidentified Fe(III) complex since treatment with Chelex resin abolishes the residual color (not shown). The results are consistent with a rapid removal of Fe(III) from bleomycin upon addition of iodosobenzene.

Changes in the ultraviolet absorbance of bleomycin A_2 were monitored in another experiment (not shown). The absorbance peak at 292 nm is abolished after treatment with iodosobenzene though the analysis of UV spectra is complicated by the strong overlapping spectra of iodosobenzene and, presumably, iodobenzene.

Fluorescence Spectrophotometry. The fluorescence of Fe(III)–bleomycin A_2, due to emission from the bithiazole moiety,[30] is quickly altered upon addition of iodosobenzene. Figure 3 shows a shift in the emission maximum from 346 to 365 nm. The same fluorescence change was observed in a sample of metal-free bleomycin A_2 treated with iodosobenzene. This demonstrates that iron is *not* required for the reaction of iodosobenzene with the drug. The observed fluorescence change could *not* be achieved when bleomycin A_2 was added to iodosobenzene incubated first with Fe(III), nor was the quenching of fluorescence known to occur with formation of Fe(III)–bleomycin[24] observed. This result suggests that a Fe(III)–iodosobenzene complex, if formed, does not interact with bleomycin A_2 and/or that iodosobenzene is quickly discharged in the presence of Fe(III).

Thin-Layer Chromatography. Bleomycin A_2 has an R_f value of 0.45 in 0.6 M ammonium acetate/0.04 M ammonium hydroxide

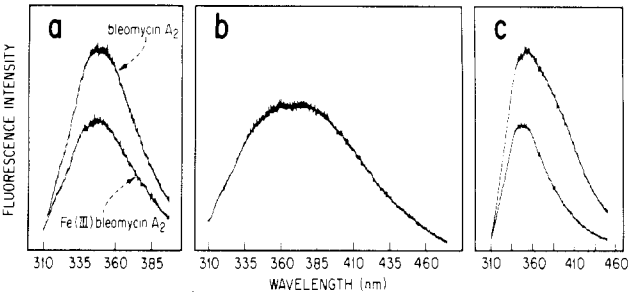

Figure 3. (a) Fluorescence emission spectrum of bleomycin A_2 and Fe(III)–bleomycin A_2. (b) Fluorescence emission spectrum of a sample of Fe(III)–bleomycin A_2 after treatment with iodosobenzene. (c) Fluorescence emission spectrum of Fe(III)–bleomycin A_2 (lower spectrum) and a sample of bleomycin A_2 added to a preincubated mixture of Fe(III) with iodosobenzene (upper spectrum). In part c, no quenching by Fe(III) is observed in the presence of iodosobenzene.

in 50% methanol. The drug spot could be visualized on TLC plates either by its quenching of the emission from the fluorescent indicator or by the drug's intrinsic fluorescence upon illumination with long-wavelength UV light. An experiment was performed in the presence of *cis*-stilbene to reconstruct the conditions for which epoxidation has been reported.[10] Here, Fe(III)–bleomycin A_2 was incubated with iodosobenzene, either in the presence or absence of *cis*-stilbene. The reaction mixture was extracted with ether and an aliquot taken from the aqueous phase was applied to a TLC plate. Fe(III) remains at the origin (its presence is confirmed by the formation of Prussian Blue with ferrocyanide), unreacted bleomycin A_2 appears as a blue spot upon long-wavelength UV illumination, and two yellow fluorescent spots (R_f = 0.51, 0.59) appear. No difference could be observed in the chromatograms of aliquots removed from reaction mixtures with or without stilbene, nor in those sampled after 5- or 30-min incubation with oxidant.

Chromatograms of products formed upon treatment of Cu(II)–bleomycin A_2 with iodosobenzene yielded slightly different results. Unlike the ferric drug complex, Cu(II)–bleomycin A_2 elutes as a metal complex, with a slightly smaller R_f value than that for metal-free bleomycin A_2. It appears as a dark spot upon long-wavelength UV illumination due to the efficient quenching of bithiazole fluorescence by Cu(II).[24] Samples of iodosobenzene-treated Cu(II)–bleomycin A_2 show a spot due to unaltered starting material and a new spot that also contains Cu(II). (Copper-containing spots were visualized by staining with diphenylthiocarbazone.) The appearance of two new spots upon treatment of Fe(III)–bleomycin A_2 with iodosobenzene and one new spot in the treated sample of Cu(II)–bleomycin A_2 suggests that the Cu complex of bleomycin A_2 is protected from damage by iodosobenzene or that Cu(II) binding to the degraded products causes the two species to run together on the TLC plate.

An optical experiment was designed to monitor the reaction of Fe(III)–bleomycin A_2 with *trans*-1,4-diphenyl-1,3-butadiene upon addition of iodosobenzene. This diene is readily epoxidized in experiments with iodosobenzene-activated iron(III) tetrakis-(2,6-dichlorophenyl)porphyrin chloride.[19] No loss of the diene could be detected optically at 350 nm (the long-wavelength edge of the diene absorption) under the following conditions: 0.1 mM diene, 1 mM iodosobenzene, 0.06 mM Fe(III)–bleomycin A_2, in 99:1 methanol–H_2O.

Conclusions

The initial goal of the experiments reported here was to compare the properties of peroxide-activated Fe(III)–bleomycin A_2 (or the equivalent oxygen-activated Fe(II)–bleomycin) with iodosobenzene-activated Fe(III)–bleomycin A_2 in the context of the known behavior of HRP or cytochrome P-450. The EPR-active "perferryl" species generated by peroxide activation of Fe(III)–bleomycin contains low-spin ferric iron and at least one oxygen atom, and may be formulated as $Fe^{III}(O–O^{2-})$ or $Fe^{III}[O]$.[11] This activated bleomycin is relatively stable in buffered solution but decays rapidly in the presence of DNA,[31] which it cleaves

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efficiently. No EPR evidence for the species characteristic of activated bleomycin nor of the compound I type species of HRP could be found with Fe(III)-bleomycin A_2 in the presence of iodosobenzene. This is consistent with the report that thymine is not released from DNA treated with Fe(III)-bleomycin plus iodosobenzene.[15] (Thymine *is* readily released from DNA treated with O_2-activated Fe(II)-bleomycin or H_2O_2-activated Fe(III)-bleomycin.[11,35])

Fe(III)- and Cu(II)-bleomycin A_2 are susceptible to damage by the oxidant, and experiments show a rapid reaction between iodosobenzene and metal-free bleomycin A_2, leading to a loss of metal binding capacity. Also, Fe(III) forms an EPR-silent complex when iodosobenzene is added to Fe(III)-bleomycin A_2, which suggests removal of the metal from the drug complex.

Iodosobenzene activation of Fe(III) in some porphyrin models is described in several accounts.[5,6,17-20,32] However, this reagent is not a benign oxygen surrogate in reactions with bleomycin. The use of iodosobenzene and related hypervalent iodine complexes in synthetic organic chemistry has been reviewed.[33] Its ability to cleave glycols (in a reaction analogous to lead tetraacetate oxidations) as well as its reactivity toward carboxylic acids, amides, and other functional groups could interfere with its usefulness as an activation surrogate in the case of bleomycin. The reports of epoxidations and other reactions using Fe(III)- and Cu(II)-bleomycin with iodosobenzene, considering the long incubations generally employed, must be viewed with caution since the drug molecule as well as the Fe(III)-drug complex breaks down rapidly. Recent reports of relatively effective and stereospecific catalysis of epoxidation by iodosobenzene in the presence of metal salts (Fe, Cu),[21,23] binuclear Cu(II) complexes,[22,34] or Zn(II)-bleomycin[21] suggest that the experimental data gathered with metallobleomycins could reflect catalysis by complexes unrelated to a putative hypervalent iron-bleomycin complex.

The disappearance of the EPR signal due to Fe(III)-bleomycin in mixtures containing iodosobenzene could be interpreted as evidence for the formation of a nonparamagnetic hypervalent iron-oxo complex, though the finding of similar behavior with Fe(III) in the absence of the bleomycin ligand, the finding that the drug cannot bind Fe(III) in the presence of iodosobenzene, and the absence of EPR signals due to other perferryl complexes argue against the notion that the loss of EPR signal represents activation of iron bleomycin.

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(35) A reviewer has suggested that activated bleomycin is probably a ferric-peroxy complex and that the species responsible for DNA cleavage or for the oxidation of small molecules may in fact be a different iron-oxo species formed subsequent to O-O bond cleavage, this second species being the analogue of activated cytochrome P-450. We suggest that this species, if it maintains the perferryl oxidation state of the original activated bleomycin and forms via heterolytic cleavage of the peroxide, should also have odd electron spin and exhibit an EPR spectrum. There is no evidence, however, that bleomycin forms a second EPR-active species after activation. Alternatively, a homolytic O-O cleavage subsequent to the formation of a ferric-peroxy complex[11] could give a ferric iron-oxy radical pair that would be EPR silent. The kinetics of decay of activated bleomycin, as stated above, argues against any intermediate between activated bleomycin and the species that attacks DNA. The reviewer further suggested that iodosobenzene generates a complex from Fe(III)-bleomycin that is equivalent to the purported species formed after bleomycin activation and that this secondary complex is "the species ultimately responsible for the observed chemistry of Fe-bleomycin". Since iodosobenzene does not activate Fe(III)-bleomycin[15] for the type of DNA cleavage characteristic of oxygen or peroxide activation in which thymine is released,[11] the interactions cannot be related. It should be noted that chain breakage of SV 40 DNA has been observed with Cu(II)- or Fe(III)-bleomycin in the presence of 100-fold excesses of iodosobenzene: Ehrenfeld, G. M.; Rodriguez, L. O.; Hecht, S. M.; Chang, C.; Basus, V. J.; Oppenheimer, N. J. *Biochemistry* **1985**, *24*, 81-92.

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Detailed Study of the Quintet ⇌ Singlet Spin Transition in Bis(selenocyanato)bis(2,2'-bi-2-thiazoline)iron(II)

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Iron(II) complexes of the series $[FeL_2X_2]$, where L = 2,2'-bi-2-thiazoline (bt) or an alkyl-substituted derivative thereof and X = NCS or NCSe, are known to exhibit thermally induced spin-state transitions in the solid state.[3] Thus the high-spin (HS, $S = 2$) ⇌ low-spin (LS, $S = 0$) transition in $[Fe(bt)_2(NCS)_2]$ has been the subject of rather detailed investigations[4] including an attempt at the rationalization of the mechanism of the transition on the basis of the domain formalism.[5] On the other hand, more detailed studies are lacking for the spin-state transition in the analogous selenocynato complex $[Fe(bt)_2(NCSe)_2]$. The transition in this complex is of particular interest since it is one of the most abrupt ones ever encountered for compounds of iron(II).

In the present paper, we report therefore studies of the HS ⇌ LS transition in $[Fe(bt)_2(NCSe)_2]$ employing the ^{57}Fe Mössbauer effect and X-ray powder diffraction.

Experimental Section

Materials. Samples of $[Fe(bt)_2(NCSe)_2]$ were prepared as described elsewhere.[3] The purity of the substance was checked by elemental analysis, magnetic measurements, and ^{57}Fe Mössbauer spectroscopy.

Mössbauer Spectroscopy. The ^{57}Fe Mössbauer spectra were measured with a spectrometer consisting of a constant-acceleration electromechanical drive and a Nuclear Data ND 2400 multichannel analyzer operating in the multiscaling mode. The source used consisted of 50-mCi ^{57}Co in rhodium at room temperature, the calibration being effected with a 25-μm iron foil absorber. All velocity scales and isomer shifts are referred to the iron standard at 298 K. For conversion to the sodium nitroprusside scale, add +0.257 mm s^{-1}. Variable-temperature measurements between 80 and 300 K were performed by using a custom-made cryostat, the temperature being monitored by means of a calibrated iron vs constantan thermocouple and a cryogenic temperature controller (Thor Cryogenics Model E 3010-II). The temperature stability was about ±0.10 K. The Mössbauer spectra were corrected for nonresonant background of the γ-rays and were least-squares fitted to Lorentzian line shapes. In order to determine values of the relative effective thickness t_i from the area of each individual Mössbauer line i, the experimental data were fitted in terms of the general expression of the Mössbauer spectrum.[6] The effective thicknesses for the quadrupole doublets of the high-spin (HS) and low-spin (LS) phases are then determined according to

$$t_{HS} = (t_1 + t_2)_{HS} = d n_{HS} f_{HS}$$

$$t_{LS} = (t_1 + t_2)_{LS} = d(1 - n_{HS}) f_{LS} \quad (1)$$

In eq 1, $d = N\beta\delta\sigma_0$ where N is the number of iron atoms per unit volume, β the isotopic abundance, δ the absorber thickness, and σ_0 the resonance cross section. In addition, n_{HS} is the HS fraction, with f_{HS} and f_{LS} being the Debye-Waller factors of the two phases.

X-ray Diffraction. Measurements of X-ray powder diffraction at variable temperatures were obtained with a Siemens counter diffractometer equipped with an Oxford Instruments CF108A continuous-flow cryostat and liquid nitrogen as coolant. The diffractometer was used in

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