Reexamining the Mössbauer Effect as a Means To Cleave DNA†

Frederick M. MacDonnell*

Department of Chemistry, Harvard University, Cambridge, Massachusetts 02138

Received May 15, 1995; Revised Manuscript Received July 21, 1995⊗

ABSTRACT: The ability of a DNA-bound Mössbauer isotope to absorb resonant γ-radiation and subsequently, upon decay, induce DNA double-strand breaks by emission of low-energy Auger electrons was examined with a simple plasmid DNA cleavage assay. This mechanism was postulated by Mills et al. [Mills et al. (1988) *Nature 336*, 787] in the observed ablation of tumor cell growth with ^{57}Fe(III)−bleomycin and Mössbauer radiation. The observed linearization of supercoiled pAA15 plasmid DNA upon treatment with five or more ^{57}Fe(III)−bleomycin per plasmid precluded its use for testing Mössbauer effect induced cleavage. An alternative ^{57}Fe−DNA-binding complex, [^{57}Fe(phen)$_2$(DPPZ)](PF$_6$)$_2$·H$_2$O (^{57}**1**), was synthesized and found to tightly bind DNA ($K = 9.8 \times 10^5$ M^{-1}) yet not induce DNA nicks or cuts at loadings of less than 500/pAA15 plasmid. Mössbauer irradiation of ^{57}**1**/pAA15 samples under frozen and solution conditions does not result in observable linearization of the plasmid DNA over control samples. Some linearization is observed in all irradiated samples but is attributed only to the photoelectric effect.

Conventional radiotherapy often requires the maximum tolerable dosage (50−80 Gy, 5000−8000 rad) to ablate tumor growth because of similar attenuation of radiation by all cellular components and tissue types (Humm, 1988). The selective irradiation of essential cellular components, such as DNA, is a highly desirable basis of therapy and has been realized with the use of certain radioactive DNA-binding drugs (Burger et al., 1981; Kassis et al., 1989; Makrigiorgos et al., 1990; Martin & Haseltine, 1981). Selected radioactive isotopes, such as ^{125}I, emit several low-energy Auger electrons upon decay which can induce DNA double-strand breaks when produced in the immediate vicinity of the DNA (10 −30 Å) (Martin & Haseltine, 1981).

In order to avoid the use of radioactive isotopes *in vivo*, Mills and co-workers replaced the radionuclide with a nonradioactive Mössbauer isotope drug. They reasoned that the Mössbauer isotope could be radioexcited by externally applying resonant γ- (Mössbauer) radiation which upon decay would emit Auger electrons (Mills et al., 1988). Specifically, they reported the ability of ^{57}Fe(III)−bleomycin [^{57}Fe(III)Blm][1] and Mössbauer resonant radiation to significantly ablate tumor cell growth both *in vitro* with human breast cancer (HTB26) cells and *in vivo* with tumor-bearing (C3H) mice. As ^{57}Fe(III)Blm binds DNA tightly, cell death is postulated to occur from DNA double-strand scission by the low-energy Auger electrons emitted from the radioexcited ^{57}Fe. The most promising clinical aspect of the Mössbauer isotope resonant absorption of γ-emission (MIRAGE) therapy was the biologically insignificant dose of γ-radiation (10^{-5} Gy, 1 mR) reported to achieve these effects. For comparison, a 100 000-fold increase in dosage (1 Gy) would be required to achieve similar effects with radiation alone. The increased sensitivity is postulated to be the result of the high-resonant-absorption cross section of the Mössbauer isotope comparted to the surrounding tissue (100 000-fold for ^{57}Fe vs H$_2$O) and the increased efficacy of Auger electrons over photons at inducing cellular damage.

These dramatic and promising results are nevertheless controversial. Humm has noted that the work lacks a decisive control experiment (Humm, 1988), and Brenner, Geard, and Hall have proposed the observed ablation effects may be due to the additive effects of the Fe(III)Blm (an antitumor agent) and the 6.4 keV X-rays from the Mössbauer source (^{57}Co diffused into Rh metal) which have high biological effectiveness (Brenner et al., 1989). Mills et al. measured the cellular proliferation of the HTB26 cell line and the tumor growth in tumor-bearing C3H mice in MIRAGE treatment and two control groups. The first control received no treatment and served as the base line; the second control was treated with only the ^{57}Fe(III)Blm (drug-alone control). The MIRAGE group was treated with both ^{57}Fe(III)Blm and resonant Mössbauer radiation. Ideally, a fourth group in which the ^{57}Fe(III)Blm is replaced with ^{56}Fe(III)Blm and similarly irradiated would have been included. The ^{56}Fe isotope cannot undergo a Mössbauer transition, and thus this last control would easily eliminate any experimental artifacts and firmly establish whether a Mössbauer event is responsible for cell death.

As neither the cellular target nor a decisive control experiment were established in the report by Mills et al., we have reexamined the MIRAGE treatment in a simple *in vitro* assay. Supercoiled plasmid DNA and ^{57}Fe−DNA-binding complexes were treated with Mössbauer resonant radiation under various physical conditions. Cleavage reactions were monitored by the formation of nicked (form II) and linear (form III) DNA. This report describes the results of these reactions using a new isotopically enriched DNA-binding complex, [^{57}Fe(phen)$_2$(DPPZ)](PF$_6$)$_2$ (^{57}**1**) (see Figure 1), and the unenriched analogue [^{56}Fe(phen)$_2$(DPPZ)](PF$_6$)$_2$ (^{56}**1**) as a control. ^{57}Fe(III)Blm was examined initially but gave too much background cleavage for use with this assay.

† This work was supported in the form of a Damon Runyon−Walter Winchell Postdoctoral Fellowship (1992−1994) to the author (DRG-1206).

* Current address: Department of Chemistry and Biochemistry, The University of Texas at Arlington, Box 19065, Arlington, TX 76019.

⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1995.

[1] Abbreviations: Blm, bleomycin; DPPZ, dipyrido[3,2-*a*:2′,3′-*c*]-phenazine; phen, 1,10-phenanthroline; bpy, 2,2′-bipyridine; MIRAGE, Mössbauer isotope resonant absorption of γ-emission.

0006-2960/95/0434-12871$09.00/0

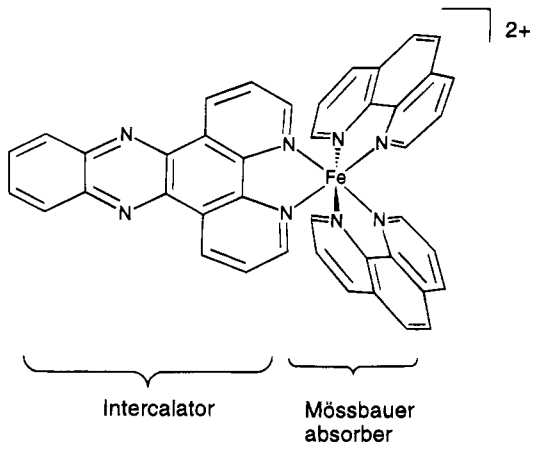

FIGURE 1: $[^{57}Fe(phen)_2(DPPZ)]^{2+}$ (^{57}**1**).

MATERIALS AND METHODS

Materials. ^{57}Fe metal (95.9% enriched), phenanthroline, and bleomycin (Bristol-Meyer Squibb, Princeton, NJ), calf thymus DNA (type 1, Sigma), agarose, low-melting temperature agarose, and buffer salts were purchased from commercial sources. Fe(III)Blm was prepared according to literature procedure (Mills et al., 1988; Rao et al., 1980). pAA15 (Ansari et al., 1992), a 4583-base pair plasmid, was obtained in the supercoiled form as a gift from Dr. Aseem Ansari (Harvard University). All aqueous solutions were prepared using distilled deionized water.

Instrumentation. ^{1}H NMR spectra were recorded with a Brucker AM 200 or 300 spectrometer. Chemical shifts were referenced to the residual solvent peak and are reported relative to tetramethylsilane. High-resolution fast atom bombardment mass spectra were obtained on a JEOL model AX-505 mass spectrometer at the Harvard University Chemistry Department Mass Spectrometry Facility. Electronic absorption spectra were recorded on a Varian Cary 13 spectrophotometer. Mössbauer spectra were collected on a modified Ranger MS-1500 Mössbauer instrument equipped with an external VT-1200L velocity transducer and a PA-1200 krypton proportional counter (Ranger Scientific, Inc., Burleson, TX). The Mössbauer source was ^{57}Co (21 mCi) diffused into a palladium matrix (Isotope Product Laboratories, Burbank, CA). Mössbauer spectra were collected in the constant acceleration mode with a symmetric triangular velocity wave form. Low-temperature data were obtained by using a Janis model 8DT superVaritemp Dewar fitted with Mylar windows. The data were analyzed and fit to Lorenztian or Viogt line shapes using WMOSS software (WEB Research Co., Endina, MN). All isomer shifts (δ) are quoted relative to Fe metal at 298 K.

Preparation of Complexes

$[^{56}Fe(phen)_2(DPPZ)](PF_6)_2 \cdot H_2O$ (^{56}1). Addition of natural abundance $Fe(SO_4)_2(NH_4)_2 \cdot 6H_2O$ (0.46 g, 1.18 mmol) in 3 mL of 1 M HCl to a solution of DPPZ (Amouyal et al., 1990) (0.50 g, 1.77 mmol) and phenanthroline (0.32 g, 1.55 mmol) in 20 mL of EtOH and 10 mL of CH_2Cl_2 immediately gave a deep red solution. After stirring for 5 min, the solution was filtered and concentrated to 5 mL total volume. Addition of 100 mg of NH_4PF_6 in 2 mL of H_2O resulted in a red precipitate which was filtered, washed with water, and dried. The crude product was purified by silica gel chromatography with 10% acetone in THF containing 1 mg/mL NH_4PF_6 eluent. The second major red band was collected and recolumned under identical conditions to give 240 mg of ^{56}**1** (20% yield): ^{1}H NMR (MeCN-d_3) δ 9.62 (dd, 2 H, J = 5.8, 3.8 Hz), 8.63 (ddd, 4 H, J = 8.1, 2.9, 0.21 Hz), 8.45 (dd, 2 H, J = 6.6, 3.4 Hz), 8.27 (s, 4 H), 8.12 (dd, 2 H, J = 6.6, 3.4 Hz), 7.86 (d, 2 H, J = 4.60 Hz), 7.75 (m, 2 H), 7.66 (d, 4 H, J = 5.3 Hz), 7.61 (dd, 4 H, J = 8.0, 5.3 Hz); FAB-MS [*m*-nitrobenzyl alcohol (NBA) matrix] m/z = 698.6 $[[^{56}Fe(phen)_2(DPPZ)]^{2+}$ requires 698.2]; UV−vis (H_2O) λ (ϵ) 358 (13 150), 374 (14 190), 429 sh (7200), 482 sh (10 140), 501 (10 675). Anal. Calcd for $C_{42}H_{28}F_{12}Fe_1N_8O_1P_2$: C, 50.12; H, 2.80; N, 11.13. Found: C, 49.90; H, 2.79; N, 11.07.

$[^{57}Fe(phen)_2(DPPZ)](PF_6)_2 \cdot H_2O$ (^{57}1). A stock solution of 56 mM $^{57}FeCl_3 \cdot xH_2O$ in 1 M HCl was prepared from dissolution of ^{57}Fe metal in HCl. Addition of 25 mg of ascorbic acid to a solution of $^{57}FeCl_3 \cdot xH_2O$ (1.3 mL, 0.073 mmol) resulted in a color change from yellow to colorless. The resulting solution was immediately added to a solution of DPPZ (36 mg, 0.127 mmol) and phenanthroline (23 mg, 0.127 mmol) in 5 mL of EtOH and 2.5 mL of CH_2Cl_2. The product was isolated as described for ^{56}**1**: yield 11 mg (15%); identical with the unenriched form by ^{1}H NMR and visible absorption spectroscopy; FAB-MS (NBA matrix) m/z = 699.6 $[[^{57}Fe(phen)_2(DPPZ)]^{2+}$ requires 699.2]; 93% enriched in ^{57}Fe.

DNA Binding Assay. Purified calf thymus DNA (Jennette et al., 1974) was dissolved in 20 mM sodium phosphate, pH 7.0 (buffer I), and the final concentration determined spectrophotometrically by assuming ϵ_{260} = 6600 M^{-1} (DNA phosphate) cm^{-1}. Absorbance titrations were performed in buffer I using a fixed concentration of ^{56}**1** to which increasing increments of DNA stock solution were added. Concentrations of ^{56}**1** were kept at ca. 0.02 mM, and Fe/DNA ratios varied between 0.05 and 15. The absorption spectrum was recorded between 330 and 650 nm, and data were analyzed using Igor Pro software.

DNA Cleavage Assay. Cleavage reactions were carried out in a total volume of 10 μL in 500 μL Eppendorf tubes. Reaction mixtures were prepared containing 19.4 μg of pAA15 DNA and 0.186 mM ^{57}**1** (~1 every 16 DNA base pairs) in buffer I and allowed to equilibrate at 23 °C for 15 min. Samples were irradiated by resting the eppendorf tube directly on the Mössbauer source for periods of up to 240 h. Temperature was varied between −196 and 23 °C, and in several experiments low-melting temperature agarose (3%) was added to the buffer solution to alter the solution viscosity. Exact temperature and solution conditions for each experiment are presented in the Results section.

At periods throughout the irradiation, 1 μL aliquots (~2 μg/μL DNA) were withdrawn and mixed with 15 μL of H_2O and 1 μL of 3 M NaOAc (pH 5.2). Samples were ethanol precipitated by addition of 50 μL of ethanol and chilling the samples on dry ice for 15 min. The precipitated DNA was dried and resuspended in 15 μL of TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and 5 μL of loading buffer (30% glycerol in water, 0.1%, w/v, xylene cyanol, 0.1%, w/v, bromophenol blue). Samples were loaded on 1% agarose gels and electrophoresed at 100 V for 1 h in TAE buffer (23 °C). DNA was visualized by staining with ethidium bromide and illuminating UV light.

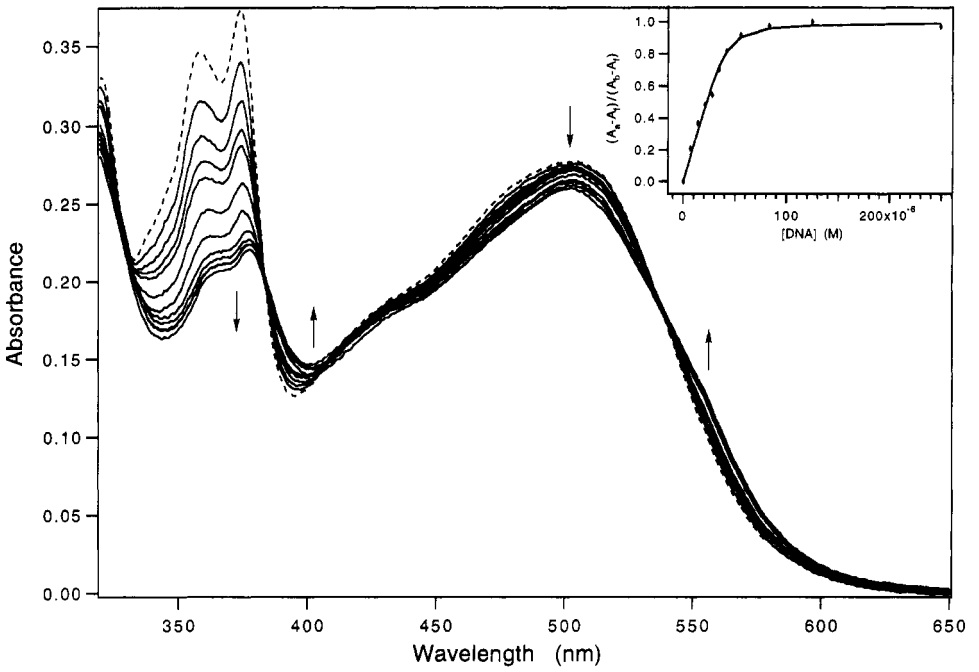

FIGURE 2: Absorption spectrum changes of 0.027 mM **1** (dotted line) upon titration with 0.50, 1.00, 1.54, 2.0, 2.6, 3.0, 4.0, 6.0, 9.2, 18.2, and 56 equiv of calf thymus DNA (solid lines) in buffer I at 23 °C. Inset shows the binding data and nonlinear least-squares fit derived from eq 1. $K = 9.8 \times 10^5$ M^{-1}, and $s = 0.94$.

A conservative estimate of the exposure rate due to the 14.4 keV photons (0.0035 Gy/h) was calculated considering the average source to sample distance (0.3 cm), source radioactivity (21 mCi), exposure time (0−240 h), sample size (10 μL), geometry (area = 0.056 cm^2, average depth = 0.134 cm), and attenuation (μ/p = 1.57 for H_2O). The Eppendorf tube wall absorbed 9% of the 14.4 keV photons as determined by measuring the count rate for the 14.4 keV photons with and without the tube wall in the path of the detector.

RESULTS

Mössbauer Complex Requirements. In the plasmid cleavage assay it is critical that the ^{57}Fe complex not cleave DNA alone. Unfortunately for our purposes, Fe(III)Blm was found to be relatively efficient at inducing DNA double-strand breaks even in the absence of external reducing agents. Titration of supercoiled plasmid DNA with Fe(III)Blm revealed that at drug loadings of four or more ^{57}Fe(III)Blm per plasmid (pAA15) significant linearization of the DNA occurs. Attempts to prevent this cleavage reaction by extended aerobic incubation (2 h, 23 °C) of the Fe(III)Blm [to oxidize any residual Fe(II)Blm] (Burger et al., 1981) prior to incubation with the DNA were only marginally successful. In order to circumvent this problem, a new ^{57}Fe−DNA-binding complex, which would not function independently as a cleaving agent, was pursued.

A review of the now extensive literature of DNA-binding transition metal complexes (Barton et al., 1984; Gupta et al., 1992; Howe-Grant et al., 1976; Jennette et al., 1974; Lippard et al., 1976; Pyle & Barton, 1990; Pyle et al., 1989) suggested the ^{57}Fe analogue of $[Ru(phen)_2(DPPZ)]^{2+}$ should have the desired properties (see Figure 1). Both the Δ and Λ enantiomers of $[Ru(phen)_2(DPPZ)]^{2+}$ strongly bind DNA through intercalation [equilibrium binding constants (K) ≥ 10^6 M^{-1}] (Friedman et al., 1990; Hiort et al., 1993). These complexes, as most $[M(phen)_3]^{n+}$ complexes, have excellent stability in aqueous solution and do not cleave DNA alone (Fleisher et al., 1986; Friedman et al., 1990). Replacement of the Ru with Fe is a trivial change with respect to the overall size, shape, and charge of the complex, and analogous DNA binding properties are expected. Furthermore, studies have demonstrated that ca. 45% of radiolabled ^{57}Co(phen)$_3^{3+}$ type complexes fragment upon radioactive decay (Nath et al., 1970). The fragmentation is attributed to radiolysis from the low-energy Auger electrons and the rapid charge buildup on the daughter ^{57}Fe ion (Fe^{8+}). These data support the choice of $[Fe(phen)_2(DPPZ)]^{2+}$ (**1**) as an attractive, synthetically accessible, alternative Mössbauer drug complex.

Synthesis and Properties. The mixed ligand complex **1** was prepared in both the unenriched (^{56}Fe) and enriched (^{57}Fe) forms. The ^{1}H NMR shows sharp, diagnostic peaks for the DPPZ and phen ligands in the expected 1:2 ratio. The complexes were further characterized by elemental analysis and mass spectrometry. The solubility in H_2O is poor unless first dissolved in a few drops of DMSO and then diluted with H_2O. Aqueous solutions (>1 mM **1**) prepared this way obey Beers law and readily pass through a 2 μm nylon filter, indicating complete dissolution. The complex undergoes ligand exchange reactions after extended incubation in H_2O, as determined by NMR and visible spectroscopy, but is stable in the presence of excess calf thymus DNA. Only an 8% drop in absorbance at 374 nm is observed after 14 days in a 10-fold excess of calf thymus DNA compared to a 45% drop in the absorbance at 374 nm without DNA in only 24 h.

DNA Binding Studies. Complex ^{56}**1** displays several intense LMCT bands in the visible region. All of these absorptions are perturbed upon addition of calf thymus DNA, with transitions assigned to the Fe(II) and DPPZ MLCT (358 and 374 nm) showing the largest perturbations. A titration of increasing [DNA] with ^{56}**1** is displayed in Figure 2 and exhibits two clean isobestic points at 383 and 538 nm. The magnitude of the red shift ($\Delta\lambda$ = 3.7 nm) and hypochroism

($H = 40\%$) observed are characteristic of an intercalative mode of binding (Long & Barton, 1990).

An intercalative binding mode and high binding constant are expected for ^{56}**1** by analogy to $[Ru(phen)_2(DPPZ)]^{2+}$ (Dupureur & Barton, 1994; Hiort et al., 1993). An expression for determining the binding constant for complexes with $K > 10^4$ M^{-1} is shown in eq 1 (Kalsbeck & Thorp, 1993):

$$\frac{\epsilon_a - \epsilon_f}{\epsilon_b - \epsilon_f} = \frac{b - (b^2 - 2K^2C_t[DNA]/s)^{1/2}}{2KC_t} \quad (1a)$$

$$b = 1 + KC_t + K[DNA]/2s \quad (1b)$$

where ϵ_a, ϵ_f, ϵ_b, C_t, and s correspond to A_{obsd}/[Fe], the extinction coefficient of the free Fe complex, the extinction coefficient of the DNA-bound Fe complex, the total [Fe], and the apparent number of binding sites per base pair of DNA, respectively. A two-parameter nonlinear least-squares fit of the data to eq 1 is shown in the inset of Figure 2 and gives $K = 9.8 \times 10^5$ M^{-1} and $s = 0.94$. These values are slightly lower than those observed for $[Ru(phen)_2(DPPZ)]^{2+}$ ($K = 10^8$ M^{-1}, $s = 2$) (Hiort et al., 1993) which may be expected as subtle structural changes can affect the binding strength [e.g., $[Ru(bpy)_2(DPPZ)]^{2+}$, $K = 4.9 \times 10^6$ M^{-1} and $s = 1.7$] (Smith et al., 1994) and the buffer conditions were not identical. In general, our values compare favorably with those observed in most DPPZ metallointercalators ($K \approx 10^6$ M^{-1} and $s = 0.9-4.5$) [Smith et al. (1994) and references therein].

Mössbauer Spectra. Mössbauer analysis of ^{57}**1** at 23 °C in the solid state gives $\delta = 0.34$ mm/s, $\Delta E_Q = 0.21$ mm/s and $\Gamma = 0.43$ mm/s. The small isomer shift and ΔE_Q are typical for low-spin Fe(II) in a symmetric ligand field (Gibb & Greenwood, 1971). A frozen solution sample of ^{57}**1** bound to calf thymus DNA ($\delta = 0.39$ mm/s, $\Delta E_Q = 0.25$ mm/s, and $\Gamma = 0.29$ mm/s at −196 °C) gives nearly identical spectra with that of a frozen solution of ^{57}**1** without DNA ($\delta = 0.40$ mm/s, $\Delta E_Q = 0.24$ mm/s, and $\Gamma = 0.30$ mm/s at −196 °C), indicating only a small perturbation of the ^{57}Fe nuclear levels upon binding DNA. The slight difference in δ at 23 and −196 °C is due to second-order temperature effects (Lang, 1970).

Experimentally, the shape and position of the Mössbauer absorption for ^{57}**1** is fortuitous. As seen in Figure 3, a large portion of the absorption peak for ^{57}**1** overlaps with the emission line for the ^{57}Co/Pd source (0.185 mm/s relative to Fe metal, $\Gamma = 0.19$ mm/s) (Gibb & Greenwood, 1971), obviating the need to apply external motion (a Doppler shift) to bring the source and sample into resonance. Because of this coincidence, DNA samples could be irradiated with resonantly tuned Mössbauer radiation simply by placing the sample near the stationary source.

DNA Cleavage Experiments. Incubation of 140 ^{57}Fe(III)-Blm/pAA15 for 10 min in buffer I results in nearly complete conversion of the supercoiled form (form I) to the nicked and linear forms, as shown in Figure 4 (lane 0). Lanes 1 (DNA only) and 2 (280 ^{57}**1**/pAA15, 10 days, 23 °C) show the stability of the plasmid DNA toward ^{57}**1** under the conditions examined during MIRAGE treatment. As can be seen, there are no excess DNA cleavage products observed in lane 2 over the control (lane 1) during the extended incubation period. Titrations of increasing ^{57}**1** with supercoiled pAA15 indicated good stability with up to 500 ^{57}**1**/plasmid (data not shown). Lanes 3−5 show the results of similarly prepared samples after extensive resonant Mössbauer irradiation (source stationary relative to the sample). After exposure to a total of 0.84 Gy of resonant 14.4 keV γ-radiation, all three lanes show some linear (type III) DNA and excess nicked (type II) DNA in comparison to the radiation-free lanes 1 and 2. The MIRAGE experiment (lane 3: ^{57}**1**, DNA, Mössbauer radiation), however, does not show any excess DNA cleavage over the control lanes 4 (no Fe) and 5 (^{56}**1**). Identical results were observed for similarly prepared samples irradiated under the conditions listed in Table 1.

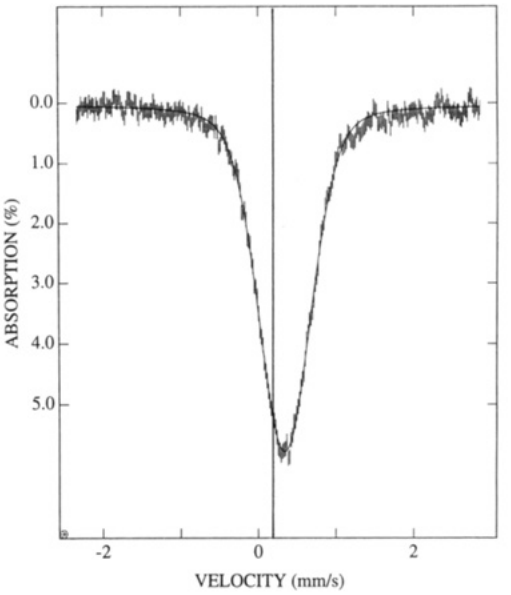

FIGURE 3: Mössbauer spectrum of **1** (solid, 23 °C) plotted relative to Fe metal. Vertical line indicates the energy of Mössbauer source γ-photons.

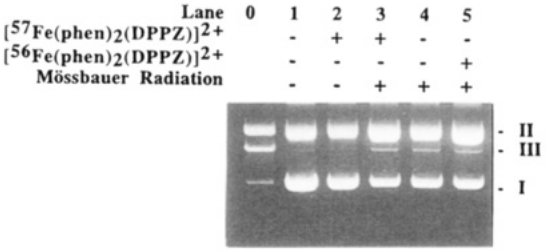

FIGURE 4: MIRAGE cleavage of pAA15 DNA (19.4 μg) with 1.86 nmol of **1**.

Table 1. Sample Conditions during MIRAGE Treatment

total dose[a] (Gy)	Fe/ plasmid	temperature (°C)[b]	physical state	clevage over controls
0.84	280[c]	23	solution	neg
0.84	280	4	solution	neg
0.84	280	4	3% agarose gel	neg
0.84	280	−15	frozen	neg
0.84	280	−195	frozen	neg
0.84[d] (0.14)[e]	280	23	solution	neg

[a] 14.4 keV radiation only. [b] Source and sample were kept at the same temperature to keep the resonant overlap conditions. [c] Corresponds to one **1** every 16 base pairs of DNA. [d] Sample irradiated with moving source ±1.51 mm/s. [e] Gy of resonant radiation from moving source.

DISCUSSION

In order to properly investigate the cleavage of DNA via a Mössbauer event, it was necessary to build a new ^{57}Fe Mössbauer isotope−DNA-binding complex which was chemically inactive toward DNA cleavage. Complex **1** met these criteria and was submitted to MIRAGE treatments. Conditions were varied from solution samples at 23 °C, to mimic the cellular milieu, to frozen solids at −196 °C to assure an appreciable recoil-free fraction for the ^{57}**1**/pAA15 DNA complex. Under no circumstances did we observe excess DNA cleavage in the MIRAGE sample (lane 3) compared to control lanes 4 and 5. These three lanes (3−5) do show the formation of linear (form III) DNA after extensive irradiation compared to the nonirradiated lanes 1 and 2. This cleavage can be attributed solely to the photoelectric effect.

Further experiments were conducted in agarose gel (3%) matrices at 4 °C in an effort to maintain solution conditions and yet restrict the diffusional mobility of the ^{57}Fe−DNA complex in order to increase the solution recoil-free fraction. The Mössbauer effect has been directly observed in viscous solutions (Gibb & Greenwood, 1971) and nonfrozen biological systems, such as *Escherichia coli* (whole cell) at 3 °C (Bauminger et al., 1976), whole red blood cells at 4 °C (Gonzer et al., 1963, 1964), deoxymyoglobin at 10 °C, chick embryo fibroblasts at 0 °C, and mycoplasma membranes at 10 °C (Bauminger et al., 1976; Nowik et al., 1983). The gel matrices were tried assuming these special conditions may be required for Mössbauer absorption and for efficient DNA cleavage by the Auger electrons (e.g., generation of diffusable free radicals). Again, the MIRAGE sample failed to show increased cleavage relative to the control samples.

Finally, it was considered that the resonance conditions were not fulfilled due to an unexpected energy mismatch between the source and the sample; therefore, an experiment was performed in which the ^{57}Co/Pd source velocity was swept between −1.51 and +1.51 mm/s about the source emission line. This dispersion of the Mössbauer radiation is wide enough to insure an appropriate resonant energy match with an appreciable dwell time [~17% assuming doublet peak line width (fwhm) = 0.5 mm/s] on the absorption peak. No DNA cleavage occurred which could be attributed to the Mössbauer effect.

From these experiments, it is obvious that the Mössbauer isotope is not attenuating the γ-radiation any better than the DNA alone. Unfortunately, it is difficult to directly compare these results with those obtained by Mills et al., in that the HTB26 and live mouse tumor cellular assays are far more complex than the simple plasmid DNA assay. Our failure to observe any Mössbauer-induced DNA cleavage does not preclude some other cellular target and/or mechanism; however, considering the pharmacologically observed affinity of bleomycin for DNA, this seems unlikely.

A comparison of the relative sensitivities of the two experiments [^{57}Fe(III)Blm/HTB26 tumor cells vs ^{57}**1**/p15 plasmid DNA] suggests they are similar. The drug loading for MIRAGE treatment used by Mills et al. was an estimated one Fe(III)Blm for every eight base pairs of DNA, on 10^{10} base pairs of DNA per mammalian cell genome. By assuming one DNA double-strand break as lethal, they calculated that one in 10^9 Mössbauer events will result in cell death, providing an extremely sensitive assay on a per event basis. No mention was made of the assumed recoil-free fraction other than the Mössbauer isotope should have a 100 000-fold increase in absorption probability over the surrounding tissue, due to the Mössbauer effect. By comparison, the plasmid DNA assay detects only one of every 280 Mössbauer events (the loading of ^{57}**1** on pAA15). To compensate for this dramatic difference in drug loadings, we adjusted experimental conditions to dramatically improve the probability of a Mössbauer absorption. We estimate a minimum 10^7-fold increase in the probability of observing a Mössbauer event was obtained by increasing the total radiation exposure (0.84 vs 1×10^{-5} Gy), the total ^{57}Fe concentration (186 vs 3.8 μM), and the recoil-free fraction (when frozen by 10−1000-fold). The last factor is estimated because the recoil-free fraction of ^{57}Fe(III)Blm bound to the cellular DNA in HTB26 is speculative. It is unlikely to be greater than 1% due to the temperature and the cellular matrix conditions; the maximum recoil-free fraction observed in whole cell *E. coli* at 4 °C was ~0.9% (Bauminger et al., 1976), whereas the recoil-free fraction of ^{57}**1** in frozen buffer solution at −196 °C is 66% as determined from the Mössbauer spectrum [for calculation method, see Lang 1970]. Even assuming the tumor cell assay were far more sensitive, our observation of equivalent amounts of DNA cleavage in lanes 3−5 due to the photoelectric effect clearly demonstrates a negligible attenuation of the γ-radiation by the Mössbauer isotope in a manner that results in DNA nicking or cleavage.

In terms of the number of plasmids which must be cut for observation, we know at least 10 ng of DNA is required for detection by gel electrophoresis (Maniatis et al., 1982). By assuming a 100 000-fold increase in the absorption probability due to the Mössbauer effect, we calculate an expected yield of 3200 ng of form II and III pAA15 DNA (0.16%) would be obtained under our experimental conditions. These numbers are derived from the fraction of plasmids (or sites) receiving one or more energy depositions (Brenner et al., 1989) which was calculated from the Poisson distribution as $1 - e^{-D/Z_{1F}}$, where D/Z_{1F} is the average number of energy depositions occurring at the plasmid. D is the absorbed dose, and Z_{1F} is the mean energy per unit mass of a single deposition at the sensitive site. For a Z_{1F} (pAA15, 4.9×10^{-18} g/molecule) calculated at 4.7×10^5 Gy, the fraction of plasmids receiving an energy deposition is 2×10^{-6}. Cutting events attributed to the photoelectric effect would yield only 0.03 ng of DNA. Our assay clearly would show Mössbauer dependent cutting events if they were occurring with the efficacy described by Mills et al. (Mills et al., 1989, 1988). Conversely our assay should not show events related to the photoelectric effect. The observation of these products is attributed to the cumulative radiation dose from the 136, 122, 14.4, and 6.5 keV photons.

As we were unable to directly examine ^{57}Fe(III)Blm for the MIRAGE treatment or exactly mimic the tumor cell assay conditions, we can only conclude that the results obtained by Mills et al. are incomplete without more decisive controls. If the tumor ablation is truly related to the Mössbauer effect, these results suggest severe restraints regarding drug design and the effectiveness of the MIRAGE treatment. Our results do support the explanation offered by Brenner et al. (Brenner et al., 1989) who postulate the tumor ablation observed by Mills et al. was not due to the Mössbauer effect but instead due to the cumulative effects of the Fe(III)Blm and the radiation acting independently. Indeed, we observe a fair amount of cleavage due to the photoelectric effect with only

a small calculated contribution from the 14.4 keV photons.

ACKNOWLEDGMENT

The author gratefully acknowledges Dr. Richard H. Holm for professional assistance, guidance, and freedom to pursue this project during a postdoctoral fellowship in his lab and Dr. Aseem Ansari for the gift of the plasmid DNA and for experimental assistance.

REFERENCES

Amouyal, E., Homsi, A., Chambron, J.-C., & Sauvage, J.-P. (1990) *J. Chem. Soc., Dalton Trans.* 1841−1845.

Ansari, A. Z., Chael, M. L., & O'Halloran, T. V. (1992) *Nature 355*, 87−89.

Barton, J. K., Danishefsky, A. T., & Goldberg, J. M. (1984) *J. Am. Chem. Soc. 106*, 2172−2176.

Bauminger, E. R., Cohen, S. G., Giberman, E., Nowik, I., Ofer, S., Yariv, J., Werber, M. M., & Mevarech, M. (1976) *J. Phys. (Paris) 37*, C6−227.

Brenner, D. J., Geard, C. R., & Hall, E. J. (1989) *Nature 339*, 185.

Burger, R. H., Peisach, J., & Horwitz, S. B. (1981) *Life Sci. 28*, 715−727.

Dupureur, C. M., & Barton, J. K. (1994) *J. Am. Chem. Soc. 116*, 10286−10287.

Fleisher, M. B., Waterman, K. C., Turro, N. J., & Barton, J. K. (1986) *Inorg. Chem. 25*, 3551−3552.

Friedman, A. E., Chambron, J.-C., Sauvage, J.-P., Turro, N. J., & Barton, J. K. (1990) *J. Am. Chem. Soc. 112*, 4960−4961.

Gibb, T. C., & Greenwood, N. N. (1971) *Mössbauer Spectroscopy*, Chapman and Hall, Ltd., London.

Gonzer, U., Grant, R. W., & Kregzde, J. (1963) *Appl. Phys. Lett. 3*, 189−191.

Gonzer, U., Grant, R. W., & Kregzde, J. (1964) *Science 143*, 680−681.

Gupta, N., Grover, N., Neyhart, G. A., Singh, P., & Thorp, H. H. (1992) *Angew. Chem., Int. Ed. Engl. 104*, 1048−1049.

Hiort, C., Lincoln, P., & Nordén, B. (1993) *J. Am. Chem. Soc. 115*, 3448−3454.

Howe-Grant, M., Wu, K. C., Bauer, W. R., & Lippard, S. J. (1976) *Biochemistry 15*, 4339−4346.

Humm, J. (1988) *Nature 336*, 710−711.

Jennette, K. W., Lippard, S. J., Vassiliades, G. A., & Bauer, W. R. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3839−3843.

Kalsbeck, W. A., & Thorp, H. H. (1993) *J. Am. Chem. Soc. 115*, 7146−7151.

Kassis, A. I., Fayad, F., Kinsey, B. M., Sastry, K. S. R., & Adlestein, S. J. (1989) *Radiat. Res. 118*, 283−294.

Lang, G. (1970) *Q. Rev. Biophys. 3*, 1−60.

Lippard, S. J., Bond, P. J., Wu, K. C., & Bauer, W. R. (1976) *Science 194*, 726−728.

Long, E. C., & Barton, J. K. (1990) *Acc. Chem. Res. 23*, 273−279.

Makrigiorgos, G., Adelstein, S. J., & Kassis, A. I. (1990) *Radiat. Environ. Biophys. 29*, 75−91.

Martin, R. F., & Haseltine, W. A. (1981) *Science 213*, 896−898.

Mills, R. L., Walter, C. W., Venkataraman, L., Pang, K., & Farrell, J. J. (1988) *Nature 336*, 787−789.

Mills, R. L., Farrell, J. L., & Reiff, W. M. (1989) *Nature 340*, 193−194.

Nath, A., Klein, M. P., Kündig, W., & Lichtenstein, D. (1970) *Radiat. Eff. 2*, 211−234.

Nowik, I., Cohen, S. G., Bauminger, E. R., & Ofer, S. (1983) *Phys. Rev. Lett. 50*, 1528−1531.

Pyle, A. M., & Barton, J. K. (1990) in *Progress in Inorganic Chemistry* (Lippard, S. J., Ed.) pp 413−476, John Wiley and Sons, Inc., New York.

Pyle, A. M., Rehmann, J. P., Meshoyrer, R., Kumar, C. V., Turro, N. J., & Barton, J. K. (1989) *J. Am. Chem. Soc. 111*, 3051−3058.

Rao, E. A., Saryan, L. A., Antholine, W. E., & Petering, D. H. (1980) *J. Med. Chem. 23*, 1310−1318.

Smith, S. R., Neyhart, G. A., Kalsbeck, W. A., & Thorp, H. H. (1994) *New J. Chem. 18*, 397−406.

BI9510892