Diffusion-Enhanced Lanthanide Energy-Transfer Study of DNA-Bound Cobalt(III) Bleomycins: Comparisons of Accessibility and Electrostatic Potential with DNA Complexes of Ethidium and Acridine Orange†

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ABSTRACT: Energy transfer in the "rapid-diffusion" limit reflects the equilibrium properties of a donor–acceptor system. Rates of energy transfer from freely diffusing terbium chelates to DNA-binding chromophores change dramatically when DNA is added; energy transfer from an electrically neutral chelate is reduced because the energy acceptor becomes partially buried in DNA, while energy transfer from a positive chelate is increased because of electrostatic attraction. The rate constants for energy transfer to DNA-bound chromophores from a positively charged terbium chelate, relative to those from a neutral chelate, were used to estimate the following values for the electrostatic potential near the surface of each DNA-bound acceptor at 298 K in the presence of 1.0 mM added salt (in units of $-e/kT$): acridine orange, 4.54 ± 0.11; ethidium, 4.66 ± 0.07; green Co(III) bleomycin A_2, 4.06 ± 0.11; orange Co(III) bleomycin A_2, 3.11 ± 0.10. Smaller numbers indicate less negative potentials; these can be due to a combination of (1) positive charge on the chromophore, (2) location of the chromophore [particularly Co(III) bleomycin] away from the DNA phosphates, and/or (3) separation of DNA phosphate negative charges by an intercalator. The magnitudes of the individual rate constants indicate that all the DNA-bound chromophores can be directly encountered by the terbium probes. Energy-transfer rate constants from a neutral terbium chelate to DNA-bound and free acceptors can provide a measure of the accessibility of the terbium probe to each bound chromophore. The ratios of these rate constants were as follows: acridine orange, 0.17 ± 0.01; ethidium, 0.27 ± 0.02; green form of Co(III) bleomycin A_2, 0.48 ± 0.06; orange form of Co(III) bleomycin A_2, 0.71 ± 0.06. These results are consistent with the probable differences in binding mechanisms for the intercalating chromophores (ethidium and acridine orange) as compared to the Co(III) bleomycins (in which the relevant chromophores are nonintercalating metal centers). In addition, all the results imply that the green Co(III) bleomycin chromophore binds closer to DNA than the orange; this provides a first step toward understanding the structural basis for the different biological properties of these metallobleomycins. Control experiments and theoretical considerations necessary to establish the validity of the results are also presented.

The binding of drugs, proteins, or other molecules to DNA can profoundly affect its biological functions. For this reason, a number of experimental methods have been developed for the study of ligand–DNA complexes. These methods include chemical techniques such as "footprinting" (Galas & Schmitz, 1978; Schmitz & Galas, 1982; Van Dyke et al., 1982) and physical techniques such as spectroscopic or hydrodynamic measurements [reviewed by Bloomfield et al. (1974), Berman & Young (1981), and Dougherty & Pigram (1982); see also Patel & Canuel (1976)].

The information provided by such procedures can be usefully complemented by experimental meausres of the electrostatic potential at the site of the bound ligand—which will depend on how the ligand is placed relative to the highly charged phosphodiester backbone of DNA and whether the bound chromophore is partly exposed on the surface or buried inside the macromolecule. Diffusion-enhanced energy transfer from lanthanide complexes (in the "rapid-diffusion" limit) has recently been demonstrated to provide information on such properties for chromophores in small molecules (Thomas et al., 1978; Meares et al., 1981), in proteins (Yeh & Meares, 1980; Meares & Rice, 1981; Wensel & Meares, 1983; see footnote 1) and in membranes (Thomas & Stryer, 1982; Stryer et al., 1982b).

Energy transfer from small terbium complexes to chromophores in solution is greatly enhanced by the fact that during its millisecond luminescence lifetime a typical excited terbium complex diffuses extensively (covering a net distance of $\sim 2 \times 10^3$ nm). Thus, under ordinary circumstances an excited terbium complex actually encounters a large number of possible energy acceptors before it decays (at 10^{-6} M, the average separation between neighboring acceptors is 65 nm). This has the effect of averaging the interactions between energy donor and acceptor(s) over all allowed directions and orientations in space [for a review, see Stryer et al. (1982a)], so that the final result reflects the *equilibrium* properties of the system under study.

Energy transfer in the rapid-diffusion limit differs from the more familiar quenching of aromatic fluorescence (Lakowicz, 1983) in several ways. The site of interest on the macromolecule need not be fluorescent but must have an absorption spectrum that overlaps the emission spectrum of terbium. A set of terbium probes having the same size and approximately spherical shape but different charges (1+, 0, 1−) permits quantitative study of electrostatic effects. Each probe has a

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[1] Note that a factor of 4/3 is missing from eq 4 of Wensel & Meares (1983).

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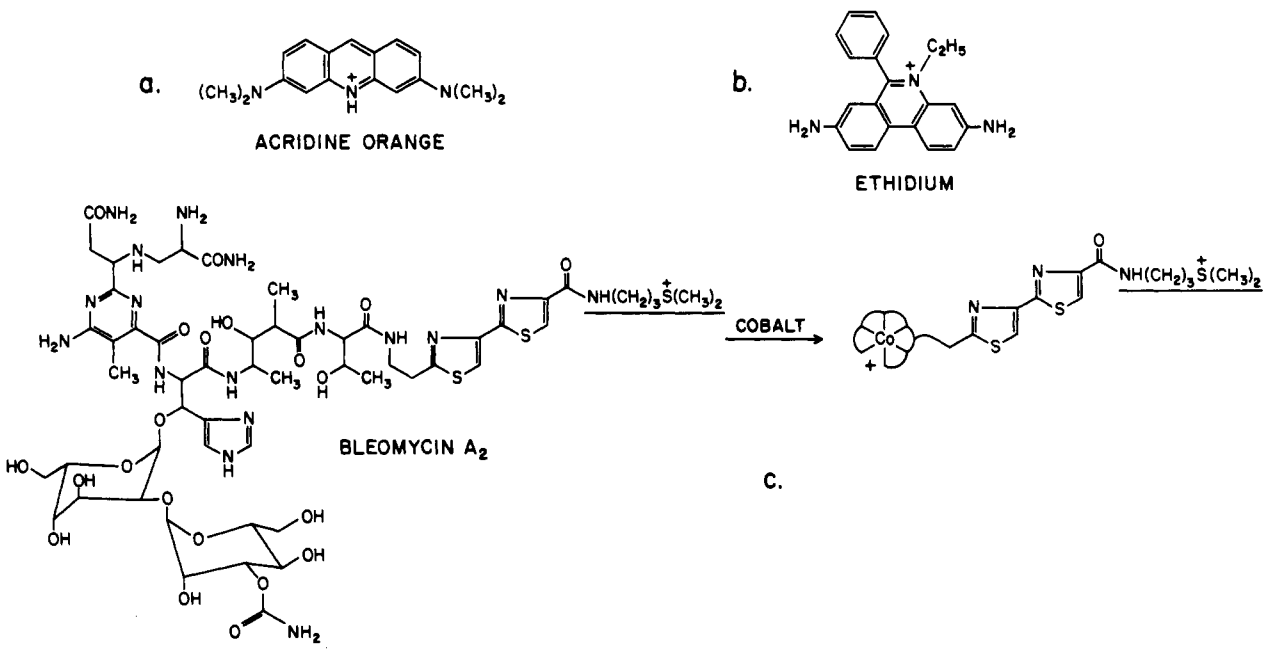

FIGURE 1: Structures of the DNA-binding compounds used as energy acceptors in this study: (a) acridine orange; (b) ethidium; (c) schematic representation of Co(III) bleomycin, showing the bithiazole moiety that is thought to intercalate into DNA and the terminal amine group of bleomycin A_2 (underlined). The curves drawn about the Co(III) center represent the metal-coordinating peptide section of the molecule, along with the disaccharide group. The groups directly coordinated to cobalt, and the geometry of the resulting complex, are still in question. It is known that the green form differs from the orange form in having a hydroperoxide ion coordinated to Co(III) (Chang et al., 1983).

4-Å radius and is quite hydrophilic and so is unlikely to be found inside proteins or membranes. Energy-transfer rate constants are typically very small compared to the diffusion-controlled value, but because of the millisecond terbium lifetime, all donors sample equivalent environments even at low quencher concentrations. Terbium luminescence is practically unaffected by heavy elements or by paramagnets such as O_2; but it is quite sensitive to the presence of H_2O molecules coordinated to the metal.

The use of this technique to detect differences in the way closely related molecules bind to DNA is of particular interest to us, because of the facts we have uncovered concerning the interactions of cobalt(III) bleomycins with DNA (Chang & Meares, 1982, 1984; Chang et al., 1983). We have found that the green and orange Co(III) bleomycins described by DeRiemer et al. (1979; Vos et al., 1980; Tsukayama et al., 1981) display strikingly different behavior. These two forms of Co(III) bleomycin (Figure 1) differ greatly in their DNA-binding affinities (Chang & Meares, 1984) and their abilities to promote light-induced nicking of DNA in vitro (Chang & Meares, 1982), as well as in their selective accumulation by tumors in vivo (DeRiemer et al., 1979). An understanding of the structural basis for these differences can provide valuable insight into the interactions of these antibiotics with DNA and may increase our understanding of their properties in vivo.

Here we describe experiments that demonstrate that the molecular probes and methods previously developed for diffusion-enhanced lanthanide energy-transfer studies of other systems can be applied to DNA. In order to do this, it was convenient to use the extensively studied DNA complexes of ethidium and acridine orange (Bloomfield et al., 1974; Jain et al., 1977; Wang et al., 1979; Patel & Canuel, 1976). Comparison of the energy-transfer properties of the four DNA ligands studied reveals structural differences that lend themselves to simple molecular interpretation.

Materials and Methods

Doubly deionized, glass-distilled water was used throughout. Labware was acid washed to prevent metal ion contamination. Unless otherwise specified, all chemicals were reagent grade.

Metal Chelates. The Tb(III) chelates of ethylenediaminetetraacetate ($TbEDTA^-$),[2] *N*-(2-hydroxyethyl)-ethylenediaminetriacetate ($TbHED3A^0$), and *N*,*N*′-bis(2-hydroxyethyl)ethylenediaminediacetate ($TbBED2A^+$), as well as $CuBED2A^0$ and Co(III)$EDTA^-$, were prepared as described (Wensel & Meares, 1983). $TbHED3A^0$ (0.10 g, 0.23 mmol) was additionally washed through a Chelex 100 (Bio-Rad) column (0.7 cm × 3.0 cm, NH_4^+ form) with water to remove traces of free Tb^{3+} and lyophilized.

DNA Ligands. Ethidium bromide was obtained from Sigma Chemical Co., and acridine orange [3,6-bis(dimethylamino)acridine] was obtained from Aldrich Chemical Co. Both were used without further purification, since thin-layer chromatography on silica gel (developed with a mixture of 65 volumes of chloroform, 25 volumes of methanol, and 4 volumes of 14.8 M aqueous ammonia) revealed negligible colored impurities for each. The green and orange forms of Co(III) bleomycin A_2 were prepared and purified as described (Chang et al., 1983).

Buffer. Because it is similar in size to the terbium chelates, Bu_4N^+ was the only cation present in the samples (other than the energy acceptors, or $TbBED2A^+$). Tetrabutylammonium bromide was obtained from Aldrich Chemical Co. Tetrabutylammonium hydroxide was prepared by passing Bu_4NBr through a hydroxide-form AG-1 (Bio-Rad) column. *N*-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid was obtained from Sigma. A stock Bu_4N^+–HEPES buffer solution was prepared by titrating a 10 mM HEPES solution with 45 mM Bu_4NOH to pH 7.3 (25 °C).

[2] Abbreviations: EDTA, ethylenediaminetetraacetic acid; $TbEDTA^-$, Tb(III) complex of EDTA; $CoEDTA^-$, Co(III) complex of EDTA; HED3A, *N*-(2-hydroxyethyl)ethylenediaminetriacetic acid; $TbHED3A^0$, Tb(III) chelate of HED3A; BED2A, *N*,*N*′-bis(2-hydroxyethyl)-ethylenediaminediacetic acid; $TbBED2A^+$, Tb(III) chelate of BED2A; $CuBED2A^0$, Cu(II) complex of BED2A; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; Bu_4N^+, tetrabutylammonium ion; AO, acridine orange; Eth, ethidium; DNAP, DNA phosphate; Co(III) blm, any complex of cobalt(III) with bleomycin.

Table I: Measured Rate Constants for Energy Transfer from TbHED3A (k_{0b} and k_{0f}) or TbBED2A$^+$ (k_{+b}) to DNA-Bound or Free Chromophores

acceptor	k_{0f} (10^6 M^{-1} s^{-1})	k_{0b} (10^6 M^{-1} s^{-1})	k_{0b}/k_{0f}	k_{+b} (10^6 M^{-1} s^{-1})	ln (k_{+b}/k_{0b}) ($\simeq\phi$)[a]
acridine orange	43.0 (±2)	7.2 (±0.5)	0.17 (±0.01)	678 (±57)	4.54 (±0.11)
ethidium	23.0 (±1)	6.3 (±0.3)	0.27 (±0.02)	663 (±33)	4.66 (±0.03)
green Co(III) blm	0.52 (±0.03)	0.25 (±0.02)	0.48 (±0.06)	14.6 (±0.6)	4.06 (±0.11)
orange Co(III) blm	0.91 (±0.05)	0.65 (±0.04)	0.71 (±0.06)	14.5 (±0.7)	3.11 (±0.10)

[a] These values may be compared to theoretical estimates for ϕ calculated for DNA without bound intercalators. Anderson & Record (1982) have reviewed various theoretical approaches and have obtained a value of $\phi \approx 8.4$ for DNA in 5 mM 1:1 added salt, from numerical solutions to the Poisson–Boltzmann equation. If counterions of finite size are considered, ϕ should be somewhat smaller (e.g., for cations of 0.4-nm radius, in 1 mM added salt, $\phi \approx 7.6$).

DNA Purification. A total of 14.7 mg of calf thymus DNA (Sigma, type I, sodium salt) was added to 146 μL of 0.1 M (sodium) EDTA, pH 8, and dialyzed against 250 mL of 0.1 M (sodium) EDTA, pH 8, for 6 days with daily changes of the EDTA solution. After 1 day of dialysis against 0.1 M tetraethylammonium bromide (Aldrich), the DNA was extracted with hot (65 °C) phenol, and then with phenol, chloroform, isoamyl alcohol, and ether, according to Maniatis et al. (1982). The DNA was concentrated to 150 μL with 2-methyl-1-propanol, and 350 μL of 0.8 M Bu_4NBr was added. The resulting solution was dialyzed for 2 days against two changes of 0.1 M Bu_4NBr, 2 days against four changes of 10 mM Bu_4NBr, and 2 days against four changes of 1 mM Bu_4NBr. The ratio A_{260}/A_{280} of the resulting Bu_4N^+ DNA was 1.9. This stock solution was stored at −70 °C in aliquots. The A_{260} measured before and after melting yielded a hyperchromicity of 29%. The DNA was tested with fluorescamine to detect bound proteins or polyamines; none was found. DNA concentrations were determined by the absorbance at 260 nm, with $\epsilon_{260} = 6.6 \times 10^3$ M^{-1} cm^{-1} (Felsenfeld & Hirschman, 1965).

Energy-Transfer Experiments. Samples for energy transfer were prepared by mixing appropriate amounts of DNA, Tb(III) chelate, Bu_4N^+–HEPES buffer, and quencher [ethidium, acridine orange, or Co(III) bleomycin] stock solutions and adjusting the pH with measured amounts of Bu_4NOH or HCl as required. The volumes of energy-acceptor stock solutions and the total sample volume were checked by weighing on an analytical balance.

On the basis of published binding constants for ethidium and acridine c nge (LePecq & Paoletti, 1967; Armstrong et al., 1970; Wil et al., 1982) and recently determined binding constants for Co(III) bleomycins (Chang & Meares, 1984), under our conditions (≥1 mM DNAP, 1 mM added salt) more than 99% of the acceptors are bound to DNA.

Rates of energy transfer were determined at 298 K by measuring luminescence lifetimes of the Tb(III) chelates in the absence (τ_0) or presence (τ) of quencher, with the instrument described by Wensel & Meares (1983). When conditions for the rapid-diffusion limit are satisfied (Thomas et al., 1978; Stryer et al., 1982a), the decay of Tb(III) luminescence is described by

$$\frac{-d[Tb^*]}{dt} = \frac{1}{\tau}[Tb^*] = \left(\frac{1}{\tau_0} + k_2[A]\right)[Tb^*] \qquad (1)$$

where [Tb*] is the molar concentration of excited donors, [A] is the molar concentration of acceptors, and k_2 (M^{-1} s^{-1}) is the rate constant for energy transfer.

Equation 1 predicts a single-exponential luminescence decay curve. As shown by the data in Figure 2, experimental observations agree quite accurately with this prediction. Equation 1 also predicts a linear relationship between $1/\tau$ and [A]; the data in Figure 3 confirm this. All the measured rate constants

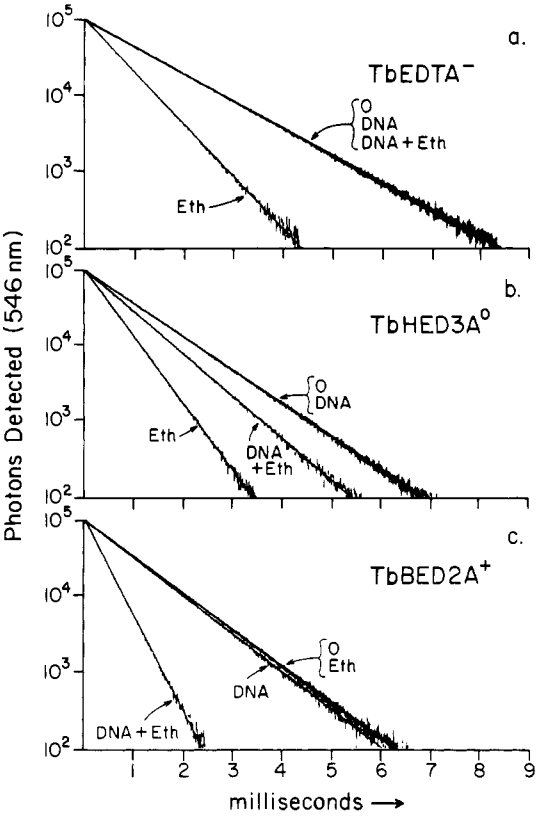

FIGURE 2: Luminescence decay data (plotted on a semilogarithmic scale) showing the single-exponential character of the decays and the effect of DNA upon the quenching properties of ethidium. All samples contained 0.5 mM Tb(III) chelate, either no DNA or 1 mM (DNAP) Bu_4N^+-DNA (calf thymus), 1.0 mM HEPES (0.5 mM Bu_4N^+), pH 7.0, and the indicated amounts of ethidium bromide. TbHED3A samples also contained 0.5 mM Bu_4NBr, so that the total added salt (not including DNA) in all samples was 1.0 mM. All lifetimes listed are accurate to within ±0.01 ms. (a) $TbEDTA^-$: no DNA, no ethidium, τ_0 = 1.20 ms; DNA added, no ethidium, τ_0 = 1.19 ms; no DNA, 19.0 μM ethidium, τ = 0.618 ms; DNA added, 19.3 μM ethidium, τ = 1.19 ms. (b) TbHED3A: no DNA, no ethidium, τ_0 = 0.948 ms; DNA added, no ethidium, τ_0 = 0.955 ms; no DNA, 45.8 μM ethidium, τ = 0.473 ms; DNA added, 44.8 μM ethidium, τ = 0.750 ms. (c) $TbBED2A^+$: no DNA, no ethidium, τ_0 = 0.889 ms; DNA added, no ethidium, τ_0 = 0.844 ms; no DNA, 2.30 μM ethidium, τ = 0.876 ms; DNA added, 2.77 μM ethidium, τ = 0.334 ms. Straight lines corresponding to the least-squares fits are superimposed on the data.

are collected in Table I. In addition, control experiments were performed with the non-DNA-binding $Cu^{II}BED2A^0$ and $Co^{III}EDTA^-$ as energy acceptors, to check for probe binding to DNA.

Tests for DNA Degradation. To test for degradation of the DNA under energy-transfer conditions, T7 DNA was used. It was prepared from bacteriophage T7 (wild type) by a method similar to that of Studier (1969). For the experiments to test for strand scission induced by the conditions of the

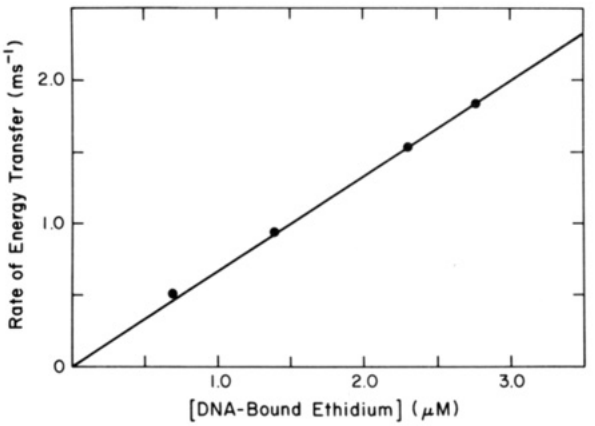

FIGURE 3: Dependence of the rate of energy transfer from TbBED2A$^+$ on the concentration of DNA-bound ethidium. All samples contained 1 mM DNAP, 0.5 mM TbBED2A$^+$ acetate, and 1.0 mM HEPES (0.5 mM Bu_4N^+), pH 7.0. The rate of energy transfer, $1/\tau - 1/\tau_0 = k_{+b}[A]$, may be found from eq 1 in the text. The straight line corresponds to the measured rate constant $k_{+b} = 6.63 \times 10^8$ M^{-1} s^{-1} (Table II).

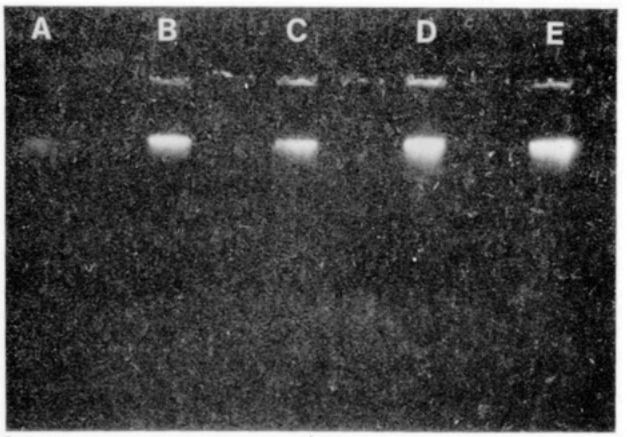

FIGURE 4: Agarose gel electrophoresis showing the lack of effect of laser irradiation on the integrity of T7 DNA in the presence of the highest concentrations of energy acceptors used in the energy-transfer experiments. The DNA bands were visualized by UV illumination of bound ethidium bromide, whose fluorescence was photographed through a red filter. (Lane a) Control, native T7 DNA with no added energy acceptors and no laser irradiation; (lane b) ethidium; (lane c) acridine orange; (lane d) green Co(III) bleomycin A_2; (lane e) orange Co(III) bleomycin A_2. All samples except for the control were irradiated for 6 min at 488 nm, the same conditions used for the energy-transfer experiments.

energy-transfer experiments, the dilute stock (~0.1 mg/mL) was concentrated by extraction with 2-methyl-1-propanol and precipitated twice with ethanol (Maniatis et al., 1982). The dried precipitate was dissolved in 1 mM $(Bu)_4N^+$–HEPES (pH 7.3) to a concentration of 30 mM DNAP. Solutions were prepared having the same concentrations of all components as those used in the energy-transfer experiments involving DNA-bound acceptors and TbHED3A^0, irradiated for 6 min (the same time as a typical energy-transfer experiment) with the same laser intensity at 488 nm as used for lifetime measurements (less than 20-mW average power). Aliquots from these samples were then electrophoresed on 0.8% agarose gels, in a buffer containing 0.089 M tris(hydroxymethyl)aminomethane–borate (pH 8.0) and 2.0 mM NaEDTA, with 0.05 μg/mL ethidium bromide.

The results of electrophoresis of T7 DNA after laser irradiation under conditions identical with those used for the energy-transfer experiments are shown in Figure 4 and reveal no double-strand breaks in the DNA. Because the energy-transfer experiments employ a great excess of DNA (10^{-3} M) to energy acceptor (~10^{-5} M), anything short of extensive degradation of the DNA to single-strand oligonucleotides is unlikely to affect the results.

T7 DNA was used for these experiments because it would be easy to detect double-strand cleavage of this intact homogenous DNA, while calf thymus DNA was used for most of the energy-transfer experiments for reasons of economy. It was found that the laser irradiation did not affect the hyperchromicity of calf thymus DNA. A few experiments with TbBED2A$^+$ as donor and ethidium as acceptor, using T7 DNA, gave results that were indistinguishable from comparable experiments using calf thymus DNA.

Test for Terbium Binding to DNA Using Precipitation from Ethanol. This experiment was carried out on DNA that had been prepared with TbBED2A$^+$ as the only counterion. This was accomplished by passing K$^+$-DNA (calf thymus), which had been dialyzed extensively against distilled water, through a TbBED2A$^+$-form AG-50 (Bio-Rad) minicolumn (~200 μL in an Eppendorf pipet tip with a glass wool plug). Control experiments indicated cation exchange was complete. The TbBED2A$^+$ (detected by luminescence in the presence of the sensitizer disodium dipicolinate) eluted with the DNA fraction (detected by A_{260}). A sample of this TbBED2A$^+$-DNA (25 mM DNAP) was precipitated with ethanol according to Maniatis et al. (1982) with sodium acetate as the supporting salt. The precipitate was washed with 70% ethanol and redissolved in water. The resulting solution was tested for dipicolinate-sensitized Tb(III) emission; none was observed.

On the other hand, when $TbCl_3$ (3.3 mM) was added to the 25 mM TbBED2A$^+$-DNA solution, the DNA immediately precipitated! It was redissolved in 0.25 M sodium acetate, precipitated with ethanol, washed with 70% ethanol, and redissolved in water. The resulting solution showed very strong Tb(III) emission.

Excitation Spectra of Terbium Probes plus or minus DNA. These were determined on a Perkin-Elmer MPF 44B spectrofluorometer, using 5-mm curvettes, 2-nm excitation slit width and 10-nm emission slit width, and a 430-nm emission cut-off filter, with emission monitored at 544 nm.

Spectral Overlaps and Values for Critical Distance for Dipolar Energy Transfer (R_0). Because the dipolar mechanism of energy transfer is familiar and well understood (Forster, 1948; Dexter, 1953; Stryer et al., 1982a), it is important to measure the experimental properties that permit its quantitative description. Absorption spectra were measured on a Hewlett-Packard 8450A UV/vis spectrophotometer. The following extinction coefficients (M^{-1} cm^{-1}) were used to determine quencher concentrations: acridine orange, 57.0×10^3 (490 nm; Schwarz et al., 1970); ethidium bromide, 5.82×10^3 (480 nm; Bresloff & Crothers, 1981); green Co(III) bleomycin A_2, 18.3×10^3 (290 nm); orange Co(III) bleomycin A_2, 18.1×10^3 (290 nm). Accurate extinction coefficients for both Co(III) bleomycins were determined by cobalt atomic absorption measurements (Chang & Meares, 1984). R_0, the critical distance for energy transfer (Förster, 1948), was calculated from the absorbance spectrum of each acceptor (in the presence and absence of DNA) and the corrected TbHED3A^0 emission spectrum (Figure 5). The relations used were

$$R_0 = 979(JQ_0\kappa^2 n^{-4})^{1/6} \text{ nm} \quad (2)$$

and

$$J = \frac{\int F(\lambda)\epsilon(\lambda)\lambda^4 \, d\lambda}{\int F(\lambda) \, d\lambda} \quad (3)$$

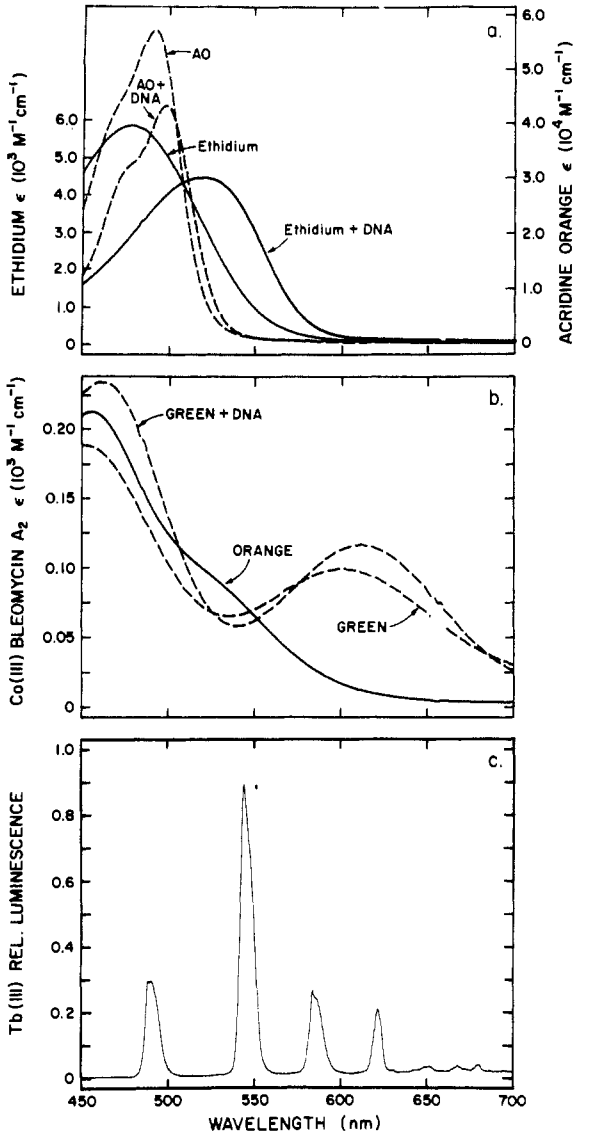

FIGURE 5: Overlap of the absorbance of the energy acceptors with Tb(III) emission: (a) absorbance spectra of ethidium (solid line, left-hand scale) and acridine orange (dashed line, right-hand scale) in the presence or absence of DNA, as indicated; (b) absorbance spectra of green (dashed line) and orange (solid line) Co(III) bleomycin A_2. The orange spectrum in the presence of DNA is indistinguishable from that shown. Note that ethidium, acridine orange, and Co(III) bleomycin are plotted on different scales and that the extinction coefficients of the absorbance maxima in the visible region decrease in the order: acridine orange > ethidium ≫ Co(III) bleomycins. (c) Corrected TbHED3A emission spectrum.

where J is the spectral overlap integral, $F(\lambda)$ is the relative fluorescence intensity of the donor at the wavelength λ (nm), and $\epsilon(\lambda)$ is the molar extinction coefficient of the acceptor at the same wavelength. Q_0 is the quantum yield of the donor in the absence of acceptor, κ^2 is the orientation factor between the donor and acceptor transition dipoles, and n is the refractive index of the intervening medium. For tabulation, the values used in the calculations were $\kappa^2 = {}^2/_3$ (Thomas et al., 1978), $Q_0 = 0.2$ (±0.05) (Yeh & Meares, 1980), and $n = 1.33$. However, the deviation of κ^2 from ${}^2/_3$ is explicitly taken into account in the derivation of eq 5 (see Appendix). The values of the spectral overlap integrals for each chromophore as acceptor and TbHED3A as donor, along with the values for R_0 calculated from them, are shown in Table II.

RESULTS

Effects of Acceptors and DNA on Donor Luminescence.

Table II: Spectral Overlaps (J) and Critical Distances (R_0) for Energy Transfer from TbHED3A to the Chromophores Studied and Estimated Rate Constants (k_d) for Dipolar Energy Transfer

acceptor	J (10^{14} M cm^{-3})	R_0 (nm)	maximum dipolar k_d (10^6 M^{-1} s^{-1})
acridine orange	6.68 (±0.27)	3.69 (±0.19)	13.1[a]
acridine orange + DNA	4.70 (±0.19)	3.47 (±0.18)	2.3[b]
ethidium	1.12 (±0.045)	2.74 (±0.14)	2.2[a]
ethidium + DNA	2.01 (±0.080)	3.02 (±0.15)	1.0[b]
green Co(III) blm	0.0784 (±0.0031)	1.76 (±0.09)	0.11[a]
green Co(III) blm + DNA	0.0865 (±0.0075)	1.79 (±0.09)	
orange Co(III) blm	0.0596 (±0.0024)	1.68 (±0.09)	0.08[a]
orange Co(III) blm + DNA	0.0596 (±0.0024)	1.68 (±0.09)	

[a] Estimated k_{0f}, calculated for TbHED3A ($R_D = 0.4$ nm), assuming a spherical energy acceptor with radius $R_A = 0.4$ nm for acridine orange and for ethidium and $R_A = 0.5$ nm for Co(III) bleomycins, from eq 4 in the text. Observed values are given in Table I. [b] Estimated k_{0b}, calculated from eq 5 in the text, using $a = 1.1$ nm. Observed values given in Table I.

Wensel & Meares (1983) found that the three terbium chelates $TbEDTA^-$, $TbHED3A^0$, and $TbBED2A^+$ behave in energy-transfer experiments as though they differ only in electric charge. The effects of ethidium and DNA on the luminescence decays of each of the Tb(III) chelates are shown in Figure 2. When the positively charged ethidium is added to a solution of negatively charged $TbEDTA^-$, the terbium luminescence lifetime decreases greatly due to energy transfer (Figure 2a). Because of their opposite charges, the rate constant for energy transfer from $TbEDTA^-$ to free ethidium is the highest of the three chelates (41.4×10^6 M^{-1} s^{-1}). However, the addition of DNA changes this situation radically; all the ethidium binds to DNA, and charge repulsion between the highly negative DNA and the negative probe prevents the probe from coming close to the bound ethidium. The rate of energy transfer becomes immerasurably small.

When ethidium is added to a solution of electrically neutral $TbHED3A^0$, the luminescence lifetime decreases due to energy transfer (Figure 2b); however, the rate constant is smaller (23×10^6 M^{-1} s^{-1}) because now there is no electrostatic attraction. The addition of DNA causes a significant decrease in the rate of energy transfer (Figure 2b) because the ethidium is almost buried in the DNA helix. As described below, the rate constants for energy transfer from the neutral terbium probe to free ethidium and to DNA-bound ethidium (Table I) can be used to estimate the accessibility of the bound chromophore.

When ethidium is added to a solution of positively charged $TbBED2A^+$, the observed rate constant for energy transfer is low (12.7×10^6 M^{-1} s^{-1}) because of electrostatic repulsion between the two cations (Figure 2c). Because oppositely charged probes should give reciprocal electrostatic effects (Wensel & Meares, 1983), the rate constants observed for $TbEDTA^-$ and $TbHED3A^0$ imply that the rate constant for $TbBED2A^+$ should be (23/41.4) (23×10^6) = 12.5×10^6 M^{-1} s^{-1}, in excellent agreement with the observed value. When DNA is added to a solution containing ethidium and $TbBED2A^+$, the electrostatic repulsion between ethidium and $TbBED2A^+$ is replaced by a very powerful electrostatic attraction between $TbBED2A^+$ and DNA-bound ethidium. Energy transfer is dramatically enhanced, with the rate constant being increased by a factor of 52 (Figure 2c). As described below, rate constants for energy transfer to DNA-

bound ethidium from the positive and neutral probes can be used to estimate the electrostatic potential at the surface of the bound chromophore.

Effects qualitatively similar to those illustrated for ethidium were observed with acridine orange, green Co(III) bleomycin, and orange Co(III) bleomycin. However, the magnitudes of the effects were different for each acceptor, and it is those differences that provide information about the properties of each DNA–acceptor complex. The relevant energy-transfer rate constants are given in Table I.

Experiments To Check for Probe Binding. A number of tests were performed to determine whether the probes, particularly $TbBED2A^+$ (which forms the weakest chelate and is electrostatically attracted to DNA), were in fact diffusing freely and interacting with DNA only through the influence of its electric field or whether they were actually binding to the DNA. In this context, the formation of short-lived ion pairs between $TbBED2A^+$ and DNA phosphate residues is a normal consequence of electrostatics and therefore is to be expected. The type of binding that is a source of concern here would involve chemical coordination of a significant fraction of the terbium probes to residues on DNA, causing a change in the properties of both; this would complicate interpretation of the results.

A sensitive indication of no significant binding was the lack of effect of DNA on the luminescent lifetime of each probe and, particularly, on the single-exponential character of its decay. Although the emission *spectrum* of Tb(III) is not sensitive to its environment, the luminescence lifetime τ is very sensitive, since energy from the luminescent excited state of Tb(III) can be efficiently transferred to vibrational modes of coordinated O–H groups (e.g., coordinated water molecules; Horrocks & Sudnick, 1979). Each probe is expected to have three water molecules coordinated to the metal, in addition to six ligands from the chelator. Displacement of just one H_2O (e.g., by DNA phosphate) should increase the Tb(III) lifetime by more than 0.3 ms (an easily measurable difference). Furthermore, such bound Tb(III) should be insensitive to the presence of bound acceptors since diffusion enhancement of energy transfer could no longer occur (see below). As shown in Figure 2, no *increase* in τ_0 in the presence of DNA, as would be expected for binding of Tb(III) to DNA, was found for any of the probes.

Figure 2 also demonstrates the single-exponential character of the luminescence decays, as expected for a homogeneous population of donors. Accurately single-exponential decay indicates that all donors experience the same environment; i.e., each donor is influenced by acceptors at all possible distances and orientations. All the lifetimes used to calculate energy-transfer rate constants reported here were computed from semilog plots that were linear over a 1000-fold range of intensity and had correlation coefficients of at least 0.999. Solutions of $TbBED2A^+$ and DNA were found to deteriorate somewhat over several days, as indicated by the appearance of a detectable long-lived component of the luminescence decay that was insensitive to added acceptors. All results reported here are for fresh samples, in whose decays this component could not be detected.

Another test for binding was carried out by studying energy transfer from $TbBED2A^+$ to the electrically neutral complex $CuBED2A^0$ in the presence and absence of 1 mM Bu_4N^+ DNA (calf thymus). If a significant fraction of $TbBED2A^+$ is bound to DNA, its accessibility to an electrically neutral acceptor (and consequently the rate constant for energy transfer) should be significantly reduced in the presence of DNA. It was found that no change occurred; $k_2 = (1.9 \pm 0.1) \times 10^6\ M^{-1}\ s^{-1}$, whether DNA was present or not.

The anionic $Co^{III}EDTA^-$ complex was also used as an energy acceptor to study the $TbBED2A^+$–DNA interaction. In this case, addition of 1 mM Bu_4N^+-DNA caused the rate constant for $TbBED2A^+$ to $CoEDTA^-$ energy transfer to decrease from $(24 \pm 0.7) \times 10^6\ M^{-1}\ s^{-1}$ to $(21 \pm 1.9) \times 10^6\ M^{-1}\ s^{-1}$, a barely significant change. Because DNA electrostatically attracts cations and repels anions, it would be expected to decrease energy transfer somewhat for this donor–acceptor pair. It is instructive to compare this result to the experiment in which DNA was added to $TbEDTA^-$ and ethidium$^+$, another oppositely charged donor–acceptor pair (Figure 2a). In that case, all the ethidium became bound to DNA, and energy transfer decreased by about 2 orders of magnitude. It is clear that nothing of that sort occurs with the $TbBED2A^+$–$CoEDTA^-$ pair, again indicating that practically all the $TbBED2A^+$ probes are free in solution.

Also, ethanol precipitation of $TbBED2A^+$-DNA did not bring down detectable quantities of terbium with the DNA. However, none of these experiments can rule out the possibility that a few percent of the probe is bound to DNA.

A test that is sometimes capable of detecting very low levels of binding was provided by examining the excitation spectra of the probes in the presence and absence of DNA. When 1 mM calf thymus DNA was added to $TbBED2A^+$, a weak terbium excitation peak at 290 nm was replaced by an 11-fold more intense peak. However, when T7 DNA was used instead of calf thymus DNA, no change occurred. Earlier studies of unchelated terbium and DNA (Ringer et al., 1978; Topal & Fresco, 1980) lead us to conclude that the sample of calf thymus DNA (but not the T7 DNA) had trace amounts of single-stranded regions containing guanosine residues to which $TbBED2A^+$ could bind. Since both calf thymus and T7 DNA gave the same results for $TbBED2A^+$–ethidium energy transfer, the difference between DNAs does not appear to be important in this context. Excitation spectra of the other probes ($TbHED3A^0$, $TbEDTA^-$) showed no such enhancement.

Discussion

Energy-Transfer Mechanisms and Chromophore Accessibility. As discussed in detail elsewhere (Meares et al., 1981; Meares & Rice, 1981; Stryer et al., 1982a; Wensel & Meares, 1983), energy transfer may occur by more than one mechanism. Therefore, the experimentally observed rate constant for energy transfer may be a sum of terms:

$$k_2 = \sum_i k_i$$

The familiar dipolar mechanism, which depends on the sixth power of distance, is the dominant one at large donor–acceptor separations (e.g., >1 nm). For separations of ≤1 nm, several other mechanisms of energy transfer— notably the exchange, dipole–quadrupole, quadrupole–quadrupole, etc.—may become important. The latter mechanisms decline very sharply with distance, and there is no simple way to distinguish between them; however, they are distinguishable from the dipolar mechanism. Thus, it is convenient to divide the possible mechanisms of energy transfer into two classes, so that the observed rate constant may be written $k_2 = k_d + k_x$ where k_d is a dipolar rate constant calculable from theory and k_x represents a sum of nondipolar rate constants. In situations where donor and acceptor are sterically prevented from close approach (for typical situations, constrained to be >1 nm apart), $k_2 = k_d$ (Meares & Rice, 1981; Stryer et al., 1982a). But if

donors can directly contact acceptors, k_x may be much larger than k_d.

In the case of dipolar energy transfer in the rapid-diffusion limit, the theory is well developed so that a rate constant can be calculated for any geometric model (Stryer et al., 1982a). For example, for dipolar energy transfer between a spherical donor and a spherical acceptor

$$k_{sph} = \frac{10^{-24}N}{\tau_0}\int_0^{2\pi} d\phi \int_0^{\pi} \sin\theta\, d\theta \int_{R_A+R_D}^{\infty} (R_0/r)^6 r^2\, dr$$

$$k_{sph} = 2.523R_0^6/[\tau_0(R_A + R_D)^3]\ M^{-1}\ s^{-1} \qquad (4)$$

where all distances are in nanometers, time is in seconds, N is Avogadro's number, and R_A and R_D are the radii of acceptor and donor.

It is shown in the appendix that for a spherical donor and an acceptor buried in the center of a long cylinder (length $\gg R_0$), with the acceptor's transition dipole transverse to the cylinder axis, the dipolar rate constant is

$$k_{cyl} = 1.672R_0^6/[\tau_0 a^3]\ M^{-1}\ s^{-1} \qquad (5)$$

where a is the minimum donor–acceptor separation (i.e., the radius of the cylinder plus the radius of the donor). Calculation of rate constants for other energy-transfer mechanisms is not practical at present. However, comparison of a calculated k_d with the observed k_2 usually makes it clear whether nondipolar energy transfer is important (Meares et al., 1981; Rice & Meares, 1981). For example, from Table I, orange Co(III) bleomycin has an observed $k_{of} = 0.91 \times 10^6\ M^{-1}\ s^{-1}$. Using eq 4, with a minimum acceptor radius $R_A = 0.5$ nm and an average donor radius $R_D = 0.4$ nm estimated from space-filling models, we find

$$k_d = 2.523(1.68)^6/[(0.95 \times 10^{-3})0.9^3] = 0.08 \times 10^6\ M^{-1}\ s^{-1}$$

Because the observed rate constant is 1 order of magnitude higher than the calculated dipolar rate constant, it is clear that nondipolar energy transfer dominates. Since dipolar energy transfer is less sensitive to steric hindrance, it might become more important for bound chromophores; however, for all eight systems studied, comparing the observed rate constants k_{of} and k_{ob} in Table I with the calculated dipolar rate constants in the last column of Table II indicates that nondiolar energy transfer plays the dominant role in each case. *Thus, all the chromophores (bound or free) are at least partly accessible to direct contact with donor molecules.*

Electrostatic Potential at the Surface of Each DNA-Bound Chromophore. The rate constant for energy transfer from an electrically charged donor to an acceptor is strongly dependent on the electrostatic potential about the acceptor. For the present case of nondipolar energy transfer, this dependence is described by (Meares et al., 1981; Stryer et al., 1982a; Wensel & Meares, 1983)

$$k_{+b} = k_{ob}\exp(\phi) \qquad (6)$$

where k_{+b} is the rate constant from a donor chelate with a single positive charge ($TbBED2A^+$) to bound acceptor, k_{0b} is the rate constant from a neutral chelate ($TbHED3A^0$) to the same bound acceptor, and $\phi = \ln(k_{+b}/k_{0b}) = -e\psi/(kT)$, where ψ is the electrostatic potential experienced by the donor when it is in contact with the acceptor and e is the magnitude of the electron charge. In the case of dipolar energy transfer, the rate constant is somewhat less sensitive to electrostatic effects (Meares et al., 1981; Wensel & Meares, 1983).

Measuring the electrostatic potential of a DNA-bound chromophore has several attractive features. Since it involves studying the same bound chromophore with two energy donors differing only in charge, problems in comparing bound and free chromophores are avoided. Since electrostatic forces depend rather weakly ($1/r$) on distance, they should be less sensitive to the molecular details of the donor–acceptor interaction than are the accessibility measurements described below. And since it is equal to the logarithm of an experimental quantity, the apparent electrostatic potential ϕ can be measured with precision.

Because of the high negative charge on DNA, k_{+b} can be 2 orders of magnitude larger than k_{0b} (Table I). The values of $\ln(k_{+b}/k_{0b})$ indicate that ϕ values for the acceptor–DNA complexes are related as ethidium ~ acridine orange > green Co(III) bleomycin A_2 > orange Co(III) bleomycin A_2. Previous work on DNA–ethidium (Mariam & Wilson, 1983) and DNA–acridines (Bustamante & Stigter, 1984) does not lend itself to direct comparison with the present measurements of electrostatic potentials. But the fact that the apparent potentials of acridine orange and ethidium are very similar is consistent with other information about their complexes with DNA. They both intercalate (Wang et al., 1979; Reddy et al., 1979; Jain et al., 1977) and spread the DNA base pairs apart by about the same amount (thus displacing phosphates and changing the electrostatic potential), and both should have a single positive charge at neutral pH. The electrostatic potential at the surface of the bound Co(III) bleomycins is less negative than that for the intercalators; even more interesting is the considerable difference between the ϕ values for the green and orange Co(III) bleomycins (Table I). A likely explanation for this difference is that the Co(III) center of the orange bleomycin complex is less closely associated with the negatively charged phosphodiester backbone of DNA.

Accessibility of Bound Chromophores. Simple geometric considerations suggest that the ratio of bound to free rate constants (k_{0b}/k_{0f}) should provide a rough measure of the bound acceptor's accessibility to the donor (Meares & Rice, 1981). This involves the assumption that the electronic properties of the bound and free acceptor are the same (not accurate for acridine orange and ethidium), and it neglects the role of detailed molecular interactions during energy transfer. These sources of error are less likely to be important for the cobalt bleomycins (whose chromophoric metal centers have relatively constant, unpolarized electronic properties) than for the planar aromatic intercalators. The four chromophores studied provide a wide range of behavior, illustrating most of the results likely to be encountered. Conceptually similar approaches have been used to interpret data on quenching of aromatic fluorophores in macromolecules by small solute molecules [see, for example, Lakowicz (1983), Perkins et al. (1984), and Eftink & Ghiron (1984)].

The case of orange cobalt(III) bleomycin is simplest to interpret. From Table I the ratio $k_{0b}/k_{0f} = 0.71$, indicating that the cobalt center remains highly accessible to the terbium probe even when bound to DNA. Also, as shown in Figure 5b, the absorption spectrum of this compound does not change upon binding to DNA; this implies that its properties as an energy acceptor are unaltered. Thus, it seems clear that DNA obscures only a small fraction of the orange cobalt center.

For green cobalt(III) bleomycin, $k_{0b}/k_{0f} = 0.48$. This number is significantly smaller than the value for the orange Co(III) bleomycin, suggesting that the green species binds with its cobalt center closer to DNA. As shown in Figure 5b, there is a small spectral change when green cobalt(III) bleomycin binds to DNA. This indicates a specific interaction with DNA, affecting the ligand field of the cobalt center. However, it does not significantly change the spectral overlap (Table II) and

so is not likely to have an important effect on the properties of green Co(III) bleomycin as an energy acceptor.

DNA-bound ethidium appears to be still less exposed, since its $k_{0b}/k_{0f} = 0.27$. As shown in Figure 5a, the absorption spectrum of ethidium changes shape considerably upon binding to DNA. The resulting increased spectral overlap is likely to make DNA-bound ethidium a significantly better energy acceptor than unbound ethidium, since all mechanisms of energy transfer depend on some type of spectral overlap (Dexter, 1953). Thus, the simple rate-contant ratio probably overestimates the accessibility of bound ethidium.

For DNA-bound acridine orange, $k_{0b}/k_{0f} = 0.17$. In this case, the absorption spectrum changes so as to decrease the spectral overlap (Figure 5a, Table II) so that the rate-constant ratio may somewhat underestimate the accessibility of bound acridine orange.

Correlation with Other Studies of DNA-Bound Ethidium and Acridine Orange. For both of these dyes there is a large body of evidence supporting intercalation of their planar ring systems between the base pairs of DNA [for reviews, see Blake & Peacocke (1968), Bloomfield et al. (1974), Porumb (1978), and Dougherty & Pigram (1982)]. Several features of the intercalated complexes have been well established. The DNA appears to be lengthened by about 3.4 Å per intercalated dye molecule (Luzzati et al., 1961; Mauss et al., 1967; Cohen & Eisenberg, 1969; Lang, 1971; Frederico & Houssier, 1972), implying that the dye separates its neighboring base pairs by this amount. The helix is unwound to accomodate the ligand (Crawford & Waring, 1967; Bauer & Vinograd, 1968; Waring, 1970). And each dye's electronic transition dipoles appear to be roughly parallel to the bases and perpendicular to the helix axis (Lerman, 1963; Neville & Davies, 1966; LePecq & Paoletti, 1967; Gardner & Mason, 1967). In addition, studies on the chemical reactivity of intercalated aminoacridines imply that the amino groups are largely inaccessible (Lerman, 1964). Fluorescence quenching of ethidium–DNA by O_2 is very ineffective, implying that the dye is buried in the DNA (Lakowicz & Weber, 1973). These properties of intercalated dyes in solution are in good agreement with expectations based on the crystal structues of dye complexes with self-complementary dinucleotides (Jain et al., 1977; Wang et al., 1979). In these structures, the chromophores' ring systems are almost completely covered by the neighboring base pairs, but it appears that the edges of the dye molecules would be partially accessible to small molecules in solution. Our results argue in favor of partial accessibility.

Besides intercalation, there is evidence for a minor, possibly "external" mode of DNA binding (LePecq & Paoletti, 1967; Blake & Peacocke, 1968; Armstrong et al., 1970; Bontemps & Fredericq, 1974; Bresloff & Crothers, 1975; Wille et al., 1982). Generally, this nonintercalative binding is much weaker than intercalation; it is primarily observed when the ratio of dye to DNAP is above 0.2 (dye/DNAP ratios used in the present work were below 0.05). Nonintercalative binding is favored by low ionic strength and is quite sensitive to ionic strength (LePecq & Paoletti, 1967; Armstrong et al., 1970; Houssier et al., 1974; Wille et al., 1982). To check for this under our experimental conditions, we measured energy transfer from neutral TbHED3A^0 to DNA-bound ethidium; the rate constant showed little change [$k_2 = (6.2 \pm 1.0) \times 10^6$ M^{-1} s^{-1}] over a range of Bu_4NBr concentrations from 10^{-4} to 0.1 M. This implies that nonintercalative binding has at most a minor effect on our ethidium experiments.

Correlation with Other Studies of Bleomycin–DNA Complexes. Along with the positively charged C-terminus, the bithiazole residues of bleomycin (see Figure 1) play a major role in DNA binding (Chien et al., 1977; Kasai et al., 1978; Povirk et al., 1979; Sakai et al., 1982; Kross et al., 1982; Booth et al., 1983). In fact, the bithiazole-containing tripeptide S is equal to intact bleomycin in its affinity for DNA (Chien et al., 1977). The analogous tetrapeptide binds less well, suggesting the importance of other residues in the bleomycin–DNA interaction (Kross et al., 1982). The role of the metal center (Figure 1) in DNA binding is less clear. We have found that metal-free bleomycin is not an energy acceptor; therefore, energy transfer to cobalt(III) bleomycin reports directly on the metal center.

There is little difference between the DNA-binding affinities of copper(II) bleomycin A_2 and the metal-free drug (Kasai et al., 1978), which have binding constants $\simeq (2–3) \times 10^5$ M^{-1} at pH 7.6, ionic strength 0.04, and 293 K. Orange Co(III) bleomycin A_2 is similar to these, having a DNA-binding constant of 1.5×10^5 M^{-1} at pH 8.0, ionic strength 0.04, and 298 K; but, green Co(III) bleomycin A_2 has a much larger binding constant, 1.3×10^7 M^{-1}, under the same conditions (Chang & Meares, 1984). This higher affinity might be due to a more attractive interaction between the green cobalt center and DNA. Such an interaction would be expected to cause the green cobalt center to bind closer to the negatively charged DNA surface and to be less accessible to probe molecules, in accord with our energy-transfer results. Preliminary studies with aquo Co(III) bleomycin suggest that it is similar to green Co(III) bleomycin.

Of course it is clear that the metal center of a DNA-bound metallobleomycin cannot be very far from the DNA, simply because the metal center is attached to the DNA-binding bithiazole group. However, there is little direct experimental evidence regarding the location of the metal center. Electron paramagnetic resonance of copper(II) bleomycin bound to DNA fibers (Shields et al., 1982) indicate that the metal center has considerable freedom to move around the long axis of DNA, while maintaining a preferred orientation along that axis. Comparison of the DNA-binding properties of iron(III) and copper(II) bleomycins indicates some metal-dependent differences (Povirk et al., 1981), and other studies indicate that binding of metallobleomycins to DNA can have some effect on the environment of the metal [Figure 5b; see also Albertini & Garnier-Suillerot (1982, 1984)].

In providing direct information about how DNA-bound orange and green Co(III) bleomycin complexes look from the outside, the present studies give an important clue to the molecular reasons for the different biological properties of these cobalt bleomycins.

Conclusions

Lanthanide energy transfer in the rapid-diffusion limit provides information about the accessibility and electrostatic potential of the chromophoric metal centers of DNA-bound green and orange cobalt bleomycins. Both measurements imply that the cobalt center of the green complex is more closely associated with the negative surface of DNA. These results fit in well with the observations that the green Co(III) bleomycin binds to DNA much more strongly than the orange and that the green complex is much more active in light-induced nicking of DNA (Chang & Meares, 1982, 1984). Since the action spectrum for light-induced nicking correlates well with a ligand to Co(III) charge-transfer absorption, it seems that the cobalt center is involved in the nicking reaction. Thus, the closer fit of the green Co(III) bleomycin suggests a simple explanation for its higher efficiency in breaking the sugar–phosphate backbone of DNA.

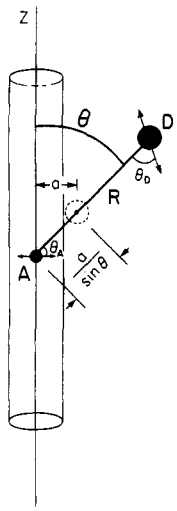

FIGURE A1: Coordinate system used for derivation of eq A7 (eq 5 in text), which describes energy transfer from a freely diffusing donor, D, to a chromophore, A, intercalated into DNA. The z axis coincides with the long axis of DNA and the x axis with the transition dipole of the acceptor. The minimum donor–acceptor separation is a; for a particular value of θ, the distance of closest approach is $a/\sin\theta$.

Our results also suggest that this energy-transfer method can be used to determine whether a DNA-binding chromophore binds by intercalation between base pairs (thus becoming much less accessible to small molecules in solution) or by association with the surface of the DNA. By comparing the positive and neutral donors, energy transfer in the rapid-diffusion limit can be useful in analyzing the electrostatic properties of DNA-bound chromophores. The electrostatic information is complementary to the accessibility and provides an independent measure of the structure of each bound complex. It is possible that polyelectrolyte theory, particularly the recently developed methods based on the detailed molecular structure of DNA (Klein & Pack, 1982; Matthew & Richards, 1984), might be used in conjunction with these results to further develop our understanding of the structures of DNA–ligand complexes in solution.

APPENDIX

Derivation of Equation 5, Describing the Rate Constant for Dipolar Energy Transfer from a Neutral Probe to an Acceptor Intercalated into DNA, in the Rapid-Diffusion Limit. The desired rate constant is given by

$$k_{db} = \frac{10^{-24}N}{\tau_0}\int_0^{2\pi} d\phi \int_0^{\pi} \sin\theta\, d\theta \int_{R_{min}}^{\infty} (R_0^6/r^6) r^2\, dr \qquad (A1)$$

where N = Avogadro's number, $R_{min} = a/\sin\theta$ (see Figure A1), distances are in nanometers, and time is in seconds [see Meares & Rice (1981) and Stryer et al. (1982a)].

Equation 2 gives the formula for R_0, which depends on κ^2. Rather than assuming $\kappa^2 = {}^2/_3$, as was done to compute the R_0 values in Table II, we shall explicitly consider the dependence of κ^2 on donor–acceptor orientation. According to Dale et al. (1979)

$$\kappa^2 = (\sin\theta_D \sin\theta_A \cos\phi_{DA} - 2\cos\theta_D \cos\theta_A)^2$$

where θ_D is the angle between the donor transition dipole and the donor–acceptor vector (Figure A1), θ_A is the angle between the acceptor transition dipole and the donor–acceptor vector (Figure A1), and ϕ_{DA} is the dihedral angle between donor and acceptor transition moments. Because lanthanides are thought to behave as isotropic donors (Horrocks et al., 1975), it is appropriate to average over all possible values of θ_D and ϕ_{DA}:

$$\langle\kappa^2\rangle_A = \frac{1}{4\pi}\int_0^{2\pi} d\phi_{DA}\int_0^{\pi} \sin\theta_D\, d\theta_D(\sin\theta_D \sin\theta_A \cos\phi_{DA} - 2\cos\theta_D\cos\theta_A)^2 = \frac{1}{3} + \cos^2\theta_A \qquad (A2)$$

At this point, averaging further over θ_A would give the usual $\kappa^2 = {}^2/_3$. However, because intercalators such as ethidium are known to bind anisotropically to DNA (with their transition dipole moments approximately perpendicular to the long axis of DNA, as shown in Figure A1), this would not give an accurate answer. Insertion of the factor

$$\frac{\langle\kappa^2\rangle_A}{\langle\kappa^2\rangle} = \frac{1/3 + \cos^2\theta_A}{2/3} = \frac{1/3 + \sin^2\theta\cos^2\phi}{2/3} \qquad (A3)$$

into equation A1 will account for the residual angular dependence of the dipolar interaction.

$$k_{db} = \frac{10^{-24}N}{\tau_0}\frac{R_0^6}{2/3}\int_0^{2\pi} d\phi \times \int_0^{\pi}\left(\frac{1}{3} + \sin^2\theta\cos^2\phi\right)\sin\theta\, d\theta \int_{a/\sin\theta}^{\infty} r^2/r^6\, dr \qquad (A4)$$

$$k_{db} = \left[\frac{10^{-24}NR_0^6}{\tau_0(2/3)}\frac{1}{3a^3}\right]\int_0^{2\pi} d\phi \times \int_0^{\pi}\left(\frac{1}{3} + \sin^2\theta\cos^2\phi\right)\sin^4\theta\, d\theta \qquad (A5)$$

$$k_{db} = \frac{10^{-24}NR_0^6}{\tau_0(2/3)(3a^3)}\int_0^{2\pi} d\phi\left(\frac{1}{3} + \frac{5}{6}\cos^2\phi\right)\frac{3\pi}{8} \qquad (A6)$$

$$k_{db} = \frac{10^{-24}N}{(2/3)3}\frac{9\pi^2}{16}\frac{R_0^6}{\tau_0 a^3}$$

$$k_{db} = 1.672R_0^6/(\tau_0 a^3)\ M^{-1}\ s^{-1} \qquad (A7)$$

If instead we had assumed that $\kappa^2 = 2/3$, the final formula would be

$$k_{db} = 1.486R_0^6/(\tau_0 a^3)\ M^{-1}\ s^{-1} \qquad (A8)$$

Thus, the experiment is not very sensitive to the detailed geometric properties of the acceptor transition moment.

Registry No. $TbEDTA^-$, 15158-65-3; $TbHED3A$, 75180-61-9; $TbBED2A^+$, 87698-05-3; acridine orange, 65-61-2; ethidium, 3546-21-2.

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