An NMR Investigation of the Kinetics of Dissociation of the Zinc(II) Complex of Bleomycin Antibiotics[1a]

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Abstract: The addition of Zn(II) to a neutral aqueous solution of bleomycin results in the observation of several new peaks in the ^1H NMR spectrum concomitant with the reduction in intensity of several of the peaks observed in the metal-free antibiotic. This observation leads to the conclusion that for some of the protons in bleomycin the rate of dissociation of the Zn(II)-bleomycin complex is much smaller than their chemical-shift difference in the free and complexed states. We have found that the Zn(II)-bleomycin complex has a 1:1 stoichiometry by measuring the relative intensity of the C4 resonance of the imidazole proton at various Zn(II) concentrations. Rates of dissociation of the complex were determined at temperatures between 303 and 353 K by means of NMR transfer of magnetization experiments. From an analysis of the temperature dependence of these rates we obtained values for the energy and entropy of activation of 16.2 kcal and 50 eu. Both the observed rates and the value of the energy of activation obtained in our analysis are more similar to parameters determined for Zn(II)-containing metalloenzymes than for low molecular weight ligands. On this basis we suggest that in the Zn(II)-bleomycin complex the Zn(II) is more likely to be in a tetrahedral environment than the octahedral environment proposed in some models for the complex.

The bleomycins are a family of glycopeptide antibiotics isolated from *Streptomyces verticillus* by Umezawa and coworkers.[2] The revised primary structures of these molecules are shown in Figure 1.[3] The various congeners of the bleomycins differ from each other in their terminal amine moiety (R group in Figure 1). Blenoxane, the commercial form of these drugs, marketed by Bristol Laboratories, contains ca. 70% bleomycin A_2 and ca. 25% bleomycin B_2 with trace amounts of other congeners. The bleomycins have been employed as antineoplastic agents in the treatment of a wide variety of human carcinomas and lymphomas.[4] It has been suggested that the mode of action of these drugs involves the scission of DNA.[5] A free-radical mechanism for this process has been suggested.[6] Recently, Sausville et al.[7] have proposed a more specific free-radical mechanism involving the formation of a complex between Fe(II) and bleomycin and its subsequent reaction with dissolved oxygen. An "active center" mechanism for the scission process involving a Zn(II) complex of bleomycin has also been suggested.[8] Other investigations have reported that polyvalent cations (e.g., Zn(II), Cu(II)) and EDTA[10] inhibit the strand scission process.[9] Thus, polyvalent metal complexes of bleomycin may play some role in the therapeutic activity of these drugs. In addition, bleomycin labeled with radioisotopes such as ^{57}Co, ^{64}Cu, ^{65}Zn, ^{67}Ga, ^{99m}Tc, ^{111}In, ^{169}Yb, ^{197}Hg, and ^{203}Pb has been employed as a tumor scanning agent in the detection of a wide variety of solid tumors and malignant lymphomas.[11]

The present investigation is part of a comprehensive program in progress in our laboratories to characterize the interactions of the bleomycins with polyvalent metal ions and nucleic acids. The properties of these antibiotics and their complexes have been investigated by a variety of spectroscopic methods including UV,[5a,12-15] IR,[16] ORD-CD,[13] fluorescence,[17] NMR,[17-19] and ESR.[14] We have previously reported the ^1H NMR assignments of the free antibiotics,[20] the ^{13}C spin-lattice relaxation times of bleomycin A_2,[21] and the pH dependence of the ^{13}C NMR shifts of bleomycin A_2.[22]

In this communication, we present the results of a ^1H NMR investigation of the interactions of Zn(II) with bleomycin. There have been several recent reports of spectroscopic investigations of the Zn(II)-bleomycin complex which were directed at determining the site of metal complexation in the antibiotic.[14,15,19] In the present study, we have determined the kinetics of dissociation of the Zn(II)-bleomycin complex from NMR transfer of magnetization experiments. These results are compared with kinetic data from the literature for small ligands and zinc-containing metalloenzymes.

Experimental Section

Blenoxane was a gift from Bristol Laboratories (Syracuse, N.Y.). The concentration of blenoxane in solution was determined spectrophotometrically using a molar absorptivity of $1.3 \pm 0.2 \times 10^4$ M^{-1} cm^{-1} at 290 nm. The concentration of Zn(II) was determined by a complexiometric titration with EDTA using Eriochrome Black T as indicator. All solutions containing Zn(II) were prepared from a stock solution of $Zn(SO_4)\cdot 7H_2O$.

NMR experiments were conducted at 90 MHz on a Bruker HX90 spectrometer operating in the Fourier transform (FT) mode. The transfer of magnetization experiments were performed on a modified Bruker HX360 (Stanford University) operating at 360 MHz in the FT mode. The spin-lattice relaxation times, at 360 MHz, were determined by the inversion recovery method using a Nicolet NTC-360 spectrometer (Purdue University). For the latter two sets of experiments, the temperature was determined to ± 1 °C from the chemical shifts of a standard sample of ethylene glycol.

Results and Discussion

90-MHz NMR Experiments. The 90-MHz ^1H NMR spectrum of 10 mM blenoxane at a pD of 6.8 is shown in Figure 2a. The addition of 5 mM of Zn(II) (Figure 2b) results in the appearance of several new peaks in the spectrum concomitant with the reduction in intensity of several peaks in the blenoxane spectrum. From this observation, we infer that for some of the protons in blenoxane the rate of dissociation of the Zn(II)-bleomycin complex is much smaller than their chemical-shift difference in the free and complexed states. Similar results have been obtained by Dabrowiak et al.[14,15] for both the ^{13}C and ^1H spectra of the Zn(II)-bleomycin A_2 complex and by Cass et al.[19] for the Zn(II) complex of blenoxane.

In order to establish the stoichiometry of the complex, we determined the relative intensities of the C4 resonance of the imidazole of blenoxane at various Zn(II) concentrations. These data are shown in Figure 3. The intercept on the abscissa of ca. 1.0 clearly demonstrates the formation of a 1:1 complex. In addition, we wish to point out that the nearly linear variation in the intensities reflects the fact that the dissociation constant for the Zn(II)-bleomycin complex is several orders of magnitude smaller than the concentration of this antibiotic used in this experiment (10 mM).

In the spectra shown in Figure 2, the largest perturbations induced by Zn(II) are downfield shifts of the resonances of the

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Structure of bleomycin

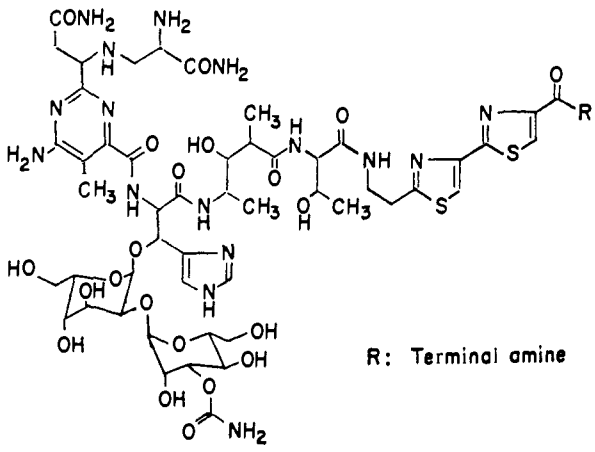
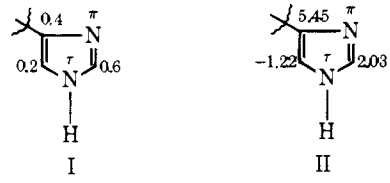


Figure 1. The primary structure of the bleomycin antibiotics. In the A_2 congener R is $-HNCH_2CH_2CH_2S^+(CH_3)_2$; in the B_2 congener R is $-HNCH_2CH_2CH_2CH_2N(H)C(NH_2)_2^+$.

C2 (~0.2 ppm) and the C4 (0.09 ppm) protons of the histidine as well as the methyl group (0.4 ppm) of the pyrimidine. It is difficult to draw any meaningful conclusions about the structure of the Zn(II)-bleomycin complex on the basis of these data alone. Dabrowiak et al.[15] have reported the shifts induced by Zn(II) in the ^{13}C resonances of bleomycin A_2. These data have been interpreted in terms of a structure for the complex in which the N^π nitrogen of the imidazole and the N_1 nitrogen of the pyrimidine participate directly in chelating the zinc ion.[14,15] We would like to present an alternate interpretation for both the ^1H and ^{13}C data. The ^{13}C shifts (ppm) induced by Zn(II) in the imidazole ring[15] are shown below (I), together with the shifts observed on protonation of the N^π nitrogen (II).[22]



I II

Note that the proposed site of metal complexation (N^π) is the same as the site of protonation. If the perturbations observed on metal complexation are purely electrostatic in origin (rather than ring current effects or changes in orbital delocalization, for example), the protonation shifts should provide a crude estimate of shifts expected on metal complexation. A comparison of the two sets of shifts indicates two discrepancies: the shifts induced by Zn(II) are much smaller than the protonation shifts and the Zn(II) shifts are all to lower field. This comparison seems to indicate that the N^π nitrogen may not be the site of metal complexation. A similar comparison made using the shifts observed on the protonation of the N^τ nitrogen[23] indicates that this nitrogen cannot be a site of metal complexation. In light of these arguments, it would seem that the imidazole moiety may not be directly involved in metal complexation. Note that both the signs and magnitudes of the shifts induced by Zn(II) in the ^{13}C and ^1H resonances of the imidazole ring are similar. These shifts might arise from an altered orientation of the side chain of the histidine with respect to other aromatic moieties of the molecule and thus be attributable to a perturbation of the ring current shifts rather than direct complexation. Evidence to support this explanation comes from a consideration of the ^{13}C spin-lattice relaxation time measurements. The latter are consistent with the existence of a folded conformation for bleomycin in solution.[21] The

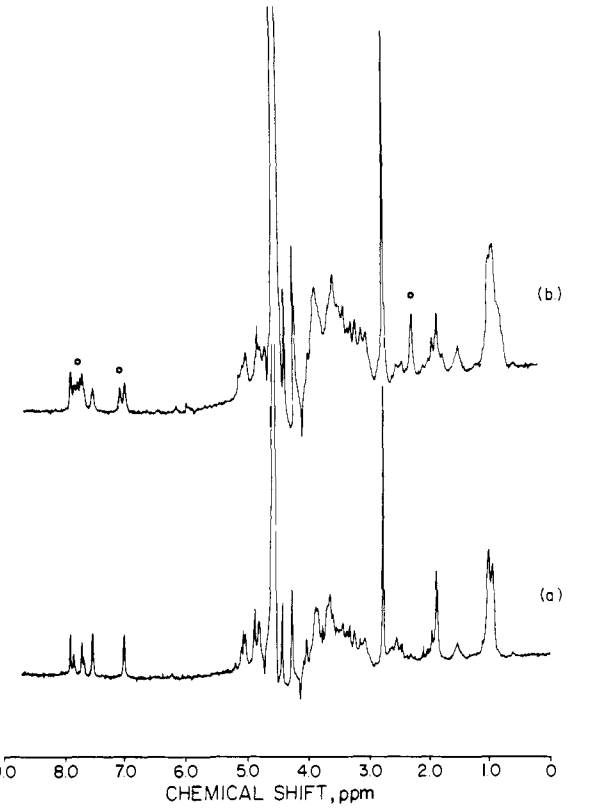


Figure 2. The 90-MHz ^1H NMR spectrum of 10 mM blenoxane at pD of 6.8 (a) in the presence of 0 and (b) 5 mM Zn(II).

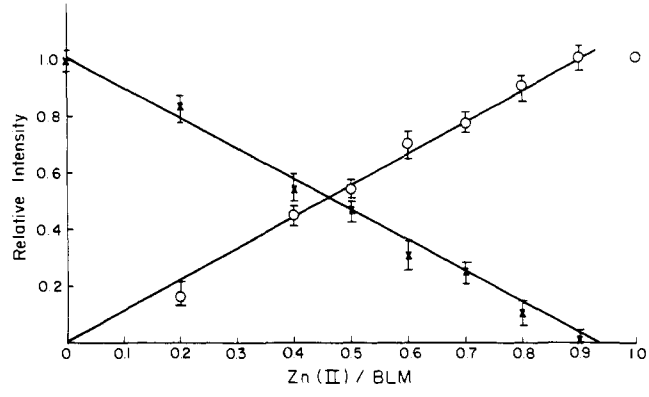


Figure 3. The variation in the relative intensity of the bound (O) and free (X) resonances of C4 proton of the imidazole as a function of the relative concentration of Zn(II) present. The data were obtained at 90 MHz in 50 mM Pipes buffer at a pD_c of 6.8.

presence of a metal ion causes some unfolding of this conformation.[24] This conformational change can be rationalized in terms of the electrostatic repulsion between the cationic tail of bleomycin and the polyvalent metal present in the complex.

We have attempted to make a similar comparison of the ^{13}C shifts observed for the pyrimidine group. However, this comparison is complicated by the fact that some of the assignments of the carbon atoms in the Zn(II)-bleomycin complex are ambiguous. Nevertheless, several interesting conclusions can still be drawn from the comparison. The site of protonation near the pyrimidine is most probably the secondary amine.[3] Takita et al. have shown that the direct protonation of the pyrimidine occurs with a pK_a of less than 1.[3]

The ^{13}C shifts observed when the secondary amine is protonated ($pK_a \sim 3$) are shown below.

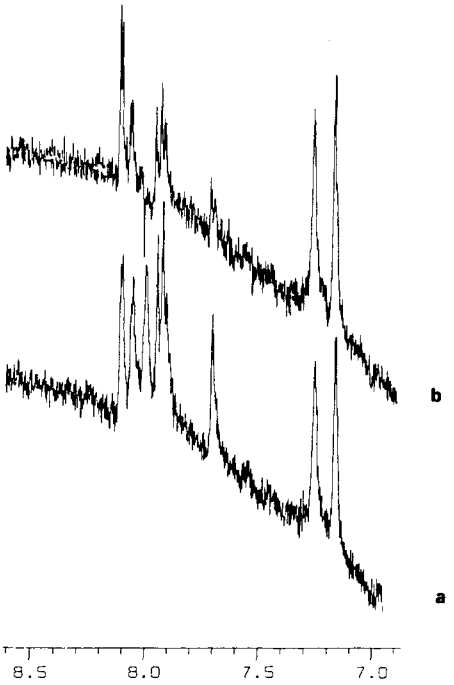
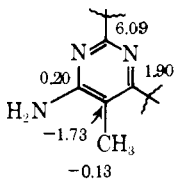


Figure 4. The 360-MHz ^1H spectrum of 10 mM blenoxane in the presence of 4 mM Zn(II) at a pD_c of 6.8 (a) no decoupling irradiation, (b) with the decoupler set at 8.04 ppm.



III

From the data it is clear that large ^{13}C shifts can arise from electrostatic interactions which do not necessarily involve coordination to the pyrimidine ring per se. Therefore, it may be possible to account for the ^{13}C shifts observed in the resonances of the pyrimidine on Zn(II) binding without proposing the direct interaction of any of the nitrogens of the pyrimidine with the Zn(II) ion. We have found that the α-amino group of the diaminopropionamide group of the bleomycin is a ligand of Ga(III) in the corresponding metal complex.[24] Note that this portion of the drug resembles part of the EDTA molecule which may account for the rather strong affinity of these antibiotics for metal ions. We have determined values for the dissociation constant of $2.0 \pm 0.4 \times 10^{-5}$ M for the Ga(III) complexes of bleomycin from fluorescence and potentiometric experiments.[24] Analysis of the results of similar experiments conducted on the Zn(II) complexes yield estimates of the dissociation constant of ca. 1×10^{-4} M.

360-MHz Transfer of Magnetization Experiments. Transfer of saturation is governed by the equation

$$(M_0^{\alpha} - M_z^{\alpha})/M_0^{\alpha} = [T_{1_\alpha}/(T_{1_\alpha} + \tau_\alpha)][(M_0^{\beta} - M_z^{\beta})/M_0^{\beta}] \quad (1)$$

which was derived from modified Bloch equations using a procedure analogous to that used by Gupta and Redfield.[25] The α and β states refer to two nuclei which are chemically exchanging. T_1, τ_α, M_z^{α}, and M_0^{α} are the spin-lattice relaxation time, lifetime, observed magnetization, and equilibrium magnetization of the α nucleus, respectively. Analogous notation is used for the β nucleus. $(M_0^{\alpha} - M_z^{\alpha})/M_0^{\alpha}$ is the fractional decrease in resonance intensity of the α resonance resulting from the double irradiation of the β resonance, whose intensity is diminished by a factor of $(M_0^{\beta} - M_z^{\beta})/M_0^{\beta}$. Complete saturation of the β state causes a fractional decrease of the α resonance equal to $T_{1\alpha}/(T_{1\alpha} + \tau_\alpha)$, which is significant only if the pseudo-first-order rate constant for exchange of the α nucleus, $1/\tau_\alpha$, is comparable to or less than its relaxation rate, $1/T_{1\alpha}$.

The aromatic region of the 360-MHz ^1H NMR spectrum of blenoxane in the presence of Zn(II) is shown in Figure 4a. The spectrum obtained on the complete saturation of the C2 proton of the Zn(II)-bleomycin complex is shown in Figure 4b. Note the significant decrease in intensity in the C2 resonance of the free bleomycin. When the frequency of the decoupler was shifted to 7.4 ppm, the spectrum obtained was identical with that shown in Figure 4a. These experiments were repeated at several different temperatures ranging from 303 to 353 K. As expected, the extent of saturation transfer was strongly temperature dependent, ranging from 0.06 at 303 K

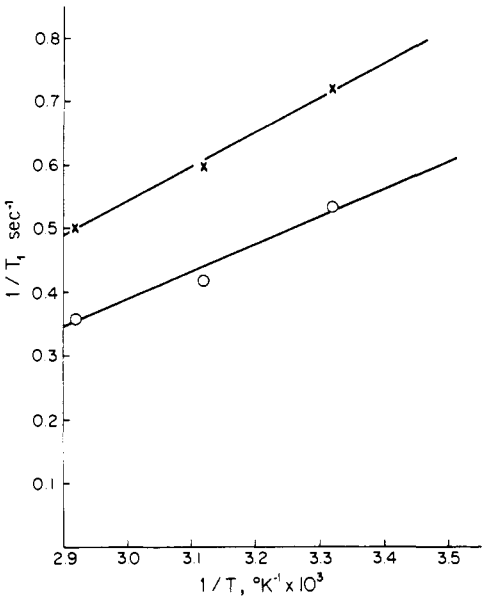


Figure 5. The variation of the spin-lattice relaxation rates with the reciprocal of the absolute temperature of the C4 (×) and C2 (○) protons of the imidazole in blenoxane. The spin-lattice relaxation times of a 10 mM blenoxane sample were measured at 360 MHz at pD_c of 6.8.

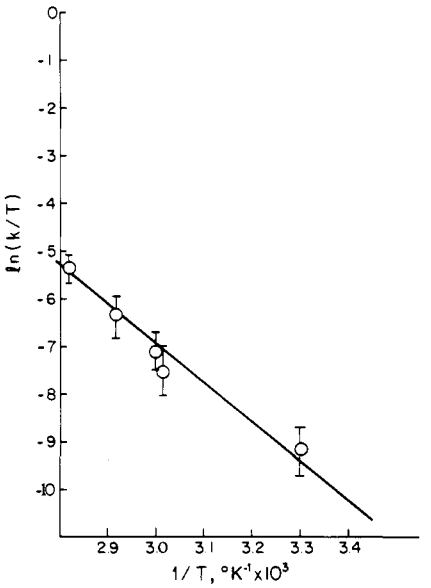


Figure 6. An Eyring plot of the kinetic data obtained for the Zn(II)-bleomycin complex (see eq 4).

to 0.84 at 353 K. The quantitative analysis of the results of these experiments (cf. eq 1) requires a knowledge of the T_1's of the free bleomycin peaks over the same temperature range. These data are shown in Figure 5. Using these results in eq 1, we obtained values for τ, the exchange lifetime, at the various temperatures. Note that this τ is the lifetime of bleomycin in its free or uncomplexed state. From the McConnell[26] development, the lifetimes of the free and bound states are related by

$$\tau_B = (P_B/P_F)\tau_F \quad (2)$$

where P_B and P_F are the fractional populations in the bound and free states, respectively. These values can be determined by measuring the integrated intensities of the appropriate peaks in Figure 4a. In this manner, it is possible to determine τ_B, the lifetime of the bleomycin molecule in the complexed state. It is often assumed that the mechanism for the dissociation step of metal complexes is first order. A result of this assumption is that τ_B is a characteristic of the system and its inverse is a true rate constant. Thus, the temperature dependence of this rate constant can be analyzed in terms of an Arrhenius relationship:[27]

$$\ln k = \ln A - E_A/RT \quad (3)$$

where k is the rate constant, T is the absolute temperature, R is the gas constant, E_A is the energy of activation for the process, and A is a constant for the system. Using eq 3, the value of E_A was found to be 16.2 kcal. An alternate method for analyzing these data is the Eyring relationship[28]

$$\ln (k/T) = -\frac{\Delta H^{\ddagger}}{RT} + \left(\ln \frac{k_B}{h} + \frac{\Delta S^{\ddagger}}{R}\right) \quad (4)$$

where $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation, respectively, and $\ln (k_B/h)$ has the value of ca. 23 eu. Using this approach (see Figure 6), the value of $\Delta H^{\ddagger}$ was found to be 15 kcal and $\Delta S^{\ddagger}$ was ca. 20 eu.

Note that since

$$E_A = \Delta H^{\ddagger} + RT \quad (5)$$

the energy of activation should be slightly larger than the value of $\Delta H^{\ddagger}$. The factors that affect the value of $\Delta S^{\ddagger}$ have been discussed in detail by Wilkins[29] and Basolo and Pearson.[30] For reactions between unlike charged species, it is often changes in solvation around each of the ions that determine the magnitude of $\Delta S^{\ddagger}$. Thus, we can explain the sizable negative value of $\Delta S^{\ddagger}$ found on the dissociation of the complex by the fact that the net charge of the Zn(II)-bleomycin complex is smaller than the net charges of the separate Zn(II) and bleomycin particles. This explanation is supported by the observation that the electrophoretic mobilities of various metal complexes of bleomycin are reduced on metal binding.[24]

Rate constants for the dissociation of Zn(II) from various ligands are given in Table I. From these data, it is clear that the rate of dissociation of Zn(II) from bleomycin falls between the rates observed for small molecules and for the enzymes. While it may be dangerous to draw firm structural conclusions on the basis of this sort of comparison, it is important to note that the zinc atom is in a distorted tetrahedral environment in both carboxypeptidase and carbonic anhydrase.[36] In addition, the energy of activation found for the Zn(II)-bleomycin complex is similar to the values reported for carbonic anhydrase.[31,32] For smaller ligands values for the energy of activation of ca. 10 kcal are expected.[31] On the basis of these two kinds of comparisons, we suggest that in the Zn(II)-bleomycin complex the zinc ion is more likely to be in a tetrahedral environment rather than in the octahedral environment proposed in several models. Experiments directed at complete identification of sites of metal complexation in bleomycin are currently in progress in our laboratories.

Table I. Rates of Dissociation of Zn(II) from Several Ligands at 25 °C

compd	rate of dissociation, s^{-1}
bipyridine[a]	25
bleomycin	$(3.0 \pm 2.0) \times 10^{-2}$
carbonic anhydrase[b]	1.5×10^{-9}
carbonic anhydrase[c]	1.1×10^{-6}
carboxypeptidase[b]	1×10^{-6}
glycine[d]	1.4×10^{3}
phenanthrolinium[a]	5.0
terpyridine[a]	2.5

[a] Taken from ref 31. [b] Taken from ref 32. [c] Taken from ref 33. [d] Taken from ref 34.

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References and Notes

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Solvent Effects on the Photophysics of Dibromoanthracene

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Abstract: The fluorescent lifetimes and relative fluorescent quantum yields of 9,10-dibromoanthracene solutions have been measured. It is found that the nonradiative transition rate of the lowest excited singlet of 9,10-dibromoanthracene is strongly dependent on the solvent and temperature, but is unaffected by the presence of heavy atoms in the solvent molecules. The temperature dependence is attributed to an endothermic transition from the lowest excited singlet state S_1 to an adjacent higher excited triplet level, T_q. The solvent dependence is due to the different extent to which S_1 and T_q are shifted by the solvent. The solvent effect on T_q is only about 30% of that on S_1. The absence of external heavy atom effect is explained on the basis that no new intersystem-crossing channel is made available by the introduction of external heavy atoms.

Introduction

It has been reported that solutions of 9,10-dibromoanthracene (DBA) in ethanol or cyclohexane exhibit increased fluorescence quantum yields when ethyl iodide, bromobenzene, or benzene are added.[1,2] The fluorescence yield of DBA in pure bromobenzene is greater than that of DBA in benzene, ethanol, or *n*-hexane.[3] These are interesting observations since normally ethyl iodide and bromobenzene are quenchers for the fluorescence of aromatic molecules. These two quenchers are believed to quench the fluorescence of organic molecules by the heavy-atom effect[4] through a route which gives exclusively the triplet state.[4-8] The presence of heavy atoms increases the overall rate of intersystem crossing from the lowest excited singlet state S_1 to the triplet manifold. This anomalous heavy-atom effect is also observed for 9-bromoanthracene.

There have been attempts to explain this anomalous heavy-atom effect. Measurements of the temperature dependence of DBA fluorescence yield in four solvents showed that the activation energies for intersystem crossing for DBA in benzene and bromobenzene were greater than that in *n*-hexane or ethanol.[3] This suggests the probable origin for the strong solvent effect on the emission yield of DBA solutions, but does not give an adequate explanation for the absence of heavy-atom quenching in the DBA solutions studied. In a more recent paper, the enhancement of fluorescent emission from DBA in cyclohexane solutions by ethyl iodide or benzene was attributed to the formation of an exciplex which had a higher emission efficiency.[2] This explanation should be examined more critically. The fluorescence spectra of DBA in cyclohexane, benzene, and ethyl iodide are very similar. They have the same vibrational structure and exhibit only a small solvent shift of 4-5 nm. The usual red-shifted, structureless emission from an exciplex is not observed for DBA in benzene or ethyl iodide solution. Since no exciplex formation can be detected in the fluorescence spectrum, it was considered advisable to investigate the fluorescence decay response from DBA solutions.

The kinetics of exciplex photophysics in solution are well known,[4,9-11] and measurement of the time profile of the DBA fluorescence would be expected to indicate whether an exciplex is responsible for the increase in the emission yield of DBA in ethyl iodide or benzene.

The purpose of this work was twofold: the first was to examine more systematically the solvent dependence of the fluorescent yields of DBA solutions; the second was to investigate the anomalous heavy-atom effect in DBA solutions with the aim of advancing a reasonable explanation for the absence of quenching by heavy atoms.

Experimental Section

Fluorescence decay measurements were made with a time-correlated single photon counting instrument.[12] A nanosecond flash lamp manufactured by Photochemical Research Associates was used. The excitation monochromator was fixed at 385 nm while the analyzing monochromator was set at the second emission maximum of DBA, which was around 433 nm. Fluorescence spectra were taken with a Perkin-Elmer MPF-4 fluorescence spectrophotometer. For relative quantum yield determinations, no correction was made for the wavelength response of the photomultiplier. Absorption spectra were recorded with a Varian-Cary 219 spectrophotometer. All DBA solutions used for emission measurements were degassed by several freeze-thaw cycles. When not specified, the experiments were performed at room temperature (about 21 °C). At all other temperatures, the sample was held in a thermostated cell holder. The concentration of DBA in the fluorescence decay measurement was about 4×10^{-5} M, while for steady-state fluorescence spectral measurements it was about 8×10^{-6} M. DBA was recrystallized and then further purified by silica gel chromatography. All solvents were purified by at least one pass through an activated silica gel or alumina column.

Results

Over a range of more than two orders of magnitude, the fluorescence decay portion of all the DBA solutions studied here could be fitted with high precision to a single exponential decay function. The reduced χ^2 for fitting to the experimental data[13] was between 1.4 and 0.9. No improvement in χ^2 was

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