TRIPLETS OF AROMATIC DYES BOUND TO DNA AS PROBES OF THE MOBILITY OF DNA-BOUND METAL IONS

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1. Introduction

The interaction of various metal ions with DNA in aqueous solutions has been studied by a variety of different techniques [1]. Metal ions bound to DNA can influence its biological activity and affect its physico–chemical properties in solution.

In this letter we describe a novel application of spectroscopic techniques to demonstrate that Mn^{2+} and Ag^{+} ions bound to DNA in aqueous solutions at room temperature diffuse from base pair to base pair along the DNA helix. The technique involves the optical excitation of triplet excited states of polycyclic aromatic molecules or dyes which are physically intercalated between the base pairs of DNA. The lifetimes of these triplets, in the absence of dissolved oxygen, may be as long as 150 ms [2] but typically are in the range of 20–30 ms. These triplets are readily quenched by metal ions which are also bound to DNA. Using the aromatic dye acridine orange (phosphate/dye binding ratio > 100) we have observed that even small amounts of bound metal ions give rise to a uniform decrease in the lifetime of the triplets of all the DNA-bound aromatic molecules. This conclusion is based on the fact that the triplet decay curves are exponential in the absence as well as in the presence of low concentrations of metal ions, showing that all of the dye molecules are equally accessible to the mobile metal ion quenchers. The base pair to base pair hopping frequency is estimated to be $\gtrsim 10^5$ sec^{-1} for Mn^{2+} and $\gtrsim 5 \times 10^2$ sec^{-1} for Ag^{+} ions bound to DNA in aqueous solution at 25°C.

2. Experimental

Acridine Orange (AO), purchased from the National Aniline Division of Allied Chemical Corporation, was recrystallized from ethanol and used as the water soluble salt. Calf thymus DNA (Type I, Sigma Chemical Company, protein $\lesssim 2\%$) was dissolved in HMP buffer, pH 6.8, consisting of 0.005 M NaH_2PO_4 and 0.0025 M Na_2HPO_4. Typical DNA concentrations used in this study were 1–3 mM phosphate groups. The AO–DNA complexes were prepared by well-known procedures [3]. Since the AO/DNA phosphate ratio was always kept below 1:100, the AO molecules are bound to DNA by the intercalation mechanism [3].

The lifetimes of the triplets of AO bound to DNA are readily observed by two different techniques. One of these involves the flash spectroscopy technique in which the decay of the triplet–triplet absorption (following excitation of the singlet with a brief laser or photoflash) is monitored using photoelectric techniques [4]. This technique is most suitable for the observation of triplets of DNA-bound polycyclic aromatic hydrocarbons where the triplet decay is non-radiative [2]. The second technique involves the observation of the E-type delayed fluorescence [5] of the aromatic dyes bound to DNA. In aromatic dyes the energy difference between the triplet T_1 and fluorescence emitting singlet excited states, is comparable to thermal energies at ambient temperatures. Thus, thermally activated emission from T_1 to S_1 results in a delayed fluorescence whose decay time is characteristic of the triplet lifetime.

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The flash photolysis apparatus for triplet–triplet absorption studies was a conventional 50 joule unit (FP-2R, Northern Precision Ltd., London). The delayed fluorescence was excited with a repetitive (~ 10 Hz) microsecond Xenon flash lamp. The photoelectric signals in both types of experiments were fed to a multichannel analyzer and signal averaging was performed as necessary. The data was obtained in digital form and decay times, as well as the form of the decay, could be analyzed conveniently.

The AO–DNA samples in aqueous solutions were bubbled with oxygen-free nitrogen (< 0.5 ppm O_2) until a limiting triplet lifetime was obtained. Metal ions in appropriate amounts were added to these samples as $AgNO_3$ or $MnSO_4$ solutions.

3. Results

The basic results are shown in figs.1 and 2. Figure 1 shows that the flash spectroscopic triplet–triplet absorption and delayed fluorescence technique yield the same type of exponential decay with the same triplet lifetime, as expected. This demonstrates that

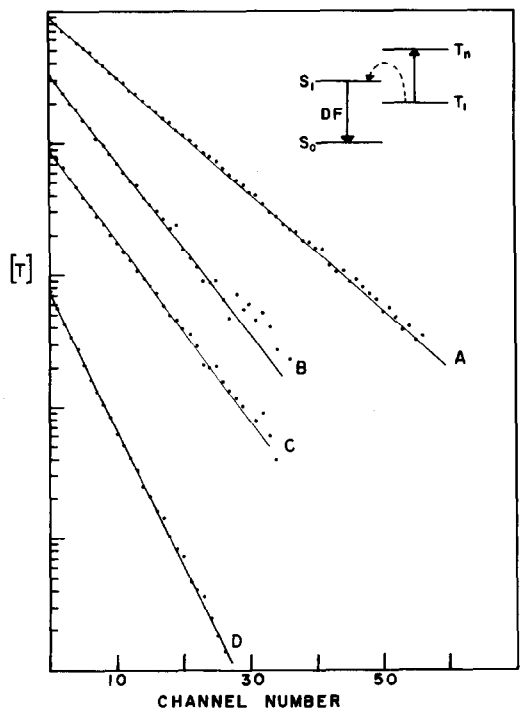

Fig.1. Triplet decay of acridine orange bound to DNA in aqueous buffer solution at 25°C. (A) In the absence of metal ions, measured by delayed fluorescence (3 ms/ch) (B) in the presence of Ag^+ (r = 0.07) measured by $T_1 \rightarrow T_n$ absorption monitored at 590 nm (2 ms/ch) (C) same sample as B measured by delayed fluorescence (2 ms/ch) (D) in the presence of Mn^{2+} (r = 0.0002) measured by delayed fluorescence (3 ms/ch). The data is taken directly from the output of the multichannel analyzer. The vertical scale represents the concentration of triplets as a function of time after the exciting flash. A simplified energy level diagram of acridine orange is provided in the insert showing the transitions involved in measuring the delayed fluorescence (DF) and the triplet–triplet ($T_1 \rightarrow T_n$) absorption. S_0 and S_1 . . . ground and first excited singlet; T_1 and T_n . . lowest and a higher excited triplet respectively.

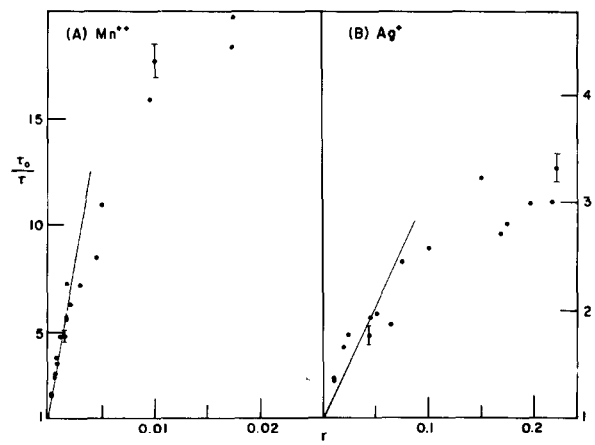

Fig.2. Ratio of unquenched to quenched triplet lifetime of acridine orange bound to DNA in aqueous buffer solution at 25°C versus r (mol metal ion/mol DNA phosphate) measured by delayed fluorescence. (A) Mn^+, (B)Ag^+.

either one or the other technique can be utilized in these experiments. Furthermore, the decay is exponential over at least three or more lifetimes both in the presence and in the absence of the metal ions.

Figure 2 shows that the decrease in the triplet lifetimes, at least in the low ranges of metal ion binding ratios r(r = metal ion added/DNA phosphate), follows a simple linear Stern-Volmer qeunching law [6].

$$\frac{\tau_0}{\tau} = 1 + p\tau_0 K\, C \qquad (1)$$

where τ_0 is the triplet lifetime in the absence of the

Table 1
Quenching of AO triplets by Mn^{2+}

	τ_0 (s × 10^3)	pK ($mole^{-1}$ liter s^{-1})
H_2O	0.89	2×10^5
DNA[a]–HMP[b]	30	7×10^7
DNA[a]–HMP[b] + 1 M NaCl	2.3	2×10^7

[a] [DNA phosphate] = 1.6×10^{-3}M
[b] HMP (0.005 M NaH_2PO_4 and 0.0025 M Na_2HPO_4 pH ~ 6.8)

metal ions and τ is the lifetime in the presence of the metal ions whose concentration is C. K is the quenching constant which is proportional to the rate of encounters between the acridine orange molecules and the metal ions and p ($\lesssim 1.0$) is the probability that a metal ion located next to a dye molecule will quench the triplet state.

The data in table 1 indicates that the quenching of the triplets of acridine orange by Mn^{2+} ions takes place with both the dye and the metal ion bound to the DNA macromolecule, rather than with both the unbound dye and the metal ion encountering each other in the solution. Table 1 shows the quenching effect of Mn^{2+} on the triplets of AO in (a) aqueous solution (without any DNA), (b) in a solution containing DNA, and (c) in solution containing DNA and NaCl. The efficiency of quenching in each case is expressed by the quenching constants pK in units of M^{-1} s^{-1} with C expressed in terms of the bulk concentration (Mol/l) of Mn^{2+} ions. Thus, the fact that the Mn^{2+} is actually bound to the DNA is not reflected in these calculations of C. We note that the quenching constant pK is approximately 350 greater for AO bound to DNA than for AO in an aqueous solution. Thus, the quenching is enhanced by the presence of DNA.

It is well known [7] that the binding of Mn^{2+} to DNA is reduced when sodium ions are also added to the solution and that the Mn^{2+} binding constant is a strong function of the NaCl concentration. It is shown in table 1 that the effective quenching ability of Mn^{2+} is reduced in the presence of 1 M NaCl, since fewer Mn^{2+} ions are bound to DNA in the presence of the sodium ions.

Furthermore, the triplet lifetime τ_0 of AO increases upon binding to DNA. This result, in addition to the enhanced quenching constants in the presence of DNA, and the effect of sodium ions on the quenching constant, show that the quenching of the triplets of AO by Mn^{2+} ions is in fact taking place within the DNA and not in the outer solution.

4. Discussion

The decay of the triplets is exponential both in the absence and in the presence of the metal ions. We thus conclude that all of the AO triplets are homogeneously quenched by the metal ions and thus all of the AO molecules are uniformly accessible to the metal ions, even at very low concentration of Mn^{2+} ions (fig.1, curve D).

This observation can be explained by two mechanisms; I: the metal ions are free to migrate along the length of the DNA molecule while the dye molecules are relatively immobile (ν(ion) >> ν(dye) where ν = velocity), and II: the dye molecules are moving with the DNA-bound metal ions relatively immobile (ν(dye) >> ν(ion)). It will now be shown that case I is the much more likely possibility than case II.

Kinetic studies of the interaction of AO with DNA have been carried out by Sakoda et al. [8] using the fluorescence stopped-flow technique. They show that the binding of AO to DNA involves a consecutive mechanism involving first the binding of AO of the outside of the DNA (rate constant k_1) followed by a slower reaction involving the intercalation of the dye between the base pairs of DNA. This consecutive intercalation mechanism is summarized by:

$$\mathrm{AO} + \mathrm{DNA} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (\mathrm{AO{-}DNA})_{\mathrm{outside}} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} (\mathrm{AO{-}DNA})_{\mathrm{intercalated}}.$$

At low DNA phosphate/AO ratios (< 10), AO dimer formation can also occur; however, this possibility need not be considered in our work because of the high DNA phosphate/AO ratio (~ 100) used here. Sakoda et al. find that $k_1 >> k_3$ and that $k_3 + k_4 \approx k_3 = 310\ s^{-1}$. From the work of Li and Crothers [9] we estimate that k_4 is at least ten times smaller than k_3 and thus $k_4 \lesssim 30\ s^{-1}$. Since $k_3 << k_2$ [8], the rate limiting step for the release of an intercalated AO molecule and its subsequent migration along the DNA is defined by the rate constant $k_4 \lesssim 30\ s^{-1}$. Since the binding constant K_b for intercalation is 10^5 [3], the AO molecule is expected to be bound again, almost immediately after its release. It is thus estimated that it can migrate at most only a few base pairs before being recaptured by the DNA. Since each AO molecule is released on the average only once very 30 ms ($k_4^{-1} \sim 0.03$ s) it appears highly unlikely that an AO molecule in its triplet excited state can travel over ~ 1000 base pairs in the case of the Mn^{2+}, or ~ 100 base pairs in the case of the Ag^+ within the triplet lifetime of ~ 30 ms. (In addition it should be noted that the triplet lifetime in the free solution is considerably shorter – see table 1). For example, in fig.1 (curve D) it is shown that the triplet decay of AO complexed to DNA is reduced to approximately one-half its value at a Mn^{2+} concentration corresponding to $r = 0.0002$ (r = metal ions added/DNA phosphate). Since the decay even at this low Mn^{2+} concentration (one Mn^{2+} ion per ~2500 base pairs) is completely exponential, each AO triplet must migrate by a metal ion at least once during its lifetime of ~30 ms. In view of the kinetic data on the rate of release of intercalated AO, this possibility seems to be highly unlikely. We thus conclude that our quenching data is much better explained in terms of case I, i.e. the velocity of the metal ions $v(\text{ion}) >> v(\text{dye})$ and that the dyes can be considered to be fixed on the kinetic time scale defined by the triplet lifetime of the acridine orange triplet. Our subsequent discussion will therefore be limited to case I.

The binding of Ag^+ and Mn^{2+} ions has been studied by a number of workers and both display complex binding equilibria and different types of binding depending on the experimental conditions. The binding of Ag^+ for $r < 0.1$ has been shown to be G–C site specific (type A) while for $r > 0.1$, Ag^+ ions are inserted between A–T base pairs with a concomitant release of a proton [10–13] (type B). Mn^{2+} ions are believed to bind primarily to the phosphate groups of DNA. More specifically there are indications that the Mn^{2+} ion tends to be chelated between the phosphate groups and a G–C pair, most likely at the N-7 position of guanine [7, 14–16]. At least two equilibrium constants are obtained for Mn^{2+} bound to DNA [15].

The Stern-Volmer relation for triplet quenching by metal ions (equation (1)) appears to hold only for relatively low ion concentrations; at higher r values a curvature in these plots can be observed. With Ag^+, the curvature appears to set in when type B binding becomes important, while for Mn^{2+} a curvature sets in when $r > 0.01$ or when at least 90% of the AO triplets are already quenched ($\tau_0/\tau \cong 10$). These effects might be due to a possible heterogeneity of bound dye molecules as well as to changes in the type of binding, each displaying slightly different p values. Thus we have limited our interpretations to quenching ranges corresponding to a ten-fold drop in the triplet lifetime in the case of Mn^{2+}, and to $r < 0.1$ in the case of Ag^{2+} when type A binding prevails.

The efficiency of the quenching by Mn^{2+} is particularly striking. Thus, the triplet lifetimes are homogeneously and uniformly shortened to one-half their value of $\tau_0 = 30$ ms for r values as low as 0.0002 (one Mn^{2+} per 2500 base pairs). Thus, the range of each Mn^{2+} ion is at least ~ 2500 base pairs within a time interval of 30 ms. If this were not the case and the mobility of the Mn^{2+} ions were restricted, the decay of the triplets would be non-exponential. Thus AO molecules bound close to Mn^{2+} ions would display a shorter decay time than those bound far away from the metal ions. In order to verify this hypothesis we have determined that the triplet decay rates are indeed non-exponential in the presence of Ag^+ and Mn^{2+} ions (at concentration $r > 0.01$) at low temperature (77°K) when the diffusion of the ions is restricted (to be published in detail elsewhere).

A lower bound of the velocity of migration of the metal ions along the length of the DNA molecule can be estimated using equation (1) and the data in fig.2. Referring to equation (1), if we express the concentration of bound metal ions as the number of ions bound per unit length of the DNA-helix (metal ion/cm), then K' (cm/sec) is the velocity corresponding to the motion of the metal ions along the DNA mole-

cule. p ($\lesssim 1.0$) is the probability that a metal ion located next to an intercalated dye molecule will quench the triplet state.

Using $C = 5.88 \times 10^7$ ion/cm for $r = 1$. We obtain the following values:

$$Mn^{2+}: (p)(K') = 2.2 \pm 0.4 \times 10^{-3} \text{ cm/s} \quad (2)$$

$$Ag^{+}: (p)(K') = 1.6 \pm 0.4 \times 10^{-5} \text{ cm/s} \quad (3)$$

Mn^{2+} ions are paramagnetic and display a quenching radius [17,18] with respect to triplets of aromatic molecules on the order of 10–20Å (spin conserving exchange interaction mechanism); however, the values of p cannot be estimated reliably from these data, particularly for the dyes bound to DNA. Keeping in mind, however, that $p = 1.0$ is an upper limit, the base pair–base pair hopping frequency of Mn^{2+} ions is estimated to be $\gtrsim 10^5$ sec^{-1}. Using the expression for a one-dimensional random walk diffusion coefficient D [19], we obtain $D(Mn^{2+}) \gtrsim 5.8 \times 10^{-11}$ cm^2/s.

The case of Ag^+, the triplet lifetime is decreased due to the heavy-atom external spin–orbit coupling effect [20] and it is again difficult to estimate values of p, but upper limits of the hopping frequency and diffusion coefficient can also be estimated. The hopping frequency is $\gtrsim 500$ sec^{-1} and the diffusion coefficient is $D \gtrsim 2.9 \times 10^{-13}$ cm^2/s. The diffusion coefficients of Mn^{2+} and Ag^+ ions in water are 0.35×10^{-5} and 1.33×10^{-5} cm^2 s^{-1} at 15°C [21]. The diffusion coefficient of these ions is reduced by 5–8 orders of magnitude upon binding to DNA.

We note that a much lower mobility is expected for the intercalated silver ions (binding constant $K_b \simeq 10^6$ M^{-1}) than for the less tightly bound Mn^{2+} ions ($K_b = 10^4$ M^{-1}). Our results are consistent with this view and indicate that the p values for Mn^{2+} and Ag^+ may not be too different.

We finally note that metal ions are present in cellular DNA in only trace amounts [22]. The mobility of these metal ions along nucleic acid chains could account for their profound biological effects even though they are present in extremely low concentrations.

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