Spin-Orbital Probes of Biomolecular Structure. A Model DNA-Acrifline System

(heavy atom effect/phosphorescence/proflavin/poly(dA-BrdU))

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ABSTRACT

Heavy atoms, such as bromine or iodine, perturb the excited-state properties of aromatic chromophores through a spin-orbital coupling mechanism. In the present work the use of specifically directed spin-orbital probes to study subtle structural relationships in biopolymers is described. Heavy atoms are introduced into defined sites in biochemical systems and the emission spectrum of a ligand or intrinsic chromophore is monitored for perturbation by the bound heavy atom. This technique is illustrated by a study of acridine dye binding to the copolymer poly(dA-BrdU). The results are interpreted in terms of an "externally" bound dye fraction whose emission is perturbed by the heavy atom in the polymer and an intercalated dye component unperturbed by bromine.

The perturbation of electronic excited-state properties of aromatic molecules through a spin-orbital coupling mechanism, mediated by solvent molecules possessing certain heavy atoms as constituents (the so-called "external heavy-atom effect"), was initially observed by Kasha (1). Since that time the influence of solvents containing heavy atoms on the spectroscopic properties of simple molecules has been studied in some detail (2-4). Experimentally, the most accessible manifestations of the external heavy-atom effect are decreases in fluorescence efficiency and phosphorescence lifetime, and an increase in the phosphorescence efficiency of the aromatic molecule. The first of these, fluorescence quenching, has been exploited in biochemical preparations by several investigators who have observed partial fluorescence quenching due to the exposure of a fraction of the emitting molecules to a solvent containing halide ion. This solvent perturbation approach has been used to estimate the number of tryptophyl residues exposed to solvent in proteins (5, 6), and it is capable of distinguishing free proflavin from the dye complexed with DNA (7). Perturbation of thymine phosphorescence by mercuric ion in stoichiometric mercury-polynucleotide complexes has also been reported (8).

In this article we wish to demonstrate how heavy atoms can be used in a more precise way as integrated probes of biomolecular structure. With a poly(dA-BrdU)-acridine preparation as an illustration, we show how specific introduction of a center of spin-orbital coupling into the biopolymer enables us to elucidate structural details of the dye binding. In this instance the uracil bromine atom, which acts as the perturbing center in the molecule, is substituted in place of the methyl group of thymine, the naturally occurring base in DNA.

The use of specifically directed spin-orbital labels is obviously extendable to other biochemical systems, for instance, interactions between enzyme and inhibitor and between substrate and coenzyme. The technique, we feel, has several attractive features: Heavy atoms such as the larger halogens, capable of producing recognizable external spin-orbital coupling perturbations, are easily incorporated into many biochemical systems. The technique is extremely sensitive to distance in that nonbonding interactions of heavy atoms and chromophores obtain only over distances of the order of van der Waal's radii. Finally, several sensitive methods are available for monitoring heavy-atom effects, including measurements of fluorescence and phosphorescence intensities and lifetimes.

EXPERIMENTAL DETAILS

The copolymers consisting of alternating adenine and 5-bromouracil deoxyribonucleotides, poly(dA-BrdU), and alternating adenine and thymine deoxyribonucleotides, poly(dA-dT), were purchased from General Biochemicals. Poly(dA-BrdU) was also prepared in this laboratory in a synthesis primed by poly(dA-dT) after the procedure of Riley and Paul (22), with Escherichia coli DNA polymerase generously provided by Dr. David Denhardt, Dept. of Biochemistry, McGill University. Proflavin was a K and K product. Acridine orange was obtained from Eastman. All other materials were of reagent grade. Samples consisted of 0.2-0.3 mM poly(dA-BrdU) in 0.01 M cacodylic buffer (pH 7.5) diluted 1:1 with an ethylene glycol or glycerol solution of dye. Ionic strengths were adjusted with sodium chloride. Spectra and lifetimes were recorded at 77°K on an instrument already described (23).

RESULTS

The changes in phosphorescence that characterize an external heavy-atom perturbation are exemplified by the influence of ethyl bromide on the phosphorescence of proflavin in propylene glycol at 77°K. The proflavin phosphorescence decay, normally exponential with a lifetime of 2.4 sec, becomes distinctly faster with the addition of the heavy-atom perturant. While the proflavin phosphorescence decay is no longer exponential, it can be represented as the sum of two exponential components (Fig. 1): an approximately normal-lived emission arising from unperturbed molecules in the system and a short-lived component with a lifetime of 0.6 sec. The observation that in a rigid medium the bromine perturbation manifests itself as an essentially two-state, all-or-none effect is typical for interactions that occur through wavefunction overlap over a rather critical interaction distance (9).

In addition to reducing the lifetime of the proflavin triplet,
the presence of 0.54 M ethyl bromide results in an enhancement of about 1.8-fold in the intensity of the proflavin phosphorescence, 51% of which is short-lived. Therefore, there is an enhancement of about 8-fold in the phosphorescence intensity for a bromine-perturbed proflavin molecule over an unperturbed molecule. If we assume (i) a random distribution of dye molecules in the rigid solution and (ii) the heavy-atom perturbation to a first approximation is an all-or-none effect, as suggested by the phosphorescence decay, the fraction of perturbed dye molecules is related to the concentration of the perturber species by the equation (10),

\[
\ln (1-P) = -\frac{4}{3} \tau r^2 N_0 M \times 10^{-3}
\]

Here \(P\) is the fraction of perturbed dye molecules, \(N_0\) is Avogadro's number, \(M\) is the molar concentration of the perturant, and \(r\) is the average radius of interaction between the perturbed dye molecule and a bromine atom. Knowing the phosphorescence enhancement factor for a perturbed dye molecule, \(P\) can be readily obtained from the percentage of perturbed short-lived intensity in the phosphorescence decay of the dye as a function of bromide concentration. Hence, a plot of \(\ln (1-P)\) against \(M\) yields \(r\) from the slope. Such a plot for proflavin is presented in Fig. 2, from which an average radius of interaction of 4.5 Å (45 nm) is obtained. This value is comparable to the sum of the van der Waals radii of bromine and proflavin (taken as a sphere) namely, 5.0 Å (30 nm). These results demonstrate the extreme short-range character of the heavy-atom effect from which its sensitivity as a structural probe is derived.

The ability of bromine to act as a spin-orbital probe of biomolecular structure can be demonstrated in the poly(dA-BrdU)-proflavin preparation. On binding to poly(dA-BrdU), the phosphorescence intensity of proflavin measured at 77 K is increased over the value obtained on binding of the dye to poly(dA-dT) under identical conditions. While the phosphorescence decay of proflavin bound to poly(dA-dT) is essentially unaltered from that observed for the free dye, the decay of proflavin bound to poly(dA-BrdU) is more rapid. Furthermore, the nonexponential decay for the phosphorescence of proflavin bound to poly(dA-BrdU) consists of two components, one with a lifetime of about 2 sec, close to that observed for poly(dA-dT)-bound dye, and a bromine-perturbed component with a lifetime of about 0.6 sec. About 30% of the total phosphorescence of proflavin bound to poly(dA-BrdU) for 5 \(\mu\)M proflavin at low ionic strength and a polymer-to-dye ratio of 20 possesses the 0.6-sec perturbed lifetime. This indicates, assuming that bound and free proflavin have comparable enhancement factors, that about 5% of the proflavin molecules are bound to poly(dA-BrdU) in such a way that they are in close contact with the bromine atoms at the uracil 5-positions of the polymer. Moreover, since it will be shown that the remaining, unperturbed proflavin molecules are also bound to the polynucleotide, the dye binding must be heterogeneous with the unperturbed dye molecules situated on the polymer at a distance greater than van der Waals' contact from the bromine atoms.

![Spin-Orbital Probe of Dye-Polynucleotide Complexes](2199)

**Table 1.** Proflavin phosphorescence in the presence of 0.14 mM poly(dA-BrdU)\(^*\)

<table>
<thead>
<tr>
<th>P/D</th>
<th>% Short-lived emission</th>
<th>Ionic strength</th>
<th>Solvent</th>
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<td>5</td>
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<td>EG-buffer</td>
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<td>0.01</td>
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<td>0.1</td>
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<tr>
<td>20</td>
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<td>1.0</td>
<td>G-buffer</td>
</tr>
</tbody>
</table>

\(P/D\), ratio of concentrations of poly(dA-BrdU) phosphate and dye; EG, ethylene glycol; G, glycerol. Buffer was 0.01 M cacodylic (pH 7.5). Ionic strengths were adjusted with NaCl.

\(^*\) Proflavin phosphorescence was excited at 436 nm and monitored at 570 nm.

**Fig. 1.** A semi-logarithmic plot of the phosphorescence intensity decay as a function of time for 1 mM proflavin in a propylene glycol solution containing 0.6 M ethyl bromide at 77 °K. The solid lines represent the resolution of the nonexponential emission decay into a sum of two exponential components. The exciting wavelength was 436 nm, and the proflavin emission decay was recorded at 570 nm.

**Fig. 2.** A plot of \(\log (1 - P)\) against \(M\), where \(P\) is the fraction of perturbed dye molecules and \(M\) is the molar concentration of ethyl bromide, is shown for 1 mM proflavin in a rigid propylene glycol solution at 77 °K.
Fig. 3. Model for proflavin intercalation between consecutive bases of the same polynucleotide strand of poly(dA-BrdU). Two adjacent base pairs in the double-stranded helix are shown in dotted and solid lines, while the dashed figure represents the dye molecule. Clearly, proflavin binding such as depicted here brings the dye molecule within van der Waal's contact of the bromine atom on the pyrimidine ring, whose van der Waal's diameter is outlined.

In view of the large body of evidence indicating that acridines form two distinct types of complexes with double-stranded polynucleotides (11-14), the proflavin-poly(dA-BrdU) preparation was investigated by varying theionic strength and polymer-to-dye ratio. Table 1 presents dye emission characteristics for proflavin bound to poly(dA-BrdU) at various polymer-to-dye ratios and ionic strengths. Table 1 shows that the fraction of perturbed proflavin phosphorescence increases with decreasing polymer-to-dye ratio, suggesting that the class of bound proflavin subject to the bromine heavy-atom effect has a larger dissociation constant than that for those bound dye molecules that are unperturbed. The fraction of perturbed dye phosphorescence also increases with decreasing ionic strength, which indicates that electrostatic forces are more important for the binding of proflavin molecules susceptible to bromine perturbation than for other types of dye binding.

Fig. 5. Model for the geometry of the weakly-bound proflavin-poly(dA-BrdU) complex. The dye ring nitrogen interacts with a phosphate group of the polymer backbone, while the dye molecule is oriented so as to overlap the pyrimidine directly beneath it, thereby coming into contact with the bromine atom at the uracil 8-position.

Given the extreme short distances over which the heavy atom effect persists, the perturbation of proflavin weakly bound to poly(dA-BrdU) and the concomitant failure of the strongly bound form of the dye to be perturbed by bromine place constraints on the geometry of both types of dye-polymer complex. These geometrical constraints comprise the most important information derivable from the technique described here and will be considered further in the Discussion.

Essential to the interpretation of the results presented in this paper is that all proflavin be bound to poly(dA-BrdU) under the described experimental conditions. A ready measure of the extent of dye binding to the polymer at 77°C is provided by the proflavin fluorescence generated by singlet-singlet energy transfer to the dye from poly(dA-BrdU). The quantity monitored is the ratio of proflavin fluorescence at 280 nm excitation, where both the dye and polymer absorb light, to the proflavin fluorescence obtained upon excitation in the visible region of the spectrum (404 nm), where only the dye absorbs. In a typical experiment, the intensity ratio of UV-to-visible induced proflavin fluorescence at 77°C for a sample containing 4 μM proflavin and 0.1 mM poly(dA-BrdU) at an ionic strength of 0.02 is 2.6 times the fluorescence intensity ratio observed for the dye alone. Furthermore, a 10-fold dilution of this same proflavin-poly(dA-BrdU) mixture leaves this intensity ratio unchanged, indicating that it is a limiting value and, therefore, that all of the proflavin is bound to the polymer under the conditions of the experiment.

The binding of the dye, acridine orange, to poly(dA-BrdU) has also been studied at polymer-to-dye ratios of 10 and 20. Acridine orange phosphorescence in the presence of poly(dA-BrdU) consists of two exponential components, the longer-lived of which exhibits the same lifetime as acridine orange bound to poly(dA-dT). Relatively more of the short-lived emission component is observed for acridine orange than for proflavin. However, the phosphorescence enhancement...
factor for bromine-perturbed acridine orange molecules is 1.8 times that for proflavin molecules. Allowing for this difference between the acridine orange and proflavin enhancement factors, under the assumption that the enhancement factors for bound and free dye are the same, we find that practically identical fractions of acridine orange and proflavin molecules bound to poly(dA-BrdU) are perturbed by bromine in comparable experiments.

DISCUSSION

The observation that a fraction of the acridine dye molecules bound to poly(dA-BrdU) exhibit a short-lived phosphorescence has been interpreted in terms of a two-state binding model featuring a strongly-bound dye fraction whose emission is unperturbed by the bromine atoms in the polymer and a weakly-bound, heavy-atom-perturbed dye fraction. The data in Table 1 are consistent with the two-state "external" and "intercalated" model for dye binding to double-stranded polynucleotides that is well documented in the literature (11-14). This first type of dye binding is thought to be an external, electrostatic binding process involving the phosphate groups along the backbone of the double-stranded helix. Characterized by a relatively large dissociation constant and a strong dependence on ionic strength, this "external" proflavin-polymer complex can be identified with the bromine-perturbed component in the proflavin phosphorescence decay. The second, "intercalated" mode of dye binding features a small dissociation constant and a reduced sensitivity to ionic strength, and it is considered to involve partial or whole insertion of planar dye molecules between consecutive base pairs. The proflavin fraction bound to poly(dA-BrdU) giving a long, unperturbed phosphorescence lifetime corresponds to this strongly bound, presumably intercalated form of the dye.

An alternating copolymer, such as poly(dA-BrdU) or poly(dA-dT), possesses two unique intercalation sites above and below a given base pair. However, preliminary measurements indicate that the dissociation constant ratio for the two types of dye-polymer binding monitored by the present technique is about 100, which is about the binding constant ratio observed for proflavin binding to DNA (15). This fact, together with the different ionic strength dependencies for the two types of dye-polymer binding observed in our experiments (Table 1), argues that the dye-binding heterogeneity disclosed by the spin-orbital probe technique corresponds to the two-state dye-polymer binding observed by other workers in several dye-polymer systems, as opposed to binding to the two distinct intercalation sites in alternating copolymers like poly(dA-BrdU).

The presence or absence of a heavy-atom perturbation by the uracil bromine atom in poly(dA-BrdU) on bound acridine dyes is dependent on the geometry of the complexes between the dye and the polymer. The data allow one to rule out some models for dye binding and to suggest others. Figs. 3-5 illustrate possible modes of dye binding to poly(dA-BrdU) based on the two-state "intercalated-external" model for the dye-polymer interaction. Note that the bromine atoms on the pyrimidines are directed into the major groove of the poly(dA-BrdU) double helix. Figs. 3-5 are drawn for only one of the two distinct binding sites between adjacent base pairs, assuming an unwinding angle of +12° upon intercalation of a dye molecule between the base pair planes. However, the generalizations made here concerning the dye binding are applicable to both intercalation sites in models proposing unwinding angles from -12° to +45° (16-18).

It has been suggested that intercalation might occur through insertion of a dye molecule between two consecutive bases of the same polynucleotide strand with the acridine ring nitrogen directed toward the near phosphate group of the sugar-phosphate backbone (19). Fig. 3 demonstrates that intercalation in this manner would bring the acridine molecule into van der Waal's contact with the bromine atom on the pyrimidine ring. Highly asymmetric dye binding of this type, at least in double-stranded polynucleotides, is precluded by our results. This conclusion is based on the assumption, of course, that dye binding to poly(dA-BrdU) is a reflection of the binding of double-stranded polynucleotides in general.

Two types of intercalation permitted by the bromine atom configuration in poly(dA-BrdU) find the acridine molecule bisected either down its width or, for certain unwinding angles, down its length by the 2-fold rotational axis, which would transform adjacent base pairs. Of these possibilities bisection down the width is the more reasonable model and places the dye molecule toward the minor groove of the double helix (Fig. 4). This mode of intercalation, first suggested by Lerman (18, 20), allows for extensive overlap between the base pairs and the planar acridine ring, while placing the dye molecule as far away as possible from the bromine atoms in the major groove. In Fig. 4, the bromine atoms are at least 2 Å beyond van der Waal's contact with the closest aromatic carbon on the acridine molecule. The second type of poly(dA-BrdU) intercalation site places the dye molecule somewhat closer to the bromine atom than does the intercalation site in Fig. 4, but still well beyond van der Waal's contact.

A recent model for proflavin binding to DNA proposed by Dalgleish et al. (14) portrays the acridine molecule intercalated at an angle between the base pairs of the polymer such that one of the dye rings is thrust toward the major groove of the double helix. This model would place the acridine much closer to the bromine atoms in poly(dA-BrdU) than the model illustrated in Fig. 4, but possibly still outside van der Waal's contact with the bromine atoms, depending on the polymer unwinding angle and the precise geometry of the dye-polymer complex.

Fig. 5 presents a model for the "external" mode of dye binding to poly(dA-BrdU). The acridine molecule in Fig. 5 is oriented so as to project its ring nitrogen toward the nearby phosphate group of the sugar-phosphate backbone, while remaining in contact with the bromine atom on the pyrimidine directly below the dye molecule. In essence, Fig. 5 illustrates the acridine molecule as partially overlapping the base pairs of the poly(dA-BrdU) copolymer, suggesting that the term "external" binding is a misnomer. This partially intercalated nature of the so-called "external" mode of dye binding to double-stranded polynucleotides has been discussed by other workers as well (21).

We feel that the use of other brominated polynucleotides in a more detailed study involving various ligands will lead to a more precise definition of the geometry of dye-nucleic acid complexes and hence to an understanding of the interactions involved in these complexes.

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