Criteria for the Mode of Binding of DNA Binding Agents

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Abstract—A complete characterization of DNA binding agents requires that their mode of binding to DNA be established. In the absence of high resolution structural data, the mode of binding is, of necessity, usually inferred indirectly from various solution studies. The purpose of this study is to show that only certain methods can be used reliably to infer the DNA binding mode. Comparative fluorescence and hydrodynamic studies using the proven intercalator ethidium and the groove binder Hoechst 33258 are described. The results of our studies show that while fluorescence intensity, polarization, and quenching measurements can detect a binding interaction of the ligand with DNA, none are sensitive indicators of the binding mode. Fluorescence contact energy transfer studies can reliably indicate intercalation, as can viscosity measurements. Our results illustrate reliable criteria that may be used to distinguish intercalation from groove binding in the absence of high resolution structural data.

Introduction

When investigating the properties of new DNA binding agents, one initial goal is to establish their mode of binding to DNA. For agents which bind to DNA noncovalently, intercalation or groove binding are the two most likely binding modes.12 The binding mode is most convincingly established by high resolution structural studies, using either X-ray diffraction methods or NMR. In the absence of such high resolution data, the mode of binding must be inferred from the results of solution studies. Possible criteria to be used in inferring the binding mode have been reviewed extensively.3-6 These criteria rest on fundamental structural differences resulting from intercalation and groove binding. Groove binding typically results in only subtle changes in structure, and the DNA remains essentially in an unperturbed 'B' form. In contrast, intercalation, in which a planar ligand moiety is inserted between adjacent base pairs, results in a substantial change in DNA structure, and causes lengthening, stiffening, and unwinding of the helix.7-9 These changes result in a pronounced alteration of the hydrodynamic properties of DNA for intercalation, but not for groove binding. Furthermore, for intercalation, the planar chromophore is in close contact with the DNA base pairs, and is oriented roughly perpendicular to the DNA helix axis. Thus, techniques that can evaluate the orientation of the ligand chromophore and its proximity to the DNA bases, like dichroism and fluorescence energy transfer methods, can potentially distinguish intercalation from groove binding.

In spite of well established criteria for distinguishing between intercalation and groove binding, considerable controversy can arise over the binding mode of new DNA binding agents. A recent example of such a controversy is the case of tris(phenanthroline)Ru(II).10-17 Such confusion over the binding mode may result when the sensitivity of particular physical methods to the key structural features that distinguish the different binding modes is not appreciated. The purpose of the studies described here is to illustrate reliable physical methods for distinguishing between intercalation and groove binding modes. Two DNA binding agents of proven binding modes were chosen for these studies. Ethidium is regarded as the standard intercalator, and its binding to DNA has been extensively studied.18-20 Hoechst 33258 is a known groove binding agent for which high resolution structural data exists.21-23 Our studies show that standard fluorescence methods such as intensity measurements, polarization, and solute quenching studies can reliably detect an interaction of both compounds with DNA, but that they are of no use in distinguishing the different modes of binding. In contrast, both fluorescence contact energy transfer studies24,25 and relative viscosity studies26 can clearly distinguish intercalation from groove binding. Our results, we hope, will serve as a convenient guide for the choice of methods to use when attempting to establish the binding mode of new DNA binding agents.

Results and Discussion

Fluorescence excitation and emission spectra for ethidium and Hoechst 33258 in the presence and absence of DNA are shown in Figure 1. Upon binding to DNA, both the intercalator and the groove binder show a red shift in their excitation spectra, and both exhibit enhanced fluorescence emission. The position of the maximum in the excitation spectrum of ethidium in the visible region shifts by approximately 40 nm (Fig. 1a). The position of ethidium's emission spectrum is unaffected by DNA binding, as expected, but there is a dramatic increase in the intensity of emitted light. The ratio of fluorescence emission intensity of the bound to the free form is 24 ± 3. The excitation and emission
spectra of Hoechst 33258 are less complicated than those observed for ethidium. The maximum in the Hoechst 33258 excitation spectrum shifts to the red by about 20 nm upon DNA binding. Fluorescence emission of Hoechst is dramatically enhanced, with a ratio of $F_b/F_f = 140 \pm 10$.

![Figure 1](image1.png)

**Figure 1.** Corrected fluorescence excitation and emission spectra of ethidium bromide (A) and Hoechst 33258 (B) obtained in the absence (-----) or in the presence (——) of excess calf thymus DNA. The scales of the y-axis were normalized by dividing by the fluorescence intensity at the peak maximum for the excitation and emission spectra. For both ethidium bromide and Hoechst 33258, a red shift in the excitation spectra is observed upon DNA binding. A shift of 40 nm is observed for ethidium, while a shift of 20 nm is observed for Hoechst 33258. No shift is observed in the emission spectra for either ligand. Fluorescence emission is greatly enhanced for both ethidium and Hoechst 33258 upon DNA binding. The ratios of fluorescence intensity of bound to free are 24 and 140 for ethidium and Hoechst 33258, respectively.

The spectral changes seen in Figure 1 arise from changes in the environment of the fluorophores upon binding to DNA. A detailed analysis and explanation of the observed spectral shifts and fluorescence enhancements can be complicated, but has been attempted for both ethidium and Hoechst 33258. What is clear, however, from the data of Figure 1 is that while the observed spectral changes provide a convenient means of monitoring the DNA binding reaction, they are of little use in distinguishing the DNA binding mode. Both the intercalator ethidium and the groove binder Hoechst 33258 show qualitatively the same behavior, a red shift in the excitation spectra and a dramatic enhancement in fluorescence emission.

Steady-state fluorescence polarization measurements monitor the rotational diffusion of fluorophores. When a fluorescent small ligand binds to a macromolecule, substantial changes in its polarization results, providing a convenient means of detecting and quantifying the association reaction. Excitation and emission polarization spectra for ethidium and Hoechst 33258 bound to DNA are shown in Figure 2. These spectra reveal rather subtle information about the angular orientation of the absorption and emission dipoles of the fluorophore. We are more concerned, however, with the changes in polarization values observed in the absence and presence of DNA. In the absence of DNA, ethidium shows a polarization of $> 0.03$ at 480 nm in BPE buffer at 20 °C. In Figure 2B, the polarization of ethidium is observed to increase to an average value of 0.28 in the presence of DNA. Ethidium is thus immobilized when bound to DNA. When bound to DNA, Hoechst 33258 shows an average polarization value of 0.38, slightly higher than ethidium. The key point revealed by the data of Figure 2 is that both the intercalator and the groove binder are immobilized upon complexation with DNA, with a concomitant increase in their polarization. DNA binding limits the rotational freedom of both ethidium and Hoechst 33258, and steady state polarization values can monitor DNA binding, but cannot alone distinguish the binding mode.

![Figure 2](image2.png)

**Figure 2.** Fluorescence polarization spectra of ethidium bromide (A) and Hoechst 33258 (B) bound to calf thymus DNA. Polarization excitation spectra are shown as solid lines. Polarization emission spectra are shown as dotted lines.

Solute fluorescence quenching provides a method for determining the accessibility of a fluorophore to an added quenching agent. The method is potentially useful for obtaining topographic information about the bound fluorophore. At first glance, it would seem to be an ideal technique for distinguishing between intercalation and groove binding, since in the former the fluorophore is buried deep within the DNA helix, and in the latter might be more exposed to the
surrounding solvent. This expectation is not borne out. The results of quenching experiments in which KI was used to probe the accessibility of free and bound ethidium and Hoechst 33258 are shown in Figure 3. Free ethidium is seen to be quenched by iodide with a quenching constant, $K_{sv}$, of 1.5 M$^{-1}$ in Figure 3A. Since the lifetime of free ethidium is 1.8 ns, this value of $K_{sv}$ arises from a bimolecular quenching constant, $k_q$, of $0.9 \times 10^9$ M$^{-1}$ s$^{-1}$, a value approaching that expected for a diffusion controlled encounter. When bound to DNA, $K_{sv}$ is essentially zero, indicating that intercalated ethidium is essentially inaccessible to added quencher, as expected, since it is buried deep within the DNA helix. Hoechst 33258 quenching is shown in Figure 3B. The slope in the initial region of the curve corresponds to $K_{sv} = 3.5$ M$^{-1}$, leading to an estimate of $k_q = 1.5 \times 10^9$ M$^{-1}$ s$^{-1}$ (assuming a lifetime of 2.8 ns for free Hoechst). Again, this corresponds closely to the expected value for a diffusion controlled collision between quencher and fluorophore. Figure 3B shows that bound Hoechst is, like ethidium, essentially completely inaccessible to added iodide, with $K_{sv} = 0$.

The results of fluorescence contact energy transfer experiments are shown in Figure 4. Fluorescence resonance energy transfer can occur between an acceptor and donor pair when certain conditions are met. First, there must be spectral overlap between the donor and acceptor. Second, the donor and acceptor must be within a certain distance of one another, and their dipoles oriented in a particular fashion. Le Pecq and Paoletti have shown that intercalators fulfill these criteria, and that the energy of UV absorbance by DNA base pairs may be efficiently transferred to an intercalated fluorophore. A practical result of this is that new bands appear in the excitation spectrum of the fluorophore in the spectral region where the DNA bases absorb. The form of the plot shown in Figure 4 is designed to emphasize such an alteration in the fluorophore excitation spectrum. In Figure 4, it can be seen that bound ethidium shows excitation near 260 nm, where the absorbance of DNA base pairs is maximal, resulting from fluorescence resonance energy transfer. In contrast, groove bound Hoechst 33258 shows no such energy transfer, since the efficiency of transfer is not great because of the greater distance between the base pairs and Hoechst, and because the orientation of dipoles is not optimal for transfer. Contact energy transfer thus reveals the close proximity of bound intercalator to the base pairs, and effectively distinguishes between intercalation and groove binding modes. Contact energy transfer measurements have proven to be a valuable tool to establish an intercalative binding mode in a number of recent cases.

A key feature of the classic intercalation model first proposed by Lerman is the lengthening of the DNA helix as the base pairs are separated to bind the ligand. Hydrodynamic methods sensitive to length changes...
have historically been among the most stringent tests of the binding mode of DNA binding agents. Either sedimentation or viscosity may be used to monitor such length changes of rodlike DNA. Viscosity changes offer several experimental advantages. First, viscosity is proportional to $L^2$ for rod-like DNA of length $L$, whereas the sedimentation coefficient is directly proportional to $L$. Viscosity is thus more sensitive to length changes than is sedimentation. Second, viscosity measurements are simply made with inexpensive apparatus, whereas sedimentation requires an analytical ultracentrifuge. The results obtained for viscosity measurements of DNA with increasing amounts of bound ethidium or Hoechst 33258 are shown in Figure 5. As expected, the intercalator dramatically increases the length of DNA, resulting in an increased viscosity. The groove binder, in contrast, does not lengthen the DNA helix, and does not increase the viscosity of DNA solutions. Viscosity provides a simple, theoretically sound means of distinguishing DNA binding mode.

![Figure 5. The relative specific viscosity of calf thymus DNA in the presence of ethidium bromide (squares) or Hoechst 33258 (triangles), shown as a function of the binding ratio.](image)

**Summary and Conclusions**

These comparative studies illustrate experimental methods that are useful in distinguishing the binding mode of DNA binding agents. The classic intercalation model predicts that the bound ligand will lengthen and unwind the DNA helix, and that the ligand will be in close proximity to the DNA base pairs. Viscosity measurements will sensitively detect the former, while fluorescence contact energy transfer experiments will sensitively detect the latter. Other fluorescence techniques (intensity measurements, polarization, and external quenching) can detect that DNA binding occurs, but provide no information whatsoever about the mode of binding.

**Experimental**

**Materials**

Calf thymus DNA was purchased from Boehringer Mannheim (Indianapolis, IN) and was prepared by the procedures previously described. DNA samples were dialyzed extensively against the appropriate buffer solutions prior to all experiments. BPE buffer consists of 6 mM Na$_2$HPO$_4$, 2 mM NaH$_2$PO$_4$, 1 mM Na$_2$EDTA, pH 7.0; BPES buffer is BPE + 0.185 M NaCl. DNA concentrations were estimated from absorbance measurements at 260 nm, assuming an extinction coefficient of 12,824 M$^{-1}$ cm$^{-1}$. Ethidium bromide was purchased from Sigma Chemical Co. (St Louis, MO). Hoechst 33258 was purchased from Molecular Probes, Inc. (Eugene, OR). Solutions of ethidium bromide and Hoechst 33258 were prepared in doubly distilled water at approximately 1 mg mL$^{-1}$ and stored in the dark at 4 °C. Concentrations were determined by absorbance measurements, using the extinction coefficients of 5600 M$^{-1}$ cm$^{-1}$ (480 nm) for ethidium bromide and 42,000 M$^{-1}$ cm$^{-1}$ (338 nm) for Hoechst 33258. Solutions of Hoechst 33258 were carefully handled to minimize adsorption, following the procedures suggested by Lootiens et al.

**Absorbance and fluorescence spectra measurements**

Absorbance spectra were measured using a Cary 3E UV/vis spectrophotometer (Varian, Inc.; Palo Alto, CA). Fluorescence measurements were performed with a Greg 200 photon counting fluorometer (ISS, Inc.; Champaign, IL), interfaced to and controlled by a Northgate 486 personal computer. Corrected excitation and emission spectra of ethidium bromide and Hoechst 33258 were obtained both in buffer alone and in the presence of excess calf thymus DNA, in order to obtain spectra for the free and bound forms of the ligand. For ethidium, excitation spectra were collected with $\lambda_{ex} = 605$ nm, while emission spectra were collected with $\lambda_{em} = 480$ nm. For Hoechst 33258, excitation spectra were collected with $\lambda_{ex} = 465$ nm, while emission spectra were collected with $\lambda_{em} = 342$ nm. All measurements were made at 20 °C.

**Fluorescence polarization**

Steady state fluorescence polarization spectra were measured automatically using acquisition and analysis software available with the Greg 200 fluorometer. An excitation polarization spectrum requires the acquisition of four different excitation spectra, each obtained at one of the four combinations of orientations of the excitation and emission polarizers. The steady state fluorescence polarization, $p$, may then be calculated at each wavelength by the expression

$$p = (I_v/I_h - I_h/I_v) / (I_v/I_h + I_h/I_v),$$

where the subscripts refer to the positions of polarizers for excitation and emission (v: vertical, h: horizontal). Equation (1) includes the necessary $G$ factor for the correction of polarization. Emission (or excitation) wavelengths were set as described above for the collection of polarization excitation (or emission) spectra for each ligand.
**Fluorescence quenching**

Fluorescence quenching experiments were conducted for ethidium bromide and Hoechst 33258 both in the absence and presence of calf thymus DNA. Fluorescence intensity was monitored as a function of added quencher (KI). Data were cast into the form of Stern–Volmer plots, according to the equation:

$$\frac{F}{F_0} = 1 + K_{sv}[Q], \quad (2)$$

where $F_0$ is the fluorescence intensity in the absence of added quencher, $F$ is the fluorescence intensity at a molar quenching concentration $[Q]$, and $K_{sv}$ is the Stern–Volmer quenching constant. In all quenching experiments, the total monovalent cation concentration was kept constant by the addition of appropriate amounts of KCl. Quenching of ligand–DNA complexes were studied at a binding ratio 0.02 ligand:bp in order to insure that ligand was fully bound.

**Fluorescence contact energy transfer**

Contact energy transfer from DNA bases and bound ligand was measured from corrected excitation spectra recorded from 240 to 350 nm at 1 nm intervals. The ratio between the quantum yield of bound ligand with excitation in the UV spectral region ($Q_\lambda$) to that at 310 nm ($Q_{310}$) was calculated from the expression

$$\frac{Q_\lambda}{Q_{310}} = \frac{I_\lambda E_{310}}{I_{310} E_\lambda} = \frac{I_{310} E_\lambda}{I_\lambda E_{310}} b f$$

where $I$ and $E$ are, respectively, the measured fluorescence and molar extinction coefficient at wavelengths $\lambda$ and 310 nm, and the subscripts b and f refer to the bound and free forms of the ligand.23 The wavelength 310 nm was chosen for the normalization because of the negligible absorbance of DNA in that region of the spectrum. Excitation spectra were corrected for the inner filter effect prior to normalization.24

**Viscosity experiments**

Viscosity experiments used an Ostward-type viscometer, immersed in a thermostatted water bath maintained at 27 °C. Titrations of ligand, ethidium bromide or Hoechst 33258, were performed by the addition of small volumes of concentrated stock solutions to the DNA sample in the viscometer. Solutions in the viscometer were mixed by bubbling nitrogen through the solution. DNA concentrations of approximately 1 mM (in base pairs) were used in viscosity experiments. Relative viscosities for DNA in either the presence or absence of ligand were calculated from the relation

$$\eta = \frac{(t-t_0)/t_0}{}$$

where $t$ is the observed flow time of the DNA containing solution, and $t_0$ is the flow time of buffer alone. Viscosity data were plotted as $(\eta/\eta_0)^{1/3}$ versus the binding ratio $r$, according to the theory of Cohen and Eisenberg.26

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**References**

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