Equilibrium Analysis of Ethidium Binding to DNA Containing Base Mismatches and Branches†

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ABSTRACT: In the processes of DNA replication, recombination, and repair, duplex DNA can transiently form branched structures, such as Holliday junctions, as well as base pair mismatches and bulges. These states have altered ligand and protein binding properties from normal double helical DNA. A variety of ligands have been reported to interact more tightly at branches and bulges than to normal duplex sites. The stoichiometry, structural basis, and thermodynamics of this effect have not been determined. We have investigated the binding of the intercalator, ethidium bromide, to several DNA constructs including base mismatches, bulges, and three- and four-arm branched structures, using chemical footprinting, titration calorimetry, and fluorescence lifetime measurements. Two classes of binding sites are detected in three- and four-arm junctions in our high ionic strength conditions: one class is characterized by a small number of ligands (2–4 per DNA), with high binding affinity ($K > 10^5$), and the second by a larger number of sites (10–12 per DNA) with lower affinity ($K \sim 10^4$). By use of appropriate control experiments, the former appear to be associated with sites at or near the branch point or mismatch, while the latter are consistent with binding to the normal duplex DNA region(s) of the molecule. Titration calorimetry indicates an enthalpy of −10 to −13 kcal/mol for binding of ethidium to a mismatch or three- and four-arm branch point. The tight binding class is associated with a fluorescence lifetime of 12–16 ns, distinct from that of free ethidium (ca. 2 ns) and the longer lifetime observed for ethidium intercalated in duplex DNA (22–26 ns).

The conformational manifold of duplex DNA includes many conformations outside the B helix that have functional importance. Branches, in which three or four duplexes intersect at a point, can arise in duplex DNA as a result of recombination, as in Holliday junctions (Kallenbach & Zhong, 1994), or repair of damaged sequences. Defects including bulges, internal loops, or base pair mismatches can occur as a consequence of chemical or physical damage to DNA, and particularly as intermediates in replication or recombination of sequences containing multiple short repeats (Gaillard & Strauss, 1994). The transient existence of nonclassical structural states in DNA raises the question of whether these states are preferential targets for interaction of drugs such as intercalators with DNA (Lu et al., 1992a), and how drugs might influence the recognition of branches by proteins such as resolvases or HMG proteins (Bianchi et al., 1989; Duckett et al., 1992). Intercalators include a variety of planar ring containing molecules capable of inserting between adjacent base pairs of duplex DNA (Wang, 1992). Examples include several important antitumor agents, such as daunomycin, transcriptional inhibitors such as actinomycin D, and ethidium bromide, which has been extensively studied as a model because of its favorable spectroscopic (Nelson, 1990) and nonaggregating properties. Ethidium is widely used as a stain for DNA, being strongly fluorescent when bound to duplex DNA, and weakly fluorescent in the free state (Lepecq & Paoletti, 1967).

Footprinting experiments using the reactive ethidium analog methidium–iron–EDTA, MPE·Fe(II) (van Dyke & Dervan, 1983), have revealed enhanced scission by this probe at sites near the branch point in DNAs containing three and four arms (Lu et al., 1992a). This enhanced reactivity has been attributed to increased affinity of the ligand in the vicinity of the branch. However, the stoichiometry of this interaction has not been determined, nor have quantitative values of the binding constant been reported. Thus one does not know whether the increased reactivity of MPE·Fe(II) corresponds to a difference in affinity or stoichiometry of binding, or possibly both. In this work, we approach this problem in two ways. First, titration calorimetry is used to estimate the affinity and stoichiometry of ethidium binding to fully duplex DNA 16mer controls, and a series of two- to four-stranded complexes containing base mismatches, branches, or both. Sequences of these molecules are shown in Figure 1. The results show that 2–4 ethidium ions associate more tightly in branched structures than to normal duplexes. In a junction lacking mismatches, these bind about 10-fold more tightly at 25 °C than to duplex DNA. In the presence of a mismatch at the branch, the interaction can increase its affinity by more than a hundred times. The thermodynamics of these sites indicate a favorable enthalpy of association in the case of normal duplexes, branches, or base mismatches, with a value from −10 to −13 kcal/mol at 25 °C. The tighter class observed differs more in entropy values than in the enthalpies of binding. Time resolved fluorescence spectroscopy resolves two classes of binding sites for ethidium in DNA containing mismatches and/or branched structures: one associated with a lifetime that is shorter (12–16 ns) than the value usually observed for intercalation into duplex DNA, 26 ns (Olmsted & Kearns 1977; Reinhardt & Krugh, 1978). We hypothesize that this lifetime corresponds to the tighter class of sites in branched DNA.

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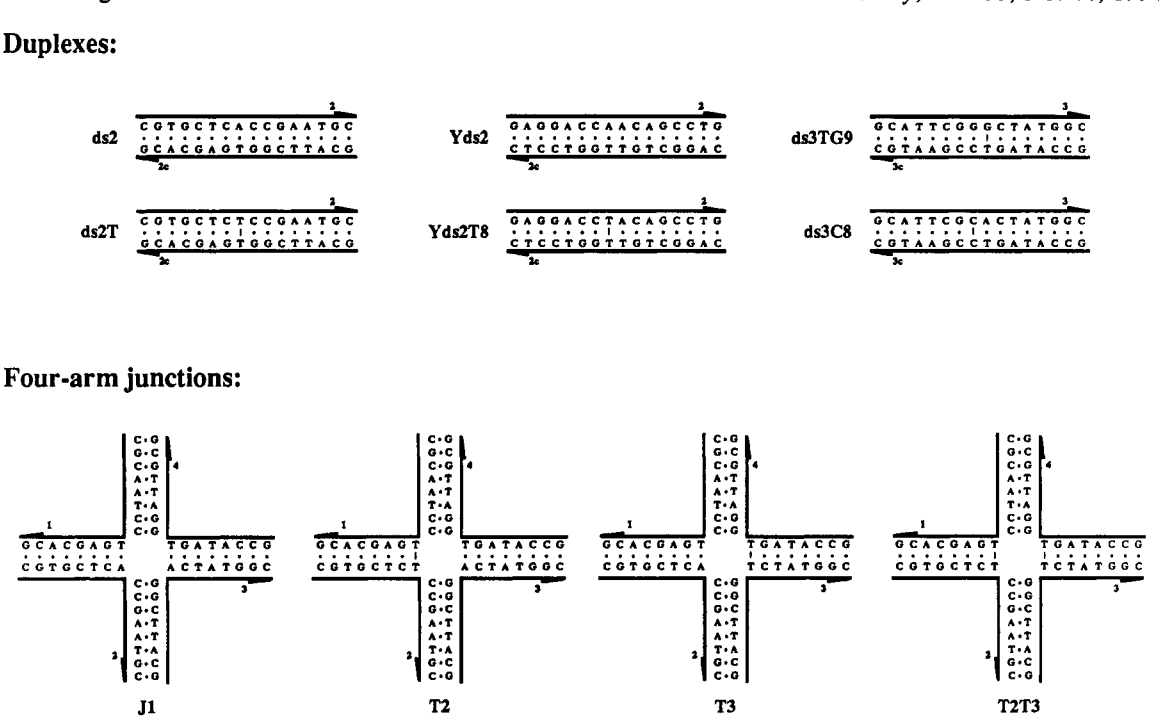

FIGURE 1: Sequences and schematic representations of the DNA molecules used in this study. The 3′ ends of the strands are indicated by half-arrowheads. The strand numbering indicated in this figure is used throughout the text.

MATERIALS AND METHODS

Synthesis and Purification of Oligonucleotides. Oligonucleotides used in this study were synthesized on an automated DNA synthesizer, deprotected by routine phosphoramidite procedures (Caruthers, 1982), and purified by ion exchange HPLC or polyacrylamide gel electrophoresis. Oligonucleotide strands were 5′-terminal labeled using bacteriophage T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ-^{32}P]ATP. The labeled strands were then purified by polyacrylamide gel electrophoresis. Strand concentration in stock solutions was determined spectrophotometrically at 260 nm and 80 °C, using the nearest-neighbor values of Cantor et al. (1970).

Annealing Reactions. Stoichiometric concentrations of the appropriate DNA strands were mixed in either 50 mM Tris·HCl (pH 7.5) with 10 mM $MgCl_2$ or 20 mM sodium cacodylate (pH 7.0) with 100 mM NaCl and 1 mM $MgCl_2$, heated to 90 °C for 2 min, and cooled slowly to room temperature to allow annealing. The solutions were finally chilled and stored at 4 °C.

Osmium Tetroxide Reaction. DNA samples (10 μM in molecules) in 50 mM Tris·HCl (pH 7.5) with 10 mM $MgCl_2$ were incubated with or without equimolar ethidium bromide at 4 °C for 20 min and then exposed to 1 mM osmium tetroxide (OsO_4) and 3% pyridine at 4 °C for 10 min (Lilley & Palecek, 1984). Reactions were stopped by two sequential ethanol precipitations, and then lyophilized. The DNA molecules were cleaved at the site of reaction by treatment with 1 M piperidine at 90 °C for 30 min and lyophilized. The reaction products were run on 20% polyacrylamide/7 M urea gels at 40 °C. Gels were dried and exposed to X-ray film at −80 °C using an intensifying screen. Autoradiograms were scanned on a Hoefer GS300 densitometer, without base-line corrections.

Titration Calorimetry. Experiments were carried out in an OMEGA titration calorimeter from Microcal Inc. (Wiseman et al., 1989). Each sample was titrated with a concentrated standard drug solution and mixed by rotating the

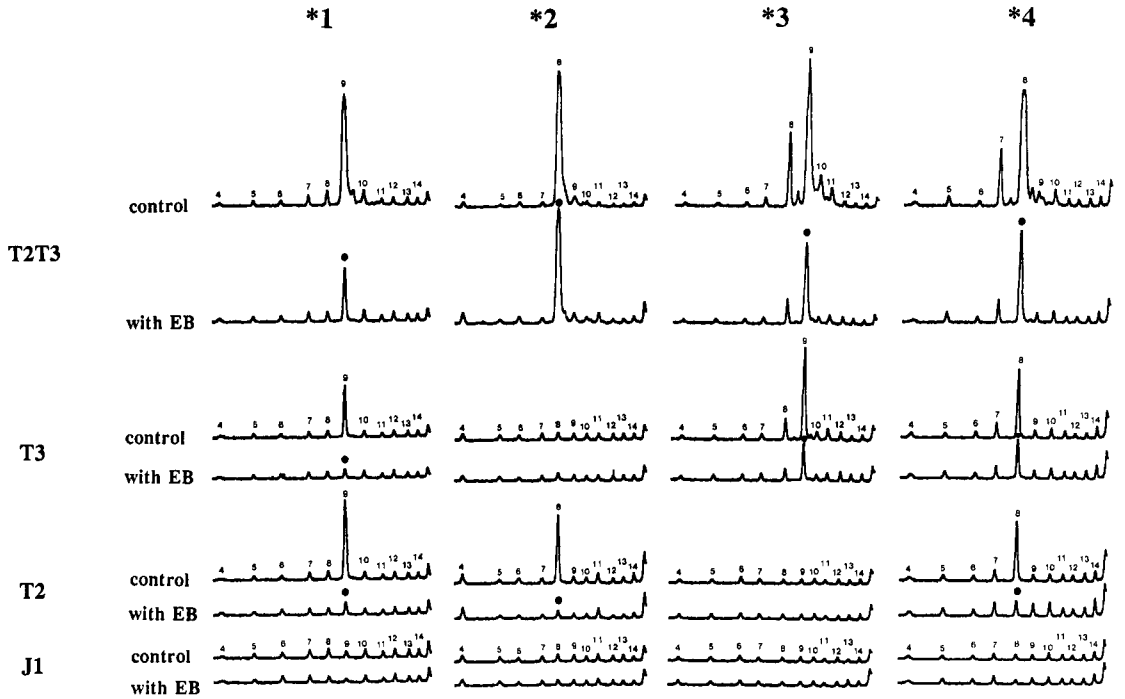

FIGURE 2: Densitometric scans of the cleavage patterns of four arm DNA junctions exposed to OsO_4–piperidine in the presence and absence of ethidium bromide. The reactions were carried out as described under Materials and Methods. Each panel of this figure corresponds to the specified strand of the junctions. Sites of differential protection in the presence of ethidium bromide relative to controls are indicated by filled circles.

syringe at 400 rpm. Typically, about 10 μM (in molecules, not strands) samples of DNA in 1.4 mL of 20 mM sodium cacodylate, 100 mM NaCl, and 1 mM $MgCl_2$ (pH 7.0) were titrated by 20–30 injections of 5–7 μL each of 1–3 mM ethidium bromide in the same buffer solution at 20 °C. The instrument was calibrated by means of a standard electric pulse. The stoichiometry, molar binding enthalpies, and binding constants were determined from the nonlinear fit of the experimental data (differential heat versus total concentration of added ethidium). The data were fitted to a single type of independent sites for the duplexes (Wiseman et al., 1989), and to two types of independent sites for the branched molecules (Lin et al., 1991).

Fluorescence Lifetime Measurements. Time domain fluorescence lifetime measurements were carried out using a time correlated single photon counting system. Samples typically contained 15 μM DNA (measured in units of duplex or complex) and 3 μM ethidium bromide in 20 mM sodium cacodylate buffer with 100 mM NaCl and 1 mM $MgCl_2$. The excitation wavelength of 330 nm was derived from a mode locked Nd:YAG laser synchronously pumping a dye laser. The dye laser was cavity dumped and frequency doubled using a lithium iodate crystal. The fluorescence was collected at 604 nm and detected using a Hamamatsu Model R2809U multichannel plate photomultiplier. Typical instrument response functions are 60 ps FWHM.

RESULTS

Mapping Tight Ethidium Binding Sites in a Four-Arm DNA Junction with a Base Mismatch Flanking the Branch. The base pairs flanking the branch point in immobile four arm DNA junctions have been found to be insensitive at low temperatures to chemical probes for bases in single strand conformations (Lilley & Palecek, 1984; Kallenbach & Zhong, 1994). By contrast, in four-arm DNA junctions containing one or two T·T mismatches flanking the branch [the sequences are illustrated in Figure 1, Zhong et al. (1992)], the mismatched thymines flanking the branch are reactive to osmium tetroxide, a chemical agent which modifies unpaired thymines, but not those base paired in duplex DNA (Lilley & Palecek, 1984). This observation provides us a potential method for locating sites of tight binding of ethidium in these DNA molecules in the sequence. We carried out osmium tetroxide–piperidine footprinting of the four arm junctions shown in Figure 1, in the presence and absence of equimolar concentrations of ethidium bromide (Figure 2). The results reveal a clear effect on the reactivity of OsO_4 at the sites of T·T mismatching adjacent to the branch, indicated as positions 7–9 in the sequences in Figure 1. This is consistent with the idea that, at low concentration, interaction of ethidium at or very close to the branch itself inhibits OsO_4 reactivity. Absence of this response at these positions in the strands making up the intact junction J1 or the strands lacking mismatches shows that differences are seen only in the presence of a mismatched T.

Titration Calorimetry of Ethidium Interaction with Duplexes and Other DNA Structures. Calorimetric titrations were carried out in order to determine the heat and affinity of ethidium binding directly. A typical titration curve obtained with the OMEGA unit as ethidium bromide is added to T·T mismatched four-arm junction T2T3 is shown in Figure 3A. Prior to saturation, the heats released in the initial injections for a particular type of binding site are independent of the total concentration of added ligand and can be used directly to estimate the molar binding enthalpy (ΔH). After correction for the heat of dilution of the ligand, measured in a separate experiment, ΔH is calculated by averaging the first three to four injections. Typical calorimetric binding isotherms are shown in Figure 3B. Upon saturation of the sites, the heat signal is reduced according to the equilibrium process(es) pertaining to the reaction, allowing determination of the binding constant. Values for the binding constant (K), molar binding enthalpy (ΔH), and stoichiometry of the complex can all be estimated from a nonlinear fit of the initial phase and

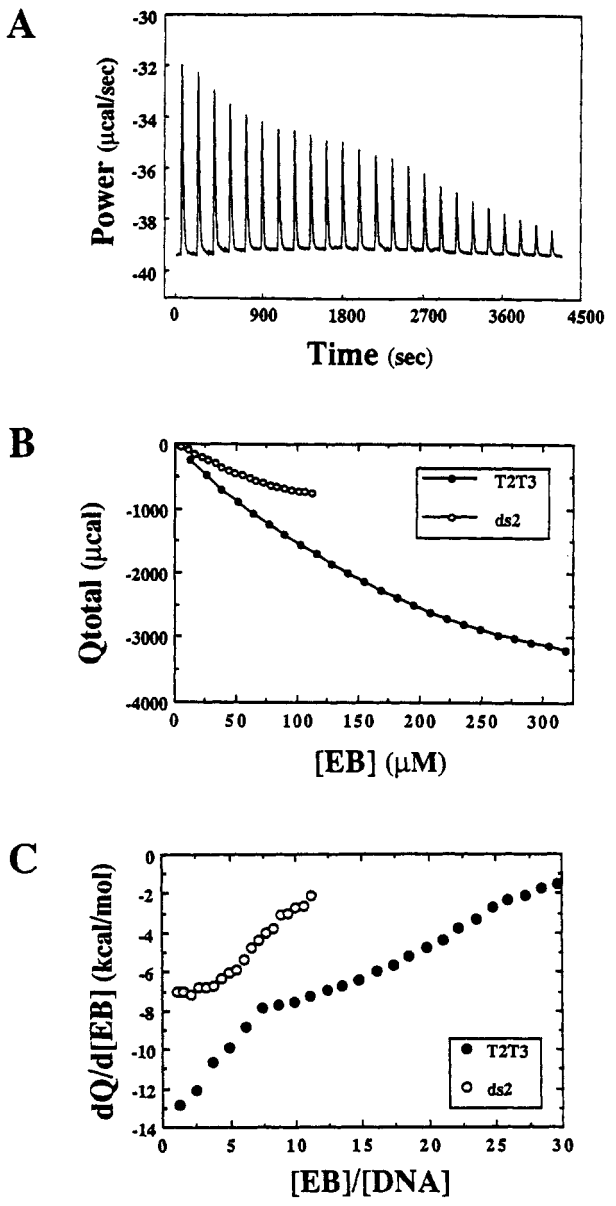

FIGURE 3: Analyses of ethidium binding by titration calorimetry. (A) Typical calorimetric titration curve of T·T mismatched four-arm DNA junction T2T3 with ethidium bromide (EB). The measurements were done in 20 mM sodium cacodylate buffer (pH 7.0), 100 mM NaCl, and 1 mM $MgCl_2$ at 20 °C. Each peak corresponds to a 6 μL injection of 2.94 mM ethidium bromide solution to 10.2 μM T2T3 (1.4 mL) in the cell. (B) Binding isotherms of ethidium bromide to T2T3 (●) and the control duplex ds2 (○). The resulting fitting curves are shown with solid lines. (C) Dependence of the differential heat on the [EB]/[DNA] ratio for the T2T3 (●) and the control duplex ds2 (○).

the enthalpically detected binding isotherm such as in Figure 3B. Panel C in Figure 3 illustrates the results of plotting the derivative of the heat profiles in Figure 3B. This plot makes it clear that two processes are present in T2T3: one is the tight binding process involving the first EB molecules, while the second resembles the binding seen in a duplex control, ds2.

Inspection of the data on duplexes in Tables 1 and 2 reveals that interaction of ethidium with the single T·T mismatch in the 16mers ds2T is not resolved as a distinct process; the mismatched duplexes cannot be distinguished from the fully paired ones. Ethidium binding to a DNA duplex is not strongly sequence dependent, and the average affinity of 8.5×10^4 and enthalpy of ca. −9 kcal/mol are in the range of previous

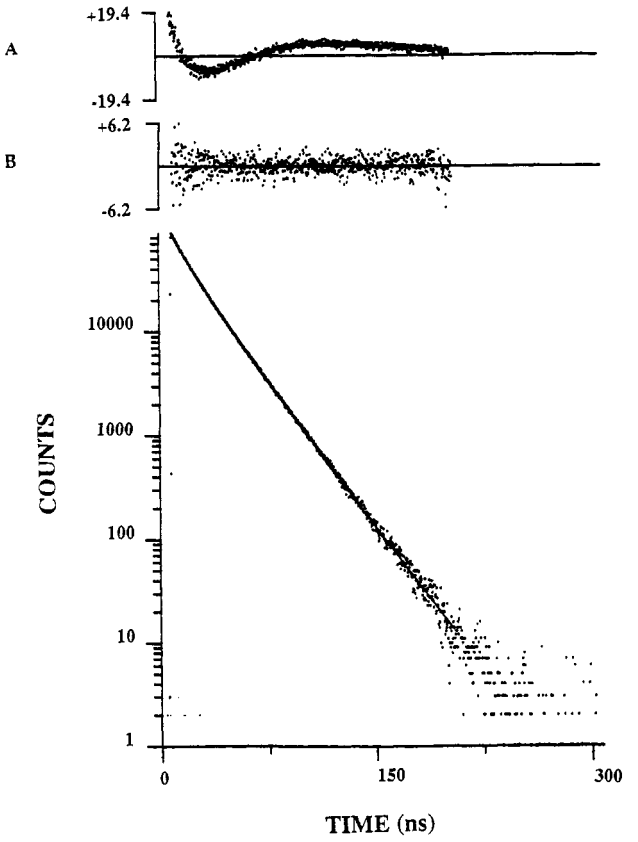

FIGURE 4: Fluorescence lifetime decay curve of ethidium bromide bound to three-arm DNA junction Y1. (A) Residuals from fit to a single exponential lifetime ($\chi^2 = 11.7$). (B) Residuals from fit to two lifetimes ($\chi^2 = 1.5$). The sample was excited at 330 nm, and emission was observed at 604 nm.

estimates of the van't Hoff heats of ethidium binding to oligonucleotide duplexes (Nelson & Tinoco, 1984). In the four-arm junctions shown, however, two classes of sites are resolved, and a mismatch near the branch can be seen to have a strong effect. A set of 3–4 ethidium molecules interact with the junctions in a binding process that is about 10-fold tighter in the four arm junction, J1, than in the larger set of weaker sites which we believe correspond to the duplex sites in the arms. The latter have a mean affinity of 4×10^4, close to the values previously determined for oligonucleotide DNA duplexes. In the junctions, one or two mismatches flanking the branch are seen to exert a dramatic effect on ethidium binding. In the presence of T·T mismatches, the stoichiometry is close to 4, with the tighter binding constant increasing to the upper limit of values that can be measured with the OMEGA apparatus (10^7–10^8) , about 3 orders of magnitude tighter than binding to normal duplex DNA and about a hundred times tighter than the stronger sites in J1. The effect in footprinting assays is obvious (Zhong et al., 1992), but previously could not be expressed quantitatively. The difference can now be seen to arise from increased affinity of the ligand, rather than a change in the stoichiometry of binding, although the latter may increase slightly, as seen in Table 1.

The interaction of ethidium with four-arm junctions differs from that in any of the three-arm junctions shown in Figure 1, as summarized by the data in Table 2. In three-arm junctions, as in J1, the tighter binding process is only about an order of magnitude stronger than the weaker background and remains so even in the presence of mismatches at the branch, with the exception of YT9. Fewer dye molecules appear to be involved in the tighter complex also, more nearly 2 than 4.

Table 1: Thermodynamic Parameters for EB Binding to DNA Duplexes and Four-Arm Junctions at 20 °C[a]

sample	n^b	K (M^{-1})	$\Delta G°$ (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	std deviation of the fit
ds2	8.3 ± 0.8	1.3 ± 10^5	−6.9 ± 0.3	−7.6 ± 0.4	−0.7 ± 0.1	3.4
ds2T	7.6 ± 0.7	8.5×10^4	−6.6 ± 0.3	−8.9 ± 0.4	−2.3 ± 0.3	1.1
J1	2.7 ± 0.1	6.4×10^5	−7.8 ± 0.4	−9.3 ± 0.5	−1.5 ± 0.2	7.4
	15.8 ± 1.5	4.8×10^4	−6.3 ± 0.3	−9.3 ± 0.5	−3.0 ± 0.3	
T2	3.7 ± 0.3	5.0×10^{7c}	−10.3 ± 0.5	−10.2 ± 0.5	+0.1 ± 0.03	9.2
	14.2 ± 1.4	3.6×10^4	−6.1 ± 0.3	−10.1 ± 0.5	−4.0 ± 0.4	
T3	4.1 ± 0.4	9.4×10^{7c}	−10.7 ± 0.5	−11.0 ± 0.6	−0.3 ± 0.1	6.8
	13.6 ± 1.3	3.2×10^4	−6.0 ± 0.3	−10.7 ± 0.5	−4.7 ± 0.5	
T2T3	4.1 ± 0.4	4.0×10^{8c}	−11.5 ± 0.6	−13.5 ± 0.7	−2.0 ± 0.2	5.4
	19.3 ± 1.9	3.1×10^4	−6.0 ± 0.3	−11.0 ± 0.6	−5.0 ± 0.5	

[a] All measurements were done in 20 mM sodium cacodylate buffer containing 100 mM NaCl and 1 mM $MgCl_2$ at pH 7.0. Sequences of the indicated DNAs are shown in Figure 1. [b] Number of binding site(s) per duplex or per DNA junction molecule. [c] These values are near the upper limits of binding constants that can be determined under our experimental conditions; with 10 μM DNA, these values are less accurate than the other constraints in this table.

Table 2: Thermodynamic Parameters for EB Binding to DNA Duplexes and Three-Arm Junctions at 20 °C[a]

sample	n^b	K (M^{-1})	$\Delta G°$ (kcal/mol)	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	std deviation of the fit
Yds2	8.7 ± 0.8	6.7×10^4	−6.5 ± 0.3	−7.8 ± 0.4	−1.3 ± 0.2	4.2
Yds2T	6.2 ± 0.6	6.1×10^4	−6.4 ± 0.3	−11.9 ± 0.6	−5.5 ± 0.6	8.9
Y1	2.1 ± 0.2	1.4×10^5	−6.9 ± 0.3	−10.5 ± 0.5	−3.6 ± 0.4	5.7
	9.8 ± 1.0	1.7×10^4	−5.7 ± 0.3	−10.7 ± 0.5	−5.0 ± 0.5	
YT8	2.2 ± 0.2	5.0×10^5	−6.3 ± 0.3	−11.4 ± 0.6	−5.1 ± 0.5	6.0
	9.7 ± 1.0	1.9×10^4	−5.7 ± 0.3	−11.0 ± 0.6	−5.3 ± 0.5	
YT9	2.2 ± 0.2	3.1×10^6	−8.7 ± 0.4	−11.3 ± 0.6	−2.6 ± 0.3	7.8
	9.6 ± 0.9	2.0×10^4	−5.8 ± 0.3	−11.0 ± 0.6	−4.2 ± 0.4	
YT8T9	2.1 ± 0.2	1.7×10^5	−7.0 ± 0.4	−11.4 ± 0.6	−4.4 ± 0.5	3.8
	9.9 ± 1.0	2.1×10^4	−5.7 ± 0.3	−11.0 ± 0.6	−5.3 ± 0.5	

[a] All measurements were done in 20 mM sodium cacodylate buffer containing 100 mM NaCl and 1 mM $MgCl_2$ at pH 7.0. Sequences of the indicated DNAs are shown in Figure 1. [b] Number of binding site(s) per duplex or per DNA junction molecule.

Table 3: Fluorescence Decay Lifetimes for EB Binding to DNA Duplexes and Their Mismatched Analogs at 20 °C[a]

molecule	pre-exponential factor (%)[b]	lifetime (ns)[c]	molecule	pre-exponential factor (%)[b]	lifetime (ns)[c]
Yds2	15.7	13.3	ds2T	19.0	16.0
	85.3	23.3		81.0	26.3
Yds2T8	24.6	19.7	ds3TG9	10.2	11.3
	75.4	28.7		89.8	24.2
ds2	11.5	11.2	ds3C8	34.0	13.3
	88.5	23.8		66.0	25.1

[a] All measurements were done in 20 mM sodium cacodylate buffer containing 100 mM NaCl and 1 mM $MgCl_2$ at pH 7.0. [b,c] The standard errors of the fit for the pre-exponential factor and lifetime values were estimated to be [b]±10.0% and [c]±1.4 ns, respectively.

Table 4: Fluorescence Decay Lifetimes for EB Binding to Three-Arm DNA Junctions at 20 °C[a]

molecule	pre-exponential factor (%)[b]	lifetime (ns)[c]	molecule	pre-exponential factor (%)[b]	lifetime (ns)[c]
Y1	40.1	13.2	NC	37.0	14
	59.9	23.0		63.0	24.3
YT8	37.8	13.4	JL1	34.4	14
	62.2	22.3		65.6	23.5
YT9	38.1	11.9	JL2	25.5	12.3
	61.9	22.2		74.5	23
YT8T9	30.9	13.9	JL3	27.6	12
	69.1	23.3		72.4	22.9

[a] All measurements were done in 20 mM sodium cacodylate buffer containing 100 mM NaCl and 1 mM $MgCl_2$ at pH 7.0. [b,c] The standard errors of the fit for the pre-exponential factor and lifetime values were estimated to be [b]±10.0% and [c]±1.4 ns, respectively.

Time Resolved Lifetime Analysis of Ethidium Fluorescence in Complexes with Different DNA Structures. Fluorescence lifetime measurements of ethidium are useful in the study of differential binding because they are responsive to the local environment of the bound dye molecules. Ethidium is susceptible to quenching when it is free in solution, and its singlet state lifetime is correspondingly short (2 ns; Olmstead & Kearns, 1977). By contrast, when ethidium is intercalated in duplex DNA, it becomes insensitive to quenchers and is characterized by a much longer singlet lifetime (26 ns; Olmsted & Kearns, 1977; Reinhardt & Krugh, 1978), consistent with the enhanced quantum yield (LePecq & Paoletti, 1967). Figure 4 shows a fluorescence decay profile for ethidium complexed to a branched DNA molecule. In binding to DNA molecules containing an "open" structure, such as a branch point (Tables 4 and 5), bulge, or unstable species of base mismatch (Table 3), a complex with a "short" lifetime component is detected (12–16 ns) in addition to the 22–26 ns lifetime observed for binding to normal duplex DNA. This short lifetime is also present in decay curves of linear molecules, but only as a minor component. This might be the result of ethidium association at sites near the frayed ends of the molecule where increased solvent exposure is possible. In general, the T·T mismatched four-arm DNA junctions have slightly higher pre-exponential factors for the short lifetime complex than three-arm junctions, the former averaging 54.9% compared to 40.6% for the latter. This observation is qualitatively consistent with the calorimetric data, which show a higher binding affinity for the tight binding class in four arm junctions than in three-arm junctions.

DISCUSSION

Selective effects in the interaction of ligands with DNA are of interest in terms of the biological role of these molecules. The existence of tight binding sites for ethidium and several other ligands in the vicinity of a branch or base mismatch in

Table 5: Fluorescence Decay Lifetime for EB Binding to Four-Arm DNA Junctions at 20 °C[a]

molecule	pre-exponential factor (%)[b]	lifetime (ns)[c]	molecule	pre-exponential factor (%)[b]	lifetime (ns)[c]
J1	11.7	13.4	T3	51.6	15.7
	88.3	24.0		48.4	24.8
J1 (w/o Mg^{2+})	19.0	13.4	T2T3	44.6	13.5
	81.0	24.0		55.4	22.6
T2	68.4	15.9			
	31.6	24.8			

[a] All measurements were done in 20 mM sodium cacodylate buffer containing 100 mM NaCl and 1 mM $MgCl_2$ at pH 7.0. [b,c] The standard errors of the fit for the pre-exponential factor and lifetime values were estimated to be [b]±10.0% and [c]±1.4 ns, respectively.

DNA has been established from footprinting experiments (Lu et al., 1992a; Zhong et al., 1992, 1993). Little information is available concerning quantitative aspects of the interaction. Qualitatively, for example, one or more mismatches flanking the branch lead to strong enhancement in the reactivity of Dervan's reagent, MPE·Fe(II), relative to a junction without mismatch (Zhong et al., 1992, 1993). The experiments reported here allow us now to address quantitative issues concerning the stoichiometry and affinity of binding of ethidium to DNAs containing branches and base mismatches. In agreement with the observation of enhanced reactivity of MPE·Fe(II), two classes of binding site in these DNAs can be demonstrated by titration calorimetry and fluorescence lifetime analysis. The calorimetric data show that one class of sites binds 2–4 ethidium molecules per DNA, with an affinity from 10 to 10^2 times stronger than to duplex DNA. The thermodynamic parameters of the interactions seen in Tables 1 and 2 do not allow us to conclude that the binding to either a mismatch or branch is more exothermic than binding to a normal duplex. Previous estimates for the enthalpy of ethidium binding to poly[d(AT)] (Marky & Macgregor, 1990) are close to the (global) average of the values shown in Tables 1 and 2 for a duplex or for the weaker binding mode in branched structures: -10.2 ± 0.3 kcal/mol. The data in Tables 1 and 2 show that the main difference between the two sites in branched DNAs is in the lower magnitude of $T\Delta S$ rather than ΔH. The thermodynamics of branch formation indicate the presence of large effects due to ion binding and solvation (Lu et al., 1992b). In the absence of Mg^{2+}, junctions assume a different structure that is much less stable than the one formed with Mg^{2+} (Cooper & Hagerman, 1989). If the tighter class of bound positive ethidium ions associate in the vicinity of the branch, it is not unreasonable to suppose that this represents a distinctive electrostatic and solvent environment from that of sites along the arms or in normal duplexes.

The footprint experiment shown in Figure 2 shows that, at a ratio of 1 ethidium per tetraplex, and 10 μM DNA concentration, the reactivity of OsO_4 for T's near the branch in junctions containing mismatches adjacent to the branch is reduced. This suggests that at least part of the tight class of binding sites seen in Table 1 is located within one residue of the branch. The result does not show that this is the exclusive region involved. However, the relatively small number of ethidium molecules in the tight class makes it unlikely that its DNA target site will be dispersed over a significant fraction of the sequence available. It is surprising, in view of the tighter binding of EB to T2T3 than T2, that the protection in Figure 2 indicates an opposite effect: scission of T2 is blocked more clearly than in T2T3. While we did not attempt to quantitate the amounts of reagents in the experiments precisely, the reversal of order seems very clear, suggesting in fact that the complexes in the two cases may differ in the binding of EB. That is, a minor difference in orientation between the drug molecules occupying the branch site in these complexes could readily explain the difference in OsO_4 reactivity that is seen.

It is interesting that, of the branched complexes, only the four-arm junction J1 lacks a large pre-exponential factor for the short lifetime component in the presence of Mg^{2+}, yet interacts strongly with ethidium. We attribute the low level of this component seen in J1 (12%) to the ends, as in the analogous duplex ds2 (Table 3). This is supported by control experiments in which the fluorescence lifetime of ethidium bound to shorter duplexes than ds2—an 8mer and a 10mer—was determined. The relative fraction of the faster component increases as the length of the duplex decreases (data not shown).

In each of the other branched structures (Figure 1, Tables 4 and 5), there is a significant population of the 13 ± 1 ns component present, which appears to involve ethidium in a more open complex than formed on normal intercalation in a duplex. The possibility that the shorter lifetime reflects resonant interactions between ethidium rings in close proximity to each other at the branch is unlikely in view of the fact that ethidium interacts tightly with J1, while the complex in this case shows no evidence of the shorter lifetime component above the level present in the duplex control. On the other hand, bases at the branch in three-arm junctions or "necks" with short duplexes emerging (Figure 1) are accessible to chemical and enzymatic probes of single strand structure (Zhong & Kallenbach, 1993), as are those at the branch in four-arm junctions with mismatches flanking the branch (Zhong et al., 1992). We therefore favor the hypothesis that the shorter lifetime represents a more solvent accessible binding mode than a traditional intercalation site in a duplex. In this connection, we note also that, as seen in Table 3, the ethidium complex with the less stable C·C mismatch has a higher population of the shorter lifetime state than those with the more stable T·G or T·T mismatches. Since the argument is by analogy, we do not consider the case proven. Equating the preferential site(s) of MPE reactivity with the tighter binding class of ethidium sites and the faster bound state lifetime of ethidium is also uncertain. This idea forms a simple working hypothesis for further analysis of these complexes. The structural basis of the tight binding interaction remains unknown, as does how the ethidium molecules which occupy this class of sites are oriented with respect to each other and the adjacent bases, or the extent of their effect on the local solvent and ionic environment. These aspects remain to be determined.

In conclusion, we find that the tighter binding mode in the four-arm junction lacking base mismatches involves 2–3 drug molecules interacting with an affinity about 10-fold tighter than normal DNA duplex binding in oligonucleotides of similar size. The tighter binding complex differs from the weaker one in its entropy rather than enthalpy of association and, from the fluorescence lifetime measurements, appears also to occur in a unique DNA environment. Mismatches adjacent to the branch enhance the affinity of binding strongly, by more than 2 orders of magnitude. In the latter case, the stoichiometry is closer to 4 ethidium molecules per DNA, and this represents a process that might be significant biologically, or in terms of ethidium binding to rRNA (White & Draper, 1987, 1989), where imperfect junctions are not an uncommon structural feature.

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