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Hydration Changes for DNA Intercalation Reactions

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Abstract: The hydration changes that accompany the DNA binding of five intercalators (ethidium, propidium, proflavine, daunomycin, and 7-aminoactinomycin D) were measured by the osmotic stress method with use of the osmolytes betaine, sucrose, and triethylene glycol. Water *uptake* was found to accompany complex formation for all intercalators except ethidium. The difference in the number of bound water molecules between the complex and the free reactants (Δn_w) was different for each intercalator. The values found for Δn_w were the following: propidium, +6; daunomycin, +18; proflavine, +30; and 7-aminoactinomycin D, +32. For ethidium binding to DNA a value of $\Delta n_w = +0.25(\pm 0.3)$ was found, indicating that within experimental error no water was released or taken up upon complex formation. Intercalation association constants measured in D_2O were found to increase relative to values measured in H_2O for all compounds except ethidium. A positive correlation between the ratio of binding constants (K_{D_2O}/K_{H_2O}) and Δn_w was found. These combined studies identify water as an important thermodynamic participant in the formation of certain intercalation complexes.

Introduction

Water is an integral part of DNA structure.[1−6] At least two hydration layers surround duplex DNA, the first of which consists of about 20 water molecules per nucleotide. Recent studies have shown that water and cations may bind in complicated, specific ways to particular DNA sequences.[7,8] Hydration plays an important role in both the binding affinity and specificity of protein−DNA interactions.[9−14] The role of water in the binding of small ligands to DNA, in contrast, is poorly characterized. For improved drug design, it is essential to clarify how water molecules participate in ligand−DNA interactions.

The osmotic stress method[15,16] has been widely used to evaluate the participation of water molecules in many biochemical reactions. In one version of the osmotic stress method, neutral solutes or cosolvents are added directly to the solution

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containing the macromolecules and ligands being studied, thereby altering water activity in the solution. These added osmolytes are assumed not interact with any of the reactants under study, an assumption that may be verified by using a variety of neutral solutes whose size and physicochemical properties differ.

"Osmotic stress", "preferential hydration", and "crowding" are all closely related phenomena, and their exact relationships are the subject of some debate.[17,18] In our recent study of crowding effects on triplex and duplex melting,[19] we were able to clearly distinguish crowding effects of added solutes from effects on water activity by using cosolutes with differing molar volumes. Small cosolutes of the type used in the present study were found to exert little, if any, crowding effects.[19]

The osmotic stress method was used to study the coupled hydration change in the binding of a netropsin analogue to DNA.[20] The surprising finding in that study was that interaction of this groove binder with DNA was accompanied by the net *uptake* of 50−60 water molecules. The osmotic stress method was recently used to study hydration changes for the intercalation of ethidium and daunomycin.[21] No change in hydration was found for the DNA binding of the simple interacalator, ethidium, but significant water *uptake* was found to accompany the binding of the more complex intercalator daunomycin, which contains not only an intercalating moiety but also a carbohydrate substituent that binds in the minor groove.

The present study builds on the preliminary results obtained for ethidium and daunomycin with use of the osmotic stress method. Sucrose, betaine, and triethylene glycol were used as osmolytes. These three osmolytes were chosen because they were recently used to study water release in DNA duplex and triplex melting reactions[19] and in the interaction of the *gal* repressor with DNA.[12] These three osmolytes vary greatly in their affects on solution dielectric constant, viscosity, and density,[10,14] and therefore offer a system in which the effect of changes in water activity can be clearly distinguished from these other physicochemical properties. Sucrose and betaine were reported to be excluded from the surface of DNA, an important prerequisite for their use in the osmotic stress strategy.[14] In this report, hydration changes that accompanied the DNA binding of five known intercalators (ethidium, propidium, proflavine, daunomycin, and 7-aminoactinomycin D) were explored. The chemical structures of these intercalators are shown in Figure 1. These intercalators differ both in size and in the complexity of their structures. Their structural differences result in DNA binding site sizes ranging from 2 bp (ethidium) to 4−5 bp (actinomcyin D).[22] The hydration changes upon ligand−DNA complex formation were found to differ for each compound studied. Except for ethidium, DNA binding affinity was found to *decrease* with decreasing water activity, indicating that water is taken up upon complex formation. Participation of water in intercalation reactions was confirmed independently by binding studies in D_2O. D_2O hydrogen bond strength is increased relative to H_2O.[23−25] If water binds specifically to DNA intercalation

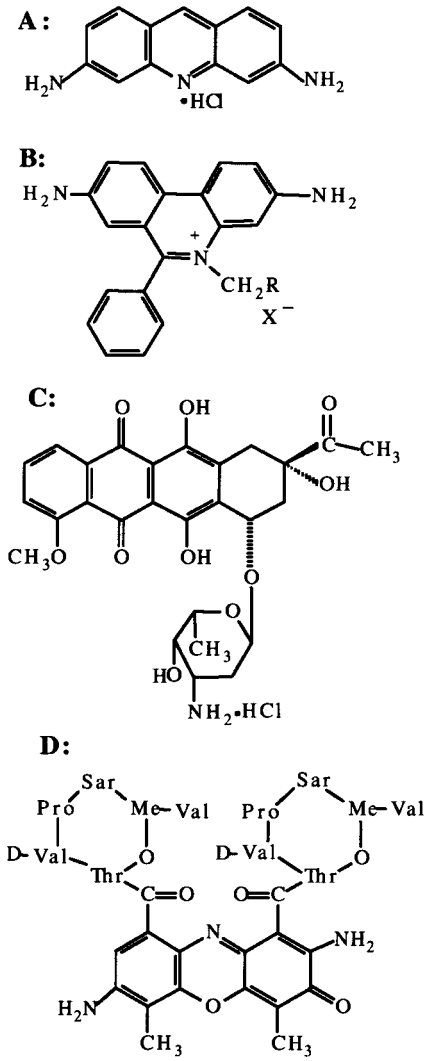

Figure 1. Chemical structures of the five intercalators studied: (a) proflavine hydrochloride; (b) ethidium bromide (R = CH_3; X = Br) and propidium iodide (R = $CH_2CH_2^+N(CH_2CH_3)_2CH_3$; X = 2I); (c) daunomycin hydrochloride; and . (d) 7-aminoactinomycin D.

complexes, substitution of D_2O for H_2O ought to increase the apparent ligand−DNA binding constant. Such an isotope effect was in fact observed. A linear correlation was found between the increase in the magnitude of ligand−DNA binding constant in D_2O and the difference in the number of bound water molecules between the complex and the free reactants, Δn_w measured by the osmotic stress method. Our combined studies clearly identify water as an hitherto unappreciated participant in intercalation reactions.

Materials and Methods

Ethidium bromide (Lot No. E−8751), daunomycin hydrochloride (Lot No. 108H1301), proflavine hydrochloride (Lot No. 50H3527), and propidium iodide (Lot No. P-4170) were obtained from Sigma Chemical Co. (St. Louis, MO) and were used without further purification. 7-Aminoactinomycin D (Lot No. 4981-2) was purchased from Molecular Probes (Eugene, Oregon). The ligand concentrations were determined by measuring their visible absorption and using the following molar extinction coefficients (with units of $cm^{-1}\ M^{-1}$): $\epsilon_{480} = 5600$ (ethidium); $\epsilon_{480} = 11500$ (daunomycin); $\epsilon_{444} = 38900$ (proflavine); $\epsilon_{493} = 5900$ (propidium); and $\epsilon_{505} = 21900$ (7-aminoactinomycin D). Sucrose (Lot No. 147816A, RNase free) was obtained from BIO-RAD (Hercules, CA). Betaine monohydrate (Lot No. 97H3349, purity >99%)

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and deuterium oxide (Lot No. D-4501, 99.8 at. % D) were purchased from Sigma Chemical Co. (St. Louis, MO). Triethylene glycol (Lot No. 112-27-6, purity >99%) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, MI). The above chemicals were used without further purification.

Calf thymus DNA (Lot No. 27-4562-02) was purchased from Pharmacia. The DNA was sonicated and purified as described earlier.[26] Before further use, the DNA was dialyzed against BPES buffer consisting of 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 0.185 M NaCl at pH 7.0 in the absence or presence of different concentration of an osmolyte (sucrose, betaine, or triethylene glycol) for 24 h. The DNA used for comparison of ligand binding affinity in H_2O and in D_2O was dialyzed against BPE buffer consisting of 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 1 mM Na_2EDTA at pH 7.0 aqueous solution or D_2O for 24 h. DNA concentration was determined by UV absorption at 260 nm, using a molar extinction coefficient of 12 824 cm^{-1} M^{-1}(bp).

Instrumentation. Absorbance measurements and melting experiments were conducted with a Varian Cary 3E UV−vis spectrophotometer (Palo Alta, CA), equipped with a Peltier temperature control accessory and interfaced to a Gateway 386 PC for data collection and analysis. Circular dichroism studies were recorded at 20 °C on a Jasco J500A spectropolarimeter. Fluorescence data were recorded with an I.S.S. Greg 200 fluorometer and Model ATF 105 automated titration spectrofluorometer (Aviv Inc., Lakewood, NJ), equipped with a NESLAB temperature control accessory. Solution osmolalities were measured with a Wescor Inc., model 5520 vapor pressure osmometer.

Determination of Binding Constants. DNA binding constants were determined by fluorescence titration as described previously.[27] Two kinds of titrations were carried out. In the first, fixed ligand concentrations were titrated by increasing DNA concentration in BPE buffer in aqueous solution or D_2O. Titration data were fit directly by nonlinear least-squares methods[27,28] to get binding constants, using a fitting function incorporated into the program FitAll (MTR Software, Toronto). Low-salt BPE buffer was chosen for these studies to maximize the magnitudes of the binding constants, in hopes of magnifying any D_2O isotope effect. In the second process, fixed concentrations of DNA were titrated with increasing ligand concentrations in BPES buffer alone or in the presence of osmolytes. Data were transformed into the form of a Scatchard plot of r/C versus r, where r is the ratio of bound ligand to the total base pair concentration and C is the concentration of free ligand. Data were fit to the McGhee−von Hippel neighbor exclusion model.[27,28] Errors were evaluated by a Monte Carlo analysis,[27,28] using a routine that has been added to the FitAll package (MTR Software, Toronto).

Results

Hydration Changes for Ligand Binding to DNA. Figure 2 shows binding isotherms for the interaction of proflavine and 7-aminoactinomycin D with calf thymus DNA in the absence and presence of an osmolyte (betaine) that perturbs water activity. The qualitative effect of the osmolyte is clear. The presence of betaine significantly decreases the apparent DNA binding affinity of both proflavine and 7-aminoactinomycin D.

Figure 3 shows the effects of added osmolytes on intercalator binding constants. The data show that as osmolyte concentration increases (and, concomitantly, as water activity decreases), the ligand−DNA binding constant decreases. The solid lines through the data in Figure 3 were obtained by global fits to the data for all three different osmolytes used. Each individual osmolyte exerts a similar effect, within experimental error, on the intercalator binding constants as inspection of the slopes collected in Table 1 shows. From the slopes of the lines in Figure 3, it is possible to obtain the stoichiometry of water binding in

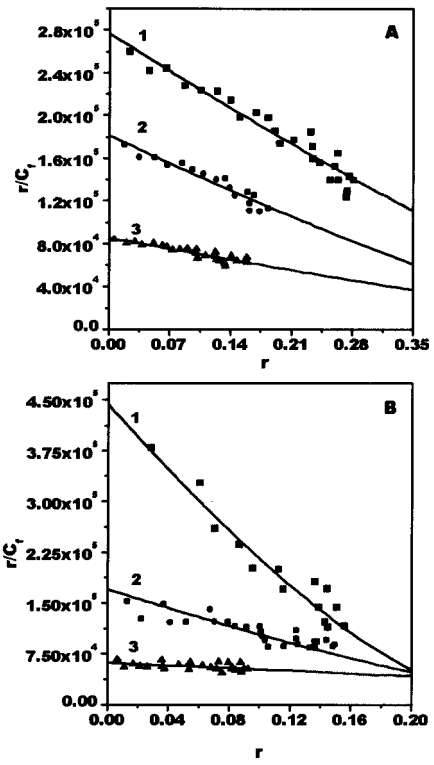

Figure 2. Binding isotherms for the interaction of proflavine (A) and 7-aminoactinomycin D (B) with calf thymus DNA in the absence and presence of betaine. The solid lines show nonlinear least-squares fits of the data to the neighbor exclusion model. Panel A: Proflavine binding to DNA in the absence of betaine (1) or in the presence of added betaine to produce 1.44 (2) or 2.48 (3) osmolal solutions. Panel B: 7-Aminoactinomycin D binding to DNA in the absence of betaine (1) or in the presence of added betaine to produce 1.44 (2) or 3.23 (3) osmolal solutions.

the formation of intercalation complexes for these five compounds. Assuming that there is no direct interaction of the osmolytes with DNA or the intercalators, the change in hydration is given by the equation

$$\partial \ln(K_s/K_0)/\partial[\mathrm{Osm}] = -\Delta n_w/55.5$$

where $\ln(K_s/K_0)$ is the change in binding free energy, "Osm" is the osmolality of the solution, and Δn_w is the difference in the number of bound water molecules between the complex and the free reactants.[15] A positive sign for Δn_w indicates the *uptake* of water upon complex formation. The negative slopes of the best-fit lines in Figure 3 indicate that Δn_w is positive and that additional water is bound upon complex formation. Hydration changes for the intercalation reactions studied are summarized in Table 2. The values found for Δn_w are the following: propidium, +6; daunomycin, +18; proflavine, +30; and 7-aminoactinomycin D, +32. The error in Δn_w estimates ranges from 2 to 17%. Within experimental error, there was no observable hydration change for ethidium binding ($\Delta n_w = +0.25 \pm 0.3$).

Appropriate controls were performed to ensure that DNA remained in standard duplex form in the presence of osmolytes over the concentration ranges used (see Supporting Information). While osmolytes decrease the DNA melting temperature,[19] the T_m remains well above the temperature used for binding studies, even at the highest osmolyte concentrations used here. Circular dichroism studies confirm that DNA retains the standard B-form spectrum in the presence of osmolytes at 20 °C (Figure S2, Supporting Information). Circular dichroism was also used to

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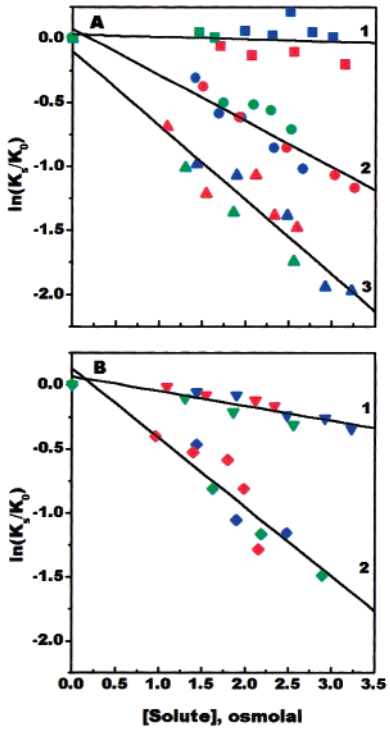

Figure 3. Dependence of DNA binding constants on osmolyte concentration. The natural logarithm of the ratio of the binding constant at a given osmolyte concentration (K_s) relative to the binding constant in BPES buffer (K_0) is shown as a function of solution osmolality. The colors indicate data obtained using a particular osmolyte: betaine (blue), sucrose (red), or triethylenglycol (green). Different symbols indicate data for different intercalators. Panel A: ethidium (curve 1, squares); daunomycin (curve 2, circles); 7-aminoactinomycin d (curve 3, triangles). Panel B: propidium (curve 1, down triangles); proflavine (curve 2, diamonds).

confirm that the intercalators studied bind to DNA by the same binding mode in the presence of osmolytes (Figure S3, Supporting Information). The induced CD spectra observed for propidium, daunomycin, and 7-aminoactinomycin D upon binding to DNA are all identical in the presence and absence of added osmolyte.

Ligand Binding in Deuterium Oxide Solution. D_2O was used to study the solvent isotope effect on ligand−DNA interactions. Hydrogen bond strength is greater for D_2O than for H_2O,[23−25] so if water is a specific participant in the formation of intercalation complexes, an increase in the apparent DNA association constant would be expected upon substitution of D_2O for H_2O. Figure 4 shows binding isotherms for the interaction of propidium, daunomycin, and 7-aminoactinomycin D with DNA in H_2O and in D_2O. The binding affinity increased by factors of 1.1, 1.7, and 2.7 for propidium, daunomycin, and 7-aminoactinomycin D, respectively. Table 3 summarizes the parameters for intercalator binding to DNA in H_2O and in D_2O. Figure 5 shows that there is a linear correlation ($R > 0.98$) between the magnitude of the relative increase of ligand binding affinity in D_2O and Δn_w. (We note that in D_2O, the intrinsic fluorescence of all the intercalators studied was enhanced (compare F_0 values in Table 3), in addition to the isotope effect on the binding constant. That is consistent with previous reports of D_2O effects on ethidium fluorescence.[29] Limiting values of fluorescence intensities were determined as fitting parameters in our analysis of binding isotherms, so D_2O effects on the optical properties of the intercalators are fully accounted for in our determination of binding constants).

Discussion

These results identify water as an important participant in DNA intercalation reactions. Separate experimental approaches (osmotic stress and deuterium isotope studies) show that water uptake accompanies the DNA binding of some (but not all) intercalators. This finding is counterintuitive since intercalation reactions are commonly thought to be accompanied by decreased solvent accessible surface areas as the planar, aromatic chromophore is buried within the DNA helix. The results reported here suggest that the situation is more complex, and must involve factors other than nonspecific binding of water to exposed surfaces. Coupled hydration changes must now be considered as an important contribution to the thermodynamics of intercalation reactions.

The osmotic stress method provides perhaps the most direct measure now possible of the hydration changes that accompany intercalator binding. Quantitative estimates for the number of additional water molecules bound upon complex formation are listed in Table 2. The osmotic stress method rests on the firm thermodynamic foundation of the Gibbs−Duhem equation.[17,18] A key assumption in the osmotic stress method is that the added neutral solutes do not themselves bind to either the DNA or the ligand.[15,16] A practical and widely accepted test of this assumption is to show that chemically different osmolytes exert the same effects on ligand binding.[15,20] Our results show that betaine, sucrose, and triethylene glycol all affect intercalator binding constants in essentially the same way. The slopes shown for the individual osmolytes in Table 1 do not vary in any systematic way for the various intercalators studied, supporting the assumption that the added solutes are not interacting with either the DNA or the ligands. More direct experimental evidence supporting the exclusion of sucrose and betaine from DNA was recently reported.[14] *If* the individual osmolytes were to interact with DNA, neglect of that interaction would render Δn_w values overestimates of the true values for the cases of water uptake observed here.[30] We emphasize that we do not believe that to be the case, since we cannot discern any differences in the effects exerted by the three different osmolytes used in either this study or our previous study of triplex and duplex melting.[19] Given the differences in the chemical properties of these osmolytes, each would be expected to interact in a unique way with DNA, which would lead to observable differences in the slopes shown in Table 1. At present, direct measurement of potential osmolyte−macromolecule interactions is a demanding and tedious task that requires enormous macromolecule concentrations.[30] While osmolyte−protein interactions have been studied to a very limited extent,[30] studies of osmolyte−DNA interactions have not yet been reported. While we hope to attempt such measurements in the future, such an effort represents a complex, arduous experimental undertaking that is beyond the scope of the current investigation.

We find that the magnitude of the deuterium isotope effect on the intercalator binding constant is directly correlated to the number of water molecules taken up as measured by the osmotic stress method (Figure 5). We interpret this finding as resulting from the increase in the hydrogen bond strength of the water molecules specifically bound within the intercalation complex. An alternate interpretation, however, could be that in D_2O solutions substituent hydrogen atoms on both the DNA and

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Table 1. Summary of $\partial\ln(K_s/K_0)/\partial[\text{Osm}]$ Values for DNA Intercalation Reactions[a]

	$\partial\ln(K_s/K_0)/\partial[\text{Osm}]$			
compd	sucrose	betaine	triethyleneglycol	global
ethidium	-0.06 ± 0.01	-0.03 ± 0.11	-0.02 ± 0.03	$-0.00_5 \pm 0.01$
propidium	-0.06 ± 0.01	-0.11 ± 0.02	-0.12 ± 0.03	-0.12 ± 0.02
daunomycin	-0.37 ± 0.03	-0.38 ± 0.05	-0.26 ± 0.02	-0.32 ± 0.01
proflavine	-0.48 ± 0.11	-0.49 ± 0.10	-0.52 ± 0.02	-0.54 ± 0.07
7-aminoactinomycin D	-0.55 ± 0.08	-0.62 ± 0.05	-0.69 ± 0.04	-0.58 ± 0.05

[a] The slopes of the lines obtained by linear least-squares fits of the data plotted in Figure 3 are shown. "Global" means that a single best-fit line was obtained by using all of the data points from all osmolytes. The individual slopes reported for each individual osmolyte for a given intercalator were obtained by linear fits to the data for that particular osmolyte. Each slope results from a linear fit to one set of colored symbols shown in Figure 3 for each intercalator.

Table 2. Comparison of Hydration Changes for Intercalator Binding to Calf Thymus DNA

ligand	K_0[a]	K_s[b]	$\partial\ln(K_s/K_0)/\partial[\text{Osm}]$[c]	Δn_w[d]
ethidium	1.1×10^5	0.9×10^5	-0.00_5	0.25 ± 0.3
propidium	5.2×10^4	4.4×10^4	-0.12	6.4 ± 1.1
proflavine	2.7×10^5	0.75×10^5	-0.54	30.0 ± 4.0
daunomycin	6.2×10^5	1.9×10^5	-0.32	18.0 ± 0.3
7-aminoactinomycin D	4.4×10^5	1.0×10^5	-0.58	32.0 ± 3.0

[a] Ligand−DNA binding constants in BPES buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, 0.185 M NaCl, pH 7.0). The binding data were obtained by fluorescence titration as described in the text and were fit to the McGhee−von Hippel neighbor exclusion model. [b] Representative ligand−DNA binding constants in BPES buffer plus sucrose. Solution osmolalities were as follows: ethidium, 3.15; propidium, 2.34; dauonmycin, 2.16; proflavine, 3.27; 7-aminoactinomycin D, 2.6. [c] The slopes ($\partial\ln(K_s/K_0)/\partial[\text{Osm}]$) were obtained by linear least-squares fits to the data shown in Figure 3. [d] $\Delta n_w = -55.5 \times (\partial\ln(K_s/K_0)/\partial[\text{Osm}])$. See text for an explanation.

intercalators exchange with deuterium, leading to stronger intercalator−DNA hydrogen bonds within the complex. If this were the case, we do not understand why there would be the correlation observed in Figure 5, so we favor the former model.

Hydration changes that accompany ligand binding to DNA can also be explored using volume, density, and ultrasound velocity methods, although these methods provide less direct measures of hydration changes in comparison to the osmotic stress method.[31] Results obtained using these alternate methods have been reported for only a few intercalation reactions. Results from volume and compressibility studies for the intercalation of ethidium and daunomycin were interpreted in terms of enhanced DNA hydration upon complex formation.[32] While that conclusion is generally consistent with the observations reported here, that study did not provide quantitative estimates for the numbers of water molecules taken up, nor was any difference between the behavior of ethidium and daunomycin noted. Volume and density measurements were recently reported that support the conclusion that intercalation of both ethidium and propidium was accompanied by the uptake of water.[33] Water uptake was greater (by about 4 water molecules) for propidium relative to ethidium, an observation in agreement with our findings. For volume, density, and ultrasound velocity measurements to be interpreted quantitatively in terms of hydration changes, assumptions must be made concerning the physical properties of "free" and "bound" water. That necessity renders these techniques more indirect, in our opinion, than the osmotic stress method, which is firmly based on the principles of linkage thermodynamics. The various experimental approaches complement one another. For the rather sparse available data on intercalation reactions, all of the various experimental methods

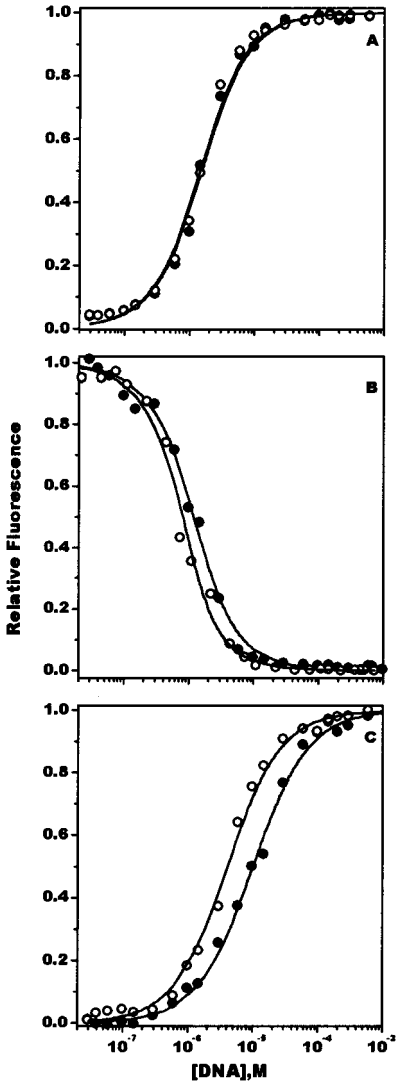

Figure 4. DNA binding isotherms for the interaction of propidium (panel A), daunomycin (panel B), and 7-aminoactinomycin D (panel C) in H_2O (solid symbols) or D_2O (open symbols). The normalized fluorescence response is shown as a function of total DNA concentration. In these titrations, the ligand concentration was kept constant at 1 μM while the DNA concentration was varied between 1 mM and 0.01 μM bp. Data fitting and determination of binding parameters (see Table 2) were carried out using nonlinear least-squares analysis, as described in the Materials and Methods section. The solid lines through the data show the best fitting curves to the binding model described in the text.

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Table 3. Summary of Thermodynamic Parameters for Intercalator Binding to Calf Thymus DNA in BPE and in DBPE (heavy water BPE)[a]

	daunomycin		ethidium		propidium		7-aminoactiomycin D		proflavine	
	BPE	DBPE	BPE	DBPE	BPE	DBPE	BPE	DBPE	BPE	DBPE
$K/10^6$, M^{-1}	1.5 ± 0.15	2.6 ± 0.50	0.93 ± 0.09	0.62 ± 0.05	0.96 ± 0.10	1.05 ± 0.10	0.10 ± 0.01	0.28 ± 0.02	0.81 ± 0.14	2.06 ± 0.37
F_b	54	124	2093	3120	4127	8605	2302	3712	152200	177900
F_0	692	2723	266	694	235	850	331	637	77610	89380

[a] Ligand−DNA binding studies were carried out in a 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 1 mM Na_2EDTA aqueous solution or D_2O solution. The ligand concentration was fixed at 1 μM while DNA concentration was varied between 0.01 μM and 1 mM. All the samples were prepared separately. The titration data were collected with the fluorescence method and used directly to get the binding constant, K, and the limiting fluorescence intensities, F_b and F_0, by using a nonlinear least-squares analysis method as described in the text.

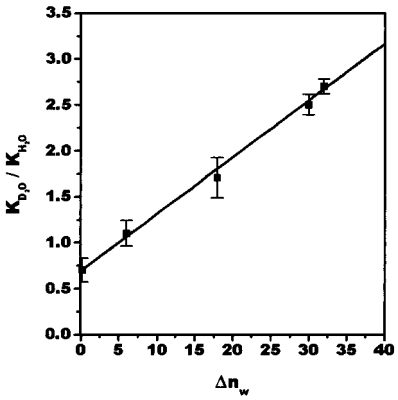

Figure 5. Correlation of the increase of intercalator binding affinity in D_2O with Δn_w, the difference in the number of bound water molecules between the complex and the free reactants. The Δn_w values were taken from Table 2. The ratio of K_{D_2O}/K_{H_2O} was calculated by using the binding constants reported in Table 3. The line through the data was obtained by linear regression ($R > 0.98$).

are generally consistent with water uptake upon intercalation complex formation.

A recent report[34] described osmotic stress studies of the binding of actinomycin D to DNA but, in contrast to our findings, concluded that water was released upon binding. At first glance, that result seems reasonable, since actinomycin D has peptide substituents that fill the minor groove and might be expected to displace groove-bound water. However, we have several concerns about the interpretation and analysis of experimental data in that report. First, the authors fit their experimental binding data to a single class of sites in the absence of osmolytes, but found it necessary to fit their data to two classes of sites when osmolytes were added to their solutions. In the only example of primary binding data provided, however, binding to what was termed the "high-affinity" class of sites was defined by only 2−3 data points in the low-saturation region of the Scatchard plot where it is most difficult to obtain reliable data. Transformation of binding data into the form of a Scatchard plot propagates error such that data at low binding ratios (which are most needed to characterize high-affinity sites) are least reliable. Binding constants for these "high-affinity" sites were used for the osmotic stress analysis, inappropriately and incorrectly in our opinion. We did not observe any indications of a multiple class of binding site in the presence of osmolytes in our studies (Figure 2). The authors also state that neighbor exclusion models could not be used to fit their experimental binding data for the interaction of actinomycin with calf thymus DNA, a situation we find peculiar. A neighbor exclusion model was first derived[35] specifically to account for the binding of actinomycin binding to calf thymus DNA,[36] and in the intervening years the neighbor exclusion model has been widely accepted as the most appropriate explanation for nonlinear Scatchard plots obtained from intercalator binding to DNA. Finally, we note that if water were in fact released upon actinomycin binding to DNA, such behavior would be inconsistent with the deuterium isotope effect we observe (Figure 5, Table 3).

Our laboratory has been devoted to understanding the molecular contributions to ligand−DNA binding free energies[37−39] and has made several attempts to parse binding free energies into their component parts.[40−42] Up until now, the contributions of water to intercalator binding were not fully appreciated and were neglected. The coupled binding of water to DNA is analogous to the coupled binding of counterions,[43] which gives rise to the salt-dependence of ligand−DNA binding constants. The dependence of ligand binding free energies on water activity is thermodynamically as significant as their dependence on salt concentration, pH, and temperature, and must be defined for a complete understanding of the binding process. Exactly how to quantitatively evaluate the energetic contribution of coupled water binding to observed binding free energies is problematic at present. Immobilization of water upon binding is expected to impose an entropic energy penalty of 0 to 2 kcal mol^{-1}.[44] For the bound water to be stable, though, a favorable enthalpic contribution from hydrogen bond formation must overwhelm the unfavorable entropy to yield a favorable, negative free energy. But the exact overall resultant free energy change is simply not known with sufficient precision to make any quantitative estimates possible for the contribution of coupled water binding to the free energies of intercalation reactions. We identify water as an important participant of intercalation reactions, and can quantitatively measure the stoichiometry of its differential uptake upon complex formation. We cannot yet calculate or estimate its exact contribution to the overall free energy of intercalation reactions.

The number of water molecules that participate in the intercalation reactions listed in Table 2 represent macroscopic, thermodynamic quantities that must be accounted for in any discussion of the underlying binding reaction mechanisms or of the structures of the complexes. These quantities pose a challenge for those studying the structures of ligand−DNA complexes. Can these numbers of specifically bound waters be identified in high-resolution structures of intercalation complexes? In general, examination of known crystal structures

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suggests that specific waters can indeed be found in a number of ligand−DNA complexes. We caution, however, that crystals are usually grown in the presence of high concentrations of osmolytes (glycerol, poly(ethylene glycol)), so the exact relationship between waters observed in high-resolution structures and the hydration changes reported here is by no means clear. With this caveat, we note the following: Crystal studies [45,46] have shown a large number of apparently specifically bound water molecules within the daunomycin−DNA complex. These include a water molecule simultaneously hydrogen bonded to the ligand O13 substituent and to a cytosine on the upper side of the intercalation site, 3−4 water molecules interacting with a sodium ion and with ligand and DNA substituents in the major groove, and several water molecules that form a "minispine" of hydration in the minor groove in the vicinity of the amine group on the daunosamine moiety. Apart from these apparently specifically bound waters in the complex, Frederick and coworkers[47] have mapped 15−20 water molecules in the first-layer solvent shell whose positions appear to be conserved over three different anthracycline crystal structures. We do not claim at all that these waters that are observed in crystal structures are the very ones counted by the osmotic stress technique, but do note that their number is generally consistent with the magnitude of $\Delta n_w = +18$. High-resolution crystal structures of dCdG−proflavine complexes at several temperatures have consistently revealed a network of 25−30 "core" water molecules that are an intrinsic part of the structure.[48,49] The number of molecules in these water assemblies ordered around the intercalation complex is remarkably (and perhaps fortuitously) close to the value of $\Delta n_w, = +30$ measured here by the osmotic stress method.

Summary

These studies identify water as an important participant in intercalation reactions. Two different experimental approaches were used, and both were consistent with the thermodynamic coupling of water and intercalator binding for four of the five compounds studied. Water binding must now be considered in any complete description of ligand binding to DNA, as important and fundamental as counterion binding.

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Supporting Information Available: UV melting curves and CD spectra of calf thymus DNA and CD spectra of propidium, daunomycin, and 7-aminoactinomycin (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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