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Nonspecific interactions in dye binding to DNA

Influence of alcohols and amides

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The binding of a few drugs (ethidium bromide, propidium diiodide, proflavine and actinomycin D) to DNA has been investigated in aqueous solutions to which cosolvents of different polarity have been added. It is found that both alcohols (less polar than water) and amides (more polar) lower the binding constant according to a linear relationship between the intercalation free energy and cosolvent concentration. The main action of cosolvents cannot be described in terms of electrostatic effects, since they predict much smaller changes in the binding constant than those observed. It appears instead that relevant solvation effects are responsible for the binding strength of the different dyes to DNA. As a general result, it is found that solvation effects largely contribute to the intercalation free energy, thereby weakening the influence of nonspecific interactions at the intercalation site.

1. Introduction

From several studies on dye binding to DNA, it is apparent that large nonspecific contributions to the intercalation free energy occur. Little DNA sequence specificity has been attributed to many dyes [1–3], and some calculations have also been performed in order to clarify the physico-chemical processes leading to specific binding [4,5]. Concerning the role of nonspecific forces instead, in the past most attention has been focussed only on long-range electrostatic effects [6]. Theoretical models have been mainly developed in order to predict the dependence of the binding constant upon ionic strength and charges (DNA and dye) [6–8].

In recent papers, the role of alcohols, as cosolvents of lower dielectric constant, in the binding of ethidium bromide (EB) to DNA has been examined [9,10] and analysis of the thermodynamics of binding has suggested that intercalation is largely determined by short range dye-solvent interactions (solvation) [10]. Microdilatometric measurements have also demonstrated large volume changes upon intercalation, probably associated with dye dehydration upon insertion into the DNA double helix [11]. Further support for the proposed relevance of solvation effects has been obtained from fluorescence studies of EB in mixed solvents [12,13].

In order to extend previous results on EB-DNA in water/alcohol solutions, more dyes have been considered in the present paper: propidium diiodide (PI), proflavine (PF) and actinomycin D (AD). PI has been chosen for both its similarity to EB and its charge, which is double that of EB, whereas AD has been selected for being neutral

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and specific (preference for G · C rich sequences) [14]. Besides alcohols, which are less polar than water, amides (formamide (FA) and *N*-methylformamide (NMF), more polar than water) have also been employed as cosolvents.

2. Experimental

2.1. Materials

Native calf thymus DNA was obtained from Sigma; EB was from Boots Pure Chemical Co., PI and AD from Calbiochem and PF from Sigma. Alcohols, NaCl and phosphate salts were from B.D.H. (AnalaR) and had a purity of 99.5% or better. FA and NMF were from Merck. Deionized water was doubly distilled prior to use. All reagents were used without further purification.

2.2. Sample preparation

DNA was dissolved in phosphate buffer (0.032 M Na_2HPO_4, 0.01 M KH_2PO_4, 0.0001 M EDTA, pH 7.45) and its double strandedness was checked by using the alkali method [15]. The concentration of DNA stock solutions was determined spectrophotometrically by employing 6600 $M^{-1}\,cm^{-1}$ for the value of the molar extinction coefficient at 260 nm [8]. Accordingly, drug concentrations were determined by employing molar extinction coefficients of 5860 at 480 nm for EB [16], 5920 at 480 nm for PI [17], 24800 at 440 nm for AD and 43000 ($M^{-1}\,cm^{-1}$) at 444 nm for PF [1].

Samples were prepared by dropwise addition of weighed amounts of alcohols and amides to the solutions in order to facilitate cosolvent solvation. The final DNA concentration P was usually maintained between 20 and 60 μM, but was raised to approx. 150 μM for low values of the binding constant. Typical drug concentrations were 2 μM in fluorescence measurements (EB, PI and PF), but around 4 μM for absorbance studies (AD). The above concentrations have been chosen in order to avoid fluorescence nonlinearities due to inner-filtering effects.

The ionic strength μ was adjusted to 0.1 M with NaCl.

2.3. Measurements

Spectrophotometric measurements were performed on a Perkin-Elmer 555 spectrophotometer and fluorescence spectra were recorded on a Perkin-Elmer 650-40 fluorometer interfaced to an AIM-65 microcomputer.

Temperature was controlled to within $\pm 0.05\,^{\circ}C$ by means of a Haake thermostat, and checked with a calibrated thermistor or thermocouple inside the cuvettes.

The optical properties of EB (excitation peak wavelength and fluorescence quantum yield) have been measured according to the procedure described in a previous report [12].

The equilibrium constants for binding of EB, PI and PF to DNA were determined by means of fluorescence measurements. The use of luminescence techniques proved to be more convenient than the more common absorption titration method because of the high sensitivity of fluorescence and the large differences in quantum yields of the dyes when free or intercalated. Excitation wavelengths (λ_{exc}) were 520 nm (EB), 535 nm (PI) and 450 nm (PF); emission wavelengths (λ_{em}) were 608 nm (EB), 615 nm (PI) and 504 nm (PF). Typical slit widths were 15 nm (excitation) and 2 nm (emission).

The binding of AD to DNA was studied by means of absorption measurements at 430 nm, since AD fluorescence is very low and is scarcely affected by intercalation into DNA.

The presence of significant amounts of dimers was excluded on the basis of the fluorescence spectral shapes, independent of the dye concentration. This conclusion is reinforced by published values for the dimerization constants of EB, PI, PF and AD, which are generally lower than 10^3 M^{-1} [18]; furthermore, the presence of alcohols appears to decrease the dimerization constants [19].

2.4. Determination of equilibrium constant

The amounts of drug bound to DNA (C_b) and free in solution ($C_f = D - C_b$, where D is the total drug concentration) were evaluated from the mea-

sured fluorescence intensities I using the relation

$$I = C_b\eta_b + C_f\eta_f$$

where η_b and η_f are the fluorescence efficiencies for bound and free dye, respectively

$$\eta(\lambda_{exc},\lambda_{em}) = I(\lambda_{exc},\lambda_{em})/D.$$

For AD, C_b and C_f were determined by measuring the absorption A according to

$$A = C_b\epsilon_b + C_f\epsilon_f$$

where ϵ_b and ϵ_f are the extinction coefficients for bound and free AD, respectively.

Both the fluorescence efficiency η_f and absorption coefficient ϵ_f of the free drugs are strongly affected by changes in the solution composition. The dye response was therefore measured as a function of cosolvent concentration (alcohols and amides) by studying solutions of known dye concentration. As an example, the quantum yield Φ_f of EB (relative to water) increases from 1 to about 2.5 on going from 0 to 1 molar fraction of FA or NMF. Similarly, Φ_f has been found to increase by a factor of between 2.5 and 3 in alcohols [12].

The fluorescence efficiencies of the bound species η_b, as well as ϵ_b for AD, were determined by carefully extrapolating the optical response to zero binding level, when practically all of the drug dissolved in solution is intercalated [10,17]. The presence of alcohols does not alter the values of η_b, within experimental uncertainty, as has been found for EB [10], suggesting that the DNA-drug complexes are well shielded from the solvent.

The values of the relative fluorescence efficiencies η_b/η_f at 25 °C are 18.4 ± 0.2 for EB, 33 ± 0.4 for PI, 0.42 ± 0.01 for PF; for AD $\epsilon_b = 11\,300 \pm 600$ M^{-1} cm^{-1} and $\epsilon_b/\epsilon_f = 0.48$ have been determined. In view of the greater difference in spectroscopic response, the data for EB and PI are subject to a smaller experimental uncertainty than those on the binding of PF and AD to DNA.

The binding constant K_{obs} was determined from the measured values of C_b, together with the known P and D values, according to the binding isotherm

$$K_{obs} = C_b/(nP - C_b)(D - C_b).$$

This rule holds true at low binding levels, where the Scatchard representation of the equilibrium is well described by a linear relationship between C_b/PC_f and C_b/P. This happens when the binding level C_b/P is lower than 0.08 for EB [9], 0.05 for PI [17] and 0.05 for AD [14]. The binding level was therefore kept below the above limits by a proper choice of the value of P.

The value of n was found to be very close to 1/6 for EB [9,10], in excellent agreement with the excluded-site model of intercalation [20]; n was found to equal 0.1 ± 0.01 for PI and 0.09 ± 0.01 for AD by graphically extrapolating to $C_b/PC_f = 0$ the linear region of the Scatchard plots presented in refs. 17 and 14, respectively. For PF, the value $n = 0.185$ was taken from ref. 21.

The ratio C_b/C_f was kept at about 1 ($0.7 \lesssim C_b/C_f \lesssim 1.3$) by appropriate selection of P, in order to decrease the statistical uncertainty in the binding constant K_{obs}. This precaution could not be followed (P was too low or too high) at very high ($K_{obs} \gtrsim 10^6$ M^{-1}) and very low ($K_{obs} \lesssim 10^4$ M^{-1}) binding strengths; therefore, the low and high values of K_{obs} shown in this paper are affected by a greater uncertainty than the intermediate ones.

3. Results

Previous results on the binding of EB to DNA in water/alcohol solutions have been extended here to other intercalating drugs (PI, PF and AD). It had been shown that the data on EB ($\ln K_{obs}$) from different alcohols could be unified by a single linear function when plotted vs. the solution dielectric constant ϵ.

The binding constants K_{obs} for the investigated drugs, EB, PI, PF and AD, are plotted in fig. 1 vs. ϵ. The data for different alcohols (including methanol, ethanol and isopropanol) fall very close together on straight lines. The data for PI are referred to a higher ionic strength (0.4 M), in order to ensure that $K_{obs} \lesssim 10^6$ M^{-1}, as found for the other drugs. At lower ionic strengths, a large uncertainty is associated with the measurements (e.g., $K_{obs} \approx 10^7$ M^{-1} for PI in water, 0.1 M NaCl). It should be noted, however, that a linear dependence of $\ln K_{obs}$ upon ϵ is observed over a

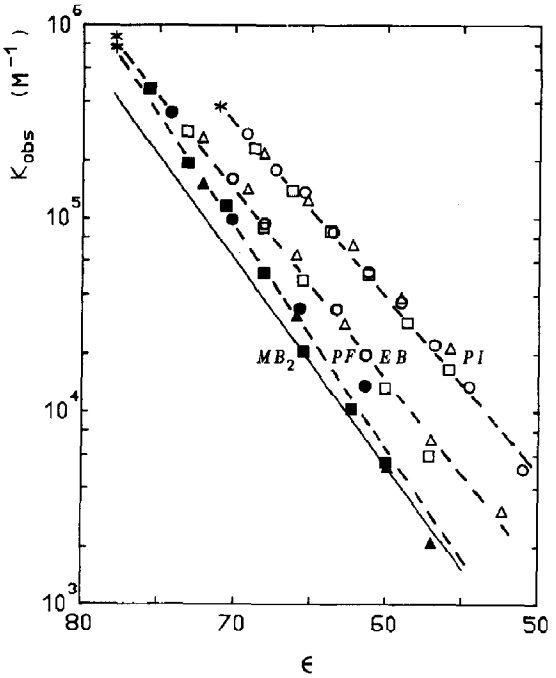

Fig. 1. (------) Binding constant of propidium diiodide, ethidium bromide and proflavine to DNA at 25°C vs. dielectric constant of water/alcohol solutions. (*) Pure water, (○) methanol, (□) ethanol, (△) isopropanol solutions. (———) Dimerization constant of methylene blue [10]. The dielectric constant was evaluated according to the alcohol and salt content [23,24].

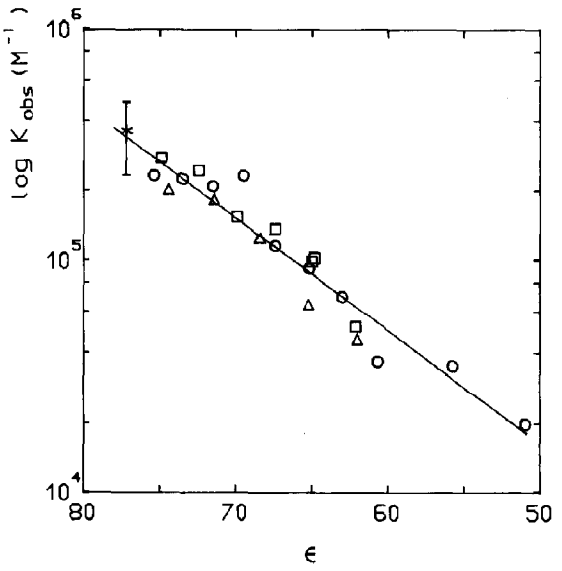

Fig. 2. Binding constant for actinomycin D-DNA binding vs. dielectric constant at 25°C of water/alcohol solutions. Symbols as in fig. 1. A typical error bar is indicated.

wide range of ionic strengths (0.01 M $\lesssim \mu \lesssim$ 0.4 M) [9], and that the data at different salt contents result in very similar slopes, each slope being peculiar to the dye. The data on AD, shown in fig. 2, are more scattered than those for the other drugs, because of the low sensitivity of AD absorption strength to intercalation (see also section 2). However, a linear decrease in K_{obs} for increasing ϵ is still observed.

The data presented in figs. 1 and 2 show that the linear dependence of $\ln K_{obs}$ upon ϵ in water/alcohol solutions is a general property of many intercalating agents.

The slopes of the ln K_{obs} vs. ϵ curves are the same for the monopositive EB and dipositive PI, the latter possessing the same heteroaromatic ring system but differing from the former only in a side chain bearing a positive charge. The log K_{obs} vs. ϵ curves display a similar trend for PF, whereas AD binding appears to be less dependent upon cosolvent concentration.

In order to determine whether the unifying role of ϵ is peculiar to water/alcohol solutions, K_{obs} has been measured for EB in water/FA and water/NMF solutions. As opposed to water/alcohol mixtures, the dielectric constant of water/amide solutions is higher than that of pure water, ranging from 80 to 110 in the amide concentration range investigated here. In fig. 3, the affinity constant for binding of EB to DNA, K_{obs}, is plotted vs. amide volume fraction v; the data for EB in water/ethanol and water/isopropanol solutions plotted vs. ϵ in fig. 1 are also shown for purposes of comparison.

The downward trend in ln K_{obs} vs. v is quite similar for both water/alcohol and water/amide solutions. It is significant, however, that ϵ increases with amide concentration [22], whereas it decreases with increasing alcohol content [23]. Therefore, the data presented in fig. 3 display a trend (increased binding at lower ϵ) which is the reverse of that shown in fig. 1. Furthermore, the data from different amides cannot be brought

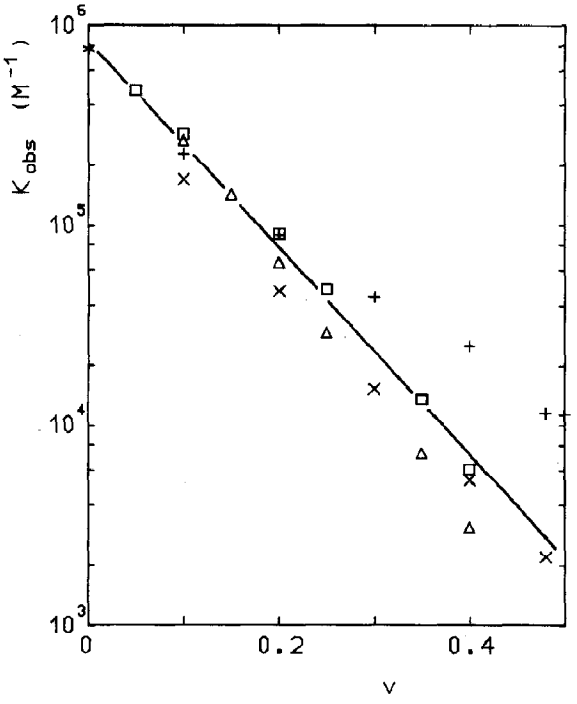

Fig. 3. Binding of ethidium bromide to DNA vs. cosolvent volume fraction at 25 °C in (+) water/FA, (×) water NMF, (□) water/ethanol and (△) water/isopropanol.

together when plotted vs. ϵ, and the linear dependence of ln K_{obs} upon ϵ seems to be peculiar to water/alcohol solutions. A few measurements on the DNA-PI system in the two water/amide solutions confirm the trend observed for EB.

In analogy with the role of water/alcohol mixtures, we have found a relationship between the intercalation free energy

$$\Delta G^0 = -RT \ln K_{obs}$$

and the optical response (wavelength at the peak in the absorption and excitation spectrum, λ_{exc}, and fluorescence quantum yield Φ_f) of EB in water/amide solutions. The data presented in fig. 4 show that the trend in ΔG^0 vs. either λ_{exc} (fig. 4a) or Φ_f (fig. 4b) is the same (essentially linear) for alcohol and amide solutions.

4. Discussion

Concerning the role of cosolvents in intercalation, few studies have appeared so far, if we neglect those on the effect of the ionic strength μ. In the presence of varying amount of salts, the decrease in K_{obs} for increasing μ has been attri-

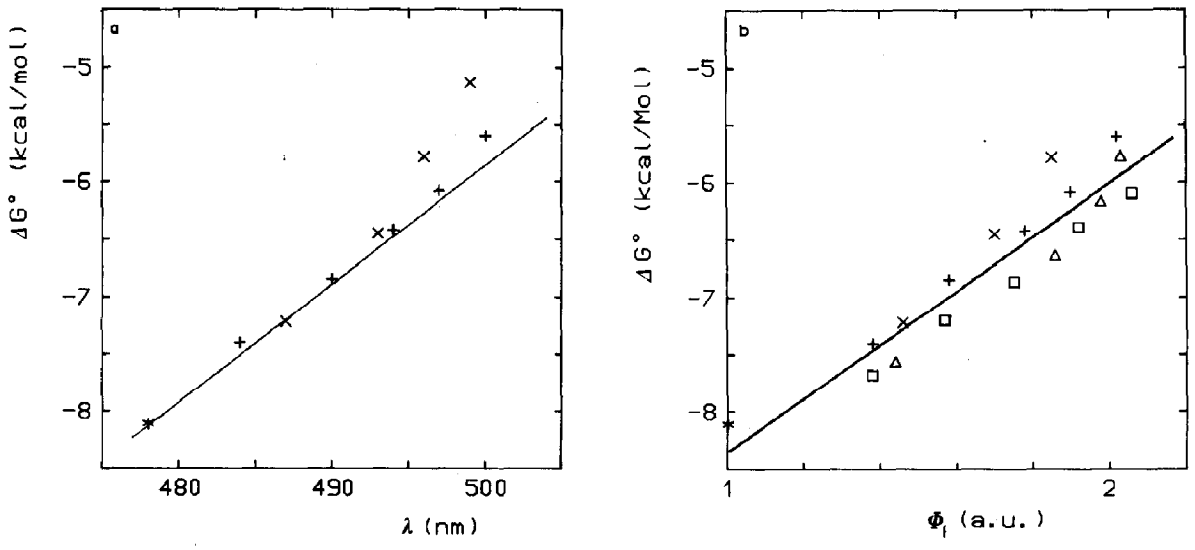

Fig. 4. (a) Intercalation free energy for EB-DNA binding at 25 °C vs. absorption peak wavelength in (+) water/FA and (×) water/NMF. The straight line is taken from linear regression of data on water/alcohol solutions. (b) Intercalation free energy for EB at 25 °C vs. EB fluorescence yield in water/alcohol and water/amide solutions. Symbols as in fig. 3.

buted to long-range electrostatic forces [6,7]. Recently, alcohols have been employed as perturbing agents in order to study solvent effects on the binding of EB to DNA [9,10]. Although the data from different alcohols can be unified by means of the common linear dependence of ln K_{obs} upon the dielectric constant ϵ, EB binding to DNA in water/alcohol mixtures does not fit the electrostatic picture [10]. In fact, the estimated electrostatic contribution to the variation of K_{obs} with ϵ represents only a few percent of the observed effect. Furthermore, the presence of alcohols (lower ϵ) should lead to increased binding if electrostatic interactions prevail.

Alcohols and amides have been employed here in order to extend previous studies on DNA-EB binding in mixed solvents to other intercalating drugs, including PI, PF and AD. The data presented in figs. 1 and 2 show that the general trend in ln K_{obs} vs. ϵ observed for EB is also found for the other drugs. The monopositive EB and dipositive PI display the same slopes, in spite of their different charges; a similar slope is found for PF, but the slope for AD is less steep. A similar behavior has also been found when analyzing the dependence of the dimerization constant of methylene blue upon alcohol content [10,19]; it should be noted that positively charged dyes are involved in the dimerization process, whereas intercalation involves oppositely charged molecules (dye and DNA). The lack of correlation between slope and dye charge seems to rule out any significant influence arising from bulk electrostatic forces. In contrast, the close similarity observed between EB and PI suggests that the weaker binding of the drugs investigated to DNA in water/alcohol solutions is concerned with the solvation of the heterocyclic rings.

The marginal role of electrostatic forces has been definitely established by also examining the interaction of EB and PI with DNA in solutions more polar than water/alcohols, employing FA and NMF. The data presented in fig. 3, when plotted vs. ϵ, show a trend which is the reverse of that expected from extrapolation of the results in water/alcohol solutions. Furthermore, the data for FA and NMF do not group together when plotted vs. ϵ.

Since long-range electrostatic interactions do not appear to be sufficient to support the intercalation process, perhaps more attention should be paid to the role played by the solvent in DNA structure. DNA is known to be sensitive to changes in the environment, although the intercalation free energy change due to solvent perturbation of DNA itself appears to be small for the cosolvent concentrations investigated here. This statement is supported by a CD investigation of the DNA spectral response in water/alcohol and water/FA solutions. The presence of formamides and alcohols as cosolvents is found to induce only a smooth transition between the very similar B and B * structures (alcohols are more efficient than formamide in promoting the B → B * transition). Finally, the similarity observed between DNA-dye binding and methylene blue dimerization [10] suggests that the major contribution to decreased binding with increasing cosolvent concentration is hardly due to DNA.

Analysis of the thermodynamics of the EB-DNA interaction has led us to suggest a primary role for solvation effects in DNA-dye binding, due to the action of relevant 'hydrophobic forces' [10]. Evidence for solvation effects has also been examined recently in a study on EB fluorescence in water/alcohol solutions [12]. The close correlation between intercalation free energy and EB optical response (fig. 4a and b and ref. 12) confirms that the fluorescence response and dye binding are controlled by similar mechanisms.

Analysis of the dependence of the EB quantum yield in mixed solvents upon the solution composition led to estimates of the dye solvation free energy [13]. At low-to-intermediate alcohol concentrations, the dye solvation free energy is lowered, owing to more favorable EB-solvent interactions [13]. The DNA-EB binding energy is then expected to be greater in water/alcohol mixtures than in water, and the binding constant K_{obs} should decrease. The correlation between intercalation free energy and EB optical response (λ_{exc} and Φ_f) shown in fig. 4a and b supports our suggestion concerning the relevance of solvation forces in the intercalation process.

The change in intercalation free energy induced by cosolvents at volume fraction v can be defined

according to:

$$\Delta\Delta G^0 = \Delta G^0(v) - \Delta G^0(\text{water}).$$

Since for cosolvent volume fractions of the order of 0.3–0.4 $\Delta\Delta G^0 \simeq \Delta G^0(\text{water})/2$, we are led to conclude that a large fraction of the overall binding energy ΔG^0 derives from dye-solvent interactions, whereas specific dye-DNA interactions (hydrogen bonding and stacking) do not appear to be as significant as is generally assumed. Within this framework, the modest specificity observed for many DNA intercalating agents can be attributed to a substantial influence of dye-solvent interactions, which makes it somewhat more difficult to single out the contribution of specific DNA-dye interactions at the intercalation site.

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