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Viscosity Dependence of Ethidium–DNA Intercalation Kinetics

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ABSTRACT: The kinetics of ethidium intercalation into double-stranded poly[d(G-C)] were investigated by use of repetitive pressure-jump chemical relaxation at 20 °C in low ionic strength (0.1 M NaCl) aqueous buffers containing either glycerol or methanol. The viscosity of the various solvents differed by more than an order of magnitude while other physical properties (e.g., dielectric constant) remained approximately constant. The single-reciprocal kinetic relaxation time (τ^{-1}) increases linearly with DNA concentration. The observed association rate constant is lower in all organic–aqueous mixtures than in water and is inversely proportional to the viscosity. These results provide evidence for an additional step in the intercalation mechanism which is identified as an obligatory DNA conformational change preceding ethidium intercalation. From the data presented, the equilibrium constant of this local conformational change is $\sim 10^{-3}$, i.e., greatly favoring the structure incapable of intercalation. The corresponding kinetics were not directly determined; however, in order to be consistent with all of the data the forward and/or reverse rate constants of the conformational change must be larger than the rate of the intercalation reaction. Thus, it is proposed that the rate of the conformational change back to the nonintercalating B-DNA structure is greater than ~ 500 s^{-1}, implying a rate of opening greater than ~ 0.5 s^{-1}, in agreement with other hydrogen exchange and NMR data. The observed overall rate constant for the dissociation of ethidium is inversely proportional to the solvent density, possibly reflecting a dependence on the solvent free volume. The overall volume change of intercalation is less negative in the organic–aqueous solvent mixtures than in water.

The physical and chemical processes leading to intercalation of a dye between successive DNA base pairs are expected to have a complex dependence on the immediate environment of the macromolecule. If the potential intercalator is charged, it will interact electrostatically with the polyanionic DNA and condensed counterions, producing at least a partial displacement of the latter. X-ray crystal structures of intercalation complexes suggest that the static conformation in the imme-

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diate vicinity of an intercalation site is quite different from that of B-DNA (Tsai et al., 1977; Jain et al., 1977; Neidle & Abraham, 1984; Quigley et al., 1986), implying that a number of conformational changes occur prior to or concomitant with intercalation (Sobell et al., 1977). In addition, the interactions of the solvent with the intercalator and DNA may influence the extent and rate of interaction. Upon intercalation, the dye is stripped of water molecules, and it seems likely that desolvation occurs prior to insertion between the base pairs (Laugâa et al., 1985). Around the intercalation site, DNA–water interactions will be influenced by the increased separation of the phosphate groups, the partial unwinding of the DNA helix, and corresponding reduced local charge density in the case of cationic dyes.

Ethidium bromide (EB)[1] intercalates between DNA base pairs (LePecq & Paoletti, 1967) and has found extensive use as a prototype for studying the modes of interaction of small molecules with DNA. Our choice of EB was due to (1) the large increase in the fluorescence quantum yield upon intercalation; (2) the relatively low susceptibility to photolysis; and (3) the large literature pertaining to the physical and biochemical properties of this dye, e.g., regarding the equilibrium properties of its complexes with DNA (LePecq & Paoletti, 1967; Pauluhn & Zimmerman, 1978, 1979; Pauluhn et al., 1979; Delben et al., 1982; Röding & Zimmermann, 1982; Winkle et al., 1982; Nelson & Tinoco, 1984; Yielding et al., 1984).

Figure 1 presents several possible reaction pathways leading to EB intercalation into an isolated region of DNA. In this figure only the most fundamental intermediates between the isolated species (B-form DNA and free EB) and the intercalated complex are represented; it is understood that each step may comprise a number of intermediates. The complications arising from nearest-neighbor interactions (excluded site effects; Macgregor et al., 1985) have been intentially omitted because they do not arise in the binding of EB to an isolated site.

We have previously examined the kinetics of EB intercalation with poly[d(A-T)] and poly[d(G-C)] in 0.1 M NaCl using pressure-jump chemical relaxation throughout a wide range of EB and DNA concentrations (Macgregor et al., 1985). Our data were consistent with a single-step bimolecular mechanism that we identified with the direct intercalation path described by the kinetic constants k_1 and k_{-1} in Figure 1. At high degrees of binding site saturation, the effects of excluded binding upon the relaxation kinetics (McGhee & von Hippel, 1974) were accounted for by assuming that the bound ligands redistribute among the available sites rapidly compared to the rate of association or dissociation (Jovin & Striker, 1977; Macgregor et al., 1985). We did not observe any other kinetic process although external electrostatic binding has been reported at the salt concentrations used in our experiments (Pauluhn & Zimmermann, 1978). Despite this, our results were consistent with those of other kinetic investigations carried out at low ionic strength (Jovin, 1975; Jovin & Striker, 1977; Mandal et al., 1980; Macgregor et al., 1985; Wilson et al., 1985); however, there was significant disparity with the results of electric-discharge temperature-jump experiments carried out at high ionic strength in which multiple relaxation times were observed (Bresloff & Crothers, 1975; Wakelin & Waring, 1980; Ryan & Crothers, 1984).

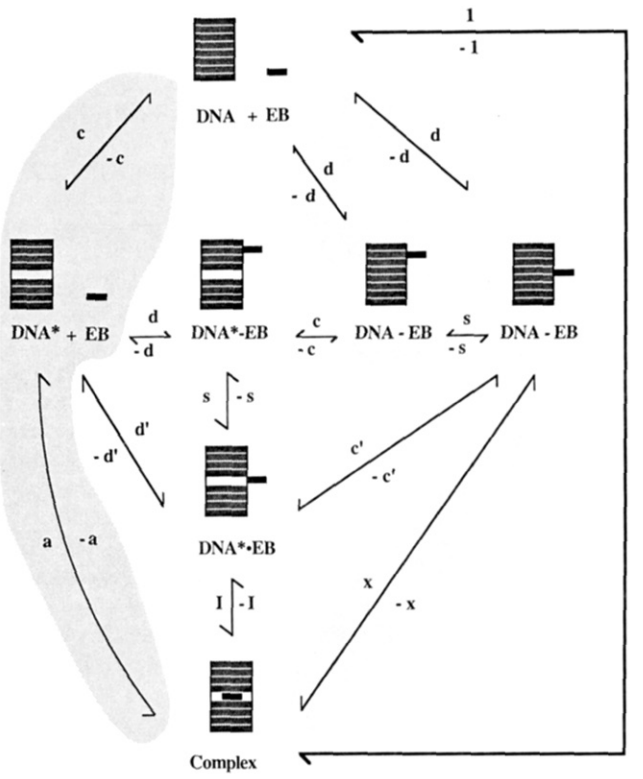

FIGURE 1: Possible reaction pathways leading to EB intercalation into an isolated, unbound section of DNA. The black rectangle represents an EB molecule, the ladderlike object is DNA, and the open gaps in some DNA molecules represent a site with the correct conformation for intercalation. If there is no physical contact between the cylinder and the rectangle, then the individual species are assumed to be free in solution with arbitrary relative orientations; otherwise, the relative orientations of the schematic representations are intended to depict the various molecular events of the kinetic processes. "Complex" refers to the intercalation complex. To make this figure more legible, the letters and numbers associated with the reactions are the subscripts of the respective kinetic constants; e.g., "1" is equivalent to "k_1" elsewhere in the text. The shaded regions emphasize the two multistep pathways discussed in detail in the text (models II and III). The steps designated by the rate constants k_s and k_{-s} refer to sliding of the EB along the DNA helix.

In addition to the direct kinetic techniques, NMR (Feigon et al., 1982) and fluorescence fluctuation spectroscopy (Magde et al., 1974) have been used to study the rate of EB binding to DNA at low ionic strength. Whereas the results of the latter study were in agreement with our results, the overall rate constant for the dissociation of EB from DNA observed by NMR is between the value we obtained and that of the temperature-jump data. Either these data are contradictory or the various experimental techniques employed are sensitive to different aspects of the reaction.

Despite the inconsistencies concerning the details of the binding reaction, it is generally agreed that at salt concentrations on the order of 0.1 M the overall bimolecular association rate constant for EB (and for many other intercalating dyes) binding to DNA is 10^6–10^7 M^{-1} s^{-1}. This value is about 3 orders of magnitude slower than expected for the diffusion limit, indicating that the observed reaction velocity is limited by additional rate controlling processes.

In the absence of evidence to the contrary, one must assume that all of the processes represented in Figure 1 take place to some extent; the problem remains to determine the major intermediates and the relative probability of the reaction proceeding along a particular pathway. The thermodynamic and kinetic parameters of the overall reaction have been measured by several workers under a variety of experimental

[1] Abbreviations: EB, ethidium bromide; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; NMR, nuclear magnetic resonance; ORD, optical rotatory dispersion; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

conditions (see references given above). These data are valuable for guiding speculation about the reaction mechanism, but additional kinetic information is required to provide details about the individual molecular reaction steps shown in Figure 1.

The solvent dependence of the equilibrium constant is difficult to interpret because it reflects the behavior of the kinetic rate constants (Löber & Klarner, 1985); therefore, it is more informative to examine the solvent dependence of the latter directly. The effect of the ionic strength upon the rate of dye intercalation has recently been reported by Wilson et al. (1985); in addition to EB, they also used propidium, a dye structurally similar to EB but with a 2+ charge. The rate constants varied as expected if the ionic effects are accounted for by their proposed extension of the ion-condensation theory (Manning, 1979), but they could not unambiguously identify the nature of the underlying kinetic steps. There is no consensus on the analysis of the ionic strength dependence; thus, Friedman and Manning (1984) were unable to reconcile equilibrium results with their own proposed extensions of the theory.

In this paper we report the pressure-jump relaxation kinetics of EB binding to poly[d(G-C)] in methanol–buffer and glycerol–buffer mixtures. The pressure-jump chemical relaxation technique is particularly well suited for studying kinetics when electric-discharge temperature-jump is impossible or inadvisable such as at very low ionic strength, in the presence of organic solvents, or when polyelectrolytes are studied (Dourlent & Hogrel, 1976). As was found for the purely aqueous system (Macgregor et al., 1985), a single-step bimolecular reaction mechanism describes the data in each of the mixed solvents. The viscosity dependence of the association constant suggests that the intercalation step is preceded by a rapid but infrequent DNA conformation change. The forward rate constant, equilibrium association constant, and molar volume change of intercalation are lowered by the addition of either organic solvent; however, these solvents have an opposite effect on the reverse rate constant.

Experimental Procedures

Concentrated stock solutions of poly[d(G-C)] (P-L Biochemicals) and EB (Serva, Inc.) were prepared in 20 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA, pH 7.2; each was divided into aliquots and stored at −20 °C. The concentrations of the stock solutions were measured spectrophotometrically with ϵ = 14 200 M^{-1} cm^{-1} at 256 nm for poly[d(G-C)] (base pairs; Pohl & Jovin, 1972), and 5850 M^{-1} cm^{-1} at 480 nm for EB (Bresloff & Crothers, 1975). DNA was exhaustively dialyzed against buffer to remove very short molecules and subsequently sized by using agarose gel electrophoresis. The polymer consisted of a broad distribution of chain lengths (data not shown).

All measurements were made at 20 °C. The amount of organic solvent in the mixtures is given in weight percent. Concentrated pH 7.2 buffer solutions were made such that, after dilution with the appropriate amount of the organic solvent, final concentrations of 20 mM Tris-HCl, 100 mM NaCl, and 1 mM EDTA were maintained. At the concentrations of organic cosolvent used in these experiments the pK_a of the Tris-HCl buffer is perturbed only slightly; we estimate the pH to be approximately 7.4 in the solvent mixtures (Douzou, 1974). The repetitive pressure-jump apparatus is described elsewhere (Macgregor et al., 1985; Clegg & Maxfield, 1976). Ethidium fluorescence was excited at 545 nm, and the emission was observed through a Schott OG-570 cutoff filter. Fluorescence excitation and emission data were recorded with an SLM Model 8000 spectrofluorometer; the emission data have been corrected for the spectral response of the detection system.

Table I: Relaxation Times of Various Reaction Mechanisms of EB Intercalation[a]

Model I: Site + EB $\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$ Complex

$$\tau^{-1} = k_1\{f(r)[DNA]_0 - f'(r)[EB]\} + k_{-1} \approx k_1[DNA]_0 \quad (I.1)$$

Model II:[b] DNA $\underset{k_{-c}}{\overset{k_c}{\rightleftharpoons}}$ DNA* $\quad K_c = \frac{k_c}{k_{-c}}$

DNA* + EB $\underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}}$ Complex

$$\tau_1^{-1} = k_c + k_{-c} \quad (II.1)$$

$$\tau_2^{-1} = k_a \frac{K_c}{1+K_c}\{f(r)[DNA]_0 - f'(r)[EB]\} + k_{-a}$$

$$\approx k_a \frac{K_c}{1+K_c}[DNA]_0 + k_{-a} \quad (for\ r \to 0\ and\ [EB] \ll [DNA]_0) \quad (II.2)$$

Model III: DNA + EB $\underset{k_{-d}}{\overset{k_d}{\rightleftharpoons}}$ DNA-EB $\quad K_d = \frac{k_d}{k_{-d}}$

DNA-EB $\underset{k_{-x}}{\overset{k_x}{\rightleftharpoons}}$ Complex

$$\tau_1^{-1} = k_d\{f(r)[DNA]_0 - f'(r)[EB]\} + k_{-d} \quad (III.1)$$

$$\tau_2^{-1} = k_x\left(\frac{K_d\{f(r)[DNA]_0 - f'(r)[EB]\}}{1 + K_d\{f(r)[DNA]_0 - f'(r)[EB]\}}\right) + k_{-x} \quad (III.2)$$

$$\approx k_x K_d[DNA]_0 + k_{-x} \quad (in\ the\ linear\ region;\ for\ r \to 0\ and\ [EB] \ll [DNA]_0)$$

[a] The term [DNA] refers to the concentration of base pairs. [b] The expressions for model II are valid for k_c and/or $k_{-c} > \tau_2^{-1}$.

Results

The effect of methanol upon the UV-ORD of DNA has been reported previously (Travers et al., 1970); at room temperature the spectrum remains substantially unchanged up to 70% (w/w) methanol, indicating that the cosolvent does not perturb the secondary structure. We observed no significant changes in the circular dichroism spectrum of poly[d(G-C)] in 60% glycerol.

In order to facilitate the discussion, Table I summarizes the concentration behavior of the inverse relaxation times (τ^{-1}) of the three kinetic mechanisms discussed below. The degree of binding site saturation r is given by

$$r = [complex]/[DNA]_0 \quad (1)$$

where [complex] is the molar equilibrium concentration of intercalated EB and $[DNA]_0$ is the total concentration of poly[d(G-C)] base pairs. The functions $f(r)$ and $f'(r)$ are as defined in Macgregor et al. (1985) and account for the excluded-site binding. Thus, $f(r)$ represents the fraction of potential sites still available for binding; $f'(r)$ is the derivative of $f(r)$. Because $r < 0.1$ in all experiments the approximate value of τ^{-1} (Table I) has been used throughout to determine the kinetic constants; in other words, by choosing DNA and EB concentrations such that $[EB] \gg [DNA]^0$, $r \to 0$, $f(r) \to$

Table II: Kinetics of Ethidium Intercalation into Poly[d(G-C)] in Organic–Aqueous Solvent Mixtures

solvent[a]	$10^{-6}k_1$ (M^{-1} s^{-1})	k_{-1} (s^{-1})	$10^{-6}K_1$[b] (M^{-1})	ΔV_1 (mL mol^{-1})	η[c] (cP)	ρ [g $(cm^3)^{-1}$]
buffer[d]	7.4	21	0.35	−9	1.0	0.999
10% glycerol	4.6	22.3	0.21	−7.2	1.29	1.022
50% glycerol	0.59	13.7	0.043	−6.2	5.98	1.127
60% glycerol	0.29	9.1	0.032	−4.3	10.7	1.154
10% methanol	4.0	37.4	0.105	−4.9	1.33	0.981
20% methanol	2.9	58.2	0.050	−6.6	1.60	0.967

[a] The concentration of the organic solvent is given in weight percent. [b] Association constant of the bimolecular reaction ($=k_1/k_{-1}$). [c] Viscosity of the solvent (Wolf et al., 1971). [d] 20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, pH 7.2, 20 °C. The same buffer was used with the mixed solvents.

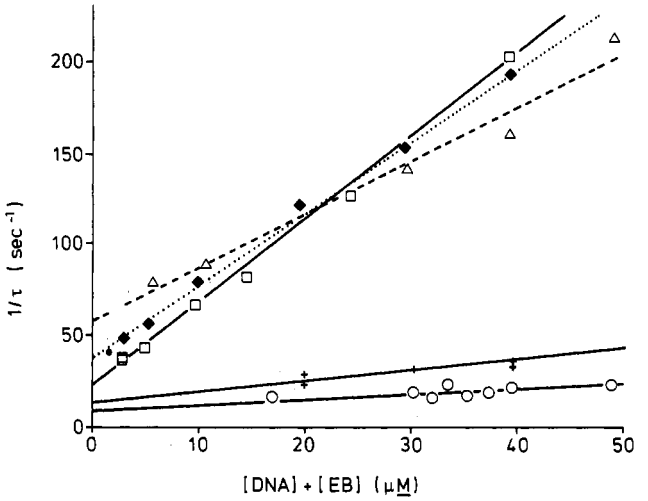

FIGURE 2: Inverse relaxation time vs. concentration for five organic–aqueous solvent mixtures: 10% methanol (◆), 20% methanol (△), 10% glycerol (□), 50% glycerol (+), and 60% glycerol (○). All organic solvent concentrations are reported as weight percent. The straight lines are least-squares fits of the data to eq I.1. The relaxation times for the 50% and 60% glycerol solutions were investigated up to $[DNA]_0$ = 120 and 240 μM, respectively. These data are not included in this figure in order to facilitate comparison with the other data.

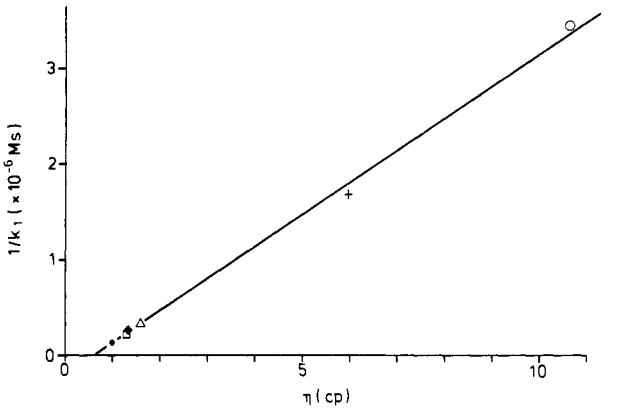

FIGURE 3: Viscosity dependence of the observed association rate constant, k_1. The symbols refer to the same solutions as those in Figure 2.

1, and $f'(r) \rightarrow -(2n-1)$ (=−3). Thus, the derived parameters are formally equivalent to those defined in general according to the excluded-site formalism.

Viscosity Dependence of Association Rate Constant. The concentration dependence of the kinetic relaxation times for each of the solvent mixtures is shown in Figure 2. The experimental values of the rate constants for the overall intercalation reaction in each solvent system are summarized in Table II. The linear dependence of the inverse relaxation time upon the DNA concentration is consistent with a single-step bimolecular intercalation reaction, model I of Table I. However, the observed forward rate constants calculated from the data are 2–3 orders of magnitude smaller than expected for a diffusion-controlled process, the rate of which can be estimated according to (Smoluchowski, 1917; Noyes, 1961; Berg & von Hippel, 1985)

$$k_d = (4\pi/1000)N_A D_{AB} R_{AB} \quad (2)$$

where N_A is Avogadro's number, D_{AB} is the sum of the diffusion constants of EB and a DNA binding site, and R_{AB} is the sum of the reactive radii of the two reactants. If D_{AB} is taken to be equal to the value of the diffusion constant for EB in water (3.0×10^{-6} cm^2 s^{-1}; Magde et al., 1974), and R_{AB} = 1.5×10^{-7} cm, then $k_d = 3.4 \times 10^9$ M^{-1} s^{-1}.

The diffusion constant of a particle is related to the solution viscosity through the friction coefficient f of the particle according to

$$D = k_b T/f \quad (3)$$

where k_b is Boltzmann's constant and T is the temperature (Einstein, 1905). For a sphere, $f = 6\pi\eta R$, where R is the radius of the particle and η is the solvent viscosity. Considering eq 2 and 3, we see that a plot of $1/k_1$ vs. η is expected to be linear for a diffusion-controlled reaction. We find that the association rate constant is in fact linearly dependent upon viscosity (Figure 3), indicating that intercalation is controlled by a diffusive process despite the quantitative disagreement with eq 2.

Resolution of this paradox requires the introduction of at least one additional step in the reaction pathway. The simplest mechanism consistent with our observation of a relatively slow but *viscosity-dependent* association rate constant is given by model II in Table I (see also Figure 1), in which DNA* is a local region of DNA with the correct conformation for EB intercalation. There is assumed to be no intercalative binding to the other form of DNA. The value of the diffusion-controlled bimolecular rate constant for the intercalation reaction (k_a) may be somewhat different from that describing diffusion to the outside bound species (k_d); however, we expect this difference to be relatively small, and assume that $k_a \approx k_d$. In deriving the equations for model II we have required that k_c and/or $k_{-c} >> k_a([DNA]^* + [EB])$ and k_{-a} because the observed kinetics do not depend upon whether EB or DNA is in excess (Macgregor et al., 1985). Expressions for the inverse relaxation time corresponding to these conditions are given in Table I. If the equilibrium constant of the DNA conformation change $K_c << 1$, the observed association rate constant will be considerably smaller than expected for a diffusion-controlled reaction but will still be proportional to the inverse of the viscosity. This is consistent with the experimental results: for example, in water k_d ($\approx k_a$) = 3.4×10^9 M^{-1} s^{-1}, and the observed association constant $k_1 = 7.4 \times 10^6$ M^{-1} s^{-1}; therefore, $K_c = 2.2 \times 10^{-3}$. Thus, the reaction is still diffusion controlled, but the number of sites capable of intercalation is much smaller than the total concentration of potential sites, thereby reducing the maximum association rate.

Considering the data acquired at all viscosities, the slope of the k_1^{-1} vs. η plot (Figure 3) is 3.5×10^{-5} M s P^{-1}. In accordance with the proposed two-step mechanism

$$\partial k_1^{-1}/\partial \eta = [(1 + K_c)/K_c](1000/4\pi N_A d_{AB} R_{AB}) \quad (4)$$

where $d_{AB} = D_{AB}\eta = 3 \times 10^{-8}$ cm^2 s^{-1} P and K_c is assumed to be independent of viscosity. Using the experimental value for the left-hand side of eq 4 and rearranging, one finds $K_c = 8 \times 10^{-4}$. Thus, the value of the rate constant in aqueous buffer and its dependence on viscosity yield similar estimates of K_c, the latter procedure being the more accurate.

Influence of Solvent Composition upon Rate of Dissociation. The rate of dissociation of the intercalated dye molecule also displays a solvent dependence; however, in contrast to the association rate, glycerol and methanol have opposite effects (Table II). Because of the chemical similarity of the solvent used and the uniform effect they exert upon the association rate constant, it is unlikely that this difference arises due to specific interactions of the organic cosolvents with the DNA. There is a linear relation between the solvent density and the dissociation constant (see Table II), and although this might reflect the sensitivity of the latter to the free volume of the solution (Clegg & Vaz, 1985), the limited range of densities employed make this relation uncertain.

ΔV in Solvent Mixtures. The molar volume change of the overall intercalation reaction (ΔV_1, Model I) was calculated from the observed relaxation amplitudes; the results are summarized in Table II. Negative ΔV values mean that the intercalated complex has a smaller molar volume than the individual species and is increasingly favored at higher pressure. Excluded-site effects were neglected because of the low degree of DNA binding site saturation. Provided that the total measured signal results from the intercalation of the dye, the relation between ΔV_1 and the volume change of the individual reactions of model II is given by

$$\Delta V_1 = \Delta V_c + [1/(1 + K_c)]\Delta V_a \quad (5)$$

where ΔV_c refers to the first step in model II and ΔV_a refers to the second step. At present we cannot resolve the relative contribution of these two reactions to the overall volume change. Because pressure was employed in the present experiments, analysis of the relaxation amplitudes yielded the reaction volumes; if instead, temperature were used to perturb the equilibrium, an equation analogous to eq 5 would be obtained with enthalpy changes (ΔH) in the place of the volume changes. Therefore, within the context of model II, previously reported ΔH_1 values depend linearly upon the enthalpy of the DNA conformation change, ΔH_c, and the enthalpy of the intercalation step, ΔH_a.

The spectroscopic properties of fluorescent molecules are sensitive to the molecular environment and could potentially be useful in the interpretation of the ΔV data. The spectral characteristics of EB in several solvents and bound to poly[d(G-C)] in buffer are summarized in Table III; they agree with similar data reported by Löber and Klarner (1985). The positions of fluorescence excitation and absorption maxima indicate that the intercalation site more closely resembles methanol or glycerol than water but that it is more polar than 2-butanol. Unfortunately, the charged EB is insoluble in nonpolar media such as cyclohexane; therefore, it is difficult to assess its spectroscopic behavior in such solvent environments. The fluorescence of EB bound to poly[d(G-C)] is shifted to higher energy (shorter wavelengths) than the emission in water, in contrast to what is observed for EB in the organic solvents. This implies either that the binding site is incapable of reorienting in response to the electronic configuration of the excited state (Macgregor & Weber, 1986) or that a different transition is enhanced in the bound state.

Table III: Spectroscopic Characteristics of EB in Various Solvents[a]

	absorption max (nm)	excitation max (nm)	emission average energy (cm^{-1})
buffer	480	476	15640
glycerol	511	510	15511
glycerol (5 °C)			15655
methanol	524	519	15400
2-butanol	546	537	15048
poly[d(G-C)]	514	521	16117

[a] All measurements at 20 °C, except as noted. The fluorescence excitation data were acquired by monitoring the fluorescence at the wavelength of maximum intensity. The fluorescence emission was excited at the wavelength of maximum absorption. The absorbance was <0.05 in all cases. The data for EB bound to DNA were determined in buffer with initial concentrations of 40 μM poly[d(G-C)] and 4 μM EB; no attempt was made to separate the relative contributions of the free dye and bound dye in the absorption spectrum.

Discussion

The modification of the physical properties of the solvent through the addition of organic cosolvents can provide insight into a reaction mechanism (Reichardt, 1979). We have used methanol and glycerol as cosolvents with water to determine which solvent properties influence the intercalation of EB into poly[d(G-C)]. The viscosity of the solution underwent by far the largest differential change and was the only solvent characteristic that could be correlated linearly or even monotonically with the association rate constant, other physical properties of the solutions remaining approximately constant. The dielectric constants of the solvents were, even in the most extreme case (60% glycerol), >62 (Janz & Tompkins, 1972), i.e., only 20% lower than the value for water. Although dielectric constants drastically different from that of water can lead to changes in the higher order structure of macromolecles, there is no evidence for such a change in poly[d(G-C)] in these solvents at the concentrations used.

The CD spectra of the poly[d(G-C)] with our solvent conditions indicate that the secondary structure of the DNA has only been minimally perturbed by the added cosolvents. In addition, we have no evidence for aggregation of the DNA based upon light scattering or ultracentrifugation (100000g). According to the work of Ivanov et al. (1973) the small variations in the CD spectrum of poly[d(G-C)] in 60% glycerol would correspond to insignificant changes in the twist angle of the DNA double helix. Transitions between the major conformation (B, Z, A) are also not seen. Thus, we assume that the structure of the DNA is independent of the cosolvent concentrations. Further evidence that there is no aggregation of the DNA (or that such a process does not play a role in the kinetics) is that the $1/\tau$ plots all vary linearly with the DNA concentration; should DNA aggregation remove or block any of the dye binding sites, and if this aggregation increases at higher cosolvent concentrations, we would observe a pronounced nonlinearity of the $1/\tau$ plots (diminishing slope) as a result of coupling to the binding step.

The observed association rate constant (k_1) for EB intercalation is several orders of magnitude slower than a single-step diffusion-controlled reaction; nevertheless, it is inversely proportional to the viscosity of the solvent. We interpret this as evidence for another step in the reaction mechanism in addition to the bimolecular association. In an earlier study we concluded that EB–poly[d(G-C)] binding kinetics were formally described by a single-step bimolecular mechanism (Macgregor

et al., 1985). This earlier work was also performed in 0.1 M NaCl and at micromolar concentrations of DNA and EB, but at a single viscosity. Our results were in agreement with previous studies in which a single-step mechanism was proposed (Magde et al., 1974; Jovin, 1975; Jovin & Striker, 1977; Mandal et al., 1980; Wilson et al., 1985).

Additional kinetic steps were observed in studies of EB intercalation carried out at high salt concentrations (Wakelin & Waring, 1980; Ryan & Crothers, 1984); however, destabilization of the complex at high ionic strength required the use of millimolar concentrations of DNA and ligand so that other kinetic steps, e.g., direct transfer of the dye between DNA strands (Ryan & Crothers, 1984), may have become important. The overall reverse rate constant, k_{-1}, measured by NMR (100 s^{-1}; Feigon et al., 1982) is 5 times greater that what we observe in aqueous buffer, a disparity that may reflect the relative sensitivities of the techniques to different aspects of the reaction. Likewise, the very short relaxation times reported in the temperature-jump studies (Waklin & Waring, 1980; Ryan & Crothers, 1984) may relate to the use of absorption instead of fluorescence to monitor the reaction kinetics. In addition, the possibility that these fast processes are due to either the high salt concentrations or the electric field used to generate the temperature jump cannot be excluded (Marcandalli et al., 1984; Dourlent & Hogrel, 1976).

There is a futher unresolved disagreement in the results of experiments carried out at higher reactant concentrations (NMR, and several temperature-jump studies). Although the lifetime of the intercalated complex as measured by NMR is 3 orders of magnitude longer than that found by electric-discharge temperature-jump, Ryan and Crothers (1984) claimed to have reconciled the temperature-jump and NMR results in a reanalysis of the data from Bresloff and Crothers (1975). To make the comparison, they selected the reaction velocity of a particular reaction in their proposed mechanism, neglecting the fact that the observed rates are the normal modes of the kinetic mechanism, i.e., linear combinations of the individual elementary steps. As their own data indicate (Bresloff & Crothers, 1975), and as supported by calculations using their reaction modes and rate constants, the expected rate of any kinetic process corresponding to their model is still much greater than those seen by NMR. Therefore, the disparity between the NMR data and the results of chemical relaxation experiments carried out at high salt and high DNA concentrations still exists. This discrepancy is not due to ionic strength effects alone since Wilson et al. (1985) found only single relaxation times in a study of the salt dependence of EB and propidium binding to DNA. The relaxations are slower than those observed in the temperature-jump experiments and agree with our results for the kinetics of EB binding to poly[d(G-C)] in 1 M NaCl with pressure jump (data not shown).

In order to resolve the difference between the expected bimolecular diffusion-controlled rate and the observed values, we have interpreted our data in terms of model II (Table I), which involves an unfavorable DNA conformational change prior to EB intercalation (Sobell et al., 1977; Dattagupta et al., 1978; Capelle et al., 1979). This is the basic mechanism usually proposed for hydrogen exchange (Englander & Kallenbach, 1984) except that the exchange reaction is replaced by the intercalation step in the present case. The mechanism represented by model II was proposed and discussed by Capelle et al. (1979) to interpret the rate of intercalative binding of bis(acridines); however, their data did not allow them to distinguish between models II and III.

According to model II the equilibrium of the local DNA conformational change greatly favors the closed B-DNA structure ($K_c \approx 10^{-3}$) which does not allow direct intercalation; this effectively lowers the binding site concentration and thereby causes the intercalation reaction to appear much slower than diffusion limited. The formation of this conformation probably involves substantial opening and unwinding of the B-DNA helix as evidenced by the crystal structure of intercalated complexes (Sobell et al., 1977; Quigley et al., 1986). As discussed by Sobell et al. (1983), the preintercalation conformational change of the DNA may be similar to that which double-stranded nucleic acids undergo during the nucleation process preceding a helix-to-coil transition or prior to hydrogen exchange. The fraction of base pairs in nonnative conformations has been estimated by using formaldehyde denaturation (McGhee & von Hippel, 1977), thermal denaturation of polynucleotides with mismatched bases (Gralla & Crothers, 1973), hydrogen exchange (Teitelbaum & Englander, 1975; Mandal et al., 1979; Leroy et al., 1985), NMR (Mirau & Kearns, 1984), and covalent drug–DNA interactions (Daune et al., 1985). There is a large difference between the various results reflecting the extent of the conformation change necesary to elicit a particular effect and the influence of other reaction steps upon the observed rate (Wartell & Benight, 1985). Our estimates of K_c agree best with those of hydrogen exchange, which show that approximately 1% of the base pairs are in an open state capable of intercalation. Recently, a reinterpretation of hydrogen-exchange kinetics of poly(rA)·poly(rU) and poly(rI)·poly(rC) led to the estimate of 10^{-3} for the equilibrium constant of base-pair opening in these polymers (Leroy et al., 1985).

The estimate of K_c depends upon the value of k_a (see Table I); we have assumed a fully diffusion-controlled bimolecular rate. Very fast reactions generally proceed by first forming an encounter complex, which is followed by the actual chemical reaction; this is depicted in Figure 1 by breaking up the reaction step (DNA* + EB → complex) into two reaction steps (DNA* + EB → DNA*·EB → complex). The association rate constant of this process is then $k_a^{-1} = k_{d'}^{-1} + k_I^{-1}$ (Noyes, 1961). This step in the pathway is fully diffusion controlled if $k_I \gg k_{d'}$ so that $k_a \approx k_{d'}$. If this condition is not met, k_a will be reduced, requiring a larger estimate of K_c; therefore, our value of K_c should be taken as a lower bound estimate.

We emphasize that if model II represents the major reaction pathway, at least one of the rate constants k_c or k_{-c} must be large compared to k_a([DNA]* + [EB]) and k_{-a} in order to account for all of the relaxation data. If [EB] ≪ [DNA]* and k_c and k_{-c} were both slower than the other rates, then the fastest normal model of model II would have a relaxation time given by eq II.2. However, if k_c and k_{-c} were both numerically small, then under conditions of excess EB, one would then find $\tau_{-1} \approx k_a$[EB] and the observed times would not be reduced by the factor $K_c/(1 + K_c)$ as is found when DNA is in excess. We have previously shown (Macgregor et al., 1985) that varying either EB or DNA has symmetrical effects upon the reaction rates, provided that excluded-site binding is accounted for. Asymmetric behavior with respect to reactant concentration is *not* observed in 0.1 M salt; therefore, because $K_c = k_c/k_{-c} \ll 1$, we conclude that $k_{-c} \gg k_a$([DNA]* + [EB]) and k_{-a}, underscoring the fact that the decrease in the observed bimolecular rate requires a rapid readjustment of the DNA ↔ DNA* equilibrium.

The viscosity dependence of k_1 implies that a diffusion-controlled process in the reaction directly influences the observed rate but does not uniquely identify the step in the

mechanism. Model III in Table I involves a rapidly formed weak electrostatic association of EB with DNA followed by a slower movement of the dye into the DNA to form the intercalation complex; this model has been proposed for the intercalation of various dyes into DNA (Li & Crothers, 1969; Ramstein et al., 1972; Bresloff & Crothers, 1975; Ramstein et al., 1980; Corin & Jovin, 1986). The first step is assumed to be diffusion controlled so that the opening of the local structure and subsequent intercalation become rate limiting. The proposed rapid binding step has not been observed for EB, so it would be assumed that the observed process corresponds to the slower reaction mode in this pathway, i.e., τ_2^{-1} of model III (Table I). The slope of the linear region of a plot of τ^{-1} vs. $\{f(r)[\mathrm{DNA}]_0 - f'(r)[\mathrm{EB}]\}$ will provide $k_x K_d$, and k_{-x} is equal to the ordinate intercept. Since the association and dissociation steps of the bimolecular reaction are assumed to be diffusion controlled, the equilibrium constant K_d would not be viscosity dependent (Eigen, 1974). Therefore, in order to be consistent with our results, this reaction mechanism *requires* that the rate of the monomolecular step k_x be inversely proportional to the viscosity. If K_d is estimated to be $\approx 10^3$ M^{-1}, then k_x would be $\approx 10^4$ s^{-1}, a value which ensures that the effective association rate constant ($k_x K_d$) is less than expected for a purely diffusion-controlled association. However, such a value for k_x (the rate of helix opening) would be orders of magnitude faster than the rates observed in NMR or hydrogen-exchange experiments. Furthermore, this second step in the mechanism is considered to involve the opening and stretching of the DNA helix so as to accommodate the dye between the base pairs; it is difficult to imagine a process involving only local conformational changes of the DNA that would be directly controlled by the viscosity of the solution and simultaneously require such a long time, ~ 100 μs, to complete. The observed viscosity independence of DNA denaturation (Crothers, 1964; Spatz & Baldwin, 1965) makes this proposal even more unlikely. For proteins, the rates of local motions directly coupled to the solvent viscosity are on the order of nanoseconds (Beece et al., 1980), much faster than those predicted by model III. Although we are unaware of analogous studies involving DNA, there is evidence for large-scale motions of the DNA bases on the nanosecond time scale from NMR (Behling & Kearns, 1986; Mirau et al., 1985), fluorescence anistropy and linear dichroism decay (Wahl et al., 1970; Millar et al., 1980; Hogan et al., 1982), and ESR (Robinson et al., 1980). Thus, even though we cannot definitively reject model III, we presently consider the evidence for model II more compelling.

Although we cannot directly determine the rate of the conformational change in model II, the observation of a viscosity-dependent association constant allows us to place limits upon it. If $K_c = 10^{-3}$ is assumed to be ≥ 500 s^{-1} (according to the model it must be faster than the observed τ), then $k_c \geq 0.5$ s^{-1}. This value would be in agreement with the majority of the reported hydrogen exchange and NMR rates (see references given above). The rates given by Leroy et al. (1985) are substantially larger than this but do not contradict our results if the reverse rate constant of the conformational change (k_{-c}) is chosen to be much larger. In summary, the conditions implicit in model II and our estimate of K_c agree with previously measured values of the rate of helix opening and closing.

Unambiguous verification of the reaction mechanism of model II will require additional experiments; however, certain implications of this model have already been addressed in other studies. For instance, the change in the DNA conformation to create an intercalation site occurs in the absence of the dye molecule so that the effective association rate constant $K_c k_a$ is directly controlled by the equilibrium properties of the DNA. Since most intercalating dyes have similar diffusion constants and, therefore, similar k_a's, the association rate constants of simple intercalators should be approximately equal, which is indeed the case (Capelle et al., 1979; Ramstein et al., 1980; Wilson et al., 1985; Laugâa et al., 1986). Any large discrepancy would be reason to suspect interactions between the dye and the DNA in addition to intercalation. A second experimental observation in agreement with model II is the finding that the charge on the intercalator does not play a major role in the observed association rate constant. Thus, the value of this parameter is approximately the same for EB, propidium, and the bis- and tris(acridines), despite the fact that at neutral pH these dyes have charges of 1+, 2+, 4+, and 7+, respectively (Wilson et al., 1985; Capelle et al., 1979; Laugâa et al., 1985). The extremely large equilibrium association constant of tris(acridine) is a consequence of the decrease in the dissociation rate constant. Of course, the particular configuration of the DNA that accepts different dyes could be quite different and influence the value of K_c as, for example, in the case of the very large porphyrin intercalators (Pasternack et al., 1983). Finally, the importance of a conformation change prior to intercalation is demonstrated by the fact that EB binds poorly to Z-DNA (Pohl et al., 1972; van de Sande & Jovin, 1982; Jovin et al., 1983), which has been shown to exchange protons with the solvent orders of magnitude slower than B-DNA (Mirau & Kearns, 1984).

The volume change of the overall intercalation reaction (ΔV_1) is less negative in the organic–aqueous solvent mixtures than in water. We have previously reported the ΔV_1 for poly[d(A-T)] to be -15 mL mol^{-1}; this is 6 mL mol^{-1} more negative than the value for poly[d(G-C)] in aqueous buffer (Macgregor et al., 1985). If the volume change associated with the actual intercalation of EB between the base pairs (ΔV_a) is dominated by the desolvation of the dye and, therefore, approximately independent of the polymer, then this difference of ΔV_1 reflects the volume change necessary to create the intercalation site in the two polymers and is presumably related to the structure and solvation changes in each case.

Conversely, we propose that the organic cosolvents predominately influence the volume change attributable to the movement of EB from the solvent into the intercalation site (ΔV_a) and that the volume change associated with the formation of the binding site (ΔV_c) is minimally altered by the solvent. This net reduction in the reaction volume arises through the ability of the organic cosolvent to form energetically favorable nonpolar contacts with EB and still hydrogen bond with the bulk solvent, a possibility which does not exist in a purely aqueous medium. These nonpolar contacts decrease the requirement for the solvent to form a cagelike structure around the free dye with the result that the partial volumes of the free and intercalated dyes become more similar and ΔV_a (and hence ΔV_1) approaches zero.

The interaction between the alkyl portion of the alcohol and EB is also reflected in the absorption and fluorescence excitation spectra; the maxima of these spectra are at approximately the same wavelength as that of EB in glycerol or methanol. However, the large blue shift of the fluorescence emission of EB bound to poly[d(G-C)] relative to any other solvent indicates that the environment of the binding site is not the same as an alkyl alcohol at 20 °C. Further experiments are necessary before these data can be employed quantitatively in the interpretation of the volume changes. It is interesting

that the ΔV_1 of the 20% methanol solution lies between the values for pure water and 10% methanol; many physical properties of alcohol–water mixtures display extrema at low alcohol concentrations (Frank & Ives, 1966).

Registry No. Poly[d(G-C)], 36786-90-0; ethidium bromide, 1239-45-8.

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Mitochondrial ATP Synthase Complex: Interaction of Its F_1 Adenosinetriphosphatase Moiety with the Heavy Atom Iodine†

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ABSTRACT: Studies were carried out to determine whether a simple electron-dense "heavy atom" like iodine could be introduced selectively into one or more of the subunits of the mitochondrial ATP synthase complex of rat liver. Surprisingly, very low amounts of iodine are incorporated into the isolated F_1 moiety of this complex under conditions which result in a marked loss of catalytic activity. ATPase activity is inactivated in a concentration-dependent manner at pH 7.5 with half-maximal inactivation occurring at about 40 μM iodine. A maximum of only 10 atoms of iodine are incorporated per F_1 molecule under conditions where inhibition of ATPase activity is linearly related to iodine incorporation. The molecular size of F_1 after iodination is unchanged, indicating that inactivation is due to modification of essential amino acid residues rather than subunit dissociation. Treatment of F_1 with 20-50 μM [^{125}I]iodine followed sequentially by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography showed that the β subunit is preferentially labeled. Significantly, about two atoms of iodine per β subunit are incorporated. Some iodine amounting to less than 23% of the total radioactivity placed on the gels is recovered in the α and γ subunits whereas no radioactivity is detected in the δ and ϵ subunits. Iodination of F_1 appears to modify essential residues other than those involved in substrate or product binding per se. Thus, nucleotide binding to F_1 is unaltered by iodine, and neither phosphate, MgADP, nor MgATP protects F_1 against inhibition by this agent. Rather, loss of ATPase activity upon iodination appears to be associated with one or more pH-sensitive groups. It seems likely that these groups are tyrosine both because tyrosine is the principal amino acid involved in the iodination of proteins and because the pK of its phenolic hydroxyl groups is dramatically altered by iodination of the associated benzene ring. These studies represent the first attempt to introduce a heavy atom into an F_1-ATPase preparation in a selective manner. The results show that at low concentrations iodine does react preferentially with β subunits of the rat liver enzyme while inactivating the catalytic capacity of the intact complex. These findings may prove useful in future studies directed at understanding structural-functional relationships within ATP synthase complexes.

Enzymes involved in ATP synthesis in biological systems consist of two major components: one called F_0 which directs protons to the second component called F_1 [for recent reviews, see Senior (1979), Dunn and Heppel (1981), Amzel and Pedersen (1983), Senior and Wise (1983), Wang (1983), and Hatefi (1985)]. It is the F_1 moiety which contains binding sites for Mg^{2+}, ADP, and P_i as well as amino acid residues essential for dehydration of the latter two substrates to give ATP. Work in a number of laboratories has focused on elucidating the structure of F_1 and relating its structural features to its function. Significantly, the F_1 moieties from bacteria, chloroplasts, and mitochondria have a molecular weight of 360000-380000 and contain five different types of polypeptide chains in the stoichiometric ratio $\alpha_3\beta_3\gamma\delta\epsilon$ (Catterall et al., 1973; Kagawa et al., 1976; Foster & Fillingame, 1979; Yoshida et al., 1979). The purified β subunit has been shown from one species, *Rhodospirillum rubrum* (Harris et al., 1985), to catalyze a very low turnover of ATP in the hydrolytic reaction, indicating that this subunit contains one or more amino acid residues involved in the catalytic event.

To date, only the F_1 moiety of the rat liver ATP synthase has been crystallized in a form which is amenable to X-ray crystallography (Amel & Pedersen, 1978). A 9-Å map has

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