Pressure-Jump Study of the Kinetics of Ethidium Bromide Binding to DNA†

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ABSTRACT: Pressure-jump chemical relaxation has been used to investigate the kinetics of ethidium bromide binding to the synthetic double-stranded polymers poly[d(G-C)] and poly[d(A-T)] in 0.1 M NaCl, 10 mM tris(hydroxymethyl)aminomethane hydrochloride, and 1 mM ethylenediaminetetraacetic acid, pH 7.2, at 24 °C. The progress of the reaction was followed by monitoring the fluorescence of the intercalated ethidium at wavelengths greater than 610 nm upon excitation at 545 nm. The concentration of DNA was varied from 1 to 45 μM and the ethidium bromide concentration from 0.5 to 25 μM. The data for both polymers were consistent with a single-step bimolecular association of ethidium bromide with a DNA binding site. The necessity of a proper definition of the ethidium bromide binding site is discussed: it is shown that an account of the statistically excluded binding phenomenon must be included in any adequate representation of the kinetic data. For poly[d(A-T)], the bimolecular association rate constant is $k_1 = 17 \times 10^6$ M^{-1} s^{-1}, and the dissociation rate constant is $k_{-1} = 10$ s^{-1}; in the case of poly[d(G-C)], $k_1 = 13 \times 10^6$ M^{-1} s^{-1}, and $k_{-1} = 30$ s^{-1}. From the analysis of the kinetic amplitudes, the molar volume change, ΔV^0, of the intercalation was calculated. In the case of poly[d(A-T)], $\Delta V^0 = -15$ mL/mol, and for poly[d(G-C)], $\Delta V^0 = -9$ mL/mol; that is, for both polymers, intercalation is favored as the pressure is increased. The magnitude of the molar volume changes suggests that they are predominately determined by the stacking interactions between ethidium bromide and the aromatic base pairs.

The noncovalent interaction of dyes and drugs with DNA has been studied by a variety of equilibrium and kinetic techniques [for recent reviews, see Neidle & Abraham (1984), Steiner & Kubota (1983), and Berman & Young (1981)]. However, there are still substantial uncertainties about the mechanism of the interaction of planar aromatic dyes with double-stranded DNA (Bresloff & Crothers, 1975; Mandal et al., 1980; Wakelin & Waring, 1980; Marcandalli et al., 1984; Ryan & Crothers, 1984). A comparison of the results is complicated by the wide range of experimental conditions, e.g., ionic strength, which have been used in the investigations. The repetitive pressure-perturbation (pressure-jump) chemical relaxation technique requires no special solvent conditions and in addition provides the molar volume change (ΔV^0) of the reaction(s). We have used this method to study the kinetics of the association of ethidium bromide (EB)[1] with the synthetic double-stranded polynucleotides poly[d(G-C)] and poly[d(A-T)].

A variety of models have been proposed to explain DNA–dye interactions. The most general analytical formalism to describe equilibrium binding experiments of molecules to DNA is that of McGhee & von Hippel (1974), which provides expressions and interpretations which take into account the phenomenon of statistically excluded binding sites on the DNA lattice. Ligands which require more than one consecutive interaction site along the DNA lattice for binding give rise to statistically excluded potential binding sites for two reasons: The potential sites are not distinct; i.e., they overlap; and the bound ligands are distributed randomly upon the DNA lattice such that gaps consisting of less than the number of interaction sites required for binding become isolated between occupied sites. This effect, which was recognized earlier by others (Latt & Sober, 1967; Crothers, 1968; Zasedatelev et al., 1971; Schellman, 1974), becomes more significant as the fraction of bound base pairs increases, such that at larger degrees of occupation the free energy required for the further binding of ligand becomes very large. The consequences of the statistical exclusion of potential binding sites are quite pronounced in equilibrium binding isotherms at high ligand concentrations when the concentration of DNA is limiting (Bauer & Vinograd, 1970); the concomitant change in the kinetic behavior under these conditions has been formulated by Jovin & Striker (1977). However, due to the difficulty of achieving the necessary experimental conditions, previous investigations of the kinetic properties of dye–DNA binding have not tested the concentration region which would yield the most information about the statistically excluded mechanism and check the consistency of the kinetic data with this model. For the case of EB–DNA intercalation kinetics, a critical examination is important because it is not clear whether reactions other than bimolecular associations between available sites and the free dye contribute to the relaxation spectrum. For example, direct ligand transfer between DNA binding sites has been proposed to explain multiple exponential relaxation kinetic data (Bresloff & Crothers, 1975; Wakelin & Waring, 1980; Ryan & Crothers, 1984). We have analyzed the data in terms of a single-step bimolecular reaction mechanism and show that the concentration region in which a relatively high fraction of lattice sites are occupied with dye molecules provides a stringent test for the statistical binding model and that no further mechanistic complications need to be invoked. When the dye concentration is limiting, two simpler binding models can be applied which do not include the concept of statistically excluded sites, but they are incapable of accounting for all the data.

Pressure perturbation offers several advantages over conventional temperature-jump techniques in the study of biological systems. The main advantages are (1) the speed of the

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[1] Abbreviations: EB, ethidium bromide; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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pressure jump is independent of solvent conditions, (2) no electric fields pass through the sample, and (3) the time domain may extend from approximately 10 μs to any desired upper limit. The main disadvantage of using pressure to perturb the equilibrium is the relative insensitivity of most reactions of biochemical interest to conveniently accessible pressures (Weber & Drickamer, 1983). Studies employing static pressure techniques employ very high pressures (on the order of several kilobars), and a number of experimental methods for making pressure jumps up to approximately 200 bar have been reported (Kegeles, 1978). In our experimental design, an adaptation of that of Clegg & Maxfield (1976), we use rapid, repetitive, 5-bar pressure perturbations generated by the expansion and contraction of piezoelectric crystals. With this method, we can spectroscopically observe the kinetics of reactions in which the magnitude of the molar volume change is less than 7 mL/mol.

Experimental Procedures

Materials. Ethidium bromide (reinst grade) was purchased from Serva (Heidelberg, FRG) and used without further purification. The concentration was determined spectrophotometrically by using an extinction coefficient of 5850 M^{-1} cm^{-1} at 480 nm (Bresloff & Crothers, 1975). In all experiments, we monitored the fluorescence of ethidium through a Schott RG-610 cutoff filter (Schott, Inc., Mainz, FRG), which passes only light of wavelengths greater than 610 nm. Excitation was at 545 nm using a spectral bandwidth of approximately 10 nm. We observed no relaxation of the free ethidium signal in the absence of DNA.

Poly[d(A-T)] (sodium salt) was purchased from Boehringer-Mannheim (Mannheim, FRG) and used without further purification. The concentration was determined by measuring the absorbance at 262 nm and using ϵ = 13 200 M^{-1} cm^{-1} (Inman & Baldwin, 1962). Poly[d(G-C)] was synthesized by a modification of the procedure of Gill et al. (1974) using *Micrococcus luteus* DNA polymerase (P-L Biochemicals) and poly[d(I-C)] as a template. The concentration was determined by measuring the absorbance at 254 nm using ϵ = 14 200 M^{-1} cm^{-1} (Pohl & Jovin, 1972).

All DNA concentrations are reported in terms of base pairs. All experiments were performed in 0.1 M NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 7.2, at 24 °C.

Pressure-Jump Relaxation Kinetic Technique. The pressure-jump cell is an improvement of that described by Clegg & Maxfield (1976). The main advantages of the new design are the substantially smaller size and easier sample loading. The rapid, repetitive pressure changes are produced by repeatedly applying and removing a high voltage to a stack of piezoelectric crystals. The high voltage causes the crystals to expand and thereby compress the sample solution in the optical cell. Upon removal of the high voltage, the piezoelectric crystals contract, and the pressure decreases to the static level. This apparatus is capable of maintaining a static pressure of approximately 120 bar. The size of the pressure jump is determined by the voltage applied to the crystals and the geometry of the sample cell. In these experiments, the static pressure was maintained at approximately 70 bar, and the magnitude of the pressure jump was 5 bar. Although in the present experiments we relied exclusively on fluorescence detection of the chemical relaxation, the design of the pressure-jump apparatus is such that any optical parameter may be monitored, if need be.

To increase the sensitivity and eliminate digitation error, the fluorescence signal is offset and amplified. The data acquisition, control of the timing, and the offset are controlled with a Data Translation Model 1711 direct-memory access, analog to digital, digital to analog converter with a maximum sampling rate of 120 kHz which is interfaced to a PDP 11/34 computer. A total of 1024 data points are acquired during each pressure cycle, during which the relaxation is observed twice, i.e., first upon application and then after removal of the excitation voltage to the piezoelectric crystals. Currently, the maximum repetition rate of this cycle is 100 Hz, which corresponds to a time per data channel of about 10 μs. The data from successive jumps are averaged until a satisfactory signal to noise ratio is achieved.

To account for nonidealities, the pressure pulse is recorded with a semiconductor pressure transducer (Kulite Semiconductor Products, Inc.). The relaxations are deconvolved from the pressure pulse and analyzed by using a nonlinear least-squares fitting program written by Leon Avery. Any number of exponentials may be fitted; however, no substantial improvement in the fit of the relaxation (as judged by the variance) could be achieved by using more than one component. A second, faster process was observed at all jump rates; neither its decay time (which was always equal to the system time constant) nor its amplitude was dependent on the concentration of DNA or ethidium bromide. We ascribe this process to an optical artifact mechanically induced by the pressure jump and will not consider it further.

Results

In order to interpret our kinetic results of EB binding to poly[d(A-T)] and poly[d(G-C)], we consider the simplest reaction mechanisms which are available. The model which defines the DNA lattice binding sites as independent and nonoverlapping is explained in the Appendix. Although we show that this simple model cannot account for all the kinetic data, we consider the limiting conditions under which the more complicated expressions of the following mechanism are indistinguishable in form from those corresponding to the simpler approach. The statistically excluded binding model defines the next level of complexity, and it is fully compatible with all our results. We do not consider any further complications, such as cooperativity effects, since this would only increase the variance of the fitted parameters without significantly improving the fits.

Statistically Excluded Binding. (A) Equilibrium State. Some aspects of the statistically excluded binding model which are required for the data analysis and the discussion are now described. The equilibrium aspects of ligand interaction with a linear array or lattice of overlapping binding sites such as that presented by DNA have been treated theoretically by Crothers (1968), Zasedatelev et al. (1971), Schellman (1974), and McGhee & von Hippel (1974). The concentration of potential binding sites, [sites], depends upon the fraction of sites already occupied such that

$$[\text{sites}] = [\text{sites}]_0 f(r) \quad (1)$$

where the subscript 0 denotes the total concentration of potential intercalation sites in the naked lattice. In this model, a site is located between any two adjacent base pairs, and $[\text{sites}]_0$ is equal to the base pair concentration provided that the number of base pairs per DNA molecule is large and end effects are ignored. Therefore, for the statistically excluded binding model, all DNA concentrations are given in base pairs. In the model of McGhee & von Hippel (1974), $f(r)$ accounts for the exclusion of potential binding sites arising from the statistical nature of the distribution of the ligands upon the binding lattice and is given by

$$f(r) = (1 - nr)^n[1 - (n - 1)r]^{1-n} \quad (2)$$

where the number of intercalation sites *directly* occupied or otherwise perturbed (i.e., removed from further binding interactions) by an isolated ligand molecule equals $2n - 1$. However, in the presence of saturating ligand concentration, potential intercalation sites are multiply excluded by flanking bound ligands so that the fractional lattice occupancy approaches the limit of $1/n$, i.e., an average of n intercalation sites per bound ligand. The fraction of binding sites occupied, r, is defined as

$$r = [X]/[\text{sites}]_0 \quad (3)$$

where [X] is the equilibrium concentration of the bound ligand species. The free ligand concentration is given by the usual conservation condition; i.e., $[L] = [L]_0 - [X]$, where $[L]_0$ is the total analytical ligand concentration.

Equations 1–3 can be used to obtain an equation with the same form as eq A1:

$$r/[L] = K_a(1 - nr)f(r) \quad (4)$$

where K_a is the association constant. For values of n greater than 1; the plot of $r/[L]$ vs. r (the conventional Scatchard plot) exhibits positive curvature. A line extrapolated from the initial slope at low r intersects the r axis at $1/(2n - 1)$. In the case of EB–DNA interactions, this intercept has been found to be 0.33 (LePecq & Paoletti, 1967; Bauer & Vinograd, 1970; Bresloff & Crothers, 1975). This implies that $2n - 1 = 3$ intercalation sites are excluded by each bound ligand at low binding levels, while a saturated lattice has a ligand attached to every other ($n = 2$) intercalation site. Thus, the intercalation of a dye molecule perturbs the structure of the two sites directly adjacent to it (nearest-neighbor sites) so as to preclude intercalation at these sites. Diffraction studies on DNA fibers and crystals have provided structural details of these effects (Bond et al., 1975; Sobell et al., 1982). This analysis is in contrast with the interpretations given in some earlier studies (LePecq & Paoletti, 1967) which were influenced by the incorrect evaluation of the extrapolated intercept according to eq A1 (see the Appendix). That the number of potential intercalation sites lost by binding one ligand to a free region of the lattice is equal to $2n - 1$ and not n arises from the formalism of the model which assumes a vectorial ligand–lattice interaction (McGhee & von Hippel, 1974). This assumption is formally equivalent to excluding an equal number of lattice sites on both sides of the bound ligand.

(*B*) *Kinetics.* Our relaxation rate data for both polymers can be represented by a single-step bimolecular reaction mechanism:

$$\text{sites} + \text{EB} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{complex} \quad (5)$$

where k_1 and k_{-1} are the forward and reverse rate constants, respectively. The dependence of the relaxation times upon the concentration of reactants for the case of statistically excluded binding has been dealt with in a manner adequate for present purposes by Jovin & Striker (1977). They found that the inverse relaxation time, τ^{-1}, for a single-step bimolecular reaction between DNA and a ligand which occupies n lattice sites is given by

$$\tau^{-1} = k_1\{[\text{sites}] - f'(r)[\text{EB}]\} + k_{-1} \quad (6)$$

where $f'(r) = df(r)/dr$ and is given by

$$f'(r) = [nr(n - 1) - 2n + 1](1 - nr)^{n-1}[1 - (n - 1)r]^{-n} \quad (7)$$

For $n = 1$, $f(r) = 1$ and $f'(r) = -1$ so that eq 6 reduces to the equation describing the kinetics for independent nonoverlapping binding sites (eq A3).

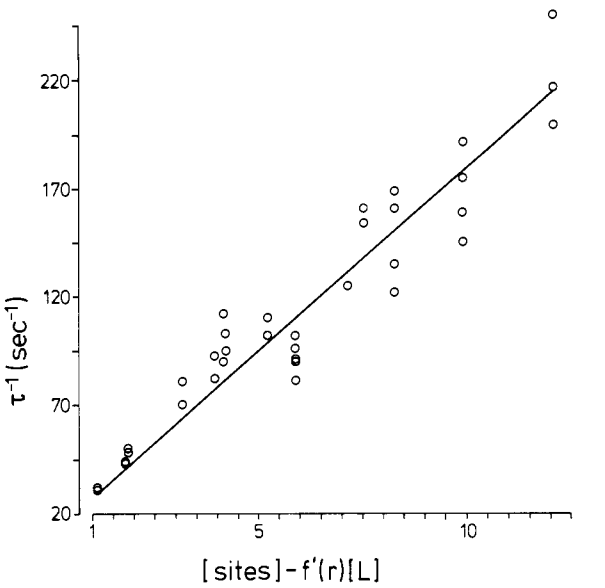

FIGURE 1: Concentration dependence of the inverse relaxation time, τ^{-1}, of EB intercalation into poly[d(A-T)]. The solid line is a fit of the data assuming the statistically excluded binding model (eq 6).

Table I: Kinetic Constants for the Ethidium Bromide–DNA Interaction Assuming Statistically Excluded Binding[a]

	k_1 ($\times 10^{-6}$ M^{-1} s^{-1})	k_{-1} (s^{-1})	K_a ($\times 10^{-6}$ M^{-1})	ΔV^0 (mL mol^{-1})
poly[d(A-T)]	17	10	1.7	−15
poly[d(G-C)]	13	30	0.43	−9

[a] At 24 °C in 0.1 M NaCl, 10 mM Tris, and 1 mM EDTA, pH 7.2. DNA concentrations given in base pairs.

Jovin & Striker (1977) also derived an expression for the concentration dependence of the relaxation amplitudes which takes into account statistically excluded binding. For a single-step bimolecular reaction, the result is the same as eq A4 except that a different concentration function, Γ^{-1}, is substituted for g^{-1}:

$$\Gamma = -f'(r)/[\text{sites}] + 1/[\text{EB}] + 1/[X] \quad (8)$$

The molar volume change of a reaction at constant temperature, ΔV^0, is related to the change in the association constant as a function of pressure by

$$(\partial \ln K_a/\partial p)_T = -\Delta V^0/RT \quad (9)$$

where p is the pressure in bar, T is the absolute temperature, and R is the gas constant (80.9 mL bar K^{-1} mol^{-1}).

Kinetics of the EB–Poly[d(A-T)] Interaction. The kinetic relaxation times are shown in Figure 1 together with the analysis according to the mechanism of eq 5. Our results are consistent with n equal to 2, in agreement with the kinetic studies of Bresloff & Crothers (1975) and previous equilibrium studies (Bauer & Vinograd, 1970). From an iterative evaluation of the slope and intercept, the kinetic constants were found to be $k_1 = 17 \times 10^6$ M^{-1} s^{-1} and $k_{-1} = 10$ s^{-1}, respectively; these values yield a kinetically determined association constant, $K_a = k_1/k_{-1} = 1.7 \times 10^6$ M^{-1} (Table I).

The difference between a model in which the statistical effects are accounted for and one in which the sites are taken to be nonoverlapping and independent becomes apparent when a substantial fraction of the binding sites are occupied. Figure 2a,b shows the dramatic effect of neglecting the statistical nature of these interactions at large r values. In Figure 2a, the data are simulated in accordance with the statistically excluded binding formalism (Jovin & Striker, 1977) using the constants given in Table I. The two simulations shown in

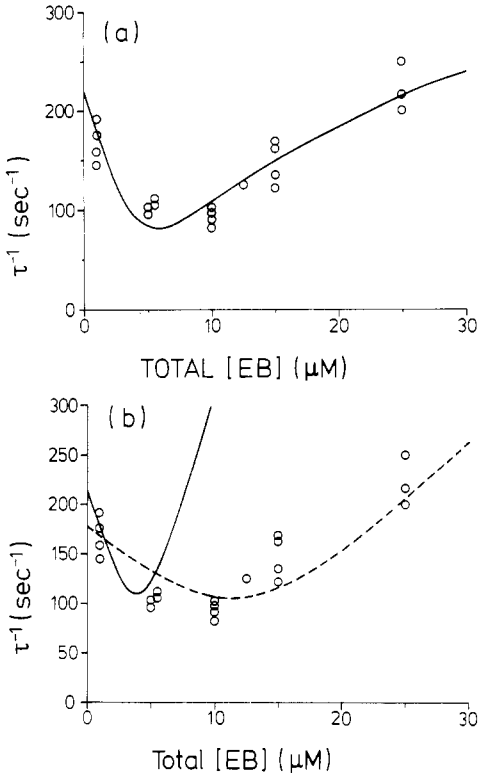

FIGURE 2: Simulations of the inverse relaxation time at large r values as a function of the total EB concentration for the three models under consideration. The total concentration of poly[d(A-T)] = 12.5 μM (base pairs). (a) Statistically excluded binding, simulated by using the constants given in Table I. (b) Data simulated for the case in which the EB binding site consists of m^{-1} independent, nonoverlapping base pairs: m^{-1} = 3 (—); m = 1 (---); see the Appendix.

Figure 2b are the results of using the kinetic constants obtained from fitting data at low r values assuming nonoverlapping, independent sites (analyses not shown; see the Appendix for a description of the model and definition of the DNA concentration units). The solid line is the case of EB binding to nonoverlapping sites consisting of three adjacent intercalation sites (k_1 = 48 × 10^6 M^{-1} s^{-1} and k_{-1} = 14.8 s^{-1}). The dashed line shows the result of assuming that all intercalation sites are potential binding sites at all levels of saturation, i.e., n = m = 1 (k_1 = 13 × 10^6 M^{-1} s^{-1} and k_{-1} = 17 s^{-1}).

The analyses of the relaxation amplitudes are presented in Figure 3a–c. The solid line in Figure 3a is a least-squares fit of ΔV^0 to eq A4, with Γ as the concentration function, using the kinetically determined K_a. In accordance with eq A4, the fit was constrained to pass through the origin. From the slope of this line, we calculate ΔV^0 = −15 mL mol^{-1} at 24 °C, the negative sign implying that the equilibrium in eq 5 shifts to the right; that is, EB intercalation is favored as the pressure is increased.

In the two cases of independent, nonoverlapping binding considered here, the amplitudes were fit by using the same K_a's as in the simulations shown in Figure 2b. The results of these fits are shown in Figure 3b,c. The relative positions of the data points in these three plots change due to the behavior of the g and Γ functions. Comparison of Figure 3a–c and the variances of the fits demonstrates that the statistically excluded binding model best describes the relaxation amplitudes.

The steady-state interaction of the dye with poly[d(A-T)] at high r values is simulated with the association constants derived from the kinetic analyses (Figure 4a–c). Each point is the average of at least two measurements. The simulations are good both for the statistically excluded binding model

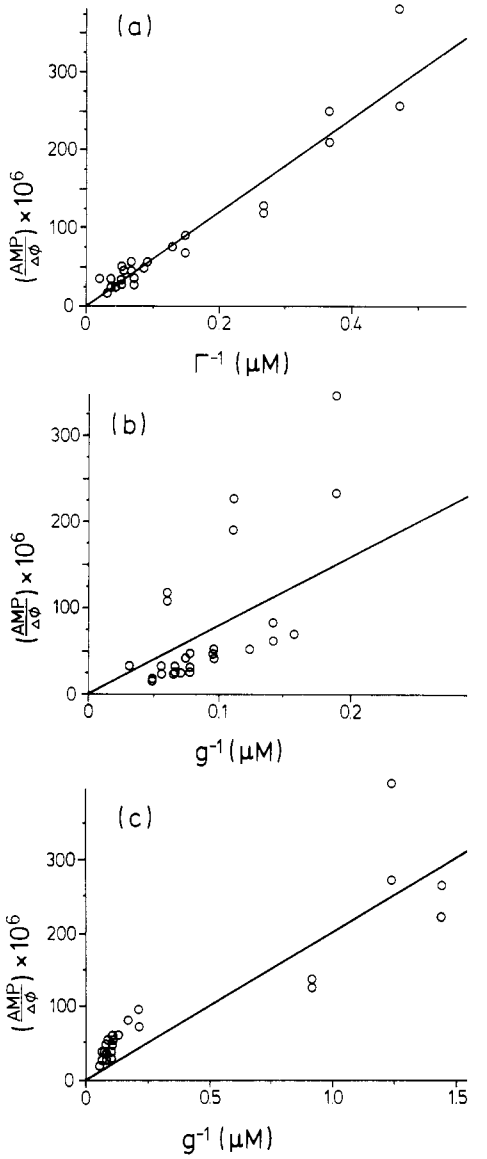

FIGURE 3: Relaxation amplitudes of EB binding to poly[d(A-T)]. The solid line is a least-squares fit of ΔV^0 using the kinetically determined K_a's for the three binding models described: (a) statistically excluded binding; (b) independent, nonoverlapping binding sites with m^{-1} = 3; (c) independent, nonoverlapping binding sites with m = 1.

(Figure 4a) and for the independent nonoverlapping site model with 3 base pairs per EB binding site (Figure 4b). However, the simulation for the m = 1 nonoverlapping, independent site model is inadequate as it overestimates the amount of binding at these concentrations.

Kinetics of EB Binding to Poly[d(G-C)]. The concentration dependence of the intercalation kinetics of EB with poly[d-(G-C)] was analyzed in accordance with a single-step bimolecular mechanism and statistically excluded binding (eq 6). The slope and intercept of the fit of eq 6 to the data (Figure 5) yield k_1 = 13 × 10^6 M^{-1} s^{-1} and k_{-1} = 30 s^{-1}, respectively. These values result in a kinetically defined association constant $K_a = k_1/k_{-1}$ = 4.3 × 10^5 M^{-1}. In agreement with the findings of Winkle et al. (1982), our results are consistent with n = 2.

For the interaction of EB with this polymer, we found ΔV^0 = −9 mL mol^{-1} (Figure 6) by fitting the amplitude data to eq A4 using Γ as the concentration function and the K_a from the kinetic analysis.

In Figure 7, we have plotted the static fluorescence intensity as a function of the total poly[d(G-C)] base pair concentration.

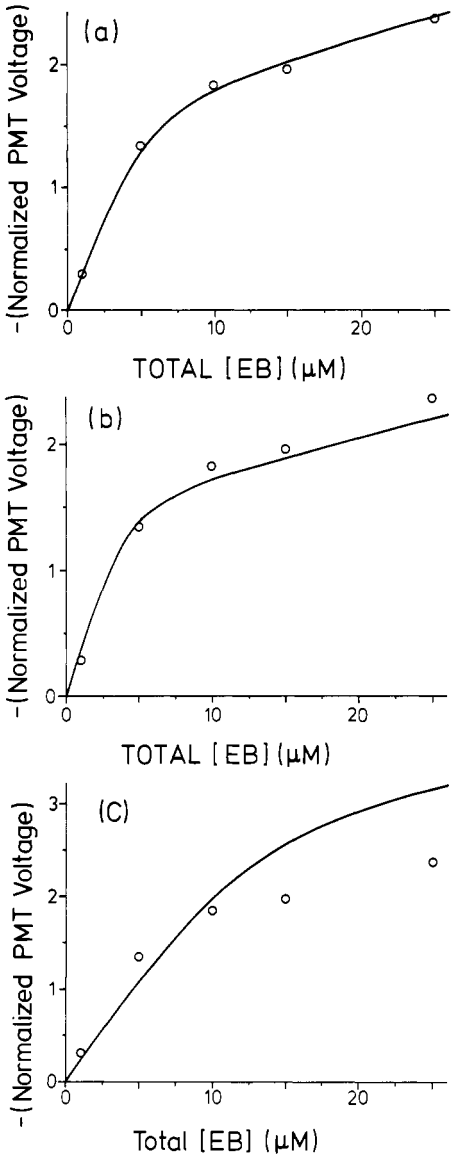

FIGURE 4: Simulations of the equilibrium fluorescence intensity of EB intercalation into poly[d(A-T)] using the kinetically determined association constants for the three cases under consideration: (a) statistically excluded binding; (b) independent, nonoverlapping binding site model with $m^{-1} = 3$; (c) independent, nonoverlapping binding site model with $m = 1$.

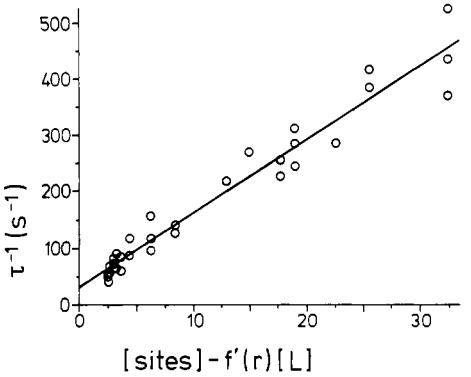

FIGURE 5: Concentration dependence of the inverse relaxation time, τ^{-1}, of EB intercalation into poly[d(G-C)]. The solid line is a fit of the data assuming the statistically excluded binding model (eq 6).

The solid line is the simulation assuming statistically excluded binding and using the association constant defined by the kinetic analysis shown in Figure 5.

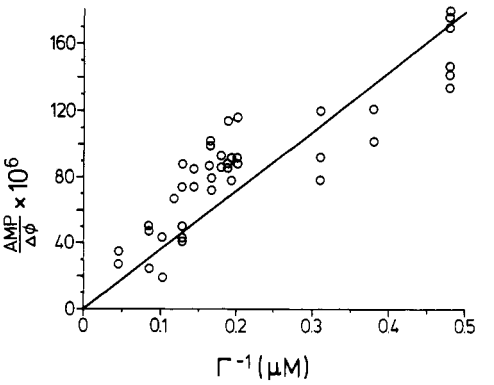

FIGURE 6: Relative amplitudes of the relaxation for ethidium bromide intercalating into poly[d(G-C)] vs. the concentration function Γ^{-1}. The solid line is a least-squares fit of ΔV^0 using the kinetically determined association constant assuming the statistically excluded site model.

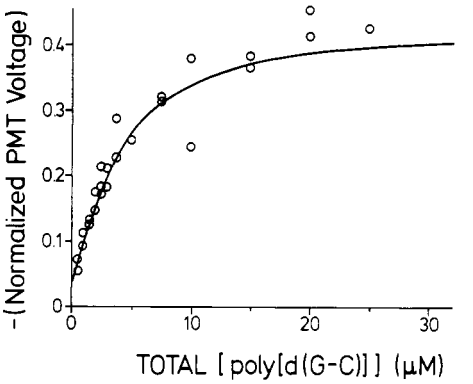

FIGURE 7: Equilibrium fluorescence intensities of EB as a function of the total poly[d(G-C)] concentration simulated by using the kinetically determined association constant assuming the statistically excluded binding site model.

Table II: Definitions and Limiting Values of Some Useful Expressions Related to Statistically Excluded Binding[a]

	r	$f(r)$	$f'(r)$
$[L]_0 \to 0$	0	1	$-(2n-1)$
$[L]_0 \to \infty$	$1/n$	0	0

$$\frac{d\tau^{-1}}{d[L]_0} = k_1\left\{\frac{2[\text{sites}]_0 f'(r) + (r[\text{sites}]_0 - [L]_0)f''(r)}{[\text{sites}]_0 + [K_d + (r[\text{sites}]_0 - [L]_0)f'(r)]/f(r)} - f(r)\right\}$$

	τ^{-1}	$d\tau^{-1}/d[L]_0$
$[L]_0 \to 0$	$k_1[\text{sites}]_0 + k_{-1}$	$-(2n-1)\left(\frac{[\text{sites}]_0 - K_d}{[\text{sites}]_0 + K_d}\right)$
$[L]_0 \gg K_d$	$k_1\{n^2\left(\frac{[L]_0}{K_d}\right)^{1/n} + 1\}$	$k_{-1}n\left(\frac{[L]_0}{K_d}\right)^{1/n}\frac{1}{[L]_0}$
$[L]_0 \to \infty$	∞	$0\ (n > 1)$ $k_1\ (n = 1)$

[a] K_d is the dissociation constant and is equal to $1/K_a$; $f''(r)$ is the second derivative of $f(r)$ with respect to r; all other terms are defined in the text.

Discussion

Examination of the data of Figures 2–4 shows that only the statistically excluded site model is consistent with all our kinetic results and demonstrates the importance of the definition of the binding site when investigating reactions involving DNA. Table II contains some general expressions and their limiting values which will be useful in the following discussion.

Kinetic Confirmation of the Statistically Excluded Binding Model. When $n = 2$, the nonlinearity of $f(r)$ (see eq 2) be-

comes observable at relatively low r values. This factor accounts for the creation of excluded binding sites arising from the distribution of the ligand within the DNA lattice. The function $f(r)$ behaves such that the concentration of EB necessary to achieve a given r value is greater than would be expected if the overlapping nature of the dye binding were not accounted for. Thus, for $n = 2$, at $r = 0.25$, $f(r)$ is 36% larger than would be expected from a linear extrapolation of the initial slope of $f(r)$. The concentration of *available* binding sites is a nonlinear function of r, although the concentration of unoccupied intercalation sites decreases linearly with increasing r.

At the lower limit of r, $f(r)$ is approximately 1; thus, if $[EB]_0 << [sites]_0$, eq 6 is identical with eq A3, for which the sites do not overlap and one intercalation site constitutes a total binding site. The kinetics of EB intercalation into poly[d(G-C)] acquired under these conditions are presented in Figure 5. Actually, any single-step bimolecular binding mechanism described by eq A3 will be consistent with the kinetic data of Figure 5, regardless of the number of intercalation sites chosen to represent one independent, nonoverlapping binding site; of course, the derived value of k_1 will depend upon the concentration units chosen. Although the results are in agreement with the tenets of statistically excluded binding, the data of Figure 5 cannot give us unambiguous confirmation of the statistically excluded mechanism. We note that it is often convenient in terms of computational effort and materials to determine the kinetic parameters of a reaction at low r, where the data analysis is straightforward.

The consequence of this statistical exclusion depends upon whether we consider equilibrium or dynamical aspects of the system. As eq 1 indicates, $f(r)$ represents the fraction of total sites which are available for binding at equilibrium. However, the kinetic formalism must express the mass action principle explicitly; thus, not only the free concentrations of the reaction species but also the stoichiometry of the dynamic interaction must be accounted for. This requirement is responsible for the presence of the factor $f'(r)$ in eq 6. The absolute value of $f'(r)$ is equal to the effective number of excluded potential intercalation sites resulting from binding one EB molecule at a given lattice saturation. At very low r values, $f'(r) = -3$, but for $r = 0.25$, addition of an EB molecule effectively removes only 2.3 intercalation sites from the population of available sites on the lattice.

The simulation of the EB–poly[d(A-T)] kinetic relaxation times using the $m = 1$, nonoverlapping, independent site model (Figure 2b) might appear adequate in the absence of the other kinetic and steady-state data (see the Appendix for the definition of m and a description of the model). However, analyses of the relaxation amplitudes (Figure 3c) and steady-state binding (Figure 4c) using the kinetically determined association constant are certainly less than satisfactory. In light of the fact that this model does not fit any equilibrium data that we are aware of, it is surprising that it describes the relaxation times so well. This can be explained by the diminishing stoichiometric participation of the available lattice sites when the reaction is perturbed at higher r values. When $f'(r) \simeq -1$ and $[sites]_0$ is less than $[L]_0$, the relaxation times will behave approximately the same for either the independent, nonoverlapping site model with $m = 1$ or the statistically excluded binding site model with $n = 2$ (compare eq 6 and A3); this is what we observe (Figure 2). Presumably, if experiments such as those depicted in Figure 2 were carried out at still larger EB concentrations, the relaxation times would also reveal the differences between these two models, and the statistically excluded binding model would more accurately account for the data. The nonoverlapping, independent site model with $m^{-1} = 3$ will not be compatible with the data under these conditions of high r since the slope is a factor of 3 too large in this concentration regime (see Figure 2b).

If $[L]_0$ is small compared to $[sites]_0$ (low r), $f'(r) \simeq -3$; in this concentration range, the slope of the $1/\tau$ vs. $[L]_0$ plot predicted by the $n = 2$ statistically excluded binding model is approximately the same as that predicted by the $m^{-1} = 3$ nonoverlapping, independent site model. We observe in Figure 2b that the slope of the $m = 1$ model seems too shallow, whereas that of the $m^{-1} = 3$ independent, nonoverlapping site model or the statistically excluded site model with $n = 2$ appears more appropriate. Very precise measurements would be required to clearly distinguish these two cases in this concentration range. The original consideration of a binding model in which the EB intercalation site consists of nonoverlapping independent groups of 3 base pairs was motivated by extrapolation from the initial slope of Scatchard plots (LePecq & Paoletti, 1967; Schellman, 1974; Bresloff & Crothers, 1975; Winkle et al., 1982).

Comparison to Similar Studies. Bresloff & Crothers (1975), Wakelin & Waring (1980), and Ryan & Crothers (1984) have reported studies on EB–DNA kinetics which have used the statistically excluded binding description in the data analysis although its necessity under the conditions used was not demonstrated in any of these cases. In addition, the factor $f'(r)$ was not included by Bresloff and Crothers in their evaluation of the kinetic data. The kinetics of EB–poly[d(A-T)] interactions were successfully analyzed without invoking excluded binding by Jovin (1975) performing temperature jump in 0.1 M NaCl; however, he did include it in subsequent analyses of EB–DNA kinetics (Jovin & Striker, 1977). Other authors have reported that it was unnecessary for data analysis (Mandal et al., 1980). That these earlier studies have not provided a clear demonstration of the kinetic manifestations of statistically excluded binding is attributable to the low fraction of sites bound in each case.

Within the framework of statistically excluded binding, our data are consistent with a single-step bimolecular reaction mechanism for EB intercalation with poly[d(A-T)] and poly[d(G-C)]. This mechanism for EB intercalation into poly[d(A-T)] was proposed earlier by Jovin (1975), Jovin & Striker (1977), and Mandal et al. (1980). Other, more complex mechanisms have also been advanced, including nonintercalative EB–DNA interactions and direct transfer of the dye between different intercalation sites (Bresloff & Crothers, 1975; Wakelin & Waring, 1980; Ryan & Crothers, 1984). These experimental differences may be due to the high salt and DNA concentrations used in these studies. The kinetic method employed in these investigations, the electric discharge temperature jump, also may have affected the course of the reaction due to the large transient electric field used to generate the temperature jump. The effect of such large electric fields upon the binding of a charged ligand like EB to a polyelectrolyte such as DNA is poorly understood. That electric fields may indeed influence the apparent course of a reaction is suggested by the results of Marcandalli et al. (1984). These authors observed a single relaxation time for proflavin intercalation into calf thymus DNA at NaCl concentrations as high as 1 M using a laser temperature jump. Studies of proflavin intercalation kinetics with natural DNAs relying upon the electric discharge temperature jump have found two observable relaxations (Li & Crothers 1969; Ramstein et al., 1980). Thus, multiple relaxation times may be an artifact of the application

of large electric fields to high ionic strength solutions. As described in the introduction, this potential problem is avoided by the repetitive pressure-jump technique.

Molar Volume of EB Intercalation. The kinetic amplitudes observed by using the pressure-jump technique enable the molar volume change of the reaction to be determined. As with all thermodynamic parameters, the molar volume change is interesting and useful when compared with similar measurements on related systems. Unfortunately, such a comparison is currently impossible on account of the paucity of pressure studies involving DNA. The intercalation kinetics of EB into calf thymus DNA were studied by Heremans & Van Nuland (1977) using a high-pressure temperature-jump method. They reported no volume change ($\Delta V^0 = 0$ mL/mol) upon intercalation of EB into DNA in the presence of 0.1 M Na^+, and $\Delta V^0 = -14$ mL/mol with 0.1 M Mg^{2+}. Delben et al. (1982) found $\Delta V^0 = -17$ mL mol^{-1} for EB binding to calf thymus DNA in 0.02 M NaCl as determined by dilatometry. From analysis of the relaxation amplitudes, we find $\Delta V^0 = -15$ mL mol^{-1} for EB intercalation into poly[d(A-T)] and $\Delta V^0 = -9$ mL mol^{-1} for poly[d(G-C)] (Table I).

Using static pressure techniques, Torgerson et al. (1980) found $\Delta V^0 = -25$ mL/mol upon binding of EB to tRNA, in 0.1 M NaCl. They proposed that a conformational change in the tRNA molecule is responsible for part of the observed volume decrease. This proposal was based on the data of Weber and co-workers, who reported ΔV^0 for the stacking of aromatic molecules in water to be approximately −5 mL/mol (Weber et al., 1974; Visser et al., 1977). Dye intercalation into a polynucleotide would be approximately twice this value if stacking alone contributes to the molar volume change since the dye molecule is effectively sandwiched between 2 base pairs. Our results provide experimental support for this conjecture. The similarity of the ΔV^0 values for the two polynucleotides we used suggests that the specific interactions reported to occur between the intercalated dye and the various nucleotides (Reinhardt & Krugh, 1978) have a limited influence on the volume change of the reaction.

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APPENDIX

Binding Models Which Do Not Account for Statistically Excluded Binding. Here we define the EB binding site and give the kinetic equations used in the nonstatistically excluded binding models. In these models, the DNA is considered to be a linear lattice of independent, noninteracting, potential EB binding sites for which equilibrium binding isotherms are described by the Scatchard representation (Scatchard, 1949):

$$r/[L] = K_a(m - r) \quad (A1)$$

where K_a is the association constant, [L] is the concentration of free ligand (EB), and, in the present case, m is the number of binding sites per intercalation site (where an intercalation site is located between 2 adjacent base pairs). The fractional saturation of the potential binding sites, r, is given by

$$r = [X]/[S]_0 \quad (A2)$$

in which [X] is the concentration of bound ligand and $[S]_0$ refers to the total analytical concentration of binding sites, so that if end effects are ignored $[S]_0 = m$[base pairs]. Thus, the binding site concentration for this model is always equal to m times the base pair concentration.

The Scatchard representation was developed to describe ligand binding to a molecular entity, such as a protein, which displays one or several, nonoverlapping, potential ligand binding sites per molecule. Although such a description is generally invalid if the potential binding sites consist of more than one intercalation site on a DNA molecule, equilibrium binding data of dyes with DNA have traditionally been presented graphically in this way. A curve is obtained if a titration of DNA with EB is presented according to eq A1; extrapolation from the initial slope (low r) to the abscissa (r axis) of such a plot gives a value of $m = {}^1/_3$, if the DNA concentration is expressed in base pairs. This has been previously interpreted to mean that 3 base pairs are occupied, or in some way removed from further binding reactions, upon binding one EB molecule (LePecq & Paoletti, 1967).

If $m < 1$ for a Scatchard analysis, then the concentration units chosen for the binding entity represent less than the total binding site. Moreover, an interpretation of the data in terms of eq A1 requires that the sites on the binding entity, protein or DNA, be segregated into physically distinct, independent, nonoverlapping clusters of m^{-1} units. Thus, if the DNA concentration unit is base pairs, then eq A1 is a valid representation only if the base pairs are somehow physically divided into groups m^{-1} base pairs long even in the absence of bound ligand (Schellman, 1974).

The kinetic relaxation time for a single-step bimolecular reaction between a ligand and independent, nonoverlapping binding sites is (Bernasconi, 1975)

$$\tau^{-1} = k_1([S] + [L]) + k_{-1} \quad (A3)$$

where [S] is the concentration of free binding sites for the model described above and k_1 and k_{-1} are the forward and reverse rate constants, respectively. The calculated forward rate constant, k_1, is always equal to m^{-1} times the k_1 which would be observed if the intercalation site were 1 base pair.

The relaxation amplitude, ΔA, for this mechanism is given by (Jovin, 1975)

$$\Delta A = -g^{-1}\Delta\phi\ \Delta \ln K_a \quad (A4)$$

where $\Delta\phi$ is the signal change for a 1 M change in the extent of reaction, and

$$g = 1/[S] + 1/[L] + 1/[X] \quad (A5)$$

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Kinetics of Creatine Kinase in Heart: A ^{31}P NMR Saturation- and Inversion-Transfer Study†

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ABSTRACT: The kinetics of the phosphate exchange by creatine kinase (CK) was studied in solution and in the Langendorff-perfused rat heart at 37 °C. ^{31}P inversion-transfer (IT) and saturation-transfer (ST) methods were applied. The kinetic parameters obtained by the two magnetization transfer methods were the same, whether in solution or in the perfused heart. Inversion transfer is the more efficient method, yielding the kinetic constants for the exchange and the relaxation rates of the transferred phosphate in both substrates, in one experiment. In solution the forward (k_F) and reverse (k_R) pseudo-first-order rate constants for the CK reaction ($k_F = k_1$[MgADP][H^+]; $k_R = k_{-1}$[creatine]) as well as the concentrations of phosphocreatine (PCr), MgATP, and creatine (Cr) remained constant between pH 6.9 and pH 7.8. Equilibrium at this pH region is therefore maintained by compensating changes in the concentration of MgADP. The forward and reverse fluxes in the perfused heart were equal with an average flux ratio ($flux_F/flux_R$) of 0.975 ± 0.065 obtained by both methods. Average values of k_F and k_R were 0.725 ± 0.077 and 1.12 ± 0.14 s^{-1}, respectively. These results clearly indicate that the CK reaction in the Langendorff-perfused heart is in equilibrium and its rate is not limited by the diffusion of substrates between different locations of the enzyme. There is therefore no indication of compartmentation of substrates of the CK reaction.

The function of creatine kinase (CK) in myocardial cells is to catalyze the transfer of a high-energy phosphate between ATP and phosphocreatine (PCr). The reaction has an apparent equilibrium constant $K_{app} = K_{eq}[H^+] = [MgATP][Cr]/([PCr][MgADP])$ of 166 at pH 7, 38 °C, ionic strength 0.25, and 1 mM Mg^{2+} (Lawson & Veech, 1979). Its rates in myocardial cells appear to be much higher than those of the myocardial ATPase or ATP synthetase reactions (Matthews et al., 1982). Therefore, when ADP ≪ ATP, as in heart, CK and the PCr pool can provide a high-energy phosphate buffer that serves to maintain fairly constant levels of ATP even during heavy periods of ATPase activity. Of the total concentration of CK, approximately 20% is known to exist as the MM isozyme bound to the myofibrils and 40% as another isozyme bound to the outside of the inner mitochondrial membrane. The remainder is found free in the cytoplasm or bound to other organelles [for a review, see Saks et al (1978)].

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