On the cooperative and noncooperative binding of ethidium to DNA

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Received 14 July 1982; Revised and Accepted 9 November 1982

ABSTRACT

The equilibrium binding of ethidium bromide to native DNAs and to poly(dG-dC)·poly(dG-dC) has been studied by both phase partition and direct spectrophometric techniques. The binding isotherms obtained from both experimental techniques show that ethidium binds in a cooperative manner to *E. coli* DNA. On the other hand, no evidence of cooperative binding was observed in the binding isotherms obtained with calf thymus, *C. perfringens*, *M. lysodeikticus*, or poly(dG-dC)·(dG-dC) under the experimental conditions used (0.1 M NaCl).

INTRODUCTION

A wide variety of techniques have been used to study the DNA binding of ethidium, a cationic dye which is widely used in physical and biochemical studies of nucleic acids (e.g., see 1-13 and references therein). Although it has been established that ethidium has a preference for binding to pyrimidine(3'-5') purine sequences when compared to purine(3'-5')pyrimidine sequences (9-10) it has also been shown that the apparent binding affinity of ethidium is not very dependent upon the overall base composition (1-2, 13). The equilibrium binding of ethidium to various native DNAs has been reported (1-12), with the binding isotherms presented in these papers being well represented by the nearest neighbor exclusion model (14-15). However, in most of the earlier studies experimental limitations have generally restricted acquisition of data to *r* values, i.e. [bound drug]/[DNA base pairs], larger than 0.05. Recent results have shown that interesting, and probably important, phenomena occur between drugs and nucleic acids at relatively low *r* values. An early example of interacting ligands was the 1977 report by Krugh

and Young (16) which showed that daunorubicin and adriamycin facilitated the binding of actinomycin D to poly(dA-dT). poly(dA-dT), a polynucleotide to which actinomycin D does not bind. Crothers and coworkers (17-18) have reported that distamycin binds cooperatively to calf thymus DNA but not to E. coli or other DNAs tested. Winkle and Krugh (19) have observed cooperativity in the equilibrium binding of actinomycin D to calf thymus DNA, and Rosenberg et al., (20) have shown that the antitumor drugs adriamycin, daunorubicin, and DHAQ (1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione) bind in a cooperative manner to native and synthetic DNAs.

Cooperative binding of ethidium to synthetic polynucleotides has been observed previously by Pohl and Jovin (21) and Bresloff and Crothers (8). The highly cooperative binding of ethidium to the left handed form of poly(dG-dC).poly(dG-dC) in 4.4 M NaCl reported by Pohl and Jovin (21) was accompanied by an ethidium induced reversal of poly(dG-dC).poly(dG-dC) to the low salt form of the complex. Bresloff and Crothers (8) observed a change in the structure of poly(dI-dC).poly(dI-dC) upon the binding of ethidium, although the structural basis for the conformational change remains unknown. Erard et al. (22) observed cooperative binding of ethidium to chicken erythrocyte nucleosome core particles, although Wu et al. (23) did not observe cooperative binding of ethidium under similar experimental conditions. The apparent discrepancy in these two results may reflect differences in the preparation of the nucleosomes, or possibly other factors. Neither Erard et al. (22), nor Wu et al. (23) observed any evidence for cooperative binding of ethidium to the DNA purified from the nucleosome preparations.

In this paper we report on the equilibrium binding of ethidium to several native DNAs and to the synthetic polynucleotide poly(dG-dC).poly(dG-dC) using both phase partition techniques and a direct spectrophotometric method to determine the equilibrium binding isotherms. The cooperative binding of ethidium by E. coli DNA was corroborated by a third technique, competition dialysis. In all cases, ethidium was found to bind to E. coli DNA in a cooperative manner which is in

marked contrast to the nearest neighbor exclusion type of binding isotherms obtained for the other DNAs studied.

MATERIALS

E. coli DNA was obtained from either Worthington Biochemicals or Sigma Chemical Company. All other DNAs were obtained from Sigma Chemical Company. Poly(dG-dC)·poly(dG-dC) was purchased from PL Biochemicals. DNA concentrations are expressed in base pairs and were determined from A_{260} measurements using $\epsilon_{260}(\text{E. coli})=13080 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{260}(\text{C. perfringens})=12000 \text{ M}^{-1}\text{cm}^{-1}$, $\epsilon_{260}(\text{M. lysodeikticus})=13200 \text{ M}^{-1}\text{cm}^{-1}$, and $\epsilon_{254}(\text{poly(dG-dC)·poly(dG-dC)})=14200 \text{ M}^{-1}\text{cm}^{-1}$. Ethidium bromide was purchased from Sigma Chemical. ^3H -ethidium bromide was kindly provided by Dr. Barbara Baser, University of Rochester. Purity of both radiolabelled and unlabelled ethidium was checked by thin layer chromatography. Specific activity of the ethidium solutions was determined by liquid scintillation counting of aqueous solutions for which the concentrations of ethidium had been determined by absorption spectroscopy using $\epsilon_{480}(\text{ethidium bromide})=5680 \text{ M}^{-1}\text{cm}^{-1}$. Specific activities ranged between $2 \times 10^{-12} \text{ mmol/cpm}$ and $7 \times 10^{-13} \text{ mmol/cpm}$.

METHODS

DNA Preparation. The DNA was dissolved in the appropriate buffer, cooled in an ice bath, and sheared using a Virtis Homogenizer. Five, one-minute periods of shearing (20,000 rpm) were used, with a five minute interval between each period. The DNA was then centrifuged at 3000 rpm for 10 minutes to clarify the solution, and after extracting 3 times with an equal volume of 24 parts chloroform plus 1 part isoamyl alcohol, the DNA was precipitated using isopropyl alcohol. For use, the DNA was redissolved in the appropriate buffer, and extensively dialyzed. E. coli DNA was individually and sequentially treated with RNase H (BRL), 0.5 mg/ml at 37°C for 30 minutes, and Proteinase K, 0.5 mg/ml at 37°C for 1 hour. Subsequent to each enzymatic step the DNA was extracted and precipitated as described above. The measured hyperchromicity (alkaline denaturation) was 25% both before and after each enzymatic treatment.

Phase Partition. Binding data were obtained using both phase partition analysis (17-19,24-26) and direct spectrophotometric analysis. In the phase partition analysis the organic phase was 1-nonanol. The aqueous phase buffer (Buffer A) was 0.01 M phosphate, 1 mM EDTA, pH 7, with the addition of NaCl as specified in the Results and Discussion section. Samples consisting of 100 μ l of each phase were shaken at 22°C on an SMI multitube vortexer for 6 cycles which consisted of 1 minute of shaking followed by 5 minutes of rest. The two phases were separated by centrifugation for 30 seconds in a clinical centrifuge. The drug concentrations in each phase were determined by putting 30 μ l aliquots of the appropriate phase into 10 ml of liquid scintillant (ACS counting fluid, Amersham), and counting on a Beckman liquid scintillation counter. The partition coefficients, (organic/aqueous), determined by shaking samples in the absence of DNA, were approximately 2.2 in 0.01 M NaCl, 1.6 in 0.1 M NaCl and 3.3 in 1.0 M NaCl, and were constant in the range of ethidium concentrations used in these experiments.

Spectrophotometric Analysis. Optical titrations were performed using a 10-cm quartz cell, wherein a known concentration and volume of DNA was titrated with a concentrated solution of ethidium. The resulting increase in the absorbance was read directly from the digital display of a Cary 219 spectrometer. Ethidium stock solutions were made immediately prior to use. An absorbance versus concentration plot was constructed for each stock solution and was used to calculate the ethidium concentration. The absorbance at the isosbestic point (512 nm) may be used to determine the total concentration of ethidium during the titration. The observed linearity of a plot of absorbance (at 512 nm) versus the drug concentration, calculated by dilution, ensured a high degree of precision in the repeated pipettings. Binding isotherms were obtained for E. coli DNA and poly(dG-dC)·poly(dG-dC) using Buffer A, as described above, with the addition of NaCl as stated in the Results and Discussion Section. For the experiments with M. lysodeikticus, C. perfringens, and calf thymus DNAs, the buffer was 0.052M phosphate, 0.10M NaCl, and 0.001M NaEDTA, pH = 7.1 (Buffer B).

Free and bound ethidium concentrations were determined from

the extinction coefficients of the free and bound ethidium. The bound extinction coefficient of ethidium was determined for each DNA by sequentially adding DNA to an ethidium solution until the DNA was present in large excess. The reciprocal of the change in the absorbance of ethidium versus the reciprocal in the DNA concentration was plotted, and the bound extinction coefficient was obtained by extrapolation to infinite DNA concentration.

In one experiment (Figure 1E), E. coli DNA was treated with S_1 nuclease at 37°C for 30 minutes, to remove single stranded regions of the DNA. Subsequent acid precipitation indicated that approximately 5% of the DNA was lost due to nuclease digestion.

Competition Dialysis. Experiments were done in a multi-chambered micro-dialysis apparatus. Calf thymus and E. coli solutions of equal concentration and volume were put into opposing chambers, separated by a semi-permeable membrane (Spectrum Medical Industries, Inc.). Ethidium was then placed on one side, and the entire dialysis apparatus was shaken for 48 hours at 22°C. To ensure that equilibrium had been reached, at least two of the thirteen cells for each experiment contained either calf thymus or E. coli DNA on both sides of the membrane. When the fluorescence intensity of the control cells reached unity, it was assumed that equilibrium was attained. By using both calf thymus and E. coli DNA in the controls, the relative fluorescence efficiencies of the two DNAs could be checked. At all bound ligand concentrations, no discrepancy in the relative fluorescence efficiency was found. For all points, duplicates were run in the same experiment. For each duplicate, the ethidium was placed in an alternate chamber of the dialysis apparatus, to ensure that the results were independent of the approach to equilibrium. The fluorescence intensity of the solutions from each side of the membrane was measured on a Perkin-Elmer 44A spectrofluorimeter. The fluorescence intensity provides a measure of the concentration of DNA bound ethidium on each side of the membrane. In these experiments the concentration of unbound ethidium was identical in each pair of chambers and always contributed less than 1% of the total fluorescence.

RESULTS AND DISCUSSION

Scatchard plots (27) for the binding of ethidium to E. coli

Figure 1. Scatchard plots for the equilibrium binding of ethidium to *E. coli* DNA(1A-1E) and the chemical structure of ethidium bromide (lower right). The data shown in Figures 1A-1C were obtained by phase partition techniques using 1-nonanol as the organic phase. Figures 1D-1E were obtained by a direct spectrophotometric technique (for details see Materials and

Methods). The curves drawn through the data in 1A-1C were calculated from the allosteric binding model of Crothers and coworkers (8,17), using the parameters listed below. Figure 1A: added [NaCl]=1.0 M. The DNA concentration ranged from 3.5 to 7.1×10^{-5} M. The total ethidium concentration ranged from 0.04 to 4.0×10^{-5} M. The parameters used to calculate the allosteric binding isotherm were: $K_1 = 4.7 \times 10^3$; $K_2/K_1 = 5.0$; $N_1 = 50$; $N_2 = 4$; $\sigma = 0.0015$; $s = 0.988$. Figure 1B: added [NaCl] = 0.1 M. The DNA concentration ranged from 1.4 to 7.0×10^{-5} M. The total ethidium concentration ranged from 0.3 to 1.3×10^{-6} M. The parameters used to calculate the allosteric binding isotherm were: $K_1 = 1.0 \times 10^5$; $K_2/K_1 = 2.0$; $N_1 = 3$; $N_2 = 3$; $\sigma = 0.002$; $s = 0.989$. Figure 1C: added [NaCl] = 0.01 M. The DNA concentration was 3.8×10^{-5} M. The total ethidium concentration ranged from 0.65 to 9.4×10^{-7} M. The parameters used to calculate the allosteric binding isotherm were: $K_1 = 3.0 \times 10^5$; $K_2/K_1 = 2.0$; $N_1 = 2$; $N_2 = 2$; $\sigma = 0.003$; $s = 0.99$. Figure 1D: Scatchard plot obtained by direct spectrophotometric titration of ethidium with *E. coli* DNA in Buffer A plus 1.0 M NaCl. Figure 1E: Scatchard plot obtained by direct spectrophotometric titration of ethidium with *E. coli* DNA in Buffer B plus 0.1 M NaCl, pH 7.1 (○, and □ are duplicate experiments). The data shown as triangles (Δ) were obtained with S_1 nuclease treated *E. coli* DNA.

DNA at differing salt concentrations (0.01 M, 0.1 M and 1.0 M NaCl) are given in Figures 1A-1E. The initially increasing slopes observed at all three salt concentrations are indicative of cooperative binding of ethidium to *E. coli* DNA. The binding isotherms for the interaction of ethidium with calf thymus DNA, *M. lysodeikticus* DNA, and *C. perfringens* DNA (Figure 2) exhibit no evidence of positive cooperative binding and appear to be well represented by nearest neighbor exclusion binding isotherms. The magnitudes of the r/c_f values are consistent with previously published data for these and other DNAs at similar ionic strengths (1-8).

As noted in the Introduction, Pohl and Jovin (21) reported that the binding of ethidium to poly(dG-dC).poly(dG-dC) in 4.4 M NaCl is a highly cooperative process, whereas a neighbor exclusion binding isotherm was observed for the binding of ethidium to poly(dG-dC).poly(dG-dC) in 1.0 M NaCl solutions. We have used both phase partition and direct spectrophotometric techniques to measure the binding of ethidium to poly(dG-dC).poly(dG-dC) in 0.1 M NaCl solutions with a special interest in obtaining data at low r values. These data (Figure 3) show no evidence for cooperative binding of ethidium to poly(dG-dC).poly(dG-dC) in 0.1 M NaCl. The binding isotherm was also measured in

Figure 2. Scatchard plots obtained by a direct spectrophotometric technique for the binding of ethidium to DNAs of varying base content in Buffer B plus 0.1 M NaCl, pH 7.1: (O) calf thymus DNA; (□) *C. perfringens* DNA, and (Δ) *M. lysodeikticus* DNA.

1.0 M NaCl (data not shown) and again no evidence for cooperative binding was observed. The absence of cooperativity under these salt conditions lends support to the interpretation that the cooperative binding observed in the 4.4 M NaCl solutions is an ethidium mediated change in the high salt structure of poly(dG-dC)·poly(dG-dC).

Binding isotherms obtained by both phase partition techniques and direct spectrophotometric titrations provide, within experimental error, identical results for both the cooperative binding of ethidium to *E. coli* DNA (Figure 1) and the non-cooperative binding of ethidium to poly(dG-dC)·poly(dG-dC) (Figure 3). Using *E. coli* DNA which has been treated with RNase H or Proteinase K, or sequentially with both enzymes, does not change the shape of the cooperative binding isotherm. We have also shown (20) that the addition of saturating concentrations 1-nonanol to calf thymus DNA does not affect or alter the cooperative binding of DHAQ, as determined by direct spectrophotometric analysis. These results allow us to rule out the possibility that the small amount 1-nonanol which is soluble in the aqueous phase is influencing the binding isotherms

Figure 3. Scatchard plot for the binding of ethidium to poly(dG-dC)·poly(dG-dC) in Buffer A plus 0.1 M NaCl, pH 7.1. The data shown as open circles (O) were obtained by a direct spectrophotometric titration using a polynucleotide concentration of 2.3×10^{-5} M (in base pairs) with the ethidium concentration ranging from 1.2×10^{-8} M. to 5.2×10^{-6} M. The data shown as open squares (□) were obtained by phase partition techniques using a polymer concentration of 3×10^{-5} M. (in base pairs) with the total ethidium concentration ranging from 0.1 to 1×10^{-6} M.

obtained by the phase partition techniques.

Competition dialysis (28-29) was used to compare the relative binding of ethidium to calf thymus DNA (which gave a neighbor exclusion binding isotherm) and *E. coli* DNA (which gave the cooperative binding isotherms). Differences in the strength of a drug binding to differing DNAs will be manifested in competition dialysis. Therefore the DNA with a higher binding affinity will sequester more drug than the DNA with the lower binding affinity. At equilibrium the free ethidium concentration on both sides of the membrane must be equal. The solid line in Figure 4, is a comparison of the r/c_f values, at equal free ethidium concentrations, derived from Figure 1E and Figure 2. This line predicts the relative binding efficiency of ethidium for calf thymus versus *E. coli* DNA. The experimental data shown in Figure 4 shows that calf thymus DNA binds a larger concentration of ethidium than the *E. coli* DNA at low r values ($r < 0.04$). At intermediate r values ($r > 0.04$) the relative concentration of ethidium bound by the two DNAs became a constant. The agreement

Figure 4. Plot of the ratio of ethidium fluorescence intensity, calf thymus to *E. coli*, as a function of [ethidium added]/[DNA base pairs]. The solid line is a comparison of r/c_f values, at equal free ethidium concentrations, derived from Figure 1E and Figure 2. Calf thymus DNA was prepared as described in the Methods section. *E. coli* DNA was prepared as follows: 1) open circles (○) no enzymatic treatment, 2) open squares (□), proteinase K, 3) open triangles (△), RNase H, 4) open diamonds (◇), RNase H treatment followed by proteinase K treatment. Competition dialysis was run in Buffer B plus 0.1M NaCl, as described in the Methods section.

between the competition dialysis results and the curve predicted by both absorptiometric and phase partition experiments shows that ethidium is indeed binding in a cooperative manner at low r values.

Furthermore, since competition dialysis is not limited by the assumptions which normally must be made with absorptiometric analysis (i.e. invariance of a bound epsilon), or phase partition analysis (i.e. possible effect of an organic solvent on the binding and/or the DNA), the agreement between competition dialysis and spectroscopic and phase partition analysis illustrates the utility of these techniques.

The solid lines drawn through the data points in Figures 1A-1C represent the "best" trial-and-error visual matches of the experimental data to binding isotherms calculated from the allosteric binding model proposed by Crothers and coworkers (8,18). In this model DNA may exist in either of two forms: form I is the "native" form (i.e., the predominant form in the

absence of a drug) and form II is an alternate form. If the drug binds preferentially to form II and induces a conversion of the DNA from form I to form II, a cooperative binding isotherm results (see also Bresloff and Crothers, 1981, reference 8). The parameters used to calculate the solid curves in Figures 1A-1C are given in the Figure legend. Although the experimental binding isotherms are adequately modeled by the calculated allosteric binding isotherms, this does not prove that ethidium induces long range allosteric transitions in E. coli DNA. For example, one may also reproduce the experimental data by assuming that E. coli DNA has limited regions to which ethidium binds in a highly cooperative manner, whereas the majority of the DNA binds ethidium in a non-cooperative manner (L.S. Rosenberg and T.R. Krugh, in preparation). Thus we do not emphasize a quantitative analysis of the data at the present time.

We note that the binding isotherm at 1 M NaCl exhibits the highest degree of curvature at low r values. As the concentration of added salt is decreased to 0.1 M NaCl and 0.01 M NaCl the degree of curvature in the binding isotherms at low r values also appears to decrease. This suggests that ethidium binds with a greater apparent cooperativity at the higher ionic strengths. These results differ from those observed for distamycin (17-18) and actinomycin D (19-20) binding to calf thymus DNA. The apparent cooperativity for distamycin is only moderately salt dependent, whereas the apparent cooperativity for actinomycin D decreases with increasing salt concentration. It is, of course, possible that different mechanisms are giving rise to the "cooperative" binding isotherms and that these various mechanisms possess differing salt dependencies. It is interesting that ethidium, a relatively simple intercalating molecule, binds cooperatively to E. coli DNA and non-cooperatively to calf thymus DNA, whereas distamycin, a non-intercalating oligopeptide, binds cooperatively to calf thymus DNA and non-cooperatively to E. coli DNA (17-18). Although at the present time we do not fully understand the factors associated with the cooperativity and allosterism observed in these and other systems, (e.g., the possible role of satellite DNA, etc.), it is likely that the phenomena which are responsible for the cooperative type binding isotherms are important in the

area of DNA structure and function (30), as well as the in vivo activity of these compounds.

ACKNOWLEDGEMENTS

This investigation was supported by PHS research grant number CA 17865, and postdoctoral fellowship award (to SAW) number CA 06756, awarded by the National Cancer Institute, DHHS. The authors thank Professor William Saunders for the use of his liquid scintillation counter.

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