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Equilibrium binding of carcinogens and antitumor antibiotics to DNA: site selectivity, cooperativity, allosterism

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ABSTRACT

The equilibrium binding of the carcinogens N-hydroxy-N-acetyl-2-amino-fluorene (HAAF) and 4-nitroquinoline-1-oxide (NQO) to ϕX174RF DNA have been studied by phase partition techniques. Both molecules bind in a cooperative manner with only a few carcinogen molecules binding to each ϕX174RF DNA molecule. The binding data for both HAAF and NQO fit a model in which two carcinogens cluster into a small number of sites - four sites for HAAF and twelve sites for NQO. Phase partition techniques were also used to study the binding of actinomycin D to both calf thymus DNA and poly(dG-dC)·poly(dG-dC) at much lower r values than had been previously reported. These data exhibit humped Scatchard plots which are indicative of cooperative binding; the overall shape of the Scatchard plots are consistent with a model for drug induced allosteric transitions in the DNA structure. The cooperativity in the actinomycin D binding to calf thymus DNA increases with decreasing sodium chloride concentration, suggesting a role for DNA flexibility in allosteric binding.

INTRODUCTION

During the last few years, it has become apparent that the DNA binding of certain carcinogens, mutagens, and antitumor drugs may be much more selective than had been previously recognized. Furthermore, the interaction may involve more than simple intercalation or binding into one of the grooves of DNA. The question of the preferential recognition and selective binding of drugs to selected sequences on DNA has been addressed by several groups. An early example of drug selectivity was the demonstration that certain drugs may preferentially bind to either A·T rich or G·C rich regions of DNA, although it is now clear that a number of small molecules also exhibit preferential binding to various base sequences (e.g., see references 1-18 and

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references therein for an introduction to this area). It is interesting to question whether carcinogenic molecules exhibit specificity in their equilibrium binding to DNA. Much of the work on carcinogen binding has centered on the nature and the specificity of covalent addition of the carcinogens with various moieties of DNA (e.g., benzo[a]pyrene metabolites and N-acetyl-2-aminofluorene metabolites) (e.g., see references 6-10, 17-18). The covalent binding of the ultimate carcinogen metabolite may likely be preceeded by equilibrium binding of the metabolite to DNA (17-18). In this paper we study the equilibrium binding to DNA of N-hydroxy-N-acetyl-2-aminofluorene (HAAF) and 4-nitroquinoline-1-oxide (NQO). HAAF is structurally similar to the covalent DNA-binding metabolite N-acetoxy-N-acetyl-2-aminofluorene, and 4-nitroquinoline-1-oxide is the parent carcinogen of its group. We show that both HAAF and NQO bind in a cooperative manner to small numbers of sites on ϕX174RF DNA (see Figure 2 for the structures of HAAF and NQO).

Poly(dGdC)·poly(dGdC) undergoes a structural transition at high salt concentrations, as evidenced by the appearance of an inversion of the circular dichroism spectrum (11). Ethidium bromide binds in a highly cooperative manner to the high salt form of poly(dGdC)·poly(dGdC) and affects the transition to the low salt form (11). In 1977, Krugh and Young (12) reported that daunorubicin facilitates the binding of actinomycin D to poly(dAdT)·poly(dAdT), which is another example of helix mediated allosteric effects. More recently, distamycin and netropsin (13,14), which are basic oligopeptides whose DNA binding probably occurs in the minor groove and does not involve intercalation, both exhibit cooperative binding to certain DNAs. Crothers and coworkers (13,14) have shown that the binding is also accompanied by structural changes in the double helix, and have interpreted these data in terms of long range drug-induced allosteric transitions in the DNA structure. We show below that the antitumor drug actinomycin D binds cooperatively to DNA and that the experimental binding isotherms may be reproduced by the drug-induced allosteric transition model of Crothers and coworkers (13,14). It now appears that cooperative binding and allosteric transitions are likely to be important aspects

in the physiological activity of a variety of compounds (the reader is referred to a review by Wells et al. (15) for a discussion of DNA structure and gene regulation).

MATERIALS AND METHODS

Materials. The ^{3}H-NQO was from the laboratory of I. Tinoco, Jr. (U. C. Berkeley), ^{14}C-HAAF was from the ICN Radiochemicals Division, and ^{3}H-Act D was from Amersham. These materials were purified on an alumina column and the purity was checked by thin layer chromatography. ϕX174RF DNA was obtained from Bethesda Research Laboratories. The ratio of form I (superhelical) to form II (relaxed circular) ϕX174RF DNA was found to be 2:1 as assayed from the relative intensities of the form I and form II bands (ethidium stained) on 1% agarose electrophoresis gels. Calf thymus DNA (Worthington) was prepared as described by Müller and Crothers (16). Poly(dGdC)·poly(dGdC) was purchased from PL Biochemicals. DNA concentrations of the stock solutions were determined from A_{260} measurements using ε_{260}(ϕX174RF) = 6600 M^{-1} cm^{-1}; ε_{260}(calf thymus) = 6250 M^{-1} cm^{-1}; ε_{254}(poly(dGdC)·poly(dGdC)) = 7100 M^{-1} cm^{-1}. Specific activities for ^{3}H-NQO, ^{14}C-HAAF and ^{3}H-Act D were determined by liquid scintillation counting of aqueous solutions for which concentrations had been determined optically (using ε_{300}(HAAF) = 14100 M^{-1} cm^{-1} (our determination); ε_{250}(NQO) = 16500 M^{-1} cm^{-1}; ε_{440}(Act D) = 24400 M^{-1} cm^{-1}.

Methods. Binding data were obtained using partition analysis methods (13,14, 19-21). The organic phase was a 1:1 mixture of n-chloroheptane and cyclohexane. For the experiments with HAAF and NQO the aqueous phase was a buffer of 0.1 M NaCl, 0.01 M Na cacodylate, 1 mM EDTA, pH 7. The buffer used in the actinomycin D experiments was 0.01 M sodium phosphate, 1 mM EDTA, pH 7, with the addition of sodium chloride as specified in the Results and Discussion section. Samples consisting of 100 µL of each phase were shaken on a mechanical twist action shaker (at 22°C for the Act D - calf thymus DNA experiments and at 5°C for the Act D - poly(dGdC)·poly(dGdC) experiments). The 5°C experiments were conducted, including workup, in a constant temperature room. NQO samples were shaken for a minimum

of 4 hours, HAAF and actinomycin D samples were shaken overnight. The two phases were separated by centrifugation for 30 seconds in a clinical centrifuge. The drug concentrations in each phase were determined by counting 20 μL aliquots diluted into liquid scintillant (ACS counting fluid, Amersham) on a Beckman liquid scintillation counter. The partition coefficients (organic/aqueous), determined by shaking samples in the absence of DNA, were approximately 13 for HAAF, 3.8 for NQO, 0.67 for Act D (0.1 M NaCl, 5°C), and 6.7 for Act D (0.1 M NaCl, 22°C).

RESULTS AND DISCUSSION

Carcinogens. The equilibrium binding isotherms for the interaction of HAAF and NQO with φX174RF DNA are plotted in the form of Scatchard (22) plots in Figure 1. As shown in the plots, the binding of both carcinogens appears to saturate at extremely low r values, which suggests that only a few carcinogen molecules

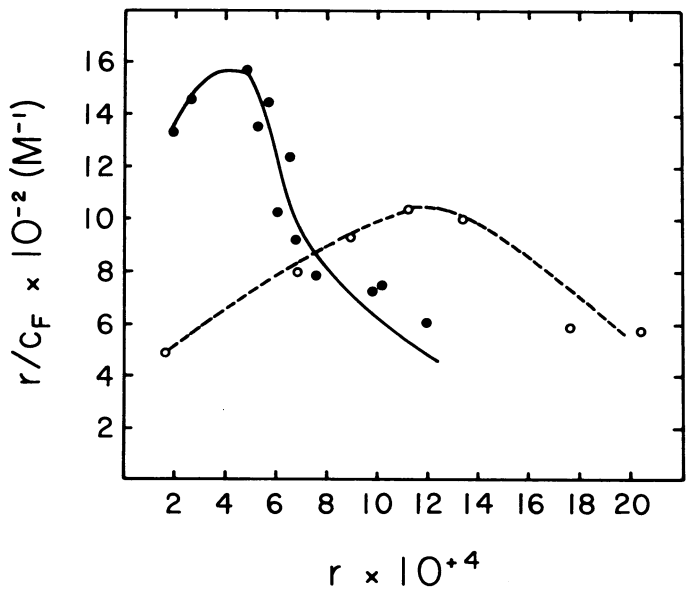

Figure 1: Scatchard plots for carcinogen binding to φX174RF DNA. HAAF + φX174RF (●——●——●); NQO + φX174RF (○——○——○). [φX174RF] = 0.1 - 1.0 x 10^{-4}M, $[HAAF]_T$ = 0.1 - 7.0 x 10^{-5}M, $[NQO]_T$ = 0.05 -5.0 x 10^{-5}M. The curves through the data are for viewing purposes.

bind per molecule of DNA. The Scatchard plots of the data could be interpreted in terms of the binding of the carcinogens to only a small number of potential sites as a result of an extremely high sequence selectivity. Alternatively, the binding of these molecules could be to some transient alternate form(s) of DNA structure. This latter explanation could include the possibility that the number of sites is limited due to long range transmission of allosteric effects (13-15).

Both sets of binding data exhibit the curvature associated with cooperative binding. One possible model to explain this cooperativity is a cluster model in which n carcinogens bind "together" or cluster into a localized site on the DNA:

$$n(\text{carcinogens}) + (\text{DNA-site}) \underset{}{\overset{K}{\rightleftharpoons}} (\text{DNA-site}) \cdot (\text{carcinogen})_n \quad [1]$$

If the binding sites are independent, and in the limit of highly cooperative binding, this model may be represented by:

$$r/(C_F)^n = (nR - r)K \quad [2]$$

where K is the equilibrium constant and R is the ratio of binding sites per residue (1/2R is the number of base pairs/site). In Figure 2 the binding data from Figure 1 are plotted according to equation [2] using a value of n = 2. Both the HAAF and the NQO binding data appear to fall on a straight line, which suggests that in both cases two molecules are binding per site. The lines drawn in Figure 2 correspond to values of K(HAAF) = 1.6×10^{13} M^{-2} with 1300 bp/site, and K(NQO) = 7.5×10^{11} M^{-2} with 460 bp/site. Both of these carcinogens bind with equilibrium constants of roughly similar magnitudes and both have a small number of binding sites on ϕX174RF. Linear ϕX174RF, produced by reacting circular ϕX174RF with the restriction enzyme Pst I (which has one cutting site on ϕX174RF), gives, for these two carcinogens, binding constants and numbers of binding sites similar to those obtained with the circular ϕX174RF. We have also obtained similar results for binding of NQO and HAAF to other DNAs. From the known size of ϕX174RF (5386 bp) (23) we calculate that there are four binding sites for HAAF and twelve binding sites for NQO per molecule of ϕX174RF.

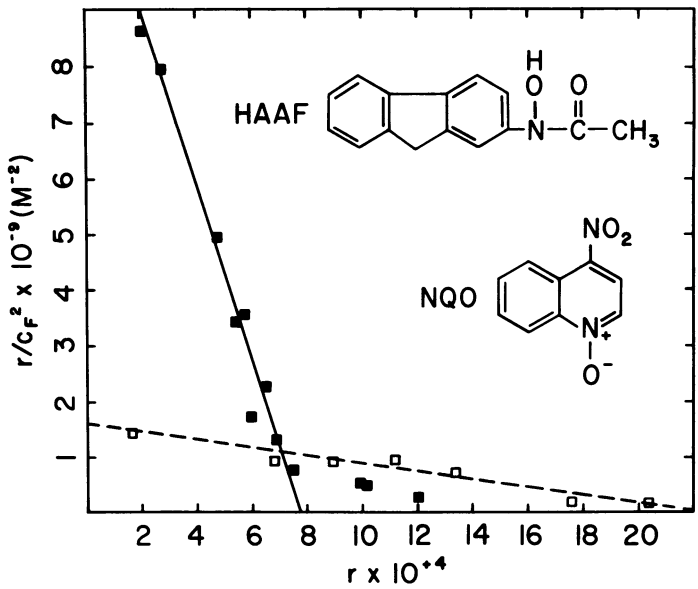

Figure 2: HAAF and NQO binding data plotted according to Equation 2. HAAF + ϕX174RF (■—■—■) (the three largest r value data points were neglected in drawing the line through the points); NQO + ϕX174RF (□—□—□).

That both NQO and HAAF exhibit relatively few binding sites on ϕX174RF (as is shown in Figures 1 and 2) raises the question of whether these two sets of binding sites might intersect. Since each of these molecules binds cooperatively, it is of interest to look for the mutual interaction of these two molecules in their binding to ϕX174RF DNA. Preliminary experiments on the binding of mixtures of HAAF and NQO (data not shown) suggest that each carcinogen enhances the binding of the other.

Actinomycin D. The binding of actinomycin D to calf thymus DNA (0.01 M, 0.1 M and 0.2 M NaCl) and to poly(dGdC)·poly(dGdC) (0.1 M NaCl) was examined. As shown in Figures 3 and 4, actinomycin D binds cooperatively to both of these DNAs. The increasing curvature in the binding data (Figure 3) as NaCl concentration decreases suggests that the cooperativity increases as the sodium ion concentration decreases. This could indicate a role for DNA flexibility and base pair orientation (which are affected by sodium ion concentration (25,29-30)) in the coopera-

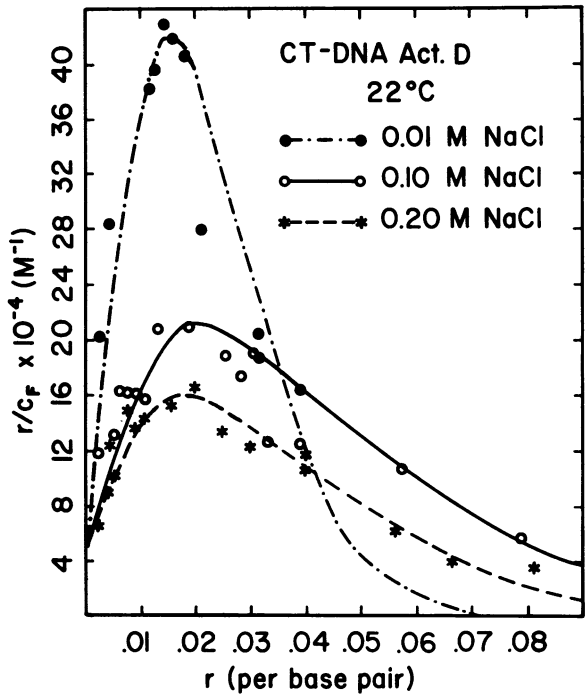

Figure 3: Scatchard plot for the binding of actinomycin D to calf thymus DNA. 0.01 M NaCl (●—•—•—●), [DNA] = 2.4 - 4.8 x 10^{-4}M, $[Act\ D]_T$ = 0.10 - 1.0 x 10^{-5}M; 0.1 M NaCl (o————o), [DNA] = 0.8 - 1.0 x 10^{-3}M, $[Act\ D]_T$ = 0.15 - 5.0 x 10^{-5}M; 0.2 M NaCl (*— — —*), [DNA] = 2.9 -5.8 x 10^{-4}M, $[Act\ D]_T$ = 0.15 - 2.5 x 10^{-5}M.

tive binding of actinomycin D to DNA.

The cooperative binding observed may be associated with drug induced allosteric transitions in the DNA structure. The cooperative binding of ethidium bromide to the high salt form of poly(dGdC)·poly(dGdC), the binding of distamycin and netropsin to calf thymus DNA (13,14), as well as the facilitation of actinomycin D binding to poly(dAdT)·poly(dAdT) by daunomycin could be, and have been, interpreted as examples of drug-induced transitions in DNA structure. Recently, Dattagupta et al. (14) outlined a theory for calculation of binding isotherms when binding is coupled to a DNA structural change. In this theoretical model for allosteric transitions in DNA, the DNA may

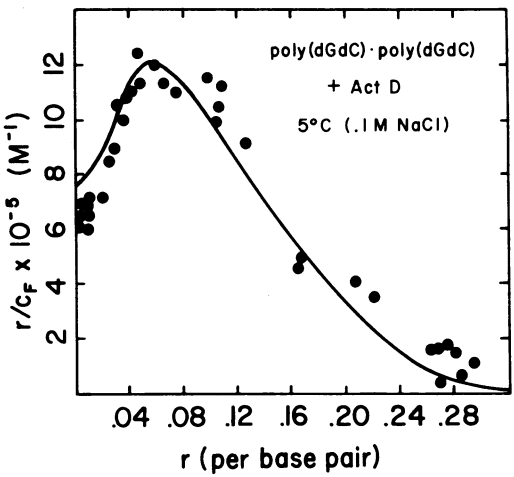

Figure 4: Scatchard plot for the binding of actinomycin D to poly (dGdC)·poly(dGdC) in the standard buffer plus 0.1 M NaCl.[poly(dGdC)·poly(dGdC)] = 1.6×10^{-5} - 2.3×10^{-3}M, $[\text{Act D}]_T = 3.5 \times 10^{-7} - 6.2 \times 10^{-4}$M.

exist in two structural forms (designated as form [1] DNA and form [2] DNA). K_1 and K_2 are the equilibrium binding constants, n_1 and n_2 are the base pair/drug ratios at saturation, for binding of the drug to form [1] and form [2] DNA,respectively. A Fortran computer program (14), which was provided by Professor D. M. Crothers, was used to calculate binding isotherms for sets of the six variable parameters. The parameters given in Table I were used to calculate the binding isotherms which are shown as the smooth curves in Figures 3 and 4, and represent our selection of the best match between the experimental data and the trial and error fit of the calculated binding isotherms. The agreement between the calculated and experimental binding isotherms suggests that the cooperative allosteric transition model may be used to describe the binding of actinomycin D to these two DNAs. The value of $n_1 = 4$ for the binding of actinomycin D to poly(dGdC)·poly(dGdC) agrees with our previous experiments (3,26). Perchance coincidentally, the value of $n_1 = 25$ in the actinomycin D - calf thymus DNA experiments corresponds approximately to the nearest neighbor frequency of

TABLE I

Parameters Used for Calculating Actinomycin D Binding Curves*

DNA	[NaCl](M)	$K_1(M^{-1})$	K_2/K_1	S	σ	n_1	n_2
poly(dGdC)	0.1	7.5×10^5	2.5	0.970	0.007	4	3
calf thymus	0.01	4.2×10^4	20.0	0.985	0.003	25	14
calf thymus	0.1	4.8×10^4	6.5	0.987	0.003	25	7
calf thymus	0.2	4.8×10^4	5.0	0.990	0.003	25	8

*S and σ are equilibrium constants describing the conversion of the DNA from form [1] to form [2]. See reference 14 for a discussion of the theory of allosteric transitions in DNA structure.

the dG(3'-5')dC sequence in calf thymus DNA (27). However, we caution against overinterpretation of the values in Table I.

It is of interest to note that if one views only the actinomycin D binding data for r values greater than 0.02(Figure 3) and 0.08(Figure 4), then the binding of actinomycin D may be well represented by the usual neighbor exclusion equations. (As a consequence, the cooperative binding equation of McGhee and von Hippel (24) does not reproduce the actinomycin D binding data over the entire range of r values studied.) Additionally, the apparent binding constant, which would be calculated from the data for r values greater than 0.02(Figure 3), increases substantially as the sodium chloride concentration is decreased, in agreement with previous reports (4).

The data in Figure 5 compare the experimental data of Müller and Crothers (4) for the binding of actinomycin C_3 to calf thymus DNA with the calculated binding isotherm for the present data. With the exception of their lowest r value point, the two sets of data agree reasonably well. We have no explanation for the apparent disagreement at the lowest point, although we note that the actinomycins used in the two experiments (C_3 and D) are not the same. We have found the present experimental results (showing cooperative binding at low r values) to be reproducible. Furthermore, we have also found cooperative

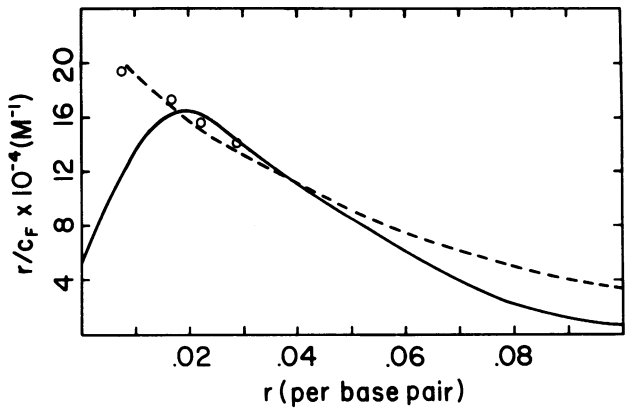

Figure 5: Comparison of the present actinomycin D - calf thymus DNA binding isotherm in (0.2 M NaCl) with the binding data of Müller and Crothers (4) for the interaction of actinomycin C_3 with calf thymus DNA (in 0.2 M Na). The circles are the four lowest r value data points of Müller and Crothers (4).
The dashed line (and the data points) are from ref.(4).

binding of daunorubicin to calf thymus DNA using solute enhanced partition experiments (in preparation, and reference 28). Thus we believe that the observation of cooperative binding and allosteric transitions in the DNA structure are likely to be important in the physiological activity of these compounds.

It is possible that the cooperativity in the carcinogen binding arises from carcinogen induced allosteric effects in the DNA structure rather than two molecules binding at each "site". However, the cooperative binding of the carcinogens occurs at a much lower range of r values (higher bp/bound carcinogen) than the range of r values for the allosteric binding observed with actinomycin D. For the carcinogens to behave allosterically, they would have to, on binding, affect the DNA structure over large distances - a minimum of several hundred base pairs. Such an alteration of DNA structure over long distances has been proposed for distamycin binding to calf thymus DNA (13) and for the cooperative binding of ethidium to poly(dGdC)·poly(dGdC) in the high salt (4M) form (11). Thus the fact that the carcinogen binding occurs at only a small number of sites does not

necessarily rule out the possibility of invoking long-range allosteric effects to explain the data. Regardless of the model chosen for the carcinogen binding, it is apparent that only a few carcinogen molecules bind to the ϕX174RF DNA. That two different carcinogens exhibit the same type of binding behavior poses intriguing questions with regard to the specific nature of their equilibrium binding sites.

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