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Mode of Reversible Binding of Neocarzinostatin Chromophore to DNA: Evidence for Binding via the Minor Groove†

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ABSTRACT: Two general approaches have been taken to understand the mechanism of the reversible binding of the nonprotein chromophore of neocarzinostatin to DNA: (1) measurement of the relative affinity of the chromophore for various DNAs that have one or both grooves blocked by bulky groups and (2) studies on the influence of adenine–thymine residue-specific, minor groove binding agents such as the antibiotics netropsin and distamycin on the chromophore–DNA interaction. Experiments using synthetic DNAs containing halogen group (Br, I) substituents in the major groove or natural DNAs with glucosyl moieties projecting into the major groove show that obstruction of the major groove does not decrease the binding stoichiometry or the binding constant for the DNA–chromophore interaction. Chemical methylation of bases in both grooves of calf thymus DNA, resulting in 13% methylation of N-7 of guanine in the major groove and 7% methylation of N-3 of adenine in the minor groove, decreases the binding affinity and increases the size of the binding site for neocarzinostatin chromophore. Similar results were obtained whether binding parameters were determined directly by spectroscopic measurements or indirectly by measuring the ability of the DNA to protect the chromophore against degradation. On the other hand, netropsin and distamycin compete with neocarzinostatin chromophore for binding to the minor groove of DNA, as shown by their decrease in the ability of poly(dA-dT) to protect the chromophore against degradation and their reduction in chromophore-induced DNA damage as measured by thymine release. Taken together with earlier evidence that neocarzinostatin chromophore intercalates DNA, these data support a binding model in which the naphthoic acid moiety of neocarzinostatin chromophore intercalates between the base pairs of DNA by way of its minor groove.

The nonprotein chromophore of the DNA-damaging antibiotic neocarzinostatin [reviewed in Goldberg et al. (1981)] binds reversibly to helical DNA in the absence of sulfhydryl activating agents (Povirk & Goldberg, 1980; Povirk et al., 1981). Physicochemical studies on the reversible binding have shown two features: (i) the chromophore has a preferred affinity for A-T base pairs over G-C base pairs (Poon et al., 1977; Povirk & Goldberg, 1980); and (ii) it binds to natural DNA by intercalation (Povirk et al., 1981). The intercalative mode of binding has been suggested by the hydrodynamic and electric dichroic properties of the DNA–chromophore complex. The complete structure of the chromophore has been reported recently. It consists of four parts: 2-hydroxy-7-methoxy-5-methyl-1-naphthoate and 2,6-dideoxy-2-(methylamino)-galactose linked to a $C_{15}H_8O_4$ substituent consisting of an ethylene cyclic carbonate group, and a highly strained ether epoxide attached to a novel bicyclo[7.3.0]dodecadiyne system (Hensens et al., 1983; Shibuya et al., 1984; Edo et al., 1985). By analogy with other intercalators, one can a priori predict the planar naphthalene aromatic ring system to be the potential

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intercalating moiety. In fact, comparison of the absorbance, fluorescence, and dichroic spectra in the region of 320–380 nm suggests that the naphthalene ring is the likely intercalating moiety (Povirk et al., 1981).

These studies, however, fail to provide any information about the geometry of the binding in terms of the position of the other substituents of the chromophore with respect to the DNA helix. In particular, no information is presently available regarding the location of these substituents in the grooves of the DNA helix. The determination of the nature of the groove in which the substituents lie is essential in understanding not only the geometry of the complex but also the molecular basis of the two reactions effected by the thiol-activated chromophore, namely, the selective oxidation of C-5′ of deoxyribose of mainly thymidylate (and to a lesser degree deoxyadenylate) residues in DNA under aerobic conditions (Kappen et al., 1982; Kappen & Goldberg, 1983) and the anaerobic formation of covalent chromophore–deoxyribose adducts (Povirk & Goldberg, 1984).

Two approaches have been taken in the present study to determine the groove—major or minor—in which the substituents of the chromophore lie during its binding to DNA: (i) comparison of the binding affinity and stoichiometry of the chromophore for various synthetic and natural DNAs in which the major groove or both the major and minor grooves are blocked by the presence of a bulky substituent and (ii) studies on the competitive binding of the chromophore and known nonintercalating, minor groove binding agents, such as the antibiotics netropsin and distamycin (Zimmer, 1975). Although it had been found earlier that intercalators such as ethidium bromide, actinomycin D, or proflavin inhibit DNA strand breakage by neocarzinostatin (Kappen et al., 1979), these results are not helpful in deciding whether the neocarzinostatin chromophore binds DNA via its minor or major groove, since any intercalator might be expected to compete for binding with another intercalator.

The polynucleotides chosen for the first kind of studies are a double-stranded alternating copolymer poly(dA-dT)·poly(dA-dT) [poly(dA-dT)],[1] poly(dA-dU), and the 5-bromo or 5-iodo derivatives of poly(dA-dU). Binding of the chromophore to DNAs from T2 and T4 bacteriophages, that have their cytosine residues partially (75% in T2) or completely (in T4) glycosylated with bulky glucosyl groups (Revel & Luria, 1970) occupying the major groove, has been compared with binding to unglycosylated DNA from *Clostridium perfringens*, containing almost the same percentage of G-C. Methylated calf thymus DNA, containing N^3-methyladenine and N^7-methylguanine, serves as the model DNA where both grooves are partially blocked.

Competitive binding studies with netropsin and distamycin have been carried out in two ways: (i) determination of the rate constants of the degradation of the labile chromophore in the chromophore–poly(dA-dT) complex in the absence and presence of netropsin and (ii) determination of DNA damage by chromophore activated by 2-mercaptoethanol in the presence and absence of netropsin and distamycin. The latter deserves special note since it can provide information on the possible role of reversible binding of the chromophore in the degradation of DNA in the presence of activating agents.

Materials and Methods

DNAs from calf thymus, *Clostridium perfringens*, and T2 and T4 bacteriophage were obtained from Sigma Chemical Co. After precipitation with ethanol, they were redissolved in 20 mM sodium acetate, pH 5.0, and extensively dialyzed against the same buffer. The synthetic polynucleotides (Pharmacia P-L Fine Chemicals) were treated similarly. The extent of double strandedness of the polymer under the experimental conditions was determined from the observed hyperchromicity of the polymer when denatured by heating. To check any possible alteration of the helix type in the presence of methanol and low pH, the circular dichroic spectra of all the polynucleotides and DNAs were recorded under the experimental conditions (20 mM sodium acetate, pH 5, containing 5% v/v methanol). The spectral profiles resembled those of normal B DNA observed at neutral pH and in the absence of methanol (Sarocchi & Guschlbauer, 1975; Wells et al., 1970). DNA concentrations were determined by using the standard extinction coefficient values reported in the literature (Wells et al., 1970; Patel & Cannel, 1979) and were expressed in terms of moles of base per liter.

The nonprotein chromophore was extracted from clinical ampules of neocarzinostatin (from Kayaku Antibiotics) according to the published method (Povirk & Goldberg, 1980) and was stored in methanol at −70 °C. The purity of the sample of extracted chromophore was checked by HPLC using a C_{18} μBondapak column. It was found to contain no more than 5% of chromophore B (Napier et al., 1981). The chromophore was checked spectrophotometrically each time prior to its use. The concentration of the chromophore was determined by adding an equimolar amount of the apoprotein and measuring the absorbance at 340 nm (molar extinction coefficient at 340 nm = 10800 M^{-1}; Povirk et al., 1981).

Netropsin was a gift of Dr. D. J. Patel of Bell Laboratories, Murray Hill, NJ. Distamycin was a gift of Dr. F. Arcamone of Farmatalia, Italy. Both agents were freshly prepared just prior to use, and the concentrations were determined spectrophotometrically. N^3-Methyladenine was a gift of Dr. D. A. Goldthwait of Case Western Reserve University School of Medicine. N^7-Methylguanine was obtained from Sigma Chemical Co.

All spectrophotometric titrations were carried out with a Perkin-Elmer Model 552A spectrophotometer. The temperature was controlled by a thermoelectric controller.

Methylation of Calf Thymus DNA. Calf thymus DNA was purified by phenol extraction and extensive dialysis against 10 mM NaCl. Purified DNA was chemically methylated by the method of Ramstein et al. (1971). A total volume of 40 μL of dimethyl sulfate was added every 30 min over 3 h to a 0.5-mL solution of the DNA (20 ODU/mL) in 1 M NaCl and 0.5 M sodium cacodylate, pH 6.6. The methylated DNA was precipitated with ethanol twice, washed with ethanol, and dissolved in 2 mL of 10 mM NaCl. It was then extensively dialyzed against the same buffer.

The extent of methylation and the mole percentages of N^3-methyladenine and N^7-methylguanine were estimated from the decrease in the melting point of calf thymus DNA (Ramstein et al., 1971) and by HPLC analysis (on a C_{18} μBondapak column) of the acid hydrolysate of the DNA (with 0.3 M HCl for 10 min at 90 °C) (Ramstein et al., 1971). The hydrolysate was loaded onto the column, followed by elution with a linear gradient of 0–70% solvent B over a period of 30 min at a flow rate of 1.0 mL/min (solvent A, 10 mM KH_2PO_4 in water; solvent B, 80:20 (v/v) methanol/water containing

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; HPLC, high-performance liquid chromatography; poly(dA-dT), double-stranded alternating copolymer poly(dA-dT)·poly(dA-dT); poly(dA-dU), double-stranded alternating copolymer poly(dA-dU)·poly(dA-dU); poly(dA-d^{5Br}U), double-stranded alternating copolymer poly(dA-d^{5Br}U)·poly(dA-d^{5Br}U); poly(dA-d^{5I}U), double-stranded alternating copolymer poly(dA-d^{5I}U)·poly(dA-d^{5I}U); A, adenine; G, guanine; T, thymine; C, cytosine.

10 mM KH_2PO_4). The HPLC profile shows the presence of N^3-methyladenine (eluting at 9 min) followed by N^7-methylguanine (eluting at 11 min) when compared with the standard markers (Thomas et al., 1982). The amounts of each modified base were quantitatively determined by cutting out the paper under the area of each peak and comparing it with the corresponding area produced by a known amount of the bases. This estimation shows that the percentages of N^3-methyladenine and N^7-methylguanine are respectively 7% and 13%. These values are in good agreement with those reported earlier (Ramstein et al., 1971).

Determination of Binding Stoichiometry. Both sample and reference cuvettes contained the same nucleic acid solution. A small volume (15 μL) of chromophore containing the same concentration of the nucleic acid was added to the sample cuvette, and the difference in absorbance at 340 and 350 nm was monitored. The difference was plotted against the concentration of the chromophore added. The binding stoichiometry (r_b) was determined from the break in the straight lines resulting from the above plot, as previously reported (Povirk et al., 1981). The ratio of the concentration of the chromophore corresponding to the break point and the concentration of the DNA gives the stoichiometry value. The nucleic acid concentration varied between 25 and 35 μM. All titrations were carried out at pH 5.0 (20 mM sodium acetate buffer containing 5% v/v methanol) and 14 °C. During the titration, the percentage of methanol changed from 5% to 6.5% (v/v).

For the determination of the binding constants, the absorbance at 324 nm was monitored, since there is a maximum change in absorbance (decrease) of the chromophore at this wavelength where DNA does not absorb. A small aliquot (usually between 3 and 6 μL) of nucleic acid solution was added each time to the sample cuvette; the nucleic acid concentration was kept constant in both cuvettes.

Spectrofluorometric studies to measure chromophore degradation were done with a Perkin-Elmer Model 512 double-beam spectrofluorometer. The temperature of the cell holder was controlled by a Lauda water circulation bath. The excitation wavelength was 380 nm, and the emission wavelength was 490 nm (Povirk & Goldberg, 1980). Usually a small volume (15 μL) of chromophore was added to the solution containing the necessary reagents, and the fluorescence was recorded after a time lag of 5–10 s.

Analysis of the Binding Data. The dissociation constant for the chromophore–DNA interaction was determined by two methods.

(i) One method involved the Li and Crothers equation (eq 1) (Li & Crothers, 1969; Bloomfield et al., 1975) where e_f = the extinction coefficient of the free chromophore, e_b = the extinction coefficient of the bound chromophore, and e_{obsd} = the extinction coefficient measured from the observed absorbance of the chromophore during the titration with DNA. K_d is the dissociation constant of the complex, and the c terms denote the concentrations of the DNA and chromophore, respectively. r_b deontes the binding stoichiometry for chromophore–DNA binding and has been determined from the titration of the chromophore in the presence of an excess fixed concentration of DNA, as mentioned previously. A plot of $1/(e_f - e_{obsd})$ against $1/(c_{DNA} - c_{chrom})$ gives a straight line; the ratio of the slope to the intercept of the line gives the K_d/r_b value. The known value of r_b gives the value of K_d. The above equation is valid under the condition $c_{DNA} \gg c_{chrom}$. The ratio of the input concentration of polymer to chromophore varied from 8 to 60, while the chromophore concentration was between 1.5 and 3.0 μM.

$$1/(e_f - e_{obsd}) = 1/(e_f - e_b) + K_d/[(e_f - e_b)(c_{DNA} - c_{chrom})r_b] \quad (1)$$

(ii) The second method utilizes the Scatchard equation (eq 2) (Scatchard, 1949) where r denotes the ratio of the concentrations of the bound chromophore and DNA and c_f is the concentration of the free chromophore. The concentration of the bound chromophore was determined from the relation c_b = the decrease in absorbance of the chromophore at 324 nm divided by $(e_f - e_b)$. A plot of r/c_f against r gives a straight line. The reciprocal of the slope of the straight line gives the K_d value. r_b is the intercept on the abscissa.

$$r/c_f = (1/K_d)(r_b - r) \quad (2)$$

Analysis of the Kinetic Data. The reciprocal of the rate constant of the chemical degradation of the chromophore in the absence and presence of the various reagents was determined from the semilog plot of the ratio of the observed fluorescence change [at 490 nm where chromophore D (Napier et al., 1981), the highly fluorescent, aqueous degradation product of chromophore A, emits] at any time to the total fluorescence change against that time. The linearity of the plot over a period of 3 half-lives of the reaction was taken as an index of the first-order nature of the degradation.

Measurement of DNA Damage. Thiol-activated neocarzinostatin chromophore induces spontaneous and alkali-dependent base release (T > A ≫ C > G) (Kappen & Goldberg, 1983). The latter is a measure of the formation of DNA strand breaks having a nucleoside 5′-aldehyde at the 5′ end; these constitute at least 90% of the strand breaks (Kappen & Goldberg, 1983). Base release experiments were done using [*methyl*-^{3}H]thymidine-labeled *Escherichia coli* DNA (6.5×10^5 cpm/mmol) (a gift of Dr. L. Povirk). Thymine release, both spontaneous and alkali-induced, was measured by paper chromatography using a solvent system of 70:30 (v/v) of ethanol and 1 M sodium acetate (Povirk et al., 1978). The composition of a typical reaction mixture (100 μL) for base release was as follows: carrier thymine, 200 μg/mL; netropsin (when present), concentration varied as indicated; *E. coli* DNA, 300 μM; [^{3}H]thymine-labeled DNA, 1.6×10^5 cpm; neocarzinostatin chromophore, 20 μM; 2-mercaptoethanol, 10 mM; and 200 mM tris-HCl buffer, pH 8.0, to make the final pH 7.5. The solutions were added in the same order as stated, and the mixture was kept at 0 °C for 15 min. It was then divided into two equal aliquots, half being treated with 6.5 μL of 1 M NaOH for 30 min at 90 °C, followed by an equal volume of 1 M HCl; to the other half was added 13 μL of water. An aliquot (40 μL) of each sample was loaded onto the paper, and after elution with the solvent, the thymine band was measured for radioactivity. Results are expressed in terms of percent of total thymine in DNA released.

Results and Discussion

The effect of the presence of bulky atoms or groups in the major and/or minor groove on the reversible binding of the chromophore to DNA can be evaluated by measuring the changes in the two parameters that characterize polymer–ligand binding: binding stoichiometry (number of chromophore molecules bound per nucleotide) and binding constant.

Binding Stoichiometry for Different Polynucleotides and Nucleic Acids. Figure 1 shows two typical titration curves, for poly(dA-dT) and T4 DNA, from which the binding stoichiometries have been calculated. The justification for measuring the difference in absorbance at 340 and 350 nm was made earlier (Povirk et al., 1981). Table I lists the binding

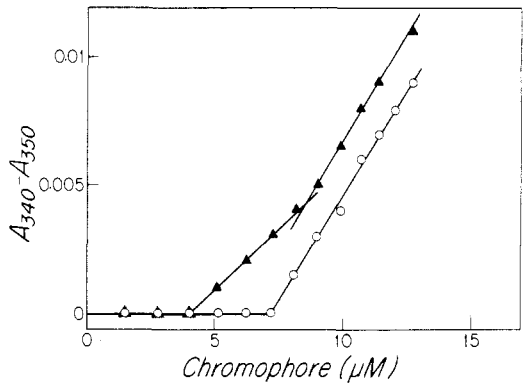

FIGURE 1: Determination of binding stoichiometry for the chromophore–nucleic acid interaction. Plot of the differences in absorbances at 340 and 350 nm ($A_{340} - A_{350}$) against the concentration of the chromophore added to poly(dA-dT) (○) and T4 DNA (▲). The concentrations of poly(dA-dT) and T4 DNA were 29.4 and 31.5 μM, respectively. r_b was calculated from the plots as described under Materials and Methods.

Table I: Stoichiometry of Binding of Chromophore to Synthetic Polynucleotides and Natural DNA[a]

DNA	stoichiometry, r_b = [nucleic acid][b] [chromophore]/ site I	site II
poly(dA-dT)	0.25	
poly(dA-dU)	0.22	
poly(dA-d^{5Br}U)	0.23	
poly(dA-d^{5I}U)	0.22	
C. perfringens	0.26	0.12
T2	0.25	0.14
T4	0.27	0.12
calf thymus	0.23	0.12
methylated calf thymus[c]	0.16	0.10

[a] Data were derived from experiments such as that shown in Figure 1. All titrations were carried out at pH 5.0 (20 mM sodium acetate buffer containing 5% v/v methanol) and 14 °C. [b] Terms in brackets denote the concentrations in moles per liter. [c] Prepared as described under Materials and Methods.

stoichiometries (r_b) for the chromophore–polynucleotide and DNA interactions. These data clearly demonstrate that the stoichiometry of chromophore–polynucleotide binding does not change significantly due to the replacement of H-5 of uracil by methyl, bromine, or the more bulky iodine atom. There is a single type of binding, and all the polymers are saturated at about one chromophore molecule per four nucleotides (r_b = 0.25). Also, in the case of the natural DNAs, the presence of the bulky glucose group in the bacteriophage DNAs does not lead to any alteration of the binding stoichiometry when compared with a DNA having almost the same percentage of G-C (30% G-C in *C. perfringens* DNA as compared to 34% G-C in T2 and T4 DNA). Furthermore, in the natural DNAs, there is a tighter binding site that saturates at a lower ratio of drug to DNA (r_b = 0.12). The finding of two binding sites has been reported earlier for calf thymus DNA (Povirk et al., 1981). Such a difference in the number of binding sites for polynucleotides and natural DNA probably reflects the sequence-specific nature of the binding of the chromophore to the natural DNA, which has heterogeneity in the types of binding sites. This heterogeneity of binding is more clearly reflected in the sulfhydryl-induced strand scission of natural DNA by the chromophore. It has been found that there is a variability in the attack rate for both T and A residues at different locations in defined-sequence DNA (Hatayama et al., 1978; Takeshita et al., 1981; Kappen & Goldberg, 1983).

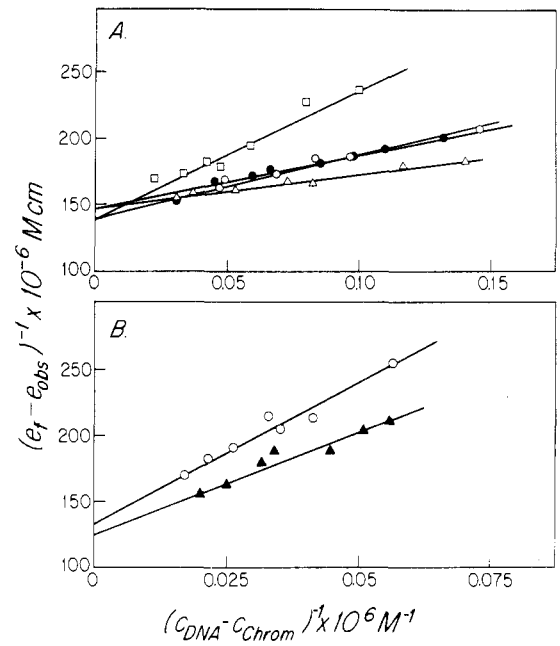

FIGURE 2: Determination of dissociation constants for the chromophore–DNA interaction by use of eq 1. Plot of $1/(e_f - e_{obsd})$ against $1/(c_{DNA} - c_{chrom})$ for the polynucleotides and the DNAs at pH 5.0, 20 mM sodium acetate buffer containing 5% v/v methanol and 14 °C: (A) Poly(dA-dT) (●), poly(dA-dU) (□), poly(dA-d^{5Br}U) (○), and poly(dA-d^{5I}U) (△). (B) *C. perfringens* DNA (○) and T4 bacteriophage DNA (▲). The method to calculate K_d from the plot is explained under Materials and Methods.

Table I further shows that although there remain two types of binding sites in chemically methylated calf thymus DNA, the size of the sites increases in both cases. For the higher affinity binding site, on the average 1 chromophore is bound per 10 nucleotides for the methylated DNA compared with 8 for the natural DNA. The lower affinity binding site also increases from four nucleotides (r_b = 0.25) in natural DNA to six nucleotides (r_b = 0.16) in its methylated counterpart. Since functional groups in both grooves of the DNA [mainly N-3 of adenine (minor groove) and N-7 of guanine (major groove)] are chemically methylated, these results do not distinguish whether partial blockade of the major or the minor groove is responsible for the observed effects. Nevertheless, these data taken together with the lack of effect of obstruction of the major groove by glucosyl moieties suggest that the neocarzinostatin binds to DNA by way of the minor groove. Efforts to determine the binding groove by the ability of the chromophore to block selectively the methylation of purine moieties facing one or the other groove were frustrated by the fact that the chromophore itself reacts with the methylating agent.

The titration described in Table I was carried up to an input ratio of one chromophore molecule per two nucleotides. There may be a weak nonspecific binding site at or beyond this value. Such a site plays very little role, if any, in determining the nature and geometry of the binding; hence, it has not been considered further.

Binding Constant and Influence of a Bulky Group in the Major Groove of Synthetic and Natural DNAs. Dissociation constants were determined by using eq 1. Figure 2 shows the plot of $1/(e_f - e_{obsd})$ against $1/(c_{DNA} - c_{chrom})$ for the polynucleotides and the natural DNAs. The linearity of the plots supports the fact that under the present experimental conditions, an independent, noncooperative type of binding takes place, supporting the validity of applying eq 1. The ratio of the slope and the intercept for the straight line gives the value

Table II: Binding Parameters for the Chromophore–DNA Interaction (by Use of Equation 1)[a]

DNA	dissociation constant, K_d (μM)	DNA	dissociation constant, K_d (μM)
poly(dA-dT)	0.83	T2	1.73
poly(dA-dU)	1.45	T4	1.75
poly(dA-d^{5Br}U)	0.78	calf thymus	2.1
poly(dA-d^{5I}U)	0.58	methylated calf thymus	3.4
C. perfringes	1.86		

[a] All the above values were measured at pH 5.0 (20 mM sodium acetate buffer containing 5% v/v methanol) and 14 °C. The r_b value necessary for the calculation of the dissociation constant was obtained from the stoichiometry data in Table I. For reasons explained in the text, the r_b for the DNA having two binding sites was taken to be the one corresponding to site II. The standard errors of deviation are of less significance because different values are compared. They are, however, ±15% of the mean value.

of K_d/r_b. The known value of r_b (from Table I) gives the value of K_d. The values for the dissociation constants for the set of polynucleotides and the natural DNAs are given in Table II. In the case of the natural DNAs, the lower value of r_b, corresponding to site II, was used for the calculation of K_d. Such a choice seems justifiable in view of the fact that the stronger binding, corresponding to the lower value for r_b, prevails under the experimental conditions where $c_{DNA} \gg c_{chrom}$. The major conclusions relevant to the objective of the present investigation, from Figure 2 and Table II, are the following. (i) For the polynucleotide set poly(dA-dT), poly(dA-dU), poly(dA-d^{5Br}U), and poly(dA-d^{5I}U), there is no increase in the dissociation constant due to the substitution of the H-5 atom of uracil by bromine, methyl, or iodine atoms. In fact, there is a decrease in the dissociation constant from poly(dA-dU) to its 5-iodo(uracil) derivative. (ii) Similar conclusions can be reached from the K_d values for natural DNAs. (iii) the K_d value for methylated DNA is higher than that for unmodified calf thymus DNA, thereby showing a decrease in affinity of the DNA for the chromophore as a result of the partial modification of A and G bases in the minor groove and major groove, respectively. Thus, occupation of the major groove of DNA by glucose does not reduce its affinity for neocarzinostatin chromophore, whereas obstruction of both grooves reduces its affinity for the chromophore.

The presence of the independent, noncooperative type of binding, indicated from the linearity of the plots according to eq 1, suggests that both r_b and K_d can also be determined from the Scatchard equation. For the polynucleotides, the Scatchard plot can give an independent method of verifying the r_b values. In the case of natural DNAs, where two types of binding sites exist, Scatchard plots should give a biphasic plot. Equation 1 determines only the stronger binding site, corresponding to the lower r_b value (site II). Since we are interested in determining the influence of glycosylation on the weaker binding site (which probably involves the G-C base pair of the DNA), attempts were made to determine K_d and r_b values corresponding to this site by using the Scatchard equation. Figure 3 shows the Scatchard plot for poly(dA-dT) and T4 DNA. As shown in the figure, for the natural DNAs, the experimentally determined r_b values lying above 0.12 have been used for the construction of the Scatchard plot (vide Table I). Points corresponding to $r < 0.1$ show a trend corresponding to a line with a higher slope and lower intercept on the r axis in accordance with the stronger binding. This indicates that there is very little influence of the stronger binding site on the calculation of K_d for the weaker site. For the synthetic polynucleotides, the r_b and K_d values obtained by this method

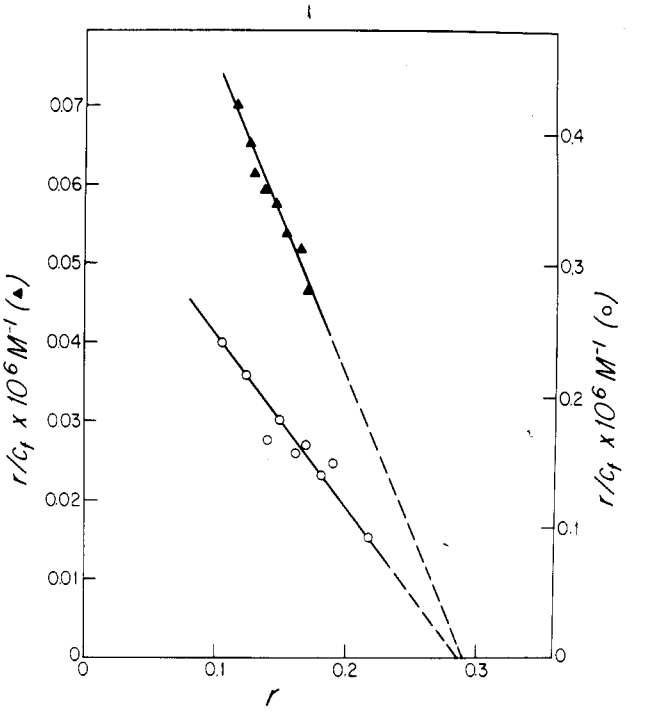

FIGURE 3: Determination of dissociation constant and binding stoichiometry for the interaction by the Scatchard equation. Plot of r/c_f against r for poly(dA-dT) (○) and T4 bacteriophage DNA (▲) under the same conditions as mentioned in the legend for Figure 2. The method to calculate K_d and r_b is explained under Materials and Methods. The K_d value for the natural DNA corresponds to the weaker binding site.

Table III: Binding Parameters for the Chromophore–DNA Interaction by Scatchard Analysis (by Use of Equation 2)[a]

DNA	dissociation constant, K_d (μM)	binding stoichiometry, r_b
poly(dA-dT)	0.74	0.29
poly(dA-dU)	1.32	0.23
poly(dA-d^{5Br}U)	0.71	0.26
poly(dA-d^{5I}U)	0.56	0.24
C. perfringens	3.53	0.27
T2	3.15	0.23
T4	3.33	0.29

[a] As described under Materials and Methods. The titrations were done at pH 5.0 (in 20 mM sodium acetate buffer containing 5% v/v methanol) and 14 °C. For the natural DNAs, the K_d values are for the weaker binding site, site I.

(Table III) agree well with the values determined by spectrophotometric titration and the Li and Crothers method, suggesting an internal consistency in the quantitation of the binding parameters. In the case of the natural DNAs, the values of r_b evaluated from the extrapolation of the straight line to the abscissa agree reasonably well with the values in Table I. The binding constants for the two types of binding sites for natural DNA differ by a factor of about 2. Comparison of the K_d values for T2, T4, and *C. perfringens* DNAs shows that the K_d values corresponding to the first type of binding site (saturated at $r_b = 0.25$) do not vary much from each other. The presence of glucose moieties in the major groove of the phage DNAs does not affect the affinity of the chromophore for the weaker binding site to any significant extent compared to *C. perfringes* DNA.

Comparison of Binding Affinities at Neutral pH from Kinetics of Degradation of Chromophore in the Presence of DNA. All the above studies were carried out at pH 5.0. The instability of the free chromophore at neutral or alkaline pH (Povirk & Goldberg, 1980) prevents direct evaluation of affinity parameters for the nucleic acid–chromophore interaction

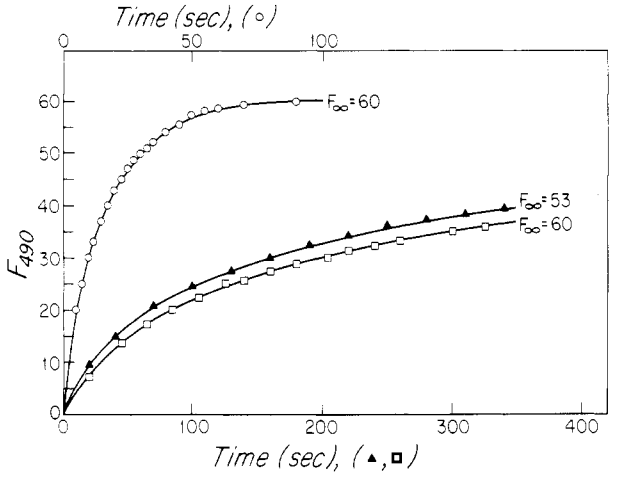

FIGURE 4: Protection of the chromophore in the presence of DNA. Change in the fluorescence of the chromophore at 490 nm (excitation at 380 nm) with time (in seconds) alone (○) and in the presence of *C. perfringens* DNA (▲) and T4 bacteriophage DNA (□) at pH 7.0 (10 mM phosphate buffer), 5% v/v methanol, and 14 °C. The concentration of the chromophore was 3.5 μM, and the DNAs were at 9.5-fold excess. F_α denotes the fluorescence change at the end of the degradation.

under these conditions. Nevertheless, as mentioned earlier, comparison of the rate constants of the degradation of the chromophore (carried out at neutral pH) in the presence of various nucleic acids and under identical conditions of pH, ionic strength, temperature, percentage of methanol, and nucleotide:drug ratio values reveals its relative affinity for them (Povirk & Goldberg, 1980). Figure 4 shows the increase of fluorescence at 490 nm, F_{490}, as a function of time for the chromophore alone and in the presence of two different nucleic acids. It is apparent that there is a decrease in the rate of the degradation in the presence of DNA; also, the magnitude of the fluorescence increase associated with the degradation remains unaltered (within the limits of experimental error) for the same concentration of the chromophore in the presence of different DNAs. These two features support the following scheme based on the assumption that the nucleic acid bound chromophore is infinitely stable:

$$\text{chrom} + \text{DNA} \rightleftharpoons \text{chrom-DNA}_{\text{complex}}$$
$$\downarrow$$
$$\text{chrom}_{\text{degraded}}$$

This scheme is supported by the finding that there is a good correlation between the protection and the fluorescence quenching (as a result of the binding) of the chromophore by calf thymus DNA at a pH low enough to monitor both processes accurately and that there is a good inverse correspondence between the extent of chromophore degradation (as measured from the F_{490} value) and the base released from DNA at pH 8.0 (Povirk & Goldberg, 1980). On the basis of this scheme, one can conclude that the relative ratio of the rate constant of the degradation of the chromophore in the presence of DNA is proportional to its affinity for the DNA. A relation has also been formulated relating the rate of the degradation in the presence and absence of DNA:

$$K_d \leq \frac{[\text{DNA}]_T}{k_D/k_0 - 1} \quad (3)$$

where k_0 and k_D are the rate constants for degradation in the absence and presence of DNA, respectively, and the term in brackets denotes the input concentration of DNA (Povirk & Goldberg, 1980). The rate constants were calculated on the basis of a first-order reaction and were found to be linear over the range of 3 half-lives. Table IV lists the time constants (reciprocals of the rate constants) thus calculated from the linear plots as mentioned under Materials and Methods. These data clearly show that there is no significant variation in the time constants within the group of polynucleotides or natural DNAs. The calculated K_d values (from eq 3) lie in the same range as those determined from the direct evaluation of K_d at pH 5.0. This supports the validity of this approach in comparing the binding affinities at neutral pH. The degree of stabilization of the chromophore by poly(dA-dU) is again observed to be comparatively less than by the other polynucleotides, consistent with what is observed from the direct determination of the binding constants. There is also a consistency in the relative binding affinities for the polynucleotides or the natural DNAs at both pHs of 5.0 and 7.0. Table IV shows further that there is a reduction in the degree of protection of the chromophore by the DNA against chemical degradation when the A and G bases of calf thymus DNA are chemically methylated.

Table IV: Kinetics of Degradation of Chromophore in the Absence and Presence of Nucleic Acids[a]

DNA	*P:D* ratio	time constant, $t = 1/k$ (s)	K_d (μM)
none		19	
poly(dA-dT)	9.5	690	0.67
poly(dA-dU)	9.5	610	0.76
poly(dA-d^{5B}rU)	9.5	708	0.65
poly(dA-d^{5I}U)	9.5	730	0.63
C. perfringens	9.5	268	1.8
T2	9.5	295	1.6
T4	9.5	285	1.7
calf thymus	10	210	2.4
methylated calf thymus	10	135	3.8

[a] *P:D* corresponds to the ratio of the concentrations of nucleic acid and chromophore. A small volume of the stock solution of the chromophore was added to the fluorescence cuvette containing the required concentration of the DNA in 10 mM phosphate, pH 7.1, containing 5% v/v methanol at 14 °C. The concentration of the chromophore was constant in all cases (2.5 μM). There was usually a time lag of 5–10 s between the addition of the chromophore and the first point of recording the fluorescence. For $t < 50$ s, there is an uncertainty of about 15% in the values, while for $t > 60$ s the uncertainty is 10%. K_d is calculated from eq 3 (Results and Discussion).

The above studies—direct comparison of the binding constants and stoichiometries at pH 5.0 and indirect comparison of the binding constants at pH 7.0—suggest that the partial or complete obstruction of the major groove in a polynucleotide or natural nucleic acid does not influence the extent of its binding with the chromophore. Thus, it may be concluded at this point either that the binding involves the unblocked minor groove or that it occurs in the major groove without unfavorable steric contacts with the bulky atoms on the pyrimidine base in the polynucleotides. The second possibility, however, appears remote, at least for the natural DNAs, because the glucose group(s) is (are) too large to allow any substituent of the chromophore to approach the major groove.

There is also a decrease in the binding affinity when the nitrogen atoms of the bases lying in both major (G) and minor grooves (A) are modified. This observation further emphasizes the participation of the minor groove. It is, however, worth mentioning that methylation of the DNA leads to a decrease (about 15 °C) in melting temperature which indicates a weakening of the secondary structure; therefore, the observed increase in the binding size or decrease in binding affinity may be a result of modification of the secondary structure in

Table V: Kinetics of Degradation of Chromophore in the Absence and Presence of Poly(dA-dT) and Netropsin[a]

system	time constant, $t = 1/k$ (s)
chromophore alone	20
chromophore + netropsin (5 μM)	22
chromophore + poly(dA-dT)	724
chromophore + netropsin + poly(dA-dT) at following *P:D* values for netropsin	
P:D = 5	41
P:D = 10	53
P:D = 20	77

[a] *P:D* corresponds to the ratio of the concentrations of DNA and netropsin. The chromophore and poly(dA-dT) concentrations are 2.5 and 25 μM, respectively. In all cases, the chromophore was added last. The experiments were carried out at pH 7.1 (10 mM phosphate buffer), 5% (v/v) methanol, and 14 °C.

methylated DNA (the CD spectrum of the methylated calf thymus DNA, however, showed the characteristic feature expected for a B-DNA structure, indicating that there is no major alteration in B-DNA structure in the modified DNA). The reduction in binding affinity raises the possibility of the involvement of the N-3 of adenine and/or the N-7 of guanine in binding, as methylation of these sites can prevent them from making hydrogen bonds to potential sites on the chromophore.

Effect of Netropsin on the Chromophore–Polynucleotide Interaction. To obtain more direct evidence concerning the involvement of the minor groove of DNA in neocarzinostatin binding, the interaction of the chromophore with DNA in the presence of the drugs netropsin and distamycin, established minor groove binding agents (Zimmer, 1975), was examined in detail.

Table V lists the rate constants of degradation of the chromophore in the presence or absence of netropsin and poly(dA-dT). The results indicate that (i) there is no change in the stability of the chromophore in the presence of netropsin alone, (ii) the protection of the chromophore [reflected in the higher t value in the presence of poly(dA-dT)] by the nucleic acids is reversed upon the addition of netropsin, and (iii) with a further increase in the ratio of poly(dA-dT) to netropsin (P/D = 10 or 20 for netropsin), there is an increase in the extent of stabilization of the chromophore, although it is still much less than the value observed in the absence of netropsin. The above results can be accounted for in two ways: (i) netropsin obstructs the potential binding site on poly(dA-dT) for neocarzinostatin chromophore, and (ii) netropsin induces an unfavorable conformational transition in poly(dA-dT), thereby preventing its binding to the chromophore, which can bind only to the B-DNA structure of poly(dA-dT). The second explanation appears to be unlikely because of two observations: (i) Previous reports on netropsin–polynucleotide binding show that it favors A to B and Z to B helix transitions in DNA. This suggests that it stabilizes the B-DNA structure (Ivanov et al., 1974; Zimmer et al., 1983). (ii) X-ray analysis of the complex of netropsin with a B-DNA dodecamer of sequence C-G-C-G-A-A-T-T-^{Br}C-G-C-G shows that the binding of netropsin does not induce any major alteration in the conformation of the B-DNA dodecamer (Kopka et al., 1985). In fact, it has recently been found that binding of netropsin to the minor groove does not prevent binding of a basic polypeptide to the major groove (Gupta et al., 1984).

Similar types of experiments on the protection of chromophore by poly(dI-dC) at neutral pH were carried out in the presence of another related drug, distamycin. The results show the same trend as observed in the case of netropsin. The stabilization of the chromophore due to its binding by poly(dI-dC) is reversed in the presence of prebound distamycin.

Table VI: Chromophore-Induced Thymine Release from *E. coli* DNA in the Absence and Presence of Netropsin[a]

[DNA]:[netropsin] ratio	% thymine release		
	spontaneous	alkali induced	total
	3.5[b]	6.4	9.9[b]
35	3.2	5.8	9.0
15	3.0	3.5	6.5[b]
5	2.5[b]	1.6[b]	4.1[b]

[a] Terms in brackets denote concentrations in moles per liter. In all experiments, the concentration of the chromophore was 20 μM, and the [DNA]:[chromophore] ratio = 15. [b] The mean of two sets of experiments with a standard deviation of 10%. The control containing no chromophore showed 0.15% total and 0.06% spontaneous base release.

Effect of Netropsin on the DNA Damage Capacity of Neocarzinostatin Chromophore. The results presented above demonstrate that under the condition of reversible binding of the chromophore to a synthetic nucleic acid, known minor groove interacting drugs inhibit the binding of the chromophore even when the major groove is available for its binding. Attempts have been made to extrapolate and examine the validity of the above conclusion when the chromophore degrades the DNA via covalent interaction in the presence of a sulfhydryl reagent. DNA damage can be quantified by measuring the combined spontaneous and alkali-labile base release. The results are summarized in Table VI. It is apparent that (i) there is a decrease in the total thymine release in the presence of netropsin, (ii) alkali-induced thymine release is more affected than spontaneous base release, and (iii) thymine release increases as the input ratio of the concentration of DNA to netropsin increases, probably as a consequence of the availability of a greater number of binding sites on the DNA for the chromophore. Distamycin also influences thymine release from DNA by the chromophore in the same way as netropsin does (data not shown). These observations also suggest a reversible, minor groove binding mode for the chromophore as a step prior to its activation.

A comparison of the inhibitory effects of netropsin on the protection of chromophore by poly(dA-dT) (Table V) and on base release by chromophore from *E. coli* DNA (Table VI) shows that the effect is more pronounced for the former. This difference in degree can be explained as follows: Netropsin binds only to A-T-rich regions in natural DNA (Lane et al., 1983). Activated neocarzinostatin chromophore, however, attacks both A-T-rich and non-A-T-rich sequences in defined-sequence DNA (Takeshita et al., 1981). Since the latter sites become preferred cleavage sites in the presence of netropsin (D. Dasgupta and I. H. Goldberg, unpublished observations), it appears that in the presence of netropsin significant thymine release occurs from these sites, but they are poorer binding sites for the chromophore and thus provide less protection. Furthermore, the observed preferential inhibition of alkali-induced thymine release in the presence of netropsin suggests that spontaneous and alkali-induced thymine release may involve different mechanisms.

Geometry of Binding and Nature of Neocarzinostatin Chromophore–Nucleic Acid Complex under Reversible Conditions. Most intercalators with bulky substituents bind to the minor groove of DNA [polyintercalators like naphthalene bisamides are, however, capable of binding to both grooves, perhaps due to their double anchorage points along the phosphate backbone of DNA (Wilson & Jones, 1982; Yen et al., 1982; Waring, 1981)]. The disruption of the stable hydration spine in the minor groove of the A-T region of B DNA by the presence of substituents can, in principle, lead

to a favorable entropic contribution to the free energy decrease associated with minor groove binding (Dickerson et al., 1982; Zakrzewska et al., 1984). Viscosity studies on the unwinding of PM2 DNA by chromophore have shown that the chromophore induces a relatively large (compared to other intercalators like proflavins or anthracyclines) unwinding angle of 21° in superhelical DNA (Povirk et al., 1981). Such a large unwinding angle may result from the opening of the minor groove to accommodate the bulky substituents of the chromophore. In addition to intercalative binding and electrostatic interactions, the chromophore has potential loci (e.g., carbonyls from naphthoate or cyclic carbonate, hydroxyls from galactose or naphthoate) for hydrogen bonding to sites on the base, resulting in further stabilization of the complex. The minor groove binding raises the possibility that N-3 of adenine and O-2 of thymine (or cytosine O-2) is such a site of hydrogen bonding. The decrease in binding affinity of methylated calf thymus DNA may indicate the involvement of N-3 of adenine in the interaction. There are reported examples of drug–DNA complexes (e.g., daunomycin–DNA complex) where the N-3 of adenine and the O-2 of thymine are sites of hydrogen bonds (Patel et al., 1981; Wilson & Jones, 1982).

Role of Minor Groove Binding in DNA Damage by Chromophore in the Presence of Sulfhydryl Reagents. During sulfhydryl-induced DNA cleavage, minor groove binding of the native chromophore (in the prebound DNA complex) or of the sulfhydryl-modified chromophore may determine the geometry of the reactive site on the chromophore for further reaction leading to strand breaks, covalent adduct formation, and/or base release from the DNA. Inspection of the sugar protons (C-1′, C-2′, C-3′, and C-4′) in a space-filling model of DNA shows that they are easily accessible to chromophore bound in the minor groove. Abstraction of hydrogen(s) from C-5′ of the deoxyribose by the chromophore appears to be responsible for the generation of a carbon-centered radical at C-5′ (Charnas & Goldberg, 1984) that may be the common intermediate in the formation of DNA damage (Kappen & Goldberg, 1985). One of the pair of hydrogen atoms at C-5′ of deoxyribose lies on the periphery of the minor groove and may be a target for the activated chromophore. Alteration of the phosphate backbone as a result of the binding of the chromophore may also bring C-5′ into a more favorable position for abstraction of the hydrogen.

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