Cryospectrokinetic Evidence for the Mode of Reversible Binding of Neocarzinostatin Chromophore to Poly(deoxyadenylic-thymidylic acid)†

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ABSTRACT: The spectra of neocarzinostatin (NCS) chromophore during its reversible association with poly(dA-dT)·poly(dA-dT) [poly(dA-dT)] were recorded (at intervals of 17 ms or more) by a cryospectroscopic method. Examination of the spectral changes of a drug during its interaction with DNA has not been previously reported. Such studies indicate binding of chromophore to poly(dA-dT) is a two-step process in which the spectral properties of the intermediate poly(dA-dT)·NCS chromophore species closely resemble those of the final equilibrium species. On the basis of cryokinetic studies (at single wavelengths) carried out at low temperature (2 °C), the following proposed mechanism of the DNA–drug (PD) interaction was quantitated:

$$\text{poly(dA-dT)} + \text{chromophore} \underset{21\ s^{-1}}{\overset{7.8 \times 10^5\ M^{-1}\ s^{-1}}{\rightleftharpoons}} (PD)_I \underset{0.75\ s^{-1}}{\overset{9.2\ s^{-1}}{\rightleftharpoons}} (PD)_{II}$$

In analogy with the other reports on the kinetics of drug–DNA interaction, $(PD)_I$ and $(PD)_{II}$ could represent externally bound and intercalated complexes, respectively. However, since the spectra of $(PD)_I$ and $(PD)_{II}$ are closely similar, it can also be proposed that $(PD)_I$ and $(PD)_{II}$ represent two forms of an intercalated complex. The rate and equilibrium constant for each step were determined by examining the kinetics of the forward and reverse reactions. This was accomplished by determining (i) the polynucleotide concentration dependence of the apparent fast and slow first-order rate constants observed during a double-exponential increase in transmittance (at 330 nm) associated with the binding and (ii) the apoprotein-induced dissociation rate constant of the chromophore from poly(dA-dT). The opportunity to use apoprotein, instead of a detergent, to follow the kinetics of the reverse reaction provides a novel approach to these studies. The slow off rate for the second step might place the chromophore on the DNA for a sufficient length of time to be activated by thiol.

Neocarzinostatin (NCS),[1] an antitumor antibiotic, consists of a labile, nonprotein chromophore noncovalently bound to a low molecular weight apoprotein (Goldberg et al., 1981; Napier et al., 1979). The chromophore consists of four parts: 2-hydroxy-7-methoxy-5-methyl-1-naphthoate and 2,6-deoxy-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; Edo et al., 1985). The DNA-damaging activity of NCS in the presence of sulfhydryl-activating agents has been ascribed to the chromophore (Kappen et al., 1980). Chromophore binds reversibly to DNA in the absence of thiols (Povirk & Goldberg, 1980). The reversible binding to DNA plays an important role in the ensuing thiol-activated processes of base-specific DNA strand breakage, selective oxidation of C-5′ of deoxyribose, base release, and formation of covalent chromophore–deoxyribose adducts, all with base specificity in the order T > A ≫ C > G (Kappen & Goldberg, 1985). Physicochemical studies have shown the following features of the reversible interaction: (i) chromophore has a preferred affinity for the A-T base pair over the G-C base pair (Povirk & Goldberg, 1980); and (ii) the planar aromatic naphthalene ring intercalates between the base pairs of natural DNA, whereas the other substituents lie in the minor groove (Povirk et al., 1981; Dasgupta & Goldberg, 1985).

To further understand the mode of the reversible binding, we undertook the study of the kinetics of association of the chromophore with poly(dA-dT), a B-DNA polymer with two sequences (ApT and TpA) (Assa-Munt & Kearns, 1984). Poly(dA-dT) has also been used as a model DNA to understand the chemistry of DNA damage by thiol-activated chromophore (Povirk & Goldberg, 1982, 1984). We would like to emphasize two novel features of the present study. The soluble nature and stability of the chromophore in methanol (Povirk & Goldberg, 1980) have allowed us to work at 0 °C or at subzero temperatures by using a suitable buffered methanol solution as a cryosolvent. Cryospectrokinetics (Auld, 1979; Hanahan & Auld, 1980; Auld et al., 1984) were employed to follow the time course of the fast association of the NCS chromophore with poly(dA-dT) by either rapid-scanning or single-wavelength techniques. This technique has the potential of spectral and hence molecular characterization of the elusive intermediates in drug–DNA interactions. A new method was also used to measure the dissociation rate of chromophore from poly(dA-dT). Instead of conventional detergent-induced (e.g., sodium dodecyl sulfate) dissociation

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[1] Abbreviations: NCS, neocarzinostatin; HPLC, high-performance liquid chromatography; CD, circular dichroism; poly(dA-dT), double-stranded alternating copolymer poly(dA-dT)·poly(dA-dT); *P*/*D*, molar ratio of DNA nucleotide phosphorus to drug.

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of the drug–nucleic acid complex (Fox et al., 1981), the high affinity of chromophore for the apoprotein [dissociation constant of 10^{-10} M (Povirk & Goldberg, 1980)] has been utilized to measure the dissociation rate of chromophore from poly(dA-dT).

Materials and Methods

Materials. NCS was provided by Dr. William Bradner of Bristol-Myers. Poly(dA-dT) was from Pharmacia P-L Fine Chemicals, Inc.

Chromophore and Apoprotein Preparation. Chromophore was obtained by extraction of the dialyzed and lyophilized NCS with pure methanol at 4 °C, according to a previously published procedure (Povirk et al., 1981). The concentration of the chromophore was determined from the absorbance at 340 nm after the addition of an equimolar amount of apoprotein (ϵ_{340} = 10.8 mM^{-1} cm^{-1}) (Povirk et al., 1981). The chromophore solution was stored at −70 °C. Its purity was checked routinely by HPLC, as reported previously (Napier et al., 1981), and found to contain no more than 5% of chromophore B. Since no difference in the kinetics was observed when purified (by HPLC) chromophore A was used, no attempt was made to further purify chromophore A from the small amounts of chromophore B. The apoprotein was prepared from the holoantibiotic by passing it through columns of (i) XAD-7 (to free it from chromophore) (Napier et al., 1980), (ii) SP-Sephadex C-25, and (iii) DEAE-Sephadex A-25. The absence of chromophore in the apoprotein was indicated by the absence of any absorbance in the region of 310–400 nm. The concentration of the apoprotein was determined from the reported value of ϵ_{278} = 14.4 mM^{-1} cm^{-1} (Povirk et al., 1981).

Poly(dA-dT) Preparation. The commercial sample was dissolved in 15 mM NaCl, sonicated for 10 min to reduce the chain length to 250 base pairs, precipitated by ethanol, and redissolved in 20 mM sodium acetate buffer, pH 5.2. It was then extensively dialyzed against the same buffer. The band shape of the CD spectrum of poly(dA-dT) in 20 mM sodium acetate, pH 5.2, containing 20% (v/v) methanol (buffer A) is identical with that of the spectrum recorded in 20 mM sodium phosphate buffer, pH 7.1 (Wells et al., 1970), indicating that there is no change in the B-DNA conformation of the polynucleotide under the present condition. The concentration of poly(dA-dT) was expressed in terms of moles of nucleotide (ϵ_{260} = 6.7 mM^{-1} cm^{-1} at pH 7.0).

Stopped-Flow Kinetics. The low-temperature stopped-flow instrument was equipped with quartz fiber optics, a Schoeffel monochromator, a 75-W xenon lamp, a low-noise, high-sensitivity detector system photomultiplier, and a Sorensen high-voltage power supply (Auld, 1979; Hanahan & Auld, 1980). The path length of the stopped-flow cell was 2 cm. The temperature in the stopped-flow apparatus was regulated to ±0.2 °C by passing cooled N_2 gas through the environmental chamber (Hanahan & Auld, 1980; Auld, 1979). The transmittance was digitized, and 1000 data (time) points were collected per experiment with the aid of a PDP 11/34 computer (DEC). Double-exponential fits were determined by a nonlinear least-squares program kindly provided by Dr. Thayer C. French. First-order rate constants were determined from least-squares analysis of plots of $-\log (T_\infty - T_t)$ vs. time where T_∞ and T_t are the transmittance at the end of the reaction and at any time t, respectively (Lobb & Auld, 1980).

Experiments were initiated by mixing the DNA solution in buffer A with an equal volume of chromophore in the same buffer. The change in transmittance was normally monitored at 330 nm. The slit width of the incident light on the reaction mixture was also kept at a minimum of ≤1 mm to prevent any photochemical degradation of the chromophore. The rate calculated for each concentration of DNA is a statistical average of five to eight individual values at that concentration. The standard deviation was in the range of 6–20%.

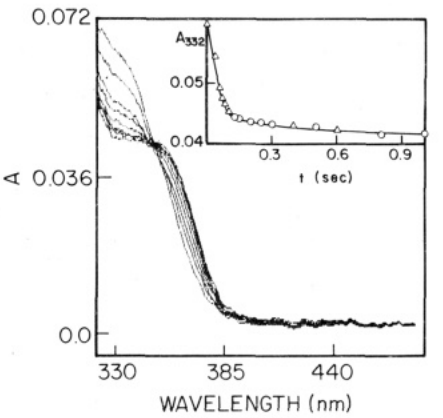


FIGURE 1: Absorption spectra of the intermediates in the reversible binding of the chromophore (5 μM) to poly(dA-dT) (30 μM) at 1.6 °C in buffer A. The spectra were recorded at 0 (uppermost), 33, 50, 67, 84, 101, 400, and 600 ms after mixing. Inset: Absorbance change at 332 nm as a function of time after mixing. The circles correspond to the data points from additional spectra not shown in the figure.

The spectra of the intermediates were scanned over a range of 315–550 nm in 16.48 ms intervals with a rapid-scanning photodiode array interfaced to the stopped-flow apparatus (Auld et al., 1984; Geoghegan et al., 1983). A filter corresponding to the cutoff wavelength of 295 nm was placed in the light path to prevent photodecomposition of the chromophore by UV light. Delays between the scans were from 17 ms to several hundred milliseconds.

Spectrophotometric titrations were carried out with a Perkin-Elmer Model 512A spectrophotometer. The temperature was controlled by a thermoelectric controller.

Results

Cryospectroscopy of Intermediates in Chromophore–Poly(dA-dT) Association. The absorption spectra of the chromophore were measured during the course of its binding to poly(dA-dT) at 1.6 °C (Figure 1). All spectra were normalized in the region of 500–550 nm, and absorbance was constant in the region 420–550 nm. The equilibrium spectrum at 600 ms closely resembled one previously reported for calf thymus DNA-bound chromophore (Povirk et al., 1981). With time, there was a progressive increase in the absorbance in the 350–385-nm region, whereas there was a time-dependent hypochromicity in the 330–345-nm region. The most characteristic feature of the spectra was the time-dependent decrease in slope in the 330–345-nm region, finally culminated in a spectrum with almost zero slope in the region 340–350 nm. The inset shows the plot of A_{332} as a function of time; the resulting curve fits a double-exponential process, suggesting at least two processes were taking place. The absorption spectra show no major change in the isosbestic point.

Kinetics of Association between Chromophore and Poly(dA-dT). Stopped-flow studies at single wavelengths were preformed to elucidate the kinetics and reaction mechanism. A representative stopped-flow trace of the transmittance change associated with the binding of the chromophore to poly(dA-dT) is shown in Figure 2. The following features are apparent from such traces. There was an increase in transmittance at 330 nm with time, consistent with the fact that the bound chromophore has a lower absorbance than the free chromophore at this wavelength, as shown by the time-

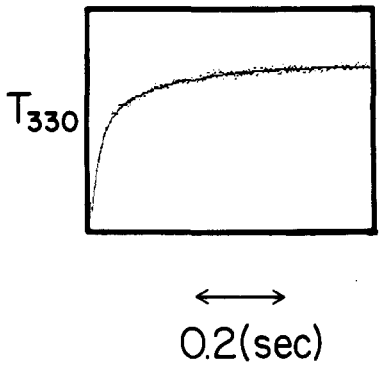


FIGURE 2: Stopped-flow kinetics trace of the change in transmittance of the chromophore at 330 nm with time, reflecting the reversible association of the chromophore, 2.5 μM, with poly(dA-dT), 25.0 μM, at 2.0 °C in buffer A. The solid line represents the theoretical double-exponential plot fit to the experimental transmittance trace (360 data points). The plot is generated from the relation $\Delta T_{330} = 0.56e^{-48.5t} + 0.44e^{-6.0t}$.

resolved spectra (Figure 1). The final transmittance of the bound chromophore was essentially the same as that for the previous reaction, indicating no photolysis was occurring. As predicted from the equilibrium and time-resolved spectra (Figure 1) of the chromophore in the presence of poly(dA-dT), a time-dependent decrease in transmittance is observed when the studies were preformed at both 368 and 380 nm (data not shown). Since the decrease was of smaller amplitude at these wavelengths than the increase at 330 nm, the latter wavelengths was preferred for the kinetic studies. The data points comprising the observed transmittance change at (ΔT_{330}) in Figure 2 could be fitted very well to a double-exponential process throughout the entire time course by a nonlinear, least-squares analysis:

$$\Delta T_{330} = a_f e^{-k_f t} + a_s e^{-k_s t}$$

where a_f and a_s are the amplitude factors and k_f and k_s are the apparent first-order rate constants for the fast and slow processes respectively. The solid line (shown in Figure 2) passing through the experimental data points represents the double-exponential fit. The observation of the double-exponential process suggests that there are two steps involved in the transmittance change, in accordance with observations from the time-resolved spectra of the binding of the chromophore to poly(dA-dT).

Dependence of Apparent Rate Constants on the Concentration of Poly(dA-dT). Concomitant with the increase in the *P/D* value, there is an increase in both the amplitude and the apparent rate of increase in the transmittance. The increase in amplitude is consistent with the fact that at a higher *P/D* value, the fraction of bound chromophore ([chrom]) increases. The mechanism of the interaction was determined by examining the effect of the concentration of poly(dA-dT) on the two apparent rate constants.

Figure 3A shows the dependence of the faster rate constant on poly(dA-dT) concentration. The linear relationship under a pseudounimolecular condition ([poly(dA-dT)] > [chrom]) suggests that the fast apparent first-order rate constant (k_f) represents a simple bimolecular reversible binding interaction (Lobb & Auld, 1984):

$$P + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (PD)_I$$

and

$$k_f = k_1[P] + k_{-1} \quad (1)$$

where [P] is the concentration of poly(dA-dT). The linear

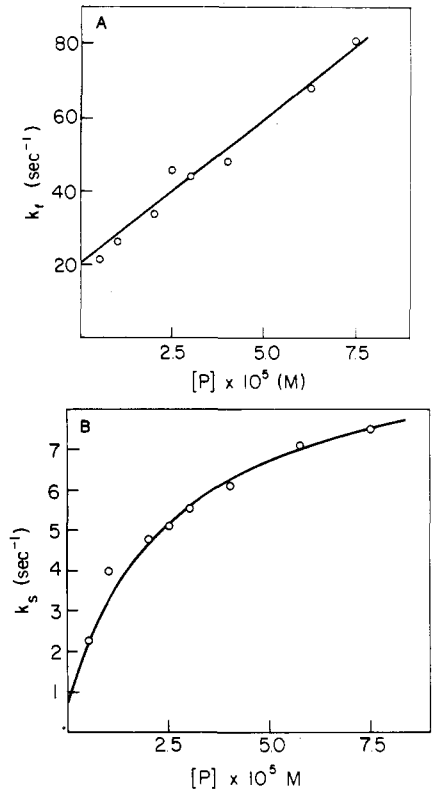


FIGURE 3: (A) Dependence of the apparent first-order rate constant (k_f) for the fast process against the concentration of poly(dA-dT), [P]. The concentration of the chromophore was kept constant at 2.5 μM. The parameters k_1 and k_{-1} are obtained from least-squares regression of the data from [P] = 20–75 μM (Table I). (B) Dependence of the apparent first-order rate constant (k_s) for the slow process against the concentration of poly(dA-dT). The best-fit curve, constructed as described under Results, is shown as a solid line.

Table I: Kinetic and Thermodynamic Parameters for Chromophore–Poly(dA-dT) Interaction[a]

parameters	values	parameters	values
k_1 (M^{-1} s^{-1})	7.8×10^5	K_1 ($=k_1/k_{-1}$) (M^{-1})	3.7×10^4
k_{-1} (s^{-1})	21	K_2 ($=k_2/k_{-2}$)	12
k_2 (s^{-1})	9.2	K_{app} [$=K_1(1 + K_2)$] (M^{-1})	4.9×10^5
k_{-2}[b] (s^{-1})	0.75	K_{app}[c] (M^{-1})	3.0×10^5

[a] The measurements were done at 2 °C in buffer A. [b] Value taken from Table II. [c] Measured under equilibrium conditions from the absorbance change of the chromophore at 324 nm (eq 4).

plot of k_f against [P] gives the forward rate constant (k_1, slope of the line) and the reverse rate constant (k_{-1}), intercept on the rate axis). These values and the value of K_1 (k_1/k_{-1}) are summarized in Table I.

The variation of the slower unimolecular rate constant (k_s) with the concentration of the polynucleotide (Figure 3B) shows that there is an initial dependence of the rate on the concentration but at high concentrations of the polynucleotide it begins to plateau. this behavior is consistent with the mechanism shown in Scheme I.

Scheme I

$$P + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (PD)_I \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (PD)_{II}$$

For Scheme I, the rate constant for the first step, k_f, will show a linear relationship (eq 1), and the first-order rate constant (k_s) for the slower unimolecular isomerization (Lobb & Auld, 1984) is given by

$$k_s = k_{-2} + \frac{k_2}{1 + K_1^{-1}/[P]} \quad (2)$$

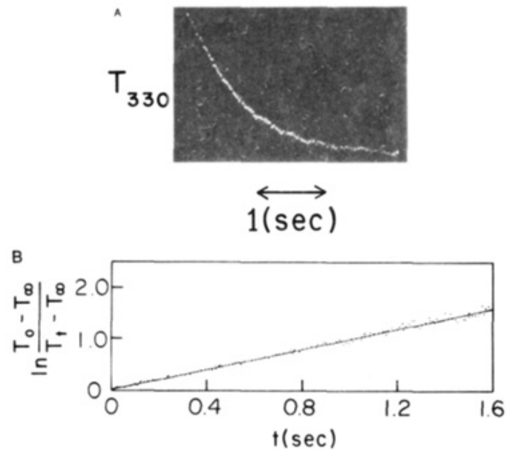


FIGURE 4: (A) Stopped-flow kinetics trace of the decrease in the transmittance at 330 nm reflecting the apoprotein (2.5 μM) induced dissociation of the chromophore (2.5 μM) from an equilibrium mixture of chromophore and poly(dA-dT) (42 μM) at 2 °C, all in buffer A. (B) Semilogarithmic first-order rate plot (using 200 data points) for the trace shown in (A).

Table II: Apoprotein-Induced Dissociation Rate of Chromophore from Poly(dA-dT)[a]

poly(dA-dT) concn (μM)	apoprotein concn (μM)	first-order rate constant (s^{-1})[b]
42	1.5	0.74 ± 0.06
42	2.5	0.77 ± 0.09
25	2.5	0.75 ± 0.04

[a] Measurements were done at 2 °C in buffer A. The concentration of the chromophore was 2.5 μM. Apoprotein was mixed with an equilibrium mixture of chromophore and poly(dA-dT). [b] The rate constants were obtained from data ranging up to 2–2.5 half-lives of the reaction.

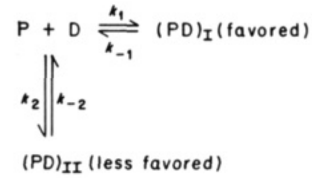
At a high value of [P], i.e., $[P] \gg K_1^{-1}$, k_s should tend toward a constant with a value equal to $k_2 + k_{-2}$ (Lobb & Auld, 1984). $K_1^{-1}(k_{-1}/k_1)$ is the dissociation constant for the first step. k_{-2} corresponds to the intercept on the abscissa (i.e., at the condition of $\lim_{[P]\to 0} k_s = k_{-2}$).

Kinetics of the Apoprotein-Induced Dissociation of the Chromophore–Poly(dA-dT) Complex. We attempted to fit the poly(dA-dT) concentration dependence of k_s (Figure 3B) to the expression given by eq 2. For this purpose, an independent determination of k_{-2} is necessary. Since k_{-2} is the rate constant for the slowest step in the process of dissociation of the chromophore from poly(dA-dT), following the reverse reaction should allow an accurate measurement of k_{-2}. The apoprotein was used as the agent to reverse the reaction. The addition of apoprotein at an equimolar concentration to the chromophore to a chromophore–poly(dA-dT) mixture results in the abstraction of the chromophore from the poly(dA-dT) to give the spectrum of the holoprotein (Povirk et al., 1981). Figure 4A illustrates a typical kinetic trace for such an addition. The following observations led to the conclusion that the rate constant calculated from the above trace represented the dissociation rate (k_{-2}) of chromophore from poly(dA-dT): (i) in accordance with the observations under equilibrium conditions, there is a decrease in the transmittance of the chromophore bound to poly(dA-dT) due to the presence of the apoprotein; (ii) the affinity of the chromophore for the apoprotein is 1000-fold higher than that for poly(dA-dT) (Povirk et al., 1981); (iii) the interaction between the apoprotein and the chromophore was over in the mixing time even at 0 °C (data not shown); (iv) data points comprising the trace in Figure 4A could be readily fitted to a first-order process (shown in Figure 4B), the rate constant of which is 0.75 s^{-1};

Scheme II

$$P' \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P + D \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (PD)_I$$

Scheme III



and (v) this rate constant is not dependent on the concentrations of poly(dA-dT) or apoprotein (Table II).

By the above means, k_{-2} was readily determined. k_{-1}/k_1 was obtained from the slope and intercept of the straight line in Figure 3A. The value of k_2 was varied until the curve shown by the solid line in Figure 3B was obtained. The average standard deviation of calculated from experimental rate constants was 5%. The results are summarized in Table II. The absence of a second step in the dissociation by apoprotein is explained by the fact that $k_{-1} \gg k_{-2}$.

Comparison of Kinetic and Equilibrium Binding Data. The overall apparent binding constant (K_{app}) for Scheme I is related to the association constants (K_1 and K_2) for the individual steps by (Li & Crothers, 1969)

$$K_{app} = K_1(1 + K_2) \qquad (3)$$

The relation shown in eq 3 provides a method to verify the proposed mechanism, because K_{app} can also be evalulated from spectrophotometric titration of the chromophore with poly(dA-dT). Under the condition $[P] \gg [D]$, K_{app} can be determined by using the equation (Li & Crothers, 1969):

$$\frac{1}{\Delta E_{obsd}} = \frac{1}{\Delta E} + \frac{1}{K_{app}\Delta E([P] - [D])} \qquad (4)$$

where ΔE_{obsd} = the observed change in the extinction coefficient of the chromophore due to addition of poly(dA-dT) and ΔE = the difference in the extinction coefficients of the free and bound chromophores. A plot of $1/\Delta E_{obsd}$ against $1/([P] - [D])$, under the experimental condition $[P] \gg [D]$, gave a straight line, the ratio of intercept to slope of which gave K_{app} (Table I). The K_{app} values, from kinetic and equilibrium studies, agree very well with each other, thereby strongly favoring the proposed Scheme I as the operating reaction mechanism.

Discussion

We have succeeded in measuring the bimolecular association kinetics of the NCS chromophore with poly(dA-dT) by means of a low-temperature stopped-flow instrument despite the major problem of the labile nature of the chromophore and its rapid rate of interaction with DNA at room temperature. The observation of a two-step process during the association might suggest other possible mechanisms: (i) preequilibrium isomerization of the polymer as in Scheme II; and (ii) instead of the sequential formation of $(PD)_I$ and $(PD)_{II}$, formation in parallel pathways as in Scheme III. Schemes I and III have been observed for other drug–nucleic acid systems (Fox & Waring, 1984). Scheme II predicts that the slower rate process will show an inverse rate constant dependence on the concentration of poly(dA-dT) (Lobb & Auld, 1984), while Scheme III predicts both rate processes will be linearly dependent on poly(dA-dT) concentration. In addition, a double-exponential rate process would occur for Scheme III when

examined in the dissociation direction. None of these predictions are observed in the present situation. Scheme II would also predict an alteration in the conformation of poly(dA-dT) upon binding of the chromophore; we could not detect any gross conformational change of poly(dA-dT) upon chromophore binding by CD spectroscopy.

Kinetic Mechanism in the Light of Mode of Binding of Chromophore. Considering the fact that the chromophore probably binds to natural DNA via intercalation, one can, by analogy with other drug–nucleic acid interactions, suggest that the fast bimolecular step proposed in Scheme I represents the formation of an externally bound chromophore (Li & Crothers, 1969; Schmechel & Crothers, 1971; Sturm, 1982; Fox & Waring, 1984; Chaires et al., 1985). This complex probably involves ionic interaction of the amino group of the sugar ring in the chromophore with the sugar phosphate backbone of DNA. $(PD)_I \rightleftharpoons (PD)_{II}$ represents the intercalation of the naphthalene moiety into the DNA helix. Thus, the proposed Scheme I may be rephrased as follows:

$$P + D \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (PD)_{out} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (PD)_{int}$$

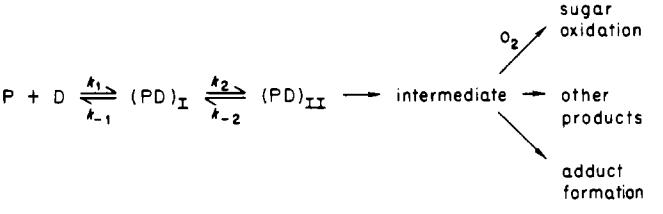
where the subscripts "out" and "int" refer to the outside and intercalative modes of binding, respectively. The observed hypochromicity in the region of 330–345 nm and the bathochromic shift of the intermediate spectra (Figure 1) would then probably arise out of both external and intercalative modes of binding of the chromophore. It was shown earlier that the naphthalene ring contributes to the absorption of the chromophore in the 320–370-nm region (Napier & Goldberg, 1983). It is reasonable to assume that for the externally bound chromophore molecules, the naphthalene rings stack upon each other along the polynucleotide helix with sufficient proximity [binding stoichiometry = 0.25 chromophore/nucleotide (Dasgupta & Goldberg, 1985)] to give rise to the above effects. The effects become more pronounced when the naphthalene ring stacks between the base pairs of poly(dA-dT). However, the first step, i.e., $P + D \rightleftharpoons (PD)_I$, can also be interpreted as the intercalation step; this is followed by a slower isomerization of the intercalated drug. This proposition appears equally plausible since the electronic absorption properties of $(PD)_I$ are closely similar to those of $(PD)_{II}$ (Figure 1).

Comparison with Other Intercalating Molecules. Previous studies of the binding drugs such as echinomycin (Fox & Waring, 1984), tilorone (Sturm, 1982), or ethidium bromide (Jovin, 1978) to polynucleotides like poly(dA-dT) suggest a simple bimolecular association involving the intercalation of the planar aromatic ring of the drug between the DNA base pairs. On the other hand, fluorescence relaxation kinetic studies of proflavin binding to poly(dA-dT) (Ramstein et al., 1980) or poly(A)·poly(U) (Schmechel & Crothers, 1971) suggest a two-step reaction with a preequilibrium similar to the present case. The observed kinetics of interaction of drugs with natural DNAs (Muller & Crothers, 1968; Li & Crothers, 1969; Ramstein et al., 1980; Fox & Waring, 1984) range from a simple bimolecular step, as observed in the case of the bisintercalator echinomycin (Fox & Waring, 1984), to a complex multistep process reported for actinomycin D (Muller & Crothers, 1968).

The magnitude of the forward rate constant ($k_1 = 0.78 \times 10^6$ M^{-1} s^{-1}) for the bimolecular reaction in the two-step scheme is comparable [taking into account the slower rate constant determined here at a temperature of 2 °C, much lower than the temperature (10–25 °C) used in other systems] with those reported for some other intercalators, e.g., proflavin (Li & Crothers, 1969; Schmechel & Crothers, 1971; Ramstein et al., 1980), ethidium bromide (Jovin, 1978), or daunomycin (Chaires et al., 1985). On the other hand, the rate constant for the association of the chromophore with poly(dA-dT) is about 100-fold higher than those for echinomycin (Fox & Waring, 1984) or tilorone (Sturm, 1982) binding to poly(dA-dT). The rate constant of the reverse reaction for the first step ($k_{-1} = 21$ s^{-1}) in the present case is somewhat slower than those for the other drugs like proflavin (10^2 s^{-1}) (Li & Crothers, 1969) or ethidium bromide (10^3 s^{-1}) (Wakelin & Waring, 1980), giving rise to a high value of K_1 (3.7×10^4 M^{-1}), the first equilibrium constant, which suggests that a majority of the free energy for the binding is derived from the first step. The magnitude of the affinity constant (K_2) for the second step is comparable to that observed for the similar step in the case of intercalators like proflavin (Li & Crothers, 1969), daunomycin (Chaires et al., 1985), or tilorone (Sturm, 1982).

The kinetic features in the present case do not suggest direct ligand transfer as reported for a monointercalator like ethidium bromide (Wakelin & Waring, 1980) or a bisintercalator like echinomycin (Fox & Waring, 1984). The absence of any influence of the preincubation time of the chromophore–poly(dA-dT) mixture upon the dissociation rate constant of the chromophore from poly(dA-dT) (D. Dasgupta, unpublished results) argues against any internal shuffling, as reported for echinomycin binding to natural DNA (Fox & Waring, 1985).

Proposed Kinetic Scheme in Relation to DNA Degradation. The reaction mechanism of the reversible chromophore–poly(dA-dT) interaction is in agreement with that suggested earlier from this laboratory (Povirk et al., 1981) and can be further extended to illustrate its relation to DNA cleavage, taking into account the recent findings of adduct formation (Povirk & Goldberg, 1984, 1985) as follows:



The slow rate for the second step ($k_{-2} \simeq 0.75$ s^{-1}) might place the chromophore on the DNA for a sufficient length of time to be activated by thiol.

Registry No. NCS chromophore, 81604-85-5; poly(dA-dT), 26966-61-0.

References

Assa-Munt, N., & Kearns, D. R. (1984) *Biochemistry 23*, 691–697.

Auld, D. S. (1979) *Methods Enzymol. 61*, 318–335.

Auld, D. S., Galdes, A., Geoghegan, K. F., Holmquist, B., Martinelli, R. A., & Vallee, B. L. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5041–5045.

Chaires, J. B., Dattagupta, N., & Crothers, D. M. (1985) *Biochemistry 24*, 260–267.

Dasgupta, D., & Goldberg, I. H. (1985) *Biochemistry 24*, 6913–6920.

Edo, K., Mizugaki, M., Koide, Y., Seto, H., Furihata, K., Otake, N., & Ishida, N. (1985) *Tetrahedron Lett. 26*, 331–334.

Fox, K. R., & Waring, M. (1984) *Biochemistry 23*, 2627–2633.

Fox, K. R., & Waring, M. J. (1985) *Nucleic Acids Res. 13*, 595–603.

Fox, K. R., Wakelin, L. P. G., & Waring, M. J. (1981) *Biochemistry 20*, 5768–5779.
Geoghegan, K. F., Galdes, A., Martinelli, R. A., Holmquist, B., Auld, D. S., & Vallee, B. L. (1983) *Biochemistry 22*, 2255–2262.
Goldberg, I. H., Hatayama, T., Kappen, L. S., Napier, M. A., & Povirk, L. F. (1981) in *Second Annual Bristol-Myers Symposium in Cancer Research*, pp 163–194, Academic Press, New York.
Hanahan, D., & Auld, D. S. (1980) *Anal. Biochem. 108*, 86–95.
Hensens, O. D., Dewey, R. S., Liesch, J. M., Napier, M. A., Reamer, R. A., Smith, J. L., Albers-Schönberg, G., & Goldberg, I. H. (1983) *Biochem. Biophys. Res. Commun. 113*, 538–547.
Jovin, T. M. (1978) in *Biochemical Fluorescence Concepts* (Chen, R. F., & Edelhoch, H., Eds.) Vol. 1, pp 305–372, Marcel Dekker, New York.
Kappen, L. S., & Goldberg, I. H. (1985) *Nucleic Acids Res. 13*, 1637–1648.
Kappen, L. S., Napier, M. A., & Goldberg, I. H. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1970–1974.
Li, H. J., & Crothers, D. M. (1969) *J. Mol. Biol. 39*, 461–477.
Lobb, R. R., & Auld, D. S. (1980) *Biochemistry 19*, 5297–5302.
Lobb, R. R., & Auld, D. S. (1984) *Experientia 40*, 1197–1206.
Muller, G., & Crothers, D. M. (1968) *J. Mol. Biol. 35*, 251–290.
Napier, M. A., & Goldberg, I. H. (1983) *Mol. Pharmacol. 23*, 500–510.
Napier, M. A., Holmquist, B., Strydom, D. J., & Goldberg, I. H. (1979) *Biochem. Biophys. Res. Commun. 89*, 635–642.
Napier, M. A., Kappen, L. S., & Goldberg, I. H. (1980) *Biochemistry 19*, 1767–1773.
Napier, M. A., Holmquist, B., Strydom, D. J., & Goldberg, I. H. (1981) *Biochemistry 20*, 5602–5608.
Povirk, L. F., & Goldberg, I. H. (1980) *Biochemistry 19*, 4773–4780.
Povirk, L. F., & Goldberg, I. H. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 369–373.
Povirk, L. F., & Goldberg, I. H. (1984) *Biochemistry 23*, 6304–6311.
Povirk, L. F., & Goldberg, I. H. (1985) *Biochemistry 24*, 4035–4040.
Povirk, L. F., Dattagupta, N., Warf, B. C., & Goldberg, I. H. (1981) *Biochemistry 20*, 4007–4014.
Ramstein, J., Ehrenberg, M., & Rigler, R. (1980) *Biochemistry 19*, 3938–3948.
Schmechel, D. E. V., & Crothers, D. M. (1971) *Biopolymers 10*, 465–480.
Sturm, J. (1982) *Biopolymers 21*, 1189–1206.
Wakelin, L. P. G., & Waring, M. J. (1980) *J. Mol. Biol. 144*, 183–214.
Wells, R. D., Larson, J. E., Grant, R. C., Shortle, B. E., & Cantor, C. R. (1970) *J. Mol. Biol. 54*, 465–497.

Rattlesnake Presynaptic Neurotoxins: Primary Structure and Evolutionary Origin of the Acidic Subunit†

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ABSTRACT: Crotoxin and homologous crotalid presynaptic neurotoxins consist of a toxic, basic subunit and a slightly smaller, nontoxic, acidic subunit. The latter, in turn, consists of three chains, interconnected by disulfide bonds. The complete sequences of two of the three acidic subunit chains of crotoxin, from the venom of the South American rattlesnake *Crotalus durissus terrificus*, have been determined. In addition, all but the ten amino-terminal residues of the third chain have been sequenced. Sequence comparison data suggest that the acidic subunit has been derived from a nontoxic, homodimeric, crotalid phospholipase A_2. When compared with sequences of phospholipases A_2, the acidic subunit lacks a 22-residue amino-terminal segment and two additional segments that are implicated in phospholipid substrate binding. However, it apparently retains an intact active site, the calcium binding loop, and segments involved in subunit binding in homodimeric phospholipases A_2. The C chain of the acidic subunit shows strong homology with mammalian neurophysins, lending possible support to the hypothesis that the acidic subunit functions as a chaperone to prevent nonspecific binding of the toxic basic subunit. Crystals suitable for X-ray diffraction studies have recently been produced [Achari, A., Radvanyi, F. R., Scott, D., Bon, C., & Sigler, P. B. (1985) *J. Biol. Chem. 260*, 9385–9387]; thus with these data it should now be possible to determine the three-dimensional structure of the intact neurotoxin and dissociated subunits.

In 1938, Slotta & Fraenkel-Conrat (1938) isolated a toxic protein from the venom of the South American rattlesnake (*Crotalus durissus terrificus*) that represented some 65–70% of the total venom protein. Named crotoxin, it proved to be a potent presynaptic neurotoxin. This discovery conflicted with prevailing herpetological dogma, which held that neurotoxins were the exclusive province of cobras and their relatives (elapids) and sea snakes (hydrophiids). Accordingly, the South American rattlesnake was viewed for many years as something of an enigma. Although it was suspected that crotoxin could be a mixture of two different proteins, its heterodimeric nature was not determined for 18 years. Fraenkel-Conrat & Singer (1956) eventually not only verified its dimeric structure but also concluded that the larger, basic subunit was rich in lysine

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