Kinetic Studies of Actinomycin D Binding to Mono-, Oligo-, and Polynucleotides†

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ABSTRACT: The kinetics of association of actinomycin D with poly(dG-dC) and the oligonucleotides d(CGCGCGCG), d(GCGCGC), d(ATGCAT), d(CGCG), and d(GC), as well as with several mononucleotides, have been investigated. In all cases, the association interaction was characterized by several slow, unimolecular processes with qualitatively similar rate constants. The activation enthalpies and entropies for the association of actinomycin with deoxyguanosine 5′-monophosphate resemble closely those typical for calf thymus DNA. This observation of little or no sequence or length dependence in the binding kinetics suggests that the slow phases arise from properties of the drug alone. These results are discussed in terms of both the shuffling model of Fox and Waring [Fox, K. R., & Waring, M. J. (1984) *Eur. J. Biochem. 145*, 579] and the model of Muller and Crothers [Muller, W., & Crothers, D. M. (1968) *J. Mol. Biol. 35*, 251] involving both rapidly forming and slowly forming complexes, with the rapidly forming species being the predominant one.

Actinomycin D (Figure 1) is a chromopeptide antibiotic currently used as a clinical antitumor agent in the treatment of a limited number of cancers. It is known to be a strong DNA binding drug, with a preference for GC base pairs, and a potent inhibitor of RNA synthesis. These properties have been described in detail in several reviews (Meienhofer & Atherton, 1977; Mauger, 1980). Spectroscopic and hydrodynamic studies have provided strong evidence for intercalation as the DNA binding mechanism for actinomycin D (Muller & Crothers, 1968; Waring, 1970). Subsequently, Jain and Sobell (1972) obtained the X-ray crystal structure of a 2:1 complex of deoxyguanosine with actinomycin D. This provided the basis for a model of the actinomycin D–DNA complex (Sobell & Jain, 1972) based on intercalation of the drug's chromophore between d(GC) base pairs with the peptide lactone rings lying in the minor groove.

More recently, the crystal structure of the complex formed between actinomycin D and the dinucleoside monophosphate d(GC) has been solved (Takusagawa et al., 1982). In that complex, a nonclassical pseudointercalated structure was observed that did not involve the formation of a helical duplex of d(GC). Two-dimensional proton NMR studies on the complex formed between actinomycin D and the hexanucleoside pentaphosphate d(ATGCAT) have been recently carried out in this laboratory (Brown et al., 1984). These experiments revealed a classical intercalation structure. This also appears to be the case for this same complex in the crystalline state (Takusagawa et al., 1984).

While much progress has been made in determining the actinomycin D–DNA structure, the kinetics of actinomycin binding to DNA remain puzzling. Early work of Muller and Crothers (1968) demonstrated that the drug binds to DNA with very slow, complex kinetics. In the binding to natural DNA, three slow unimolecular rate processes were seen in both association and sodium dodecyl sulfate (SDS) driven dissociation measurements. Muller and Crothers proposed that a series of conformational changes in the drug was responsible for the complicated, slow kinetics. On the basis of results of kinetics experiments on a variety of actinomycin analogues, we proposed that cis–trans isomerization about one or more peptide bonds in the pentapeptide lactone rings of the drug gave rise to the observed kinetic behavior (Shafer et al., 1980; Mirau & Shafer, 1982).

Studies by Krugh et al. (1980) indicated that it was site heterogeneity in natural DNA that led to the multistep nature of the slow dissociation of the drug–DNA complex. They also reported only one slow process in the dissociation of actinomycin D from poly(dG-dC), although the corresponding rate constant depended on the initial DNA phosphate to drug ratio (P/D). More recently, Fox and Waring (1984) have proposed that the multiple unimolecular processes in the association of actinomycin D with DNA reflect shuffling of the drug from an initial binding site to another of higher affinity. This model appears to explain the binding kinetics for calf thymus DNA but not for poly(dG-dC) nor for the smaller systems described in this work.

Our recent two-dimensional (2-D) NMR work on the actinomycin D–d(ATGCAT) complex has shed some light on the question of binding kinetics (Brown et al., 1984). In particular, we found no significant conformational differences between the free and bound drug. This rules out the possibility that the slow kinetics are due to large-scale conformational changes in the drug upon binding. In the experiments described below, we show that the slow kinetics nevertheless appear to be due to properties of the drug rather than the DNA. In addition, we demonstrate that the association process is characterized by several slow steps for homogeneous binding lattices such as short $(dG\text{-}dC)_n$ oligomers and certain mononucleotides as well as poly(dG-dC). This implies that, while site heterogeneity may play a role in the multistep nature of the binding process, other factors are also involved.

Materials and Methods

Actinomycin D was purchased from Sigma or obtained as a gift from Merck Sharp & Dohme. Purity was checked by thin-layer chromatography (TLC). Nucleotides were obtained from Collaborative Research or Sigma, except for d(ATGCAT), which was synthesized by Drs. K. Mullis and C.

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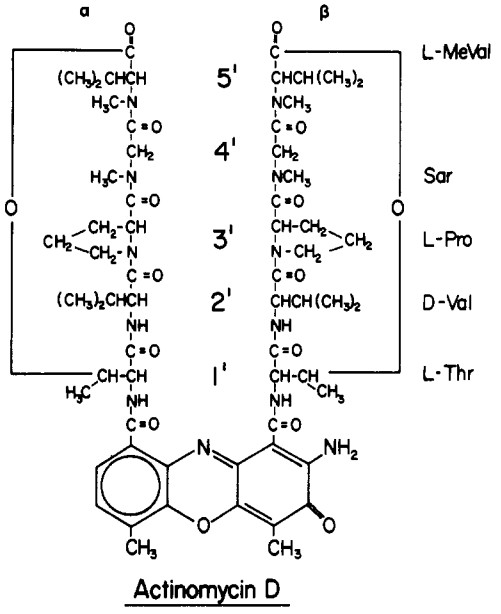

FIGURE 1: Structure of actinomycin D (ActD).

Levenson, Cetus Corp. (Brown et al., 1984). All oligonucleotides were run on polyacrylamide gels, demonstrated single bands as detected by ethidium fluorescence, and migrated according to their appropriate molecular weight. Mononucleotide purity was checked by TLC on poly(ethylenimine) (PEI) plates developed by LiCl. Poly(dG-dC) was obtained from P-L Biochemicals. Concentrations of actinomycin D were determined spectrophotometrically with a molar extinction coefficient of 24 450 at 440 nm. Nucleotide concentrations were determined with molar extinction coefficients at 260 nm supplied by the manufacturers. Poly(dG-dC) concentrations were measured on the basis of a molar extinction coefficient of 7100 at 254 nm, while calf thymus DNA concentrations were determined with an $\epsilon(260)$ of 6600. All solutions were in BPES buffer [0.080 M Na_2HPO_4, 0.020 M NaH_2PO_4, 0.180 M NaCl, 0.010 M disodium ethylenediaminetetraacetate (Na_2EDTA), pH 7.0], filtered through 0.22-mm filters and degassed by stirring under vacuum for 30 min prior to use.

Association kinetics were followed by mixing equal volumes of actinomycin D and nucleotide solutions in a Durrum D-110 stopped-flow mixing chamber outfitted with a visible lamp and monochromator manufactured by Instruments SA. Binding was usually monitored by measuring the absorbance at 425 nm as a function of time. Occasionally, binding was monitored at 469 nm to check for possible artifacts resulting from a decrease in complexed drug concentrations in the optical cell. The output from the photomultiplier was sent directly to a Northstar Horizon microcomputer supplied by On-line Instrument Systems (OLIS). Data acquisition was performed by using the OLIS Model 3820 software package. Instrument dead time was 4 ms, and stability was ±0.002 absorbance units over a period of 2000 s. Typically, 256 data points were collected during 500 s, and the base line was measured following another 500 s. The resulting kinetic curves were subjected to multiexponential analysis with a locally written nonlinear least-squares regression program based on the Marquardt–Levenberg algorithm.

The total absorbance changes ΔOD_{tot} were measured by comparing the absorbance of a solution of actinomycin D in buffer alone with that of the complex at equilibrium. UV/visible spectra were determined on either a Cary 118 or Gilford 2600 spectrophotometer, and circular dichroism (CD) spectra

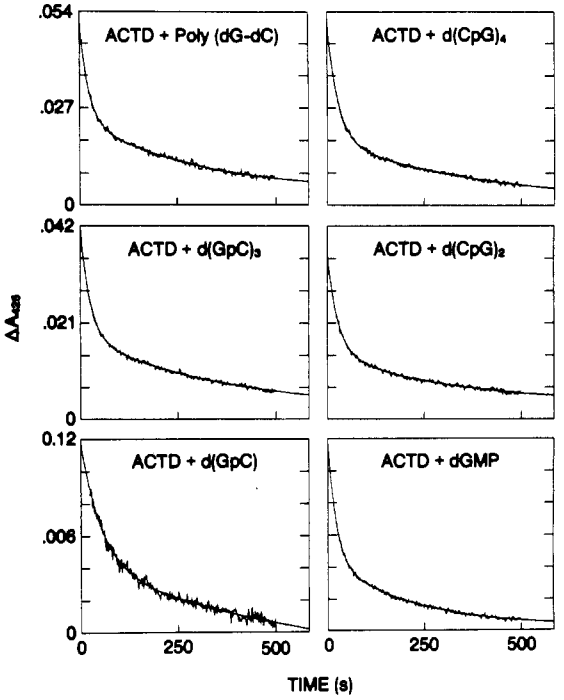

FIGURE 2: Representative kinetic traces monitoring association of actinomycin D with the indicated nucleotide systems at 20 °C. Smooth line represents the best fit to the experimental data using a two-exponential nonlinear regression model.

Table I: Unimolecular Rate Constants for Association of Actinomycin D with Calf Thymus DNA and Poly(dG-dC)

	P/D	10^3k_1 (s^{-1})	% ΔOD_{tot}	10^2k_2 (s^{-1})	% ΔOD_{tot}
DNA (T = 23 °C)	18	5.0 (±0.9)	4	4.2 (±0.4)	9
	27	4.5 (±0.2)	6	4.2 (±0.2)	9
	34	3.7 (±0.3)	6	4.4 (±0.2)	7
	46	5.0 (±0.6)	8	3.9 (±0.3)	9
	65	4.7 (±0.3)	6	3.8 (±0.7)	9
poly(dG-dC) (T = 20 °C)	6–41	3.8 (±0.5)	7	4.6 (±0.7)	14

were obtained on a JASCO 500A spectropolarimeter in Professor J. T. Yang's laboratory, Department of Biochemistry and Biophysics, UCSF. Final drug concentrations ranged from 1.5×10^{-5} to 4×10^{-5} M. Final nucleotide concentrations can be obtained from the P/D (phosphate to drug) or S/D (strand to drug) ratios. Unless otherwise stated, experiments were carried out at 20 °C. The temperature dependence of the rate constant k for association with deoxyguanosine 5′-monophosphate (5′-dGMP) was analyzed according to the equation $k/T = (k_B/h)e^{-(\Delta H^* - T\Delta S^*)/RT}$, where k_B is Boltzmann's constant, h is Planck's constant, and R is the gas constant.

RESULTS

Association kinetics of actinomycin D to calf thymus DNA were investigated first to provide a reference for comparison with other nucleotide systems. As reported previously (Muller & Crothers, 1968; Bittman & Blau, 1975), three slow unimolecular processes and two fast bimolecular processes were observed. Kinetic data are displayed in Figure 2, and the results of these studies are summarized in Table I. Only the two slowest unimolecular rate processes for DNA and poly(dG-dC) are listed since only these are of interest for the present discussion. It is interesting to note that the fraction of the total absorbance change due to each of the slow processes did not vary significantly with P/D. Similar results were reported by Bittman and Blau (1975). Fox and Waring (1984), however, reported that relative amplitudes for the two

Table II: Unimolecular Rate Constants for Association of Actinomycin D with Oligonucleotides at 20 °C

	S/D[a]	10^3k_1 (s^{-1})	% ΔOD_{tot}	10^2k_2 (s^{-1})	% ΔOD_{tot}
d(CGCGCGCG)	0.6–2.4	4.0 (±0.5)	5	5.5 (± 0.7)	8
d(GCGCGC)	2.0–9.2	3.0 (±0.2)	6	3.4 (±0.6)	9
d(ATGCAT)	3.0–8.0	2.55 (±0.2)	2.4	3.8 (±0.7)	8
d(CGCG)	3.4–9.2	3.6 (±0.5)	4	4.2 (±0.4)	9
d(GC)	40–52	3.1 (±0.8)	3	5.8 (±1.2)	4

[a] Concentration ratios as oligonucleotide strands to drug.

Table III: Unimolecular Rate Constants for Association of Actinomycin D with Mononucleotides at 20 °C

	K_a[a]	[N]/[ActD]	10^3k_1 (s^{-1})	% ΔOD_{tot}	10^2k_2 (s^{-1})	% ΔOD_{tot}
5′-dGMP	7×10^3	25–1000	3.8 (±0.8)	4	3.7 (±1.0)	6
dG	2×10^3	100–207	2.7 (±0.2)	4	2.1 (±1.7)	4
5′-GMP	9×10^2	250–500	3.1 (±0.7)	3	4.6 (±0.1)	4
5′-dAMP	7×10^2	227	4.4 (±0.6)	2	4.5 (±0.7)	4

[a] Equilibrium affinity constants from Auer et al. (1978).

slowest association steps varied with P/D. This experimental discrepancy may be due to the fact that our results, as those of Bittman and Blau (1975), have been normalized to the total absorbance change, which does not appear to be the case for the data of Fox and Waring (1984).

The simpler, alternating synthetic polynucleotide poly(dG-dC) was examined next. In this case, we also observed three slow, unimolecular processes in the kinetic traces for association, as indicated in Figure 2 and Table I. Previous studies by Bittman and Blau (1975) reported a multiexponential process for this reaction, but no details were provided. We also note that Fox and Waring (1984) obtained similar multiexponential results for the association of actinomycin D with poly(dG-dC). The observation of three slow steps in this relatively homogeneous lattice containing only two classes of sites suggests that site heterogeneity alone cannot be the underlying reason for such complicated kinetics.

Shorter segments of DNA were studied in an effort to delineate the DNA length dependence as well as sequence dependence of the multiexponential nature of the binding kinetics. The following oligonucleotides were examined: d(CGCGCGCG), d(GCGCGC), d(CGCG), d(GC), and d-(ATGCAT). Results from association studies of these oligomers with actinomycin D are shown in Figures 2 and 3 and summarized in Table II. In the oligonucleotides containing GC only, at least two unimolecular processes were found [the small amounts of $(dG\text{-}dC)_n$ available did not permit a clear determination of a third process], while in the d(ATGCAT) system, three such terms were observed, similar to what is found for the calf thymus DNA. Here, as in the case of poly(dG-dC), relatively simple nucleotide sites gave rise to multiexponential behavior for the association kinetics. As indicated in Tables II and III, there appears to be little or no dependence of either rate constants or fractional absorbance changes on P/D values for the unimolecular steps.

The results for d(ATGCAT) are quite similar to those found for calf thymus DNA (Table II). This oligonucleotide contains a single strong binding site for actinomycin D. Because of this, and its kinetic behavior parallel to that of DNA, we chose d(ATGCAT) as a model system for DNA in our 2-D NMR analysis of the actinomycin-bound complex (Brown et al., 1984). Even the dinucleoside monophosphate d(GC) gave rise to slow association kinetics. Earlier kinetic studies by Davanloo and Crothers (1976) using temperature-jump kinetics quantitated the rapid phases of the binding kinetics of actinomycin D to dinucleotides, but no evidence was reported of any very slow stages in the binding process. These would not be detected in a temperature-jump measurement. The secondary structure of the shortest oligomers in solutions before and after mixing with drug was not rigorously measured.

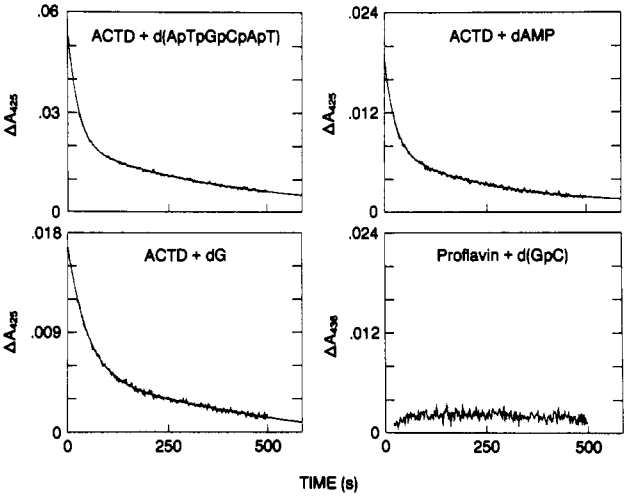

FIGURE 3: Same as in Figure 2, except for addition of a control trace obtained from the mixing of proflavin with d(GC).

The results in general give similar rate constants for all the oligonucleotides studied and clearly indicate little or no dependence on sequence. In particular, the interactions with d(ATGCAT) and d(GCGCGC) are characterized by similar rate constants, although the amplitudes for the slower process are different. This difference in amplitude may reflect changes in binding affinity due to a difference in sequence at sites neighboring the intercalation site.

We extended these studies to the mononucleotide level to see if slow phases in the binding process could still be observed. As with the larger systems, there were two slow steps in the association kinetics at sufficiently high P/D values, which can be seen in Figures 2 and 3. These studies also included the nucleoside dG. Results from these experiments are presented in Table III. The threshold of nucleotide concentrations required for observation of the slow processes parallels the K_a values for the nucleotides studied, since a minimal concentration of complex is required at equilibrium in order to raise the absorbance change associated with the slow processes to detectable levels. In all cases, both rate constants and relative amplitude changes are found to be independent of P/D ratios, as observed for the other systems described above. As demonstrated by others, the guanine requirement for complex formation with actinomycin D is relaxed at the nucleotide level (Schara & Muller, 1972; Krugh & Neely, 1973; Auer et al., 1978). This is also reflected in the kinetic properties of complex formation, as dAMP also produces two slow kinetic phases. However, neither dC nor dT shows any evidence of

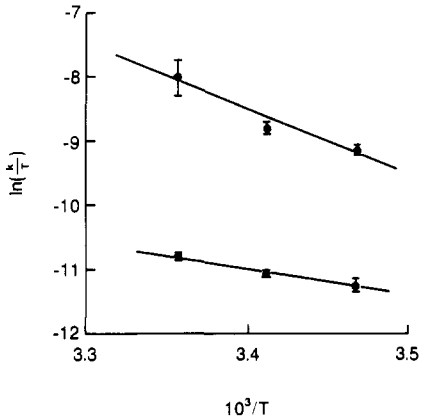

FIGURE 4: Temperature variation of the slow rate constants found for association of actinomycin D with 5′-dGMP at P/D = 186.

Table IV: Thermodynamic Activation Parameters for Association of Actinomycin D with DNA and 5′-dGMP

	process	$\Delta G^{\ddagger}$	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)
calf thymus DNA[a]	k_1	21.0	13.4 (±1.3)	−26.0
	k_2	19.4	14.2 (±0.9)	−17.7
5′-dGMP (P/D = 370)	k_1	20.3	9.5 (±2.6)	−37.0
	k_2	19.0	16.4 (±2.4)	−9.0
5′-dGMP (P/D = 186)	k_1	20.3	7.9 (±3.2)	−42.3
	k_2	18.6	16.8 (±2.0)	−6.1

[a] From Bittman and Blau (1975).

slow kinetics, nor do they show any appreciable binding affinity.

Under the circumstances, many control studies were done to ensure that we were not measuring an artifactual event. Control studies were run with several different systems: proflavin + d(GC) (see Figure 2), actinomycin D + buffer alone, actinomycin D + dCMP, and actinomycin D + dTMP. In all these systems, no evidence of any slow effects was found. Additional controls were done by measuring the absorbance increase at 469 nm which results from complex formation. Although the signal to noise ratio was not as high as for the observations at 425 nm, several slow, unimolecular rate processes were observed at this wavelength for the association of actinomycin D with d(GC) and 5′-dGMP. Rate constants derived from these data were fully comparable to those obtained for the same samples at 425 nm. Thus, artifacts arising from any process that would cause a drop in concentration of either the free or complexed drug in the optical cell during the course of these observations can be ruled out.

The temperature dependence of the two slow kinetic phases for the association of actinomycin D with 5′-dGMP was determined in order to provide further characterization of these nucleotide systems and to compare the activation parameters with those typical of large DNA. The temperature range covered in these studies was 15–25 °C. When temperatures further away from ambient were tried, we found artifacts in the results that were traced to small temperature differences between the loading syringes and the observation cell in the stopped-flow mixing chamber. Because of this relatively narrow range in temperature, the uncertainties in the resulting thermodynamic activation parameters are higher than usual.

Results from the temperature-dependence experiments on the association of actinomycin D and 5′-dGMP are presented in Figure 4 and in Table IV, which also provides a comparison with data reported for the association of actinomycin D with calf thymus DNA (Bittman & Blau, 1975). The data on the mononucleotide system show a very strong similarity to those

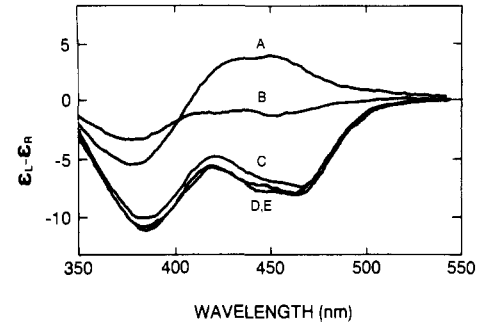

FIGURE 5: Circular dichroism spectra at 20 °C of free actinomycin D (B) and actinomycin D in the presence of 5′-dGMP, P/D = 380 (A); poly(dG-dC), P/D = 28 (C); calf thymus DNA, P/D = 80 (D); and d(ATGCAT), S/D = 20 (E). In all cases, concentration of actinomycin D was 1.89×10^{-5} M.

found for DNA. Both $\Delta H^{\ddagger}$ terms are quite large, and the $\Delta S^{\ddagger}$ terms are large and negative. This similarity of kinetic behavior in these two very differently sized systems suggests some degree of similarity in the binding interaction. However, the CD spectrum of the actinomycin D–dGMP complex is quite different from those exhibited by the larger systems such as d(ATGCAT), poly(dG-dC), and calf thymus DNA, as illustrated in Figure 5.

Discussion

The experiments presented above provide the first systematic examination of the DNA length dependence for the association kinetics of actinomycin with DNA. A large number of studies have appeared that treat the equilibrium aspect of actinomycin D binding (Meienhofer & Atherton, 1977; Mauger, 1980) to various sizes of nucleic acids, but few have explored this aspect of the binding kinetics. Perhaps the most surprising result of our study is the observation of two or more slow, unimolecular phases in the binding of this drug to systems ranging from polymers to oligomers to mononucleotides and mononucleosides. The temperature-jump studies of Davanloo and Crothers (1976) on actinomycin D–dinucleotide systems provided no evidence of such behavior. However, a report has appeared by Krugh (1974) that describes the need to wait a long time for the absorbance of a mixed solution of actinomycin D and guanine nucleotides to stabilize.

The relative constancy of the unimolecular rate parameters for the association of actinomycin D with various-sized oligomers and polymers suggests that the slow binding processes can be described in one of two ways. The first is that the slow kinetic phases are due to properties of the drug alone and hence remain essentially the same for any mono- or oligonucleotide binding partner. The second is that the slow phases arise from the binding interaction in a fashion that is independent of either base sequence or oligonucleotide length. In either case, the molecular processes underlying such a slow phase in systems as small as actinomycin D–dG may still involve conformational changes (see below). Alternatively, the slow kinetics could arise from solvent interactions in the binding process, e.g., desolvation of the drug.

Our results for the association of actinomycin D with poly(dG-dC) are interesting in light of those reported by Krugh et al. (1980) for the SDS-induced dissociation from poly(dG-dC). They found only single exponential behavior in their dissociation curves, from which they concluded that the multiexponential dissociation curves for natural DNA must arise from site heterogeneity. This would imply that association curves for poly(dG-dC) should also be single exponential, or double exponential at most, which, as described above, is

not the case. In their data, Krugh et al. (1980) found that the single exponential dissociation curve had a rate constant that varied with the initial P/D ratio while we found no variation in the three unimolecular rate constants for association.

It is possible that the variation in observed rate constant for dissociation from poly(dG-dC) is related to the cooperativity in binding to poly(dG-dC) of actinomycin D reported by Winkle and Krugh (1981). If the cooperativity were manifested only in the dissociation process and not in the association process, then the model of Lohman (1983) for dissociation of cooperatively bound ligands would be applicable. In that model, at fractional binding densities less than 1, dissociation occurs primarily from the end of clusters of bound ligands with single exponential behavior. The rate constant, however, increases with decreasing initial binding density. This is what Krugh et al. (1980) observed for poly(dG-dC). While this provides an explanation of the binding data for poly(dG-dC), it does not seem to apply in the case of natural DNA. Winkle and Krugh (1981) also reported cooperativity in the binding of actinomycin D to calf thymus DNA, but this system shows multiple exponential dissociation behavior with amplitudes that vary with the initial P/D but with rate constants independent of P/D. Perhaps the nature of the positive cooperativity in binding to calf thymus DNA is different than that to poly(dG-dC). An additional complication with poly(dG-dC) dissociation experiments is that we have observed that 2% SDS does not completely dissociate the drug from this polynucleotide, as determined by absorbance spectra, at concentrations typically used in such studies (data not shown). This may influence the P/D dependence of the measured off rates.

Both our studies and those of Fox and Waring (1984) reveal two or more unimolecular steps in the association of actinomycin D with poly(dG-dC). It is hard to imagine this degree of complexity in such a uniform lattice of binding sites if the slow processes were a manifestation of shuffling along the polymer. When one considers the binding kinetics observed for short oligonucleotides and mononucleotides in the present study, the shuffling model does not appear applicable here, either. It is possible, though, that the shuffling model of Fox and Waring (1984) applies to the binding of actinomycin D to natural DNA while some other process underlies the multiple slow association phases observed in poly(dG-dC) and the smaller systems we have examined here. The model proposed by Sobell (1974) and Bittman and Blau (1975) attributing the slow kinetics in the actinomycin D–DNA system to unwinding or some other conformational change in the DNA is not supported by the data presented above on the small nucleotide systems.

The results described above for the mononucleotide and nucleoside interactions with actinomycin D are interesting in light of previously reported results on small systems. As already mentioned, temperature-jump studies with dinucleotides (Davanloo & Crothers, 1976) showed only fast kinetics. Equilibrium studies on the interaction of actinomycin D with dG and 5′-dGMP (Gellert et al., 1965) revealed very different thermodynamic properties, e.g., negative enthalpy and entropy changes, compared to those found for DNA (Quadrifoglio & Crescenzi, 1974). Similarly, the CD data presented above show very different results for actinomycin D complexed with 5′-dGMP than with larger oligo- and polynucleotides. Yet, at sufficiently high P/D values, these small nucleotides show the same slow kinetics characteristic of large polynucleotide binding. These results can be explained in terms of the original model proposed by Muller and Crothers (1968) in which a series of slow conformational changes follows the initial formation of a complex. At equilibrium, there can be several species such as the rapidly forming complex as well as one or more slowly forming complexes. The relative equilibrium populations of these various species depend on the detailed rate constants and can be expected to vary from one nucleotide to another.

What is required to interpret our data described above with this model is an equilibrium population of the slowly forming complex that is considerably smaller than the rapidly forming complex. Thus, the bulk of the bound drug is involved in a rapidly forming complex. Only at higher P/D is the equilibrium shifted to sufficient levels of the slowly forming complex(es) such that their observation is possible. The CD signal from this complex, measured at equilibrium, may be overwhelmed by that of the rapidly formed complex, whereas the latter would not contribute any time-dependent signal in kinetic measurements at long times after mixing, thereby allowing detection of the slowly formed species in the presence of an excess of the rapidly formed one.

This model can also be considered in the case of actinomycin D binding to larger oligonucleotide and polynucleotide systems, both synthetic and naturally occurring, i.e., poly(dG-dC) and calf thymus DNA. Here, the relative stability of the two classes of complexes could vary from case to case. The amplitude associated with the slow processes would depend on the relative equilibrium populations of the various complexes in the different cases. This would be consistent with the observations of Fox and Waring (1984) that the amplitudes associated with the slow phases were different for binding to calf thymus DNA and poly(dG-dC). Our two-dimensional NMR analysis of the complex formed between actinomycin D and the d(ATGCAT) hexamer (Brown et al., 1984) failed to demonstrate the existence of an altered conformation in the pentapeptide portions of the bound drug. However, this could have been due to a relatively small population of complexes containing such changes that was simply not detected. We might point out that is not possible to determine the fraction of such complexes from the relative amplitude changes associated with the slow processes in spectrophotometric measurements since the relevant extinction coefficients are not known.

In summary, we are left with several possible interpretations of the available experimental data. The shuffling model put forward by Fox and Waring (1984) could be valid for the binding of actinomycin D to calf thymus DNA but not to the more homogeneous systems such as poly(dG-dC) or the smaller nucleotide systems containing a single site. The data for these cases requires another mechanism of binding. The model described above, based on an equilibrium population of complexes, one or more of which gives rise to slow kinetics, can explain the kinetic data for all types of drug binding sites. One problem associated with this model is the difficulty of verifying its validity experimentally. Finally, other effects may play a role, such as solvent interactions. The unambiguous resolution of this issue awaits additional work.

ACKNOWLEDGMENTS

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Registry No. dG, 961-07-9; d(GC), 23405-83-6; dGMP, 902-04-5; GMP, 85-32-5; dAMP, 653-63-4; d(GCGC), 58927-25-6; d-

(ATGCAT), 53263-13-1; d(GCGCGC), 76186-50-0; d(CGCGCGCG), 89991-79-7; poly(dG-dC), 36786-90-0; actinomycin D, 50-76-0.

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Haloperoxidase-Catalyzed Halogenation of Nitrogen-Containing Aromatic Heterocycles Represented by Nucleic Bases

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ABSTRACT: The enzymatic halogenation of nitrogen-containing aromatic heterocycles catalyzed by two different types of haloperoxidases, the chloroperoxidase of *Caldariomyces fumago* (heme type) and the bromoperoxidase of *Corallina pilulifera* (non-heme type), has been studied. Chloroperoxidase catalyzed the chlorination of uracil and pyrazole, the bromination of cytosine, uracil, thymine, cytidine, 2'-deoxyuridine, guanosine, and pyrazole, and the iodination of uracil and pyrazole to yield the respective halogenated products. The bromoperoxidase also catalyzed the bromination of cytosine, uracil, cytidine, and pyrazole and the iodination of uracil and pyrazole to form the same products as in the chloroperoxidase reactions. A slight difference in the reactivity toward these substrates was observed between the two haloperoxidases. The results of product and halogenation intermediate analyses suggested that the bromination reaction of the bromoperoxidase occurs at the active site of the enzyme. On the contrary, the halogenation by the chloroperoxidase was found to involve the formation of a molecular halogen and its release into the solution. On the basis of the results, we discussed the abilities of the haloperoxidases as halogenating reagents.

Enzyme-catalyzed halogenation is a common biological phenomenon. Previously, various halometabolites, including chloramphenicol, pyrrolnitrin, etc., were isolated from microbial sources and identified (Neidleman, 1975). In the marine environment, many halogenated compounds such as bromophenols have been found in marine plants (Faulkner, 1970; Fenical, 1974, 1975). In mammals, enzymatic halogenations are important in the biosynthesis of the thyroxine hormone and in biological defense mechanisms (Morrison & Schonbaum, 1976).

Detailed studies of the enzymes participating in the biological formation of halometabolites have been few, being limited to those on the chloroperoxidase of the fungus *Caldariomyces fumago* (Shaw & Hager, 1959, 1961; Shaw et al., 1959) and the thyroid peroxidase involved in the synthesis of thyroxine (Morrison & Schonbaum, 1976). Hager and coworkers presented information on the enzymic properties (Morris & Hager, 1966), kinetic mechanism (Hager et al., 1966), and reaction mechanism (Libby et al., 1982) of the chloroperoxidase. In the haloperoxidase reaction, a halide anion (X^-, X; Cl, Br, I) is activated to the halonium cation (X^+) through hydrogen peroxide dependent oxidation, and then the halonium cation is transferred to a halogen acceptor molecule. There have been many reports concerning chloroperoxidase substrates, as follows: β-keto acids (Shaw & Hager, 1961); cyclic β-diketones (Hager et al., 1966); steroids (Neidleman et al., 1966; Neidleman & Oberc, 1968; Levine et al., 1968; Neidleman & Levine, 1968); substituted phenols such as tyrosine (Taurog & Howells, 1966) and anisole (Brown & Hager, 1967); thiols (Silverstein & Hager, 1974), thiazoles (Neidleman et al., 1969); alkenes (Geigert et al., 1983a); alkynes; cycloalkanes (Geigert et al., 1983b,c); and several

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