Site exclusion and sequence specificity in binding of 9-aminoacridine to the deoxytetranucleotide dpApGpCpT

(intercalation/NMR/multiple equilibria)

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ABSTRACT The interaction of the mutagenic dye 9-aminoacridine (9AA) with the self-complementary tetranucleotide dpApGpCpT has been studied by a combination of proton NMR titrations and thermal denaturation experiments. A minimum of three complexes of well-defined stoichiometry can be demonstrated in this system. Complex I is a 1:2 9AA/tetranucleotide structure occurring in the presence of excess tetranucleotide. The dye appears to intercalate within the GpC/GpC site of a tetranucleotide duplex. Complex II is a 2:2 9AA/tetranucleotide structure, with two dyes intercalated at the ApG/CpT sites of the duplex. Complex III is a low-temperature 4:2 9AA/tetranucleotide structure containing two dye molecules stacked over the terminal A-T residues of the duplex in addition to those present in complex II. These results show that both sequence selectivity and site exclusion can occur in this model system.

The ability of a number of acridine dyes to induce frameshift mutations in phages has been known for many years and, indeed, led to the earliest evidence for a triplet genetic code (1, 2). Precisely how acridines, and a large number of polycyclic mutagens as well as carcinogens do this is unclear, despite a wealth of data indicating that these molecules can interact strongly with DNA to form intercalation complexes (3-5). These are structures in which the planar ring systems of the polycyclic molecule lie sandwiched between adjacent base pairs of the double helix. It has been argued (J. Owen and G. Streisinger, personal communication) that intercalation *per se* may be insufficient to explain several features of frameshift mutagenesis, including the dose-response curve, the predominance of insertions over deletions, and the activity of a number of non-planar molecules such as steroid diamines as frameshifting mutagens. Nevertheless, the correlation between intercalative or partial intercalative binding and activity of frameshift mutagens merits investigation, particularly if some variant binding modes related to classical intercalation prove to be important.

On the basis of the observation that the dye 9-aminoacridine (9AA) reverts one particular *his*⁻ frameshift in *Salmonella* and not another (6), it has been suggested that there is a sequence specificity in the mode of action of this molecule. The molecule is highly active in reverting the *his* C mutation, occurring at a GpGpGp/CpCpCpC site, and is completely inactive against the *his* D mutation, representing a GpCpGpCpGpC/CpGpCpGpCpG site. We have investigated the basis for this behavior by studying the interaction of 9AA with a series of self-complementary or mixed complementing dinucleoside phosphates of different sequence (7) and find evidence from NMR studies for intercalation with binding constants in the range 2.5 to 5 × 10⁵. Sequence preferences among the sequences examined (GpC:GpC, CpG:CpG, ApG:CpT, GpG:

CpC) are found, but these seem inadequate to account for the striking selectivity observed in the Ames test system.

However, complementary dinucleotide substrates can be questioned as a suitable model for DNA binding because they do not form a duplex in the absence of an intercalated 9AA (8). It thus is difficult to assess the effects of competing dinucleotide-dinucleotide (e.g., GpG self-interaction) and dinucleotide dye (1:1) complexation on the binding parameters. Furthermore, the dinucleotide studies do not permit evaluation of the potentially important effects of site exclusion, in which intercalation into one site inhibits occupation of adjacent site(s) of the duplex. Hence, it is desirable to extend detailed binding studies to longer oligonucleotides in which a duplex is present without addition of the intercalator (9-11).

In this article, we present a detailed ¹H NMR study of the interaction between 9AA and a self-complementary deoxytetranucleotide, dpApGpCpT. We find evidence in this case for an effect consistent with site exclusion in longer duplexes as well as a strong interaction between terminal phosphates and purines and the acridine molecule. An indication that, in the presence of excess tetranucleotide, a GpC/GpC site is favored over the ApG/CpT site is also seen.

MATERIALS AND METHODS

The tetranucleotide dpApGpCpT and dinucleotides dpApG, dGpC, and dCpT were synthesized by Collaborative Research Waltham, MA, and provided as the lyophilized ammonium salts. Purity was checked by NMR spectroscopy and by ascending paper chromatography when necessary. Concentrations were determined by weighing samples; the values compared closely with those calculated from the manufacturer's molecular absorption coefficient ($\epsilon = 30,880$ per tetramer in H₂O). 9AA was purchased from Sigma (grade II), passed through Chelex to remove divalent ion impurities, and lyophilized before use. ²H₂O was purchased from Wilmad (99.8%) and vacuum distilled in glass before use, to remove trace impurities.

NMR spectra were recorded on the Bruker WH-360 spectrometer at the Middle Atlantic NMR facility. The reference compound for protons was 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), purchased from Stöhler Chemicals (Waltham, MA). For all binding and melting studies, temperature was controlled via the Bruker temperature regulator.

Binding experiments were performed by mixing two ²H₂O solutions of 0.4 mM 9AA in 20 mM potassium phosphate at pH 7.0, one containing 6 mM dpApGpCpT. All pH values are direct readings obtained from the deuterated solutions.

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Abbreviations: 9AA, 9-aminoacridine; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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RESULTS

Assignment of ^1H NMR Spectrum. The base resonances in dpApGpCpT have been assigned by comparing the spectra of dpApG, dGpC, dCpT, and dpApGpCpT at 70°C in $^2\text{H}_2\text{O}$ titrated to pH 7.0. At this temperature the component bases of the dinucleotides and tetranucleotide are relatively unstacked and the chemical shifts obtained for each base proton resonance are almost identical for all molecules in which that base occurs (compare ref. 12). There is no overlap of resonances.

The melting profiles of the tetranucleotide proton resonances at pH 7.0 are shown in Fig. 1. Most of the protons underwent a transition in the range 10–30°C. Further details of assignment and melting of this tetranucleotide will be described elsewhere, but initial conformational analysis results are consistent with the formation at low temperature of a B-DNA helix with C2'-endo sugar puckering at all four residues.

Binding of 9AA to dpApGpCpT. The effect of addition of dpApGpCpT on the proton chemical shifts of 0.4 mM 9AA at 10°C is illustrated in Fig. 2 and plotted in Fig. 3A. The dye resonances can be followed through most of the titration. (For the assignment of dye protons see refs. 13 and 14.)

At the beginning of the titration, both doublet (H1,8/H4,5) and triplet (H3,6/H2,7) dye resonances shifted upfield and indicated strong complex formation with apparent stoichiometries of 1.75:1 (9AA/tetranucleotide) and 2:1, respectively. However, further addition of tetranucleotide led to a change in the apparent end points that suggested a transition to or increase in relative population of a different complex or com-

FIG. 1. Melting profiles of dpApGpCpT proton resonances. Conditions were 9 mM tetranucleotide, pH 7.0 (no buffer). Assignments were made as described in text.

FIG. 2. Effect of addition of dpApGpCpT on the proton chemical shifts of 9AA in 20 mM sodium phosphate buffer (pH 7.0). Top spectrum, 6 mM dpApGpCpT/0.4 mM 9AA. Middle spectrum, 0.26 mM dpApGpCpT/0.4 mM 9AA. Bottom spectrum, 0.4 mM 9AA.

plexes in which the ratio of 9AA to tetranucleotide presumably is lower. In contrast, some of the nucleotide protons [A-H8, T-(CH₃)5, and the A/T and G/C ribose 1' resonances] indicated a 1:1 stoichiometry (Fig. 3B).

Melting experiments performed at an approximately stoichiometric ratio (9AA/tetranucleotide, 3:2) and in the presence of excess tetranucleotide in both cases revealed cooperative melting of the dye protons (Fig. 4) indicative of 9AA intercalation and suggesting that the stoichiometries of complexation are actually 4:2 and 2:2 (9AA/tetranucleotide) as monitored by the 9AA and nucleotide resonances, respectively, in Fig. 3. These stoichiometries are consistent with intercalation of 9AA into the two ApG-CpT sites concomitant with terminal stacking of two 9AA molecules over the ends of the miniature tetranucleotide duplex, the latter probably stabilized by the terminal phosphate groups. The latter binding mode is expected to be weaker than intercalation, based on dinucleotide studies (7), so that addition of further intercalative sites in the form of excess tetranucleotide will compete and result in a transition to a 2:2 complex, leaving only the 9AA molecules intercalated into the ApG-CpT sites. The triplex 9AA protons (H3,6 and H2,7) and

FIG. 3. Proton chemical shift behavior of 9AA (A) and dpApGpCpT (B) at 10°C on addition of dpApGpCpT to 0.4 mM 9AA in 20 mM sodium phosphate buffer (pH 7.0).

the nucleotide protons (Fig. 3 A and B, respectively) presumably are less sensitive to the terminally stacked 9AA than the 9AA doublet resonances (H1,8 and H4,5).

A suggestion of nonsigmoidal character in the doublet resonances (H1,8 and H4,5) of the stoichiometric complex can be seen in Fig. 4. This also occurs in certain nucleotide resonances

FIG. 4. Melting profiles of 9AA protons in 9AA/dpApGpCpT mixtures in 20 mM sodium phosphate buffer (pH 7.0). Closed symbols, 0.4 mM 9AA/0.26 mM dpApGpCpT; open symbols, 0.4 mM 9AA/4.91 mM dpApGpCpT.

not shown here. Investigation of the binding curve at 26.5°C, in which this nonsigmoidal behavior seems most apparent, is illustrated in Fig. 5A. The 9AA doublet resonances (H1,8/H4,5) show a 2:2 stoichiometry, consistent with the loss of stacked 9AA on increasing the temperature from 10°C to 26.5°C while keeping the two intercalated 9AA molecules.

That the effect seen is not simply a consequence of a change in binding constant is shown by the Scatchard plots in Fig. 6, in which the difference in stoichiometries is obvious and separable from the difference in affinity.

On the other hand, the A-H8 and T-H6 protons suggest complex formation with a stoichiometry of 1:2 (9AA/tetranucleotide) (Fig. 5B). This ratio is difficult to reconcile with intercalation at a single ApG/CpT site because of symmetry considerations. Binding into one such site of the tetramer for example might exclude two neighbor sites, but this would make the 4:2 and 2:2 complex stoichiometries previously observed hard to explain. Therefore, this ratio would seem to indicate intercalation into the central GpC/GpC site. The difference in the stoichiometries monitored by the AH-8 resonance at 10°C and 26.5°C then must be due to changes in the relative populations of 9AA intercalated into ApG/CpT or GpC/GpC sites. This implies that 9AA intercalated into the ApG/CpT site melts at a lower temperature than 9AA intercalated into the GpC/GpC site within this tetranucleotide.

Given that intercalation into both ApG/CpT and GpC/GpC sites has been detected, it is important to know which is preferred in the presence of excess tetranucleotide, the presumed "physiological" condition.

Table 1 compares the chemical shifts of 9AA on intercalation into various DNA sites as determined from dinucleoside phosphate binding studies (7) to those obtained from the dpApGpCpT titration. Intercalation into a GpC/GpC site leads to chemical shift changes of 9AA that are twice those observed on intercalation into ApG/CpT. The shift changes in excess of

FIG. 5. Proton chemical shift behavior of 9AA (A) and dpApGpCpT (B) at 26.5°C on addition of dpApGpCpT to 0.4 mM 9AA in 20 mM sodium phosphate buffer (pH 7.0). (A) Assignments of the 9AA protons are as in Figs. 3 and 4.

tetranucleotide (tetranucleotide/9AA = 15) therefore support substantial GpC/GpC intercalation. This is consistent also with the values of the *ratio* of complexation chemical shifts, $(\Delta\delta_{3,6} + \Delta\delta_{2,7})/(\Delta\delta_{1,8} + \Delta\delta_{4,5})$ which are insensitive to extent of binding (7). The ratio in excess tetranucleotide is intermediate between the ratios obtained for the corresponding ApG-CpT and GpC dinucleoside phosphate sequences (7) (Table 1).

In contrast the "stoichiometric" mixture (tetranucleotide/9AA = 0.67) has a much lower chemical shift change for the 9AA triplet protons (H3,6/H2,7) and a lower ratio of $(\Delta\delta_{3,6} + \Delta\delta_{2,7})/(\Delta\delta_{1,8} + \Delta\delta_{4,5})$, which is consistent with greater occupancy of ApG/CpT and terminally stacked sites (7).

DISCUSSION

We have shown that a number of different binding modes of 9AA to the tetranucleotide dpApGpCpT are demonstrated by combining proton NMR mixing and thermal transition experiments. Fig. 7 summarizes our interpretation of some of these modes of complexation, including the self-association of 9AA which influences the binding isotherms at low nucleic acid concentration.

The difference in stoichiometries between binding of dye to tetranucleotide at 10°C and 26.5°C (Fig. 7) is attributed to an end-binding interaction at the lower temperature involving the 5'-terminal phosphates and the terminal A-T base pairs of the pApGpCpT duplex and two acridine molecules. Because this interaction involves only one "face" of the dye and base, we predict, on the basis of dinucleotide studies (7), that it is labile and dissociates more readily than do intercalative complexes at either ApG/CpT or GpC/GpC sites of the duplex. From similar binding studies of 9AA and dinucleoside phosphate of different sequence it was not possible to anticipate a strong preference for one site over the other. Although we find indications for occupation of the ApG/CpT site in complexes near stoichiometric ratios, in the presence of excess tetranucleotide the binding seems to involve a contribution from the GpC site. Thus, it seems that the number of binding sites as saturation is reached is as fundamental an issue as the intrinsic affinity of different sites present.

These results raise a number of issues. First, in isotherms of this kind, the stoichiometry observed depends on the relative sensitivity of the proton being monitored to the binding modes

Table 1. Chemical shifts from free 9AA

DNA site	Chemical shift, ppm*				Ratio $\frac{\Delta\delta_{3,6} + \Delta\delta_{2,7}}{\Delta\delta_{1,8} + \Delta\delta_{4,5}}$
	$\Delta\delta_{1,8}$	$\Delta\delta_{3,6}$	$\Delta\delta_{4,5}$	$\Delta\delta_{2,7}$	
GpC/GpC†	0.51	0.64	0.49	0.66	1.30
ApG/CpT†	0.29	0.23	0.34	0.25	0.8
pApGpCpT (tetra/9AA = 15)	0.59	0.54	0.57	0.60	1.0
pApGpCpT (tetra/9AA = 0.67)	0.66	0.44	0.61	0.45	0.7

* All shifts are measured at 10°C in the buffer conditions defined in *Materials and Methods*.

† These are intrinsic upfield shifts derived from computer fits to binding data (see ref. 7).

FIG. 6. Scatchard plot of binding of 9AA to dpApGpCpT at two different temperatures as determined from the 9AA H3,6 resonances. r , Ratio of 9AA bound to total nucleotide concentration; c , free 9AA concentration.

available. The highest dye/nucleotide stoichiometry to which a proton is responsive may or may not correspond to the predominant species of complex in solution, depending on the relative chemical shift contributions for different complexes. The problem is analogous to differences in binding isotherms that can arise depending on whether absorbance or fluorescence of dye is used to follow the binding process (15). Only when several different resonances exhibit consistent stoichiometric behavior can conclusions about the binding process be reasonably secure. Caution must be exercised in interpreting isotherms in terms of the predominant complexes present.

The evidence from chemical shifts of 9AA and thermal denaturation leads us to conclude that no complexes that violate exclusion of the central GpC/GpC site on occupancy of either ApG/CpT site or vice versa are present. This is not to say that we can rigorously rule out complexes in which adjacent site binding occurs. However, there is no indication for 3:2 or 5:2 stoichiometries which would be expected in the absence of site exclusion effects, and, given the large upfield shifts expected for the dye protons in these complexes, they could only go unnoticed if the overall binding constant were relatively weak. We believe that, as diagrammed in Fig. 7, site exclusion can occur in a tetranucleotide unit as it appears to in longer duplexes

FIG. 7. Diagram of complexes in the dpApGpCpT/9AA system for which evidence is found in the present study. A ratio of $[dpApGpCpT]/[9AA] = 0$ represents 9AA alone; the same ratio $= \infty$ represents dpApGpCpT alone. The tetranucleotide is represented as a spindly line (unstacked) or a rectangular block with three possible intercalation sites (duplex). 9AA is represented by a small rectangle which can dimerize.

(14, 16). Further analyses of stoichiometric complexes (e.g., the 4:2 9AA/tetranucleotide complex) may clarify the mechanism responsible for site exclusion.

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