Complexity in the binding of minor groove agents: netropsin has two thermodynamically different DNA binding modes at a single site

Edwin A. Lewis1, Manoj Munde2, Shuo Wang2, Michael Rettig2, Vu Le1, Venkata Machha1 and W. David Wilson2,*

1Department of Chemistry, Mississippi State University, Mississippi State, MS 39762 and 2Department of Chemistry, Georgia State University, Atlanta, GA 30302, USA

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ABSTRACT

Structural results with minor groove binding agents, such as netropsin, have provided detailed, atomic level views of DNA molecular recognition. Solution studies, however, indicate that there is complexity in the binding of minor groove agents to a single site. Netropsin, for example, has two DNA binding enthalpies in isothermal titration calorimetry (ITC) experiments that indicate the compound simultaneously forms two thermodynamically different complexes at a single AATT site. Two proposals for the origin of this unusual observation have been developed: (i) two different bound species of netropsin at single binding sites and (ii) a netropsin induced DNA hairpin to duplex transition. To develop a better understanding of DNA recognition complexity, the two proposals have been tested with several DNAs and the methods of mass spectrometry (MS), polyacrylamide gel electrophoresis (PAGE) and nuclear magnetic resonance spectroscopy in addition to ITC. All of the methods with all of the DNAs investigated clearly shows that netropsin forms two different complexes at AATT sites, and that the proposal for an induced hairpin to duplex transition in this system is incorrect.

INTRODUCTION

Compounds that bind in the minor groove are of current research interest for their therapeutic potential against diseases from cancer to a variety of infectious diseases and for biotechnology applications (1–15). For example, the DNA minor groove continues to be an important target for the development of anti-cancer (1–5,15), anti-microbial and anti-viral compounds (6–9,12,14). Minor groove binders also have a number of other properties, such as inhibition, as well as activation of transcription that have provided fundamental new information on gene expression with the potential for development of entirely new and highly specific methods of disease treatment (4–6,16–18). The development and analysis of sequence-specific, minor groove-targeted compounds is of further timely importance given our passage into the post-genomic era and accelerating efforts toward the understanding of the genomes of many organisms and how they can be selectively targeted.

Minor groove binders typically have a number of common structural features; a curved shape or the ability to adopt such a shape, groups with positive charges, H-bond donating ability and a relatively flat conformation that has some flexibility in adjustment of intramolecular dihedral angles (19–21). Many of these compounds exhibit a preference for binding to AT sequences of at least four consecutive AT base pairs in the binding site (1–8,19,22). A number of X-ray crystallographic structures have been obtained with compounds bound to the minor groove of a four base pair AATT sequence in a self-complementary duplex, d(CGCGAATTCGCG)2 (19,23). The close match of the compound curvature or compound–water complexes to the minor groove shape is important and the weak binding to the minor groove of compounds without the critical structural features to complement DNA can be understood from the DNA complex structures (19,23). The compounds form H-bonds with acceptors on the base pair edges at the floor of the groove, they effectively stack with the walls of the narrow minor groove in AT sequences and they have a positive charge to offset the phosphate charges and negative electrostatic potential of the groove.

Given these excellent structural models for minor groove complex formation, we were surprised to find two quite different binding enthalpies in a 1:1 complex in high-resolution isothermal titration calorimetry (ITC)
studies of the classical minor groove agent, netropsin, binding to a hairpin duplex with the well-studied AATT binding site (24–26). The unexpected and complicated binding profile, with two distinct regions at a single binding site, clearly implies that there are two different binding complexes for netropsin in the AATT site. We proposed that this observation was due to netropsin bound in different modes in the 1:1 AATT complex but also pointed out possible other models, for example, different DNA conformations, could explain the results (14,24–26). Other groups subsequently took up the study of the two complex observation in a single binding site. Petty and coworkers (27) confirmed our two-site observation with netropsin and showed that the magnitude of the thermodynamic differences determined in ITC experiments is dependent on the GC sequences flanking the AATT binding site. It has also been proposed that the two observed complexes are not due to netropsin binding modes in the hairpin duplex but are due to a conversion from a hairpin duplex to a two-stranded duplex with an internal bulge in place of the hairpin loop (28).

Given the importance of minor groove binding compounds and interactions, described above, for our fundamental understanding of DNA interactions, in the chemical biology of gene expression and in development of new therapies, it is essential to test the quite different proposals for the molecular basis of the two complexes at a single site in more detail and establish a clear and unified explanation for the observation. To do this we have conducted several complementary, high-resolution studies of the netropsin complex in AATT binding sites in DNA hairpin duplexes with systematic changes in flanking sequence and length (Figure 1). Sequences with G or C bases directly flanking the AATT binding site were included in the experiments. In addition to high resolution ITC, the methods of mass spectrometry (MS) analysis, native polyacrylamide gel electrophoresis (PAGE) and nuclear magnetic resonance spectroscopy (NMR) were used to investigate netropsin: DNA complexes.

We confirm the presence of the two complexes at a single binding site in ITC titrations and extend the understanding of the dependence of the thermodynamic profile on the sequences flanking the binding site. In addition, the MS and PAGE results clearly show that the proposal of a netropsin induced hairpin to duplex transition is not correct, only the hairpin conformation is present in these systems for all DNA sequences at all concentrations and netropsin ratios. The NMR studies provide important additional information and show two bound species of netropsin. All results, thus, strongly support our original model with netropsin in different binding modes as the explanation for the two different thermodynamic signatures. We speculate on how the erroneous proposal for a netropsin to duplex conformation, as the explanation for the unusual thermodynamics, may have occurred and what might be the basis for the two binding modes.

**MATERIALS AND METHODS**

**Compound and DNA samples**

Netropsin was purchased from Sigma-Aldrich (St Louis, MO, USA), and was prepared as 1 mM stock solution in double distilled water and kept frozen at −4°C away from light. The structure and purity were confirmed by NMR and MS. Lyophilized hairpin DNA oligomers containing an AATT netropsin binding site were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA) with HPLC purification (Figure 1). MS, PAGE and NMR confirmed the sequences and purities of the DNA samples.

**ITC**

All ITC experiments were carried out as previously described (24–26) with a Microcal VP-ITC (Northhampton, MA, USA). Each of the titration experiments was conducted by filling the ITC cell with ~1.5 ml of a dilute hairpin DNA solution (nominally 25 μM) and adding up to 45 (5 μl) injections of a netropsin solution (nominally 550 μM). The 1.5 ml added to the VP ITC cell overfills the cell so that there is no air or bubbles in the chamber. As a volume of titrant (netropsin) is added, an equivalent volume of the titrated solution is displaced from the cell. The DNA concentration at each point in the titration is then corrected for the loss of solution from the calorimeter cell. A small well at the top of the ITC cell collects the displaced solution. Injections were done at 180 s intervals and all ITC experiments were done at 25°C. Three or more replicate titrations were performed for each of the four hairpin DNAs and the data from each experiment fit independently. The integrated heat/injection data obtained in each ITC titration were fit to a fractional sites model and a nonlinear regression algorithm developed in our lab with Mathematica 6.0 software (24–26).

**PAGE**

10% native polyacrylamide gels (1.5-mm thick, 20-cm long) were prepared from a 40% (w/v) acrylamide

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**Figure 1.** Netropsin structure and DNA sequences. The names of the target hairpin DNAs refer to the 5'-nucleotides flanking the AATT binding site.
solution (29:1, bis-acrylamide: acrylamide, EMD, Gibbstown, NJ, USA) in TBE buffer (0.089 M Tris, 0.089 M boric acid, 2.0 mM EDTA, pH 8.3). Before sample loading, the gels were subjected to a pre-run for 40 min at 150 V and 20°C. The final DNA concentrations in different experiments varied from 1 to 100 μM with different volumes added to the gel lanes to prevent sample overloading in the gel. An appropriate amount of netropsin was added to achieve the desired ligand: DNA molar ratios. A commercially available 20-bp duplex marker (Bayou Biolabs, Harahan, LA, USA) was also loaded onto the gels for a size standard. Electrophoresis was performed at 150 V for 120 min at 20°C in TBE buffer. After electrophoresis, the gels were stained with SYBR Gold Nucleic Acid Gel Stain (Invitrogen, Carlsbad, CA, USA) at the concentration preferred by the manufacturer. Stained gels were imaged using an UltraLum Omega 10 gD Molecular Imaging System (UltraLum, Claremont, CA, USA).

**Electrospray ionization–MS**

All electrospray ionization mass spectrometry (ESI-MS) experiments were carried out on a Waters Micromass Q-TOF micro (ESI-Q-TOF, Milford, MA, USA) instrument. The instrument was used in the negative ion mode with ‘soft’ ionization conditions: the capillary voltage set at 10 kV, source block and desolvation temperatures were set to 70 and 100°C, respectively. The instrument was used in the negative ion mode with ‘soft’ ionization conditions: the capillary voltage set at 10 kV, source block and desolvation temperatures were set to 70 and 100°C, respectively. All the DNA sequences tested with MS were desalted three times by dialysis with a 1000 Da cut-off membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) in 0.15 M ammonium acetate buffer at a 5 M ammonium acetate buffer at a 5 μM hairpin DNA concentration and appropriate amounts of netropsin were added to give the desired molar ratios to DNA. The samples were introduced into the ion source by direct injection at a 10 μl/min flow rate. Full scan MS spectra for the DNA and complex ions were recorded in the mass/charge region 400–4000. MassLynx 4.1 software was used for processing the data.

**NMR**

All NMR experiments were performed using a Bruker Avance 600 MHz spectrometer. The DNA was dialyzed three times against water, lyophilized and dissolved in a 10% D2O/90% BPES buffer (20 mM NaCl, 20 mM phosphate, 0.1 mM EDTA) solution. The pH of the solution was adjusted to 7.0 and the final single strand DNA concentration was determined spectrophotometrically to 0.87 mM. All NMR experiments were performed at 278 K with the water signal serving as an internal reference (5 ppm). Netropsin was dissolved in D2O and added stepwise to the DNA solution until saturation was achieved. Volume changes were <11%. Water suppression was achieved by applying a 1–1 pulse sequence.

**RESULTS**

**ITC**

High-resolution ITC was used to systematically investigate the minor groove binding of netropsin to the four target hairpin DNAs in Figure 1. ITC is a well characterized technique for analysis of the binding enthalpy and equilibrium constant for DNA minor groove binding compounds (30–33). The method can be exploited to provide a complete thermodynamic description of the formation of ligand: DNA complexes in dilute solution in a single experiment. All of the netropsin titrations of the target hairpin DNAs exhibit a prominent dip in the titration curve immediately prior to the titration endpoint (Figures 2 and 3) and two sets of binding parameters are needed to fit these data within experimental error. The dip provides a clear indication that two unique netropsin complexes are being formed in these experiments in agreement with previous experiments on hairpin DNAs (24–27).

Since the equilibrium constants for formation of Complex I, \( K_1 \) and Complex II, \( K_2 \), are only different by about a factor of 10 (Table 1), both complexes are being formed throughout most of the titration. Therefore, the enthalpy changes shown in Figure 3 are the summation of the enthalpy changes for the formation of the two complexes times the fraction of each complex formed at that point. The effect is that the enthalpy changes plotted in Figure 3 are the weighted average of the two enthalpy changes. At the beginning of the titration, the average \( \Delta H \) is only slightly greater than the fit value for \( \Delta H_1 \). However, toward the endpoint, the average value of \( \Delta H \) is significantly less than the fit value \( \Delta H_2 \). If \( K_1 \) and \( K_2 \) were identical the ITC isotherm would show only one

![Figure 2](https://academic.oup.com/nar/article-abstract/39/22/9649/2409478/figure2)
average value for $\Delta H$ throughout the titration even if the values of $\Delta H_1$ and $\Delta H_2$ were different.

The data shown in Figure 2 are for four different netropsin titrations of the GCG target hairpin. The four data sets were fit independently and the average fit parameters used to produce the line in the figure. The data shown in Figure 3 are for a single netropsin titration done on each of the four target hairpins. Obviously all four target hairpins exhibit two binding events and all of the titration data are well fit with our two overlapping site model with $n_{\text{total}}$ constrained to a value of 1.0 (Table 1).

The last column in Table 1, $n_1/n_2$, represents the relative amounts of the two different netropsin–hairpin complexes that are formed. In each case approximately 6 mol of Complex I are formed for each mole of Complex II. In general, all four target hairpin DNAs behave similarly in that all appear to form two unique netropsin: DNA complexes. Subtle differences in binding energetics with respect to hairpin length and sequence will be the focus of future work. However, it appears that the shorter 20-nt hairpins exhibit marginally lower initial, type I, binding affinities with more favorable enthalpy changes than the longer 24-nt hairpins. With the weaker binding, type II, the shorter hairpins exhibit slightly higher affinities than the longer hairpins. In summary, the ITC studies clearly show that there are two different netropsin complexes with all of these DNA sequences. Although our fitting results provide support for the two binding mode model, it is impossible to determine from the ITC experiments alone whether netropsin is binding in two different modes to a hairpin DNA or whether the DNA exists in hairpin and double helix structures, each binding netropsin with a unique thermodynamic signature. Methods that do resolve these questions are presented below.

### PAGE

Gel electrophoresis is a sensitive method to separate and detect mixtures of hairpin and duplex DNA structures (34). We have conducted experiments with all four DNAs of Figure 1 at different concentrations and ratios of netropsin to DNA binding sites. As shown in Figure 4, the four target DNA sequences at a concentration of 1 µM were loaded on the gel with netropsin at ratios of 0:1, 1:1, 2:1 and 3:1 netropsin: DNA along with the 20 bp duplex marker. From the figure it is clear that after binding with netropsin only the hairpin band is observed and there is no duplex band for any DNA sequence. If any duplex DNA had formed on addition of netropsin, its band would move more slowly, at about the same rate as the corresponding marker band with the same molecular weight. It is known from PAGE ligation ladder experiments that netropsin stays bound to AT DNA binding sites under these conditions (35,36).

In order to determine whether formation of a hairpin to duplex transition occurs at much higher concentrations, PAGE results for addition of netropsin to the representative sequence, CG, at DNA hairpin concentrations of 10, 50 and 100 µM are shown in Figure 5 at the same ratios as in Figure 4. The gels in Figure 5 are overloaded with DNA but even under these extreme conditions, no conversion of hairpin to duplex is observed on addition of netropsin. The addition of netropsin to the hairpin DNAs does not cause a significant change in mobility under these conditions. This is in agreement with results on DNA ligation ladder PAGE results which show that addition of compounds to DNAs below about 50 bp does not change their mobilities, if they do not change the DNA conformation by a significant amount (35,36). In summary, even over a 100-fold concentration range and at netropsin concentrations three times higher than the AATT binding sites, PAGE results clearly indicate that all of the DNA complexes remain in a hairpin conformation.

### Table 1. Best fit thermodynamic parameters for a two competing site model for binding netropsin to four different hairpin DNAs

| Oligo | $K_1$ ($\times 10^7$ M$^{-1}$) | $\Delta H_1$ (kcal/mol) | $-T\Delta S_1$ (kcal/mol) | $K_2$ ($\times 10^7$ M$^{-1}$) | $\Delta H_2$ (kcal/mol) | $-T\Delta S_2$ (kcal/mol) | $n_1/n_2$ |
|-------|-------------------|-----------------|-----------------|-------------------|-----------------|-----------------|-------------|
| GCG   | 4.50 ± 1.15       | -7.18 ± 0.24    | -3.22           | 8.75 ± 1.16       | -21.53 ± 0.55   | 13.45           | 6.3         |
| CGC   | 2.88 ± 0.13       | -8.89 ± 0.04    | -1.29           | 8.75 ± 1.63       | -20.69 ± 0.03   | 12.59           | 6.8         |
| CG    | 1.75 ± 0.76       | -9.13 ± 0.13    | -0.71           | 9.10 ± 1.13       | -21.20 ± 0.56   | 15.15           | 6.7         |
| GC    | 1.29 ± 0.04       | -9.44 ± 0.08    | -0.22           | 11.10 ± 1.69      | -21.22 ± 0.70   | 13.01           | 5.2         |

Three replicate titrations were performed on each oligonucleotide. The error limits in the table are the standard deviation of the mean parameters for the three titrations fit individually.
ESI–MS

ESI–MS is a reliable method to determine the binding affinity, stoichiometry and sequence selectivity of minor groove complexes (37–40). Our MS experiments were conducted with the same hairpin duplex DNA sequences (Figure 1) as in PAGE to further test whether a transition from hairpin to duplex could be detected. Each target AATT DNA at 5 μM was injected at three concentration ratios 1:1, 1:2 and 1:3 DNA:netropsin (Figure 6A–D).

It should be noted that the conditions used in the MS experiments are quite gentle and have been validated for almost 20 years to leave DNA duplexes and complexes intact (for example, reviewed in 37,38,40). Our conditions are even milder than in many studies on DNA duplexes.

All the DNA sequences showed very strong affinity for netropsin with ions corresponding to free DNA and 1:1 netropsin: hairpin complexes at charges of $z = \frac{4}{C_0}$, $\frac{5}{C_0}$ and $\frac{6}{C_0}$. If the hairpin DNA unfolds into a single strand and anneals with another unfolded single strand to form a duplex with an internal loop and two bound netropsins, the 2:1 netropsin: duplex complex will possess double the molecular weight of the 1:1 netropsin: hairpin complex. In this case, netropsin: duplex complexes would have charges of $z = \frac{8}{C_0}$, $\frac{10}{C_0}$ and $\frac{12}{C_0}$, double the charges of $\frac{4}{C_0}$, $\frac{5}{C_0}$ and $\frac{6}{C_0}$ for hairpin complexes in Figure 6, in order to give the same m/z as the hairpins. The fact that no odd-numbered charge states of $\frac{7}{C_0}$, $\frac{9}{C_0}$ or $\frac{11}{C_0}$ were observed clearly shows that the species detected are for hairpin complexes (40).

In Figure 6, the free DNA in all spectra has a molecular weight characteristic of the hairpin structure as expected. On initial addition of netropsin, a strong hairpin DNA peak at a 1:1 ratio is seen with the presence of a small free DNA peak. On increasing the amount of netropsin to DNA, only the 1:1 bound hairpin complex, based on the molecular weight, was observed at the 1:2 and 1:3 added ratios, in agreement with the expected stoichiometry of the complex. Changing the flanking sequence and length had no effect on the amount of hairpin DNA and, as with the ITC and PAGE experiments, no evidence of a netropsin induced hairpin to duplex transition was seen in any of these spectra at any ratio. The ESI–MS spectra of all target DNA sequences and complexes evaluated, plotted according to the total mass, are shown in Supplementary Figures S1–S4. These figures emphasize that only the hairpin species are present.
Figure 6. ESI–MS of netropsin bound with (A) CG (B) GCC (C) GC (D) CGC at three ratios of compound to DNA: 1:1, 2:1 and 3:1. Each sample was prepared as described in the ‘Methods’ section. The labels HP and [1:1] stand for the hairpin DNA and netropsin–DNA complex at three charge states (z = −6, −5 and −4), respectively. These results were converted to full mass spectra for ease of visualization and are plotted in Supplementary Figures S1–S4. The experiments were in 0.15 M ammonium acetate buffer at a 5 mM hairpin DNA concentration with appropriate amounts of netropsin added to give the molar ratios to DNA shown in the figure.
NMR

Imino proton 1D NMR is especially useful for detection of multiple species in a hairpin to duplex conversion or of more than one bound species of netropsin. NMR titrations of the 20-mer hairpin DNA sequence CG (Figure 1) with netropsin were performed and the changes in the imino proton region of this AATT sequence DNA, upon addition of the compound at 278 K, are shown in Figure 7. As expected for a hairpin structure with 8 bp and a TCTC loop, seven peaks translating to eight imino protons, due to signal overlap at 13.8 ppm, can be identified for the free DNA (top part of the figure). With respect to their chemical shifts the resonances ranging from 12.7 to 13.2 ppm and 13.8 to 13.95 ppm can be assigned to guanine and thymine imino protons, respectively. As the terminal GC base pair is prone to fraying it gives rise to the broadest signal among this group of protons at 13.15 ppm. Due to their high solvent exchange rate, the non-hydrogen bonded imino protons of the loop region are severely broadened, but can still be detected at a chemical shift of around 11 ppm (data not shown).

The intensity of free DNA signals present in the top spectrum gradually decreases with increasing netropsin:DNA ratio until the original peaks are gone in the bottom spectrum of Figure 7 and this clearly shows the absence of any free DNA after the final titration step. Also, no changes can be seen in the loop region but new sharp peaks can be observed in both the guanine and the thymine imino proton regions and are in agreement with the strong interaction between netropsin and AATT DNA sequences. Signals for both the free DNA and the netropsin:DNA complex are observed until the 1:1 ratio is reached. This result shows that at the macroscopic level netropsin exchanges slowly between the complex and the free DNA.

In general, the unique interaction of netropsin with a hairpin DNA should not change the number of imino proton signals visible in the hairpin DNA NMR spectrum at a 1:1 ratio. However, in a real case scenario peak overlap or signal broadening due to altered dynamics might lead to a reduction of observable peaks. Interestingly, the overall number of peaks visible in these imino spectral regions significantly increases on addition of netropsin, as expected for two bound species of the compound. At least 11 easily distinguishable resonances can be seen at a 1:1 ratio and the number of signals certainly exceeds the 8 imino proton signals visible for the free DNA. The extra imino signals clearly rule out the existence of just a single netropsin–hairpin DNA complex. In addition, the bound loop signal, with no observable change in chemical shift or intensity, is still present at the 1:1 ratio and indicates no hairpin to duplex transition has occurred.

In an equilibrium between a monomolecular hairpin and a bimolecular duplex DNA, the former is generally favored at lower and the latter at higher concentrations. Thus, lowering the DNA concentration should shift the equilibrium toward the monomolecular hairpin, if any duplex is present. To investigate the influence of concentration on the existing netropsin:DNA complex, an additional spectrum of the 1:1 ratio at a 10 times lower concentration was obtained. The dilute sample gave essentially the same imino spectrum (Supplementary Figure S5) indicating no significant shift of the conformation over this 10-fold change, 0.09–0.9 mM, in DNA strand concentration. This finding is in agreement with PAGE (up to 0.1 mM; Figure 5) and MS results which also show that addition of netropsin to the DNA hairpin duplexes does not result in any conversion to a double-stranded duplex. In summary, the NMR results for a hairpin DNA–netropsin complex clearly show that there is more than one netropsin species bound in a 1:1 total complex stoichiometry.

Figure 7. NMR titration of netropsin with the CG target sequence (0.9 mM) at 278 K in BPES buffer containing 20 mM NaCl. Compound to DNA ratios are indicated on the right.
DISCUSSION

As noted in the ‘Introduction’ section, this research was initiated to resolve two mutually exclusive explanations for the observed two complexes of netropsin at a single AATT binding site: (i) two netropsin binding modes (24–27) or (ii) a netropsin induced hairpin to duplex conversion (28). To clearly resolve this important question for understanding minor groove complexes, hairpins with AATT binding sites and flanking sequences that differ in length and sequence (Figure 1) were selected for evaluation since both of these factors can affect binding modes and hairpin to duplex conversion. Several experimental methods, each of which can unequivocally distinguish between the two proposals, were used to monitor complex formation on netropsin binding.

ITC studies of netropsin binding to the four target duplexes clearly show the formation of two netropsin complexes in all cases. The fitting results show that the second binding event has a lower equilibrium constant than the initial binding but the dip is easily observed by ITC because of the larger favorable enthalpy of netropsin binding to the second site. The enthalpy difference from above −10 kcal/mol to below −20 kcal/mol (Table 1) is surprisingly large for two binding modes to an AATT site and it is accompanied by a shift from small favorable to large unfavorable TΔS contributions. The large negative ΔH and negative ΔS suggest that a significant amount of water is captured in the second complex. Such a proposal is in agreement with the osmotic stress results of Petty and coworkers who showed that netropsin binding to an AATT site results in an uptake of over 25 water molecules with an even larger water uptake for other minor groove binders (41).

MS is a sensitive method that can resolve small molecular weight differences in DNA complexes (37–40). The free DNA for all four oligomers has the hairpin molecular weight, as expected, and there is no evidence of any duplex. On addition of netropsin to a 1:1 level, the free hairpin DNA concentration drops to a low level and a new species appears at the molecular weight of the 1:1 netropsin: hairpin DNA complex. Even at added ratios of 2:1 and 3:1 netropsin: DNA only a 1:1 complex is observed. The MS results clearly emphasize that only a 1:1 complex is formed, in spite of the two different netropsin: DNA species observed, and that DNA in the two complexes has the same molecular weight as for the free hairpin DNAs. The hairpin species have continuous charges of −4, −5 and −6. A duplex of the same m/z would have twice the molecular weight and thus twice the charge. The charges become unrealistically high, but more importantly, the possibility of duplex species is eliminated by the absence of complexes with odd numbered charges, discontinuous charge states (40).

PAGE is also a very useful method for detection of a netropsin induced hairpin to duplex transition since there are significant differences in mobility between the species (34). PAGE experiments were conducted at DNA concentrations to 100 μM and netropsin ratios up to 3:1 to try and force a hairpin to duplex transition in any of the DNAs. Even at the high concentrations and overloaded gels in Figure 5, however, only hairpin DNA complexes can be detected. We originally thought that concentration differences might, at least, partially account for the different explanations for the two 1:1 netropsin complexes (24–26,28) but no transition is observed even at very high (100 μM) concentration of DNA strands. The published proposal for a netropsin-induced hairpin to duplex (28) transition is primarily based on a single gel experiment, which shows a weak and poorly defined gel band that migrated behind the hairpin. Since no molecular weight standard, as in Figures 4 and 5, was included in that report, it is not possible to accurately determine the molecular weight of the trailing band. The low intensity of the published gel band, even in the presence of excess netropsin, does not support a significant netropsin-induced hairpin to duplex transition. We speculate that a better explanation of the trailing band (28), given the definitive results against the hairpin to duplex proposal presented here, is that there was improper annealing of the sample, some aggregation on addition of netropsin, or some impurity which separated in the presence of netropsin to give the reported PAGE results.

Since all results point to two different bound species of netropsin 1D imino proton NMR experiments were conducted with the CG hairpin to determine whether the different species of netropsin could be observed. The spectrum of the free hairpin has eight imino proton signals for the eight different base pairs in the duplex stem of the hairpin and addition of netropsin should not change this since the hairpin and netropsin are not symmetric. Based on this reasoning and the increased number of imino proton peaks (Figure 7), there are two different netropsin complexes at the single AATT site. In summary, NMR spectra over a 10-fold concentration range (Figure 7 and Supplementary Figure S5) agree with PAGE and MS that no hairpin to duplex transition can be detected on addition of netropsin.

It is interesting that in a very early NMR experiment with netropsin bound to a symmetric AATT duplex, 2D NMR experiments clearly showed that there were two bound species of netropsin at a 1:1 ratio that could be identified by exchange crosspeaks (42). The ITC results of Petty and coworkers (27) also with duplex DNAs that have an AATT site clearly show that two complex forms of netropsin can exist in duplex as well as in hairpin DNAs. It is thus apparent from the results presented here and in the literature that two bound forms of netropsin can form in both hairpin and duplex DNAs while no hairpin to duplex transition can explain the two bound species of minor groove complexes.

In conclusion, ITC and NMR results clearly show that there are at least two netropsin complexes present in the 1:1 total complex of netropsin at AATT. PAGE, MS and NMR conclusively show that the two complexes are not due to a hairpin DNA to duplex transition. All results strongly support the presence of two different modes of netropsin binding to AATT. NMR results show clear differences for the two complexes that suggest different structures for the two bound forms. The structures of the two species are not determined by these methods, however, and will require additional detailed structural experiments.
on the DNA hairpin–netropsin complex. Results from our (24–26) and the Petty laboratory (27) suggest that more water interacts with the second, weaker complex and can account, at least partially, for the large thermodynamic differences for binding of netropsin to the same AATT site.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures S1–S5.

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