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Design of Distamicin Analogues to Probe the Physical Origin of the Antiparallel Side by Side Oligopeptide Binding Motif in DNA Minor Groove Recognition

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Oligopeptides distamycin and its analogues can bind cooperatively to the minor groove of certain DNA sequences in a closely compacted antiparallel side by side motif, where the positively charged ends are located in the carboxyl termini of such peptidic structures. In order to dissect its physical underpinning, the role of charge in maintaining the integrity of this novel motif is explored by using three judiciously designed distamycin analogues possessing either no charge or with charge ends located in the amino termini. Preliminary experiments by CD and ethidium fluorescence displacement suggested that the charge plays an role in influencing the degree of binding cooperativity as well as binding strength, although qualitatively the dimeric binding remains cooperative.

Naturally occurring peptidic antibiotics distamycin A and netropsin have served as paradigms in designing DNA minor groove recognizing molecules in the past decade,[1] since it was clearly revealed that their biological activities are correlated with their DNA template blocking function[2] and the structures of their oligonucleotide complexes become available.[3] More recently, a new structural motif was defined from solution NMR studies.[4] In this new motif, two peptidic molecules are simultaneously located in the minor groove in an antiparallel side by side manner with each positively charged end of each peptide strand pointing outward to the 3′ end of its neighboring DNA strand (Scheme 1). Ensuing studies established the generality of this new motif and provided a broadened picture.[5,6,7,8] Notably, the heterodimeric motif where two different peptide molecules coexist are more stable and cooperative than the corresponding homodimeric ones in the same site.[5d,6b] Each binding molecule interacts specifically with only one DNA strand, which constitutes a unique strand specific information reading pattern. Overall, the dimeric binding motif opens up possibilities of the minor groove information readout, since structurally controllable variables are now doubled. We have successfully designed cross-linked oligopeptides to effectively enforce the side by side antiparallel binding motif.[9]

Despite the revelation of the detailed anatomy of this unique motif, little has been advanced on its physical basis. In-depth understanding of four outstanding and very likely related features of this motif, including the relative orientation of two peptide strands, the relative orientation of each peptide and its neighboring oligonucleotide strand, the close packing of two peptide molecules and the cooperativity of binding, may lay a foundation for the design of the next generation of sequence-specific DNA minor groove binders. To this end, we are undertaking an experimental approach to dissecting the physical origin of the motif. Because all oligopeptides in the motif contain a positively charged moiety at the carboxyl end, residing either in amidinium group or dimethylammonium group, the role of charge in maintaining the integrity of the motif becomes our initial focal point.

By virtue of the antiparallel orientation of two peptide strands, positively charged moieties in the dimeric motif are widely separated. It was suggested that the antiparallel orientation of two peptides is favored because it reduces charge-charge repulsion between two positively charged moieties.[4a]

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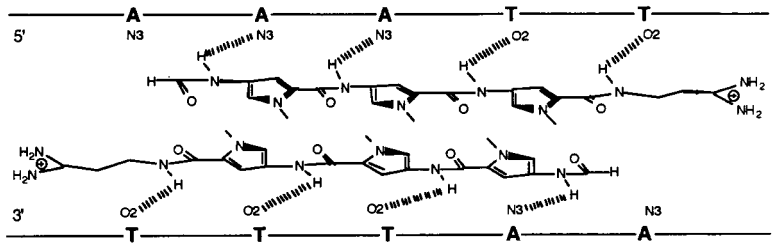


SCHEME 1. The antiparallel side by side binding motif composed of two distamycin molecules.

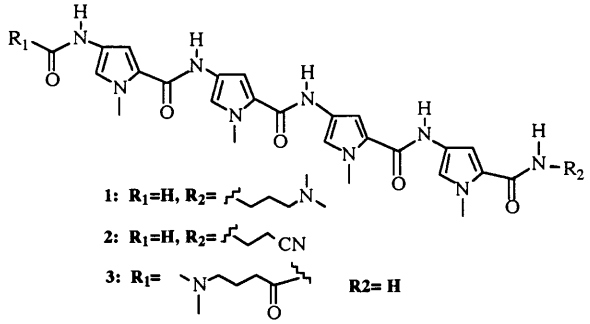
However, we reason that the orientation could also be decided by another factor, that is, the maximization of stabilizing electrostatic interactions between two peptide strands. In the dimeric motif, each pyrrole ring in one peptide strand stacks over a 3′-shifted amide linkage in the opposite strand. Quite likely, this orientation has the stabilizing electrostatic complementarity, originating from the specific polarizations of both the pyrrole ring and the amide linkage.[10] If peptides in the motif are free of any charge, the potential contribution from any formal charge-charge repulsion in the peptide assembly will be eliminated, permitting an assessment of the influence of charge in maintaining the antiparallel orientation of two peptide strands. Thus, the first probe molecule **2** was designed to be charge neutral, in contrast to its parent molecule **1.**

Previous theoretical and experimental studies have established that the surrounding bases of a binding site may influence the orientation of distamycin and its analogues in 1:1 complexes.[11,12] The theoretical studies further suggested that the orientational specificity is mainly derived from the electrostatic interaction between the positively charged moiety of the ligand and the surrounding bases of the binding site.[11] In contrast, present experimental results suggest that the surrounding bases of a binding site and thus the electrostatic factor just mentioned play a relatively insignificant role in determining the relative orientation of a peptide strand and its adjacent DNA strand in a 2:1 complex. In every 2:1 complex so far structurally characterized, the N-terminus to the C-terminus direction of the peptide strand is always parallel to the 5′ terminus to 3′ terminus direction of the neighboring DNA strand. Therefore, it seems that the inherent compatibility between the charge free peptide moiety of a ligand and the DNA backbone is a decisive factor. Although neutral probes such as **1** could provide some support for this contention, an even more stringent test may come from charge-switched probes such as **2,** in which the charge moiety is located at a similar distance from the tetra(aminopyrrolecarboxyl) moiety to that of the parent molecule **1** but in the opposite amino terminus. It is envisaged that if the charge dependent electrostatic interaction factor were of great importance, the relative orientation of the peptide strand and its neighboring DNA strand in a new 2:1 complex would be reversed. The entire side chain in each carboxyl terminus is eliminated to avoid any steric complication.

Presently available NMR evidence suggests the applicability of the antiparallel side by side motif in the tetra(aminopyrrolecarboxyl) system.[5f,8] Because our interest in extending the antiparallel side by side motif to the specific recognition of longer sequences, the tetra(aminopyrrolecarboxyl) system instead of the tri(aminopyrrolecarboxyl) one was chosen. An additional practical consideration is that pallindromic oligonucleotide sequences can be utilized in NMR studies to simplify the spectral analysis and subsequent model building, since the 2:1 complexes of these peptides should occupy an even number of base pairs, i.e., six. Accordingly, three probe molecules **1, 2** and **3** of the tetra(aminopyrrolecarboxyl) system were synthesized. For comparison purposes, their counterparts in the tri(aminopyrrolecarboxyl) system were also prepared.

MATERIALS AND METHODS

Compounds **1, 2** and **3** were characterized by ^{1}H-NMR, UV, IR, and fast atom bombardment MS and shown to be homogeneous.



SCHEME 2. Design of distamycin analogues in probing the role of charge.

A buffer solution (pH = 7.00) containing Tris-HCl (10.0 mM), sodium chloride (10.0 mM) and EDTA sodium salt (1.0 mM) was prepared with distilled deionized water. This buffer was used in the preparation of all DNA and ligand stock solutions for CD and ethidium displacement experiments. DNA polymers were purchased from the Sigma company and stored at −5°C. A mixture of methanol/buffer (1/2, v/v) was used to prepare a homogeneous stock solution of the neutral ligand **2.** The amount of methanol introduced into the titration was shown to be inconsequential by a calibration experiment.

Circular dichroism was measured on a Jasco-ORD/UV5 polarimeter at a constant temperature of 23°C. Concentrations of DNA were determined from UV absorption at 260 nm with ϵ_{260} = 6750 and 6000 for poly (dA-dT)-poly (dA-dT) and poly (dA)-poly (dT) respectively. The DNA concentration per nucleotide was maintained at ~80 μM for all titrations while the concentration of ligand was twenty times this value. Fluorescence was measured on a digital Sequoia-Turner fluorometer (model 450) at ambient temperature. Excitation and emission wavelengths were set at 550 nm and 600 nm respectively. Readings were taken at least nine times for each ligand concentration to give an average value. For displacement experiments, the ethidium bromide solution before addition of DNA was taken as blank and concentrations of DNA and ethidium bromide were 1.00 μM per nucleotide and 1.26 or 2.52 μM respectively.

RESULTS AND DISCUSSION

Our preliminary structural analysis by NMR spectroscopy indicated that the parent molecule **1** adopts a canonical highly cooperative antiparallel side by side motif in the minor groove of oligodeoxynucleotides $(5'CGTATATAGC3')_2$ and $(5'CGCAAATTTGCG3')_2$, covering six central AT base pairs.[16] While the structural studies are continuing with ligands **2** and **3,** we have also characterized the interactions of these three oligopeptides with DNA polymers such as poly (dA-dT)-poly (dA-dT) and poly (dA)-poly (dT) by CD and ethidium fluorometry methods, which are described in detail below.

Previous CD studies demonstrated that the alternating AT polymer poly (dA-dT)-poly (dA-dT) can accommodate the dimeric binding of distamycin and its analogues while the homo AT polymer poly (dA)-poly (dT) cannot, before the detailed structure of the dimeric motif became available (Scheme 2). [13] The studies on the cross-linked lexitropsins provide further support for this con-

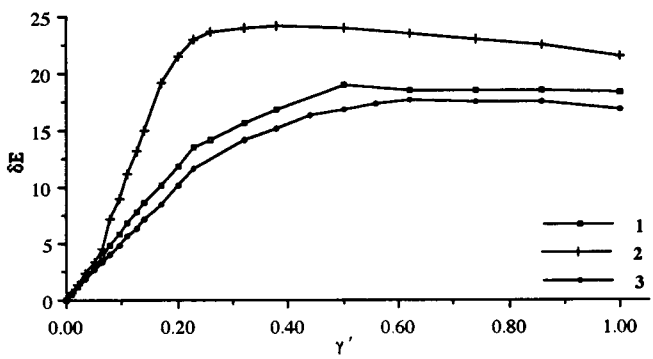


FIG. 1. CD titration curves with poly (dA-dT)-poly (dA-dT).

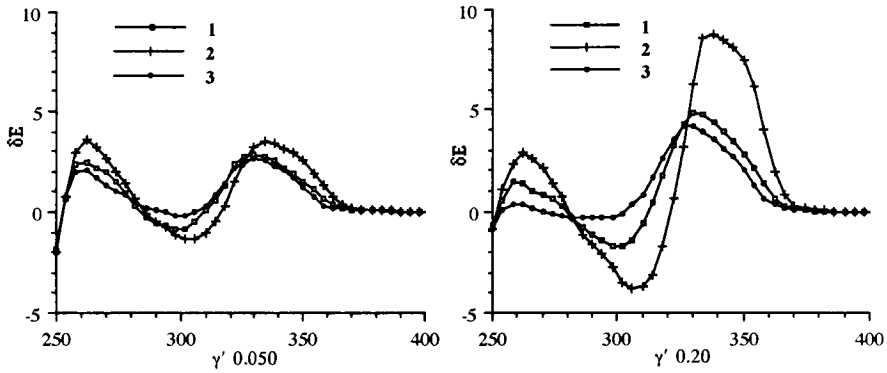


FIG. 2. CD titration spectra with poly (dA-dT)-poly (dA-dT).

clusion.[9] Therefore, it can be conjectured that CD titration studies with the alternating AT polymer would be informative. CD titration curves at 332 nm as plotted in Figure 1 present a snapshot view of the binding of three oligopeptides.[14] Charged ligands **1** and **3** are grossly similar to each other throughout the titration, suggesting the similarity of their binding interaction with DNA. The titration curve of the charge-switched ligand **3** is somewhat below that its parent **1,** which could be accounted for by its weaker overall binding. The neutral ligand **2** has its titration curve coincident with those of the other two ligands up to [Ligand]/[DNA] (i.e., γ') equal to 0.065. Because it is the 1:1 binding mode that dominates in low ligand concentration, this similarity suggested an analogous 1:1 binding mode among three ligands. However, the titration curve quickly turns upward after this point and results in a considerably different end point. This may be explained by either an inherent greater extinction coefficient of the 2:1 binding motif for the neutral ligand **3** and/or the 2:1 binding is significantly more cooperative which prompts the earlier saturation of the DNA matrix. The titration curve of **2** levels off at a substantially lower γ' (0.32), corresponding to the earlier appearance of turbidity in solution. The turbidity is probably due to DNA precipitation, which is caused by the increased hydrophobicity of the complexed DNA matrix. The fact that ligand **2** cannot increase the hydrophobicity of the DNA matrix by charge neutralization suggests an overall faster saturation of DNA by the ligand. Taking into account that **2** is a weaker binder than **1** in the 1:1 mode (discussed below), we may infer that the dimeic binding of **2** and DNA is significantly more cooperative.

A more detailed view of the CD titration is presented in Figure 2 where CD spectra at $\gamma' = 0.050$ and 0.20 are ploatted respectively. Notably, the neutral ligand **2** has its induced band at $\sim$330 nm shifted to longer wavelength, which perhaps reflects the change of local electrostatic potentials due to charge removal.

Binding constants of these ligands to both poly (dA)-poly (dT) and poly (dA-dT)-poly (dA-dT) polymers were determined by the ethidium displacement assay.[15a,b] Displacement experiments were carried out to determine the required total ligand concentrations to achieve a 50% reduction in fluorescence intensity in the presence of ethidium (1.26 μM) and DNA (1.00 μM), the C_{50} values, from which binding constants are calculated by using the McGhee-von Hipple

TABLE 1
Binding Constants to Poly(dA)-poly(dT)

ligand	**1**	**2**	**3**
$C_{50} \times 10^2$ (μM)	7.00 ± 0.46	19.9 ± 1.5	26.1 ± 1.6
K ($\times 10^6$)	39.4 ± 3.5	6.29 ± 0.62	4.48 ± 0.33

TABLE 2
Binding Constants to Poly(dA-dT)-Poly(dA-dT)

ligand	**1**	**2**	**3**
$C_{50} \times 10^2$ (μM)	9.67 ± 0.41	23.7 ± 2.3	57.8 ± 2.5
$C_{25} \times 10^2$ (μM)	5.06 ± 0.51	14.3 ± 2.0	31.2 ± 2.1
K ($\times 10^6$)	268 ± 20	77.1 ± 9.0	27.9 ± 1.3
k_{11} ($\times 10^6$)	38.2 ± 3.9	6.04 ± 0.84	4.26 ± 0.29
k_{21} ($\times 10^6$)	1866 ± 79	984 ± 95	184 ± 8
α (k_{21}/k_{11})	49	163	43

equations.[9a,15c] The ligand concentrations required to achieve a 25% reduction in fluorescence intensity, the C_{25} values, were also determined for the binding to the alternating polymer in order to provide a minimal data set for calculations of two stepwise binding constants k11 and k21.[9a]

Intrinsic binding constants K with the homo polymer are calculated by the hypobolic equation $K = \frac{0.9670 \times 10^6}{C_{50} - 0.04544}\ M^{-1}$ and the results are shown in Table 1. Both the neutral ligand **2** and the charge-switched ligand **3** are significantly weaker binders than the parent molecule **1.** While it is expected that **2** is weaker than **1** due to charge removal, it is somewhat surprising that the binding strength of **2**is still comparable with that of the charge-switched ligand **3.**

Binding constants of **1, 2** and **3** to poly(dA-dT)-poly(dA-dT) are shown in Table 2. The averaged binding constants *K,* the square root of the product of two stepwise binding constants k11 and k21, can be readily derived from the formula: $K = \frac{15 \times 10^6}{C_{50} - 0.04080}\ M^{-1}$. The order of averaged binding strength qualitatively follows the same trend as in the case of binding to the homo- AT polymer. The k11 and k21 sets that best fits the data points are derived using the previously reported method.[9a,15c] The cooperativity factor α for each ligand is computed as the ratio of k21 to k11.

It is evident that the removal of charge increases the cooperativity of the 2:1 binding mode from 49 to 163, which is consistent with the CD titration result that the saturation end point is approached faster by the neutral ligand **2.** The main source of increased cooperativity comes from the greater reduction of binding strength in the 1:1 binding mode. The switching of charge from one side to another decreases the binding strength in both the 1:1 mode and the 2:1 mode. The binding cooperativity is somewhat reduced, consistent with the slower approaching to the saturation end point by the charge-switched ligand **3,** as demonstrated in the CD titration experiments. The greater reduction of binding strength in 2:1 mode plays a more important role in the cooperativity decrease.

In summary, we present the design of distamycin analogues to probe the physical origin of the antiparallel side by side binding motif, which represents the first experimental attempt to our knowledge. Preliminary evaluation of the interactions between these distamycin analogues and DNA polymers with CD and ethidium fluorescence displacement methods indicates that the charge plays an role in influencing the degree of binding cooperativity as well as the binding strength, although qualitatively the dimeric binding mode remains cooperative. Structural studies by 2D NMR spectroscopy and molecular modeling are in progress to provide the full details of the dimeric binding interaction in terms of orientational specificities in the minor groove and will be reported in due course.

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