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DNA sequence recognition by bis-linked netropsin and distamycin derivatives

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Abstract We studied the interaction of *cis*-diammine Pt(II)-bridged bis-netropsin, *cis*-diammine Pt(II)-bridged bis-distamycin and oligomethylene-bridged bis-netropsin with synthetic DNA fragments containing pseudosymmetrical AT-rich nucleotide sequences and compared it with the interaction of the parent compounds netropsin and distamycin A. For fragments containing multiple blocks of $(A/T)_4$ and $(T/A)_4$ separated by zero, one, two and three GC-base pairs, DNase I footprinting and CD spectroscopy studies reveal that 5′-TTTTAAAA-3′ is the strongest affinity binding site for *cis*-diammine Pt(II)-bridged bis-netropsin and bis-distamycin. They both bind less strongly to a DNA region containing the sequence 5′-AAAATTTT-3′. Netropsin, distamycin A and oligomethylene-bridged bis-netropsin exhibit far less sequence discrimination.

Key words: DNA-drug interaction; Distamycin; Bis-netropsin; Bis-distamycin; d(TTTTAAAA) sequence

1. Introduction

There is much interest in design and synthesis of biologically active compounds that can bind to DNA with a high affinity and specificity. Cell-permeable sequence-specific DNA-binding ligands have a potential to control gene expression and to serve as prototypes of gene-selective drugs. Netropsin and distamycin A are among the best characterized sequence-specific ligands with respect to their mode of interaction with DNA ([1] and literature cited therein). X-ray [2–4] and NMR studies [5] show that these antibiotics bind in the minor groove of double-helical DNA at sites of 4 and 5 successive AT base pairs [2–5].

An obvious way to enhance the binding specificity shown by netropsin or distamycin A is to synthesize dimer compounds (bis-netropsins and bis-distamycins) in which two monomers are linked in head-to-head, head-to-tail and tail-to-tail manners. Since the first communications on the design and DNA binding properties of synthetic ligands of this class [6,7] a considerable progress has been achieved [8–17]. It was shown that some of these compounds exhibit a high binding specificity and inhibit selectively initiation of transcription directed by certain procaryotic promoters [7]. Bis-linked netropsin derivatives selectively inhibit activity of topoisomeases I and II [18,19] and HIV-I reverse transcriptase [20]. It was found that two distamycin (or lexitropsin) molecules can be packed in the minor groove, with the peptide group of one drug molecule stacked on the aromatic ring of the other [21–23]. Antiparallel hairpin motifs containing different sequences of *N*-methylpyrrole and *N*-methylimidazole residues can recognize a broad category of nucleotide sequences in the minor groove [13–17]. Recently it has been reported that two parallel oligopyrrolecarboxamide strands can be sandwiched in the minor DNA groove [24].

In the present work, we report on the DNA-binding properties of *cis*-diammine Pt(II)-bridged bis-netropsin (←**Nt-Pt(NH_3)$_2$-Nt**→), *cis*-diammine Pt(II)-bridged bis-distamycin (←**Dst-Pt(NH_3)$_2$-Dst**→) and oligomethylene-bridged bis-netropsin (←**Nt-(CH_2)$_5$-Nt**→) (Fig. 1). Interaction of netropsin and distamycin with DNA has been previously studied by quantitative footprinting [8,9]. However, there have been no reports which examine systematically the affinities of bis-linked netropsin (distamycin) derivatives to different AT-rich sequences. To facilitate comparison of the binding of sequence-specific ligands to different AT-rich sites we have used cloned synthetic DNA fragments as substrates for footprinting studies.

2. Materials and methods

2.1. Ligands

Distamycin A and netropsin were purchased from Sigma. ←**Nt-Pt(NH_3)$_2$-Nt**→, ←**Dst-Pt(NH_3)$_2$-Dst**→ and ←**Nt-(CH_2)$_5$-Nt**→ (Fig. 1) were synthesized and purified as described elsewhere [25]. The concentrations of ←**Nt-Pt(NH_3)$_2$-Nt**→ and ←**Nt-(CH_2)$_5$-Nt**→ were determined spectrophotometrically, using a value for the molar absorbance at 297 nm of 42 000 M^{-1} cm^{-1}. The concentration of ←**Dst-Pt(NH_3)$_2$-Dst**→ was determined at 303 nm, using a value for the molar absorbance of 60 000 M^{-1} cm^{-1}. The CD spectra were recorded on a Jasco Model 720 instrument using 0.1-, 0.2- and 1.0-cm cells.

2.2. DNA fragments and DNase I footprinting studies

The oligonucleotides containing pseudosymmetrical nucleotide sequences were synthesized on the phosphoramidite chemistry by using an automated synthesizer from Applied Biosystems. Each oligonucleotide was cloned in the polylinker region of plasmid pGEM7Z(f+) (Promega). The ligation mixtures were transformed into *Escherichia coli Sure* and successful clones were isolated in the usual way as white colonies from agar plates containing X-gal and IPTG. The modified plasmid containing inserted oligonucleotide was used in a second round of cloning with another oligonucleotide. The plasmid containing all the inserts was digested by *Xba*I restrictase (Promega), labelled at the *Xba*I site with [^{33}P]CTP using Klenow fragment (Pharmacia) and then digested with *Nsi*I restrictase (Promega). DNase I footprinting was performed according to the protocol outlined in [15]. Samples (5 μl) of radioactively labelled *Xba*I-*Nsi*I restriction fragment (around 1.5 μM base pairs) in 10 mM Tris-HCl buffer (pH 7.6) were mixed with a solution (5 μl) of the appropriate ligand in 10 mM Tris-HCl buffer (pH 7.6) in the presence of 0.5 M NaCl and kept at 20°C for

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20 min and then digested with 10 μl of DNase I solution (0.5 μg/ml) in DNase buffer (10 mM Tris-HCl buffer (pH 7.6), 0.25 M NaCl and 5 mM $MnCl_2$) for 3 min at 20°C. The reaction was stopped by adding 90 μl of the solution containing 50 mM Tris-HCl buffer (pH 7.5), 0.15 M NaCl, 10 mM EDTA and 10 μg/ml tRNA.

2.3. Determination of thermodynamic parameters

The occupancy of the 12-mer DNA oligomer containing eight successive AT-base pairs by bis-netropsin bound in the extended conformation (R_1), hairpin (R_2) and dimer (R_3) forms can be calculated from the following equations:

$$R_1 = K_1 m/Z \quad (1)$$

$$R_2 = (5K_2 m + 2K_2^2 m^2)/Z \quad (2)$$

$$R_3 = 2Wm^2/Z \quad (3)$$

$$m = C - O(R_1 + R_2 + R_3) \quad (4)$$

$$W = K_d K_3 \quad (5)$$

$$Z = 1 + K_1 m + 5K_2 m + K_2^2 m^2 + Wm^2 \quad (6)$$

$$\Delta D/O = \Delta\varepsilon_1 R_1 + \Delta\varepsilon_2 R_2 + \Delta\varepsilon_3 R_3 \quad (7)$$

where C is the concentration of the ligand; Z is the grand partition function for the system under study; K_1 and K_2 are the affinity constants for binding of a bis-netropsin to the 12-mer DNA oligomer in the extended conformation and hairpin form, respectively; K_d is the dimerization constant; K_3 is the binding constant of dimer species to the DNA; $\Delta D/O$ is the measured CD amplitude at 310 nm expressed per mol of DNA oligomer and 1 cm pathlength cell; O is the molar concentration of the DNA oligomer; $\Delta\varepsilon_1$ and $\Delta\varepsilon_2$ are the molar dichroism values for bis-netropsin binding in the extended conformation and hairpin form, respectively; $\Delta\varepsilon_3$ is the molar dichroism value of antiparallel 2:1 complex between bis-netropsin and DNA oligomer. The molar dichroism values at 310 nm were found to be $\Delta\varepsilon_1 = 85 \pm 5$, $\Delta\varepsilon_2 = -5 \pm 1$, and $\Delta\varepsilon_3 = 16 \pm 1$ [24]. Thermodynamic parameters were determined from CD titration curves at 310 nm by iterative least square fitting of the experimental plots of $\Delta D/O$ versus C/O to Eqs. 1–7 [24].

3. Results

It is of interest to compare the sequence specificity exhibited by *cis*-diammine platinum-bridged bis-netropsin (distamycin) with that of netropsin and distamycin A under the same conditions. In order to accomplish this we used in the footprinting studies a specially constructed DNA fragment containing a series of potential interaction sites for netropsin, distamycin A and bifunctional derivatives. Fig. 3 presents an autoradiograph of DNase I protection patterns observed in the presence of various concentrations of ←**Nt-Pt(NH$_3$)$_2$-Nt**→, ←**Dst-Pt(NH$_3$)$_2$-Dst**→, **Nt** and **Dst**. Netropsin and distamycin protect from DNase I cleavage a series of regions on the DNA fragment whose sequence is shown in Fig. 2, as is made clear in Fig. 3. Each protection zone is associated with a cluster of four or more AT-base pairs. Looking at the results for Pt-bridged bis-netropsin and bis-distamycin, it can be seen that only a few protection zones are produced. Clear footprints are produced around 5′-TTTTAAAA-3′ at low ligand concentrations (0.2–0.5 μM), while footprints at sites with sequences 5′-AAAAATTTTT-3′ and 5′-TTTTGAAAA-3′ require higher ligand concentrations. The strongest affinity site has the sequence 5′-TTTTAAAA-3′. Some protection at this DNA region is seen in the presence of 0.1 μM bis-netropsin. Footprinting patterns obtained for ←**Dst-Pt(NH$_3$)$_2$-Dst**→ do not differ significantly from those obtained with ←**Nt-Pt(NH$_3$)$_2$-Nt**→. The binding of ←**Dst-Pt(NH$_3$)$_2$-Dst**→ seems to be more tolerant to insertion of single GC pairs in AT-tracks. As with bis-netropsin, the preferred binding sequence for Pt-

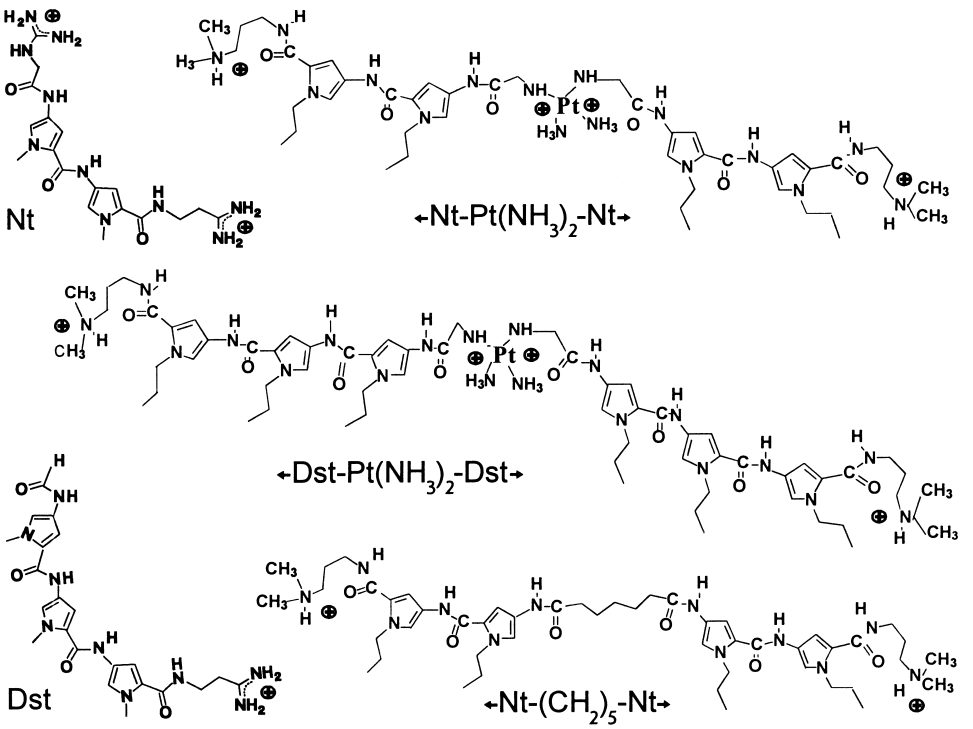

Fig. 1. Chemical structures of netropsin (**Nt**), distamycin A (**Dst**), *cis*-diammine Pt(II)-bridged bis-netropsin (←**Nt-Pt(NH$_3$)$_2$-Nt**→), *cis*-diammine Pt(II)-bridged bis-distamycin (←**Dst-Pt(NH$_3$)$_2$-Dst**→) and oligomethylene-bridged bis-netropsin (←**Nt-(CH$_2$)$_5$-Nt**→). The arrows indicate directions from the N-terminus to the C-terminus in each peptide fragment.

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5′-GCGAAAAGGTTTTACGCGCGATTTCGAACCCCGAAAAGGGTTTTCGGGGGTACCCC
3′-CGCTTTTCCAAAATGCGCGCTAAAGCTTGGGGCTTTTCCCAAAAGCCCCCATGGGG

   CGCTTTTTAAAAAGCGGGTTCGAAATCGCGCAAAAATTTTTGCGCGATAAGCTTGG
   GCGAAAAATTTTTCGCCCAAGCTTTAGCGCGTTTTTAAAAACGCGCTATTCGAACC

   ATCTCCGGACGTTTTTGAAAAACGGATCCGCGATATCCATATCGCGGATCCAAGCT
   TAGAGGCCTGCAAAAACTTTTTGCCTAGGCGCTATAGGTATAGCGCCTAGGTTCGA

   CGCGTTTTGGAAAACGCGAGCCGTTTTGCAAAACGCGATCCGTTTTGGGAAAACGC
   GCGCAAAACCTTTTGCGCTCGGCAAAACGTTTTGCGCTAGGCAAAACCCTTTTGCG

   GAATTCGGTACCCCCGCTATAGGTATACGCGGGGTTCGAAATCGGGGAAAAAAAAA
   CTTGAGCCATGGGGGCGATATCCATATGCGCCCCAAGCTTTAGCCCCTTTTTTTTT

   CCCCGGGGTTTTCTTTTCCCCGGGGAAAAGAAAACCCCGATAAGCTTGGATCCGG-3′
   GGGGCCCCAAAAGAAAAGGGGCCCCTTTTCTTTTGGGGCTATTCGAACCTAGGCC-5′
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Fig. 2. Nucleotide sequence of the *Xba*I-*Nsi*I restriction fragment derived from of a modified plasmid pGEM7Z(f+). The strand shown is the one seen by labelling the fragment at the 3′-end of the *Xba*I site. Indicated are the oligonucleotide inserts which serve as substrates in footprinting studies.

bridged bis-distamycin on the DNA fragment is 5′-TTTTAAAA-3′. However, X-ray-induced cleavage diagrams show that the most preferred site for binding of ←**Dst-Pt(NH$_3$)$_2$-Dst**→ involves a longer sequence 5′-TXTTTA-AAYA-3′, where X and Y are either A or T ([11] and unpublished data). Netropsin and distamycin A exhibit far less sequence discrimination than Pt-bridged bis-netropsin and bis-distamycin. Bis-derivatives do not practically bind to DNA regions in which two blocks of four or five AT-base pairs are separated by CC, CG, GC, GG and GGG steps. In contrast, netropsin and distamycin bind to clusters of AT-pairs in all these regions, irrespective, in the first approximation, of the flanking sequences. In addition, at low concentrations of netropsin and distamycin (0.1 μM) clear footprints are produced around tetramers 5′-AAAT-3′ and 5′-AATT-3′. These sites are not protected by Pt-bridged bis-netropsin (distamycin) at the highest concentrations used (1 μM).

Our observations that DNA regions with symmetry-related nucleotide sequences 5′-TTTTAAAA-3′ and 5′-AAAAT-TTT-3′ have different affinities for Pt-bridged bis-netropsin are supported by CD studies on binding of the synthetic ligand to 12-mer duplexes with sequences 5′-CGTTTTAA-AACG-3′ (**I**) and 5′-CGAAAATTTTCG-3′ (**II**). Fig. 4 shows the CD spectra for complexes between DNA oligomer **I** and ←**Nt-Pt(NH$_3$)$_2$-Nt**→ as well as difference CD spectra obtained by subtracting the CD spectra of DNA oligomers **I** and **II** from the CD spectra of corresponding complexes with the synthetic ligand. For a complex with duplex **I**, the maximum of the CD band occurs at 316 nm. The molar CD absorbance at 316 nm per mol of DNA oligomer (75 ± 1) is approximately twice that found for binding of a netropsin analogue to poly(dA)·poly(dT), 33 ± 2 [15]. This indicates that both monomer fragments of the bis-netropsin molecule are bound to DNA. Fig. 5 shows the typical plots of the CD amplitude at 310 nm expressed per mol of DNA oligomer ($\Delta D/O$) against the molar ratio of added ligand to the DNA oligomer (C/O). We found that ←**Nt-Pt(NH$_3$)$_2$-Nt**→ binds more strongly to oligomer **I** than to oligomer **II**. The complex with oligomer **I** is stable in the presence of 3.0 M NaCl, whereas the complex with oligomer **II** is found to be less resistant to the salt administration. Approximately half of the total amount of the bis-netropsin complexed with the duplex **II** in 1 mM sodium cacodylate buffer (pH 7.0) in the presence of 0.1 M NaCl dissociates in the presence of 0.9 M NaCl (Fig. 5b). The higher affinity of ←**Nt-Pt(NH$_3$)$_2$-Nt**→ to the duplex **I** can be attributed to the conformational properties of the DNA oligomer which contains a centrally located TpA step and may have a widened minor groove [26].

The binding specificity of Pt-bridged bis-netropsin and bis-distamycin may be influenced in some way by the *cis*-diammine platinum group. To check the validity of this proposal we studied the interaction with the oligomers **I** and **II** of another bis-netropsin (←**Nt-(CH$_2$)$_5$-Nt**→) in which two netropsin residues were bridged by an aliphatic linker (Fig. 1). Fig. 5 shows the CD titration curves obtained from binding of ←**Nt-(CH$_2$)$_5$-Nt**→ to the oligomers **I** and **II**. As before, the binding approaches saturation when one bis-netropsin mole-

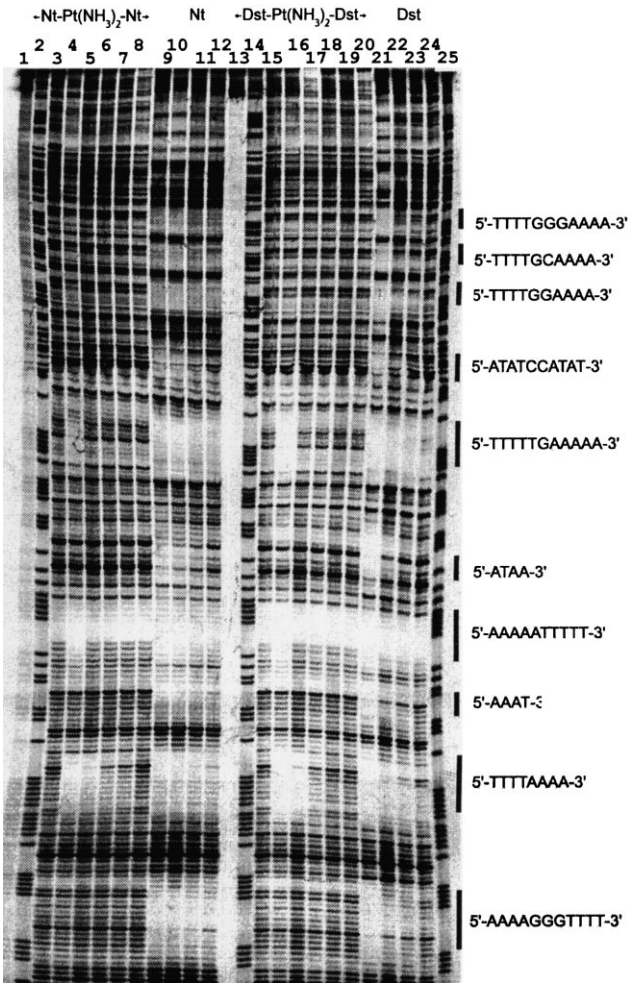

Fig. 3. DNase I cleavage protection patterns generated by treatment of the DNA fragment with DNase I in the presence of increasing concentrations of ←**Nt-Pt(NH$_3$)$_2$-Nt**→, **Nt**, ←**Dst-(NH$_3$)$_2$-Dst**→ and **Dst**. Lanes 1 and 13: Free DNA fragment; lanes 2, 14 and 25: Maxam-Gilbert's purine sequence markers; lanes 3, 8, 15 and 20: DNase I cleavage products of the free DNA; lanes 4–7: cleavage products generated by DNase I treatment in the presence of Pt-bridged bis-netropsin at concentrations 1, 0.5, 0.25 and 0.125 μM, respectively; lanes 9–12: DNase I cleavage products generated in the presence of 1, 0.5, 0.25 and 0.125 μM netropsin; lanes 16–19: cleavage products generated in the presence of 1, 0.5, 0.25 and 0.125 μM Pt-bridged bis-distamycin; lanes 21–24: cleavage products generated in the presence of distamycin at concentrations of 1, 0.5, 0.25 and 0.125 μM, respectively. Sequences on the right side correspond to the oligonucleotide inserts into the DNA fragment.

cule is bound per duplex. Examination of the CD spectral profiles obtained for complexes of ←**Nt-Pt(NH$_3$)$_2$-Nt**→ with duplex **II** reveals that at occupancy approaching the saturation level of binding the CD spectra can be well represented as a linear combination of two reference CD patterns: the CD spectrum characteristic of bis-netropsin binding in the extended conformation and CD spectrum corresponding to a complex containing antiparallel dimer motif. At $C/O = 1.2$ about 26% of bound bis-netropsin exist as side-by-side dimers (Fig. 4c). The same situation is observed for complexes of ←**Nt-(CH$_2$)$_5$-Nt**→ with the duplex **I**, but in this case the fraction of the bis-netropsin bound in a dimer form is even greater (about 45%). Decomposition of the CD spectrum for a complex between ←**Nt-(CH$_2$)$_5$-Nt**→ and DNA oligomer **II** at $C/O = 1.1$ shows that fractions of the extended conformation, hairpin and dimer forms are 40, 16 and 44%, respectively. This indicates that each bis-netropsin in the extended conformation, hairpin and dimer forms exhibits different sequence preferences. The magnitudes of binding constants determined

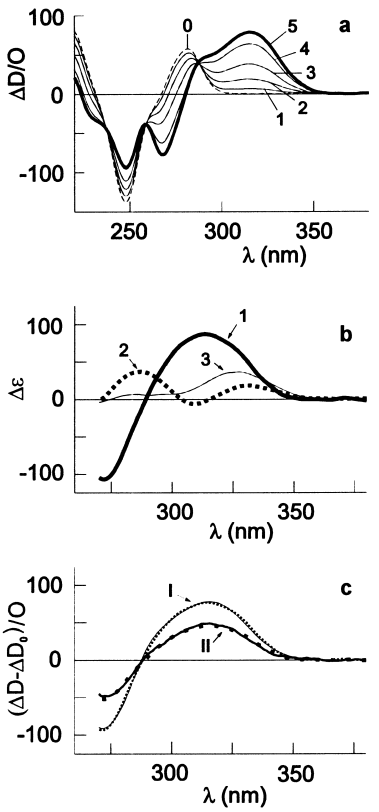

Fig. 4. a: CD spectra for complexes of ←**Nt-Pt(NH$_3$)$_2$-Nt**→ with DNA oligomer **I** (28 μM) at different molar ratios of ←**Nt-Pt(NH$_3$)$_2$-Nt**→ to the duplex (C/O). The C/O values were as follows: 0, 0; 1, 0.09; 2, 0.27; 3, 0.55; 4, 0.91; 5, 1.37; 6, 1.91. The CD spectra were recorded in 1 mM sodium cacodylate buffer (pH 7.0) in the presence of 0.1 M NaCl at 20°C. b: Difference CD patterns calculated for binding of ←**Nt-Pt(NH$_3$)$_2$-Nt**→ in the extended conformation (1), hairpin (2) and dimer (3) forms. c: Decomposition of the difference CD patterns **I** and **II** obtained by subtracting the CD spectra of oligomers **I** (19 μM) and **II** (13 μM) from the spectra of their complexes with ←**Nt-Pt(NH$_3$)$_2$-Nt**→ at C/O values of 1.4 and 1.2, respectively. The experimental and calculated CD patterns are shown by solid and dotted lines, respectively. The contents of the extended conformation, hairpin and dimer forms were 0.82, 0.18 and zero for ←**Nt-Pt(NH$_3$)$_2$-Nt**→ complexed with the duplex **I** and were 0.74, zero and 0.26, respectively, for ←**Nt-Pt(NH$_3$)$_2$-Nt**→ bound to the duplex **II**.

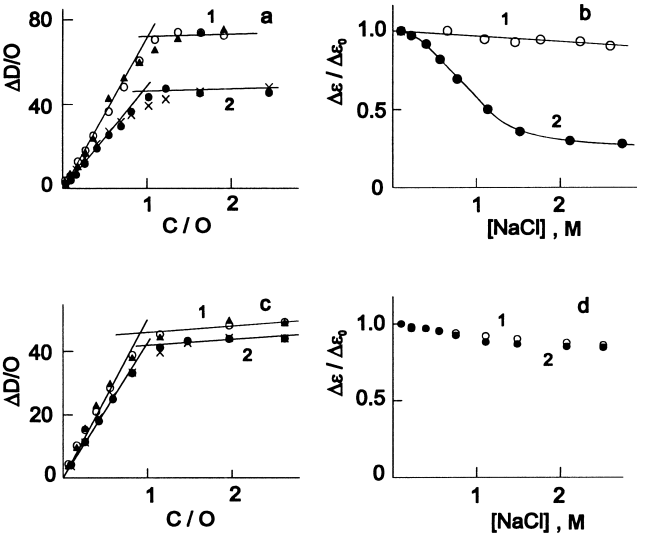

Fig. 5. CD titrations of duplexes **I** (26 μM) and **II** (17.5 μM) by ←**Nt-Pt(NH$_3$)$_2$-Nt**→ (a) and duplexes **I** (14 μM) and **II** (14 μM) by ←**Nt-(CH$_2$)$_5$-Nt**→ (c). ○ and ●, Data points from titrations of duplexes **I** (1) and **II** (2), respectively. ▲ and ×, Theoretical values of $\Delta D/O$ calculated from Eqs. 1–7 at different C/O values, using the best fit values for binding parameters. Panel a: $K_1 = 6.4\times10^5$ M^{-1}, $K_2 = 5\times10^3$ M^{-1} and $W = 1.0$ M^{-2} for binding to duplex **I** and $K_1 = 1.8\times10^5$ M^{-1}, $K_2 = 5\times10^3$ M^{-1} and $W = 7\times10^9$ M^{-2} for binding to duplex **II**. Panel c: $K_1 = 2.2\times10^5$ M^{-1}, $W = 1.6\times10^{10}$ M^{-2} and $K_2 = 1.0$ M^{-1} for binding to duplex **I** and $K_1 = 2.1\times10^5$ M^{-1}, $K_2 = 1.5\times10^4$ M^{-1} and $W = 3.6\times10^9$ M^{-2} for binding to duplex **II**. The mean square deviations of the experimental and calculated $\Delta D/O$ values were 9.4 and 6.5 for complexes of ←**Nt-Pt(NH$_3$)$_2$-Nt**→ with duplexes **I** and **II**, respectively, and were 1.1 and 0.6, respectively, for complexes of ←**Nt-(CH$_2$)$_5$-Nt**→ with the same duplexes. Stabilities of (b) ←**Nt-Pt(NH$_3$)$_2$-Nt**→ and (d) ←**Nt-(CH$_2$)$_5$-Nt**→ complexes with duplexes **I** (○) and **II** (●) as functions of NaCl concentration. $\Delta\varepsilon_0$ is the CD amplitude measured at 310 nm in the presence of 0.1 M NaCl. $\Delta\varepsilon$ is the same quantity measured at higher NaCl concentrations. Concentrations of DNA oligomers **I** and **II** were equal to 17 μM (panel b, $C/O = 2$) and 12.7 μM (panel d, $C/O = 2.6$).

from the CD titration curves by iterative least square data fitting procedure [24] are consistent with the results of deconvolution analysis (see legend to Fig. 5). We found that Pt-bridged bis-netropsin in the extended conformation exhibits three times higher affinity to DNA oligomer **I** than to oligomer **II**. In contrast, ←**Nt-(CH$_2$)$_5$-Nt**→ in the extended conformation binds to duplexes **I** and **II** with nearly the same affinity. Interestingly, both complexes are stable in the presence of 3.0 M NaCl (Fig. 5d).

4. Discussion

Footprints produced by Pt-bridged bis-netropsin and bis-distamycin on the DNA fragment exhibit only a few protection zones in the presence of 0.25 M NaCl. Each protection zone corresponds to the preferred interaction site for a synthetic ligand. The strongest affinity site for ←**Nt-Pt(NH$_3$)$_2$-Nt**→ has the sequence 5′-TTTTAAAA-3′ [11] which differs only in one position from that found in a strong interaction site for ←**Nt-Pt(NH$_3$)$_2$-Nt**→ in the regulatory region for human MPA-kinase gene [27]. The inappropriate expression level of the gene could be responsible for pathology caused by Alzheimer disease [27]. Quantitative DNase I footprinting

showed that tetramers AATT and AAAA are better binding sites for distamycin and netropsin than TTAA and TATA [28]. Pt-bridged bis-netropsin and bis-distamycin exhibit a higher binding specificity than netropsin and distamycin, in spite of the fact that two monomers in the bis-netropsin molecule have a lower affinity for DNA than netropsin itself. At low extents of binding both Pt-bridged bis-netropsin and bis-distamycin bind to DNA predominantly in the extended conformation. Each bound molecule covers approximately one turn of the DNA helix in such a way that both netropsin (distamycin)-like fragments are implicated in specific interaction with DNA base pairs. The observed preference of Pt-bridged bis-netropsin and bis-distamycin for binding to DNA regions with sequences 5′-TTTTAAAA-3′ or 5′-TTTTTAAAAA-3′ and their lower affinities to sites in which blocks of Ts and As are interchanged can be explained by variation in the minor groove width and/or sequence-dependent deformability inherent to these DNA regions. The wider minor groove is observed in the centre of the dodecamer duplex d(CGCGTTAACGCG)$_2$ as compared with the width of the minor groove in the oligomer d(CGCGAATTCGCG)$_2$ [26]. It is well-documented that TpA steps are associated with the capacity of DNA to be bent in such a way that the minor groove is widened upon interaction with the groove binders [29,30]. The increased width of the minor groove might be needed to accommodate simultaneously the *cis*-diammine platinum residue and two netropsin-like fragments of the bis-netropsin molecule in the minor groove and to form specific contacts with both parts of the DNA target site. ←**Nt-(CH$_2$)$_5$-Nt**→ containing a flexible aliphatic linker exhibits less sequence discrimination.

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