Single and Bis Peptide Nucleic Acids as Triplexing Agents: Binding and Stoichiometry

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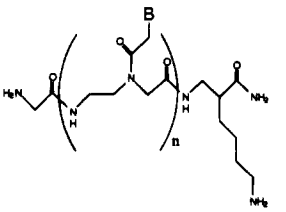
Homopyrimidine peptide nucleic acids (PNAs) form stable triplexes with single-stranded (ss) oligonucleotides and invade double-stranded (ds) DNA.[1−3] The resulting triplexes consist of two PNAs bound to one DNA strand. A number of oligonucleotide-dependent enzymatic functions are inhibited by PNA/DNA or PNA/RNA complexes, including restriction enzyme cleavage, transcription, reverse transcription, and translation.[4−6] These results have made PNAs attractive agents for oligonucleotide research and therapeutics.

The slow rate of strand invasion at physiological-level ionic strengths needs to be addressed in order to further the use of triplexing PNAs as research tools and antigene agents.[7] One approach links the two PNA strands together to reduce entropy and convert binding into a bimolecular process. Egholm et al. have described the use of two N-terminus to C-terminus linked PNAs (bis-PNA) for triplex formation.[8] We have prepared bis-PNAs connected by either a neutral (poly(ethylene glycol)-based) or a positively charged (lysine/aminohexyl) linker and utilized gel shift experiments to determine the relative affinities and binding rates of these PNAs for ds and ss DNA. Additionally, we determined the stoichiometry of the complex between PNA and DNA by direct observation via electrospray mass spectrometry.

The four PNAs shown in Figure 1 were synthesized by standard methods[9−11] and analyzed by HPLC and electrospray mass spectrometry. The bis-PNA **1** containing the positively charged linker gave substantially better strand invasion of the ds target than either the single PNA **3** or the PEG-linked bis-PNA **2**, as measured by the concentration at which 50% of target ds DNA was bound (Table 1). The time course for invasion at a fixed PNA concentration was also examined. At PNA concentrations of 250 nM, **1** bound 50% of the target in 19 h, whereas **3** showed only 2% binding after 96 h (Figure 2a); at higher PNA concentration (10 μM), **1** bound 50% of the target in less than 5 min.

The exceedingly slow off-rate demonstrated in Figure 2b for both **1** and **3** complexed to a target indicates that the data plotted in Figure 2a measure an on-rate. Nielsen et al. have proposed a model for PNA invasion of ds DNA in which the first PNA strand binds reversibly to duplex DNA while the second strand "locks" the complex in a triplex in an essentially irreversible step.[5,12] Although the exact mechanism for PNA invasion of ds DNA is not yet understood, the rapid binding of bis PNA **1**



Above is a generic PNA structure with amino acid end-groups to enhance solubility and stability. B is the nucleobase unit (N^1-alkylated cytosine, N^9-alkylated adenine, etc.).

PNAs

1 H-Gly-TTC-TCT-CTC-T-Lys-Aha-Lys
H_2N-Lys-TTC-TCT-CTC-T-Lys-Aha

2 H-Gly-TTC-TCT-CTC-T-3PEG
H_2N-Lys-TTC-TCT-CTC-T-3PEG

3 H_2N-Lys-TTC-TCT-CTC-T-Gly-H*

4 Ac-Lys-Aha-Lys
H_2N-Lys-TTC-TCT-CTC-T-Lys-Aha

*written carboxy to amino for sequence comparison

DNA Targets

A 65mer duplex containing target site: 5'-----AAGAGAGAGA-----3'
3'-----TTCTCTCTCT------5'

B 65mer duplex containing target site: 5'-----AGAGAGAGAA-----3'
(reverse orientation) 3'-----TCTCTCTCTT------5'

C single stranded target 5'-TAAGAGAGAGATGTTA-3'

D single stranded target 5'-ATTGTAGAGAGAGAAT-3'
(reverse orientation)

Figure 1. PNA and DNA sequences. Aha, 6-aminohexanoic acid; 3PEG, $H_2N(CH_2CH_2O)_3CH_2COOH$.[14]

Table 1. EC_{50} for Binding of PNA to Double- and Single-Strand DNA Targets[a]

Strand Invasion		
PNA	target **A**	target **B**
1	50 nM	40 nM
2	22 μM	40 μM
3	28 μM	65 μM
4	6 μM	6 μM

Single-Strand Binding		
PNA	target **C**	target **D**
1	300 pM	275 pM
2	225 pM	250 pM
3	20 nM	12 nM
4	100 nM	200 nM

[a] PNA binding was measured using a gel mobility assay.[15] We define EC_{50} as the PNA concentration at which 50% of the target is bound. Radiolabeled target (20 pM) was incubated for 3 days (ds target) or 1 day (ss target) with increasing concentrations of PNA at 37 °C in 100 mM Na^+, 10 mM phosphate, and 0.1 mM EDTA, pH 7.0 (charge balance made up by Cl^-).

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(9) Solid phase PNA synthesis used the "in situ neutralization" approach[10] as optimized for PNA by L. Christensen et al. Christensen's work was presented in the 3rd Solid phase Symposium in Oxford, U.K., in September 1994 and is available as Technical Information Fax No. 1800 from Millipore Corp., Bedford, MA 01730. PNA monomers were purchased from Millipore Corp. with *tert*-butyloxycarbonyl protection of the aliphatic primary amine and benzyloxycarbonyl protection of the nucleobases' exocyclic amines. Glycine, lysine, and 6-aminohexanoic acid were coupled using the same procedure and also bore *tert*-butyloxycarbonyl protection of the backbone aliphatic amino groups; the ϵ-amino group of lysine was protected as the 2-chlorobenzyloxy carbamate. A methylbenzhydrylamine-derivatized polystyrene support was used. For further details, see supplementary material. Deprotection and cleavage from support was accomplished via the low/high TFMSA method.[11]
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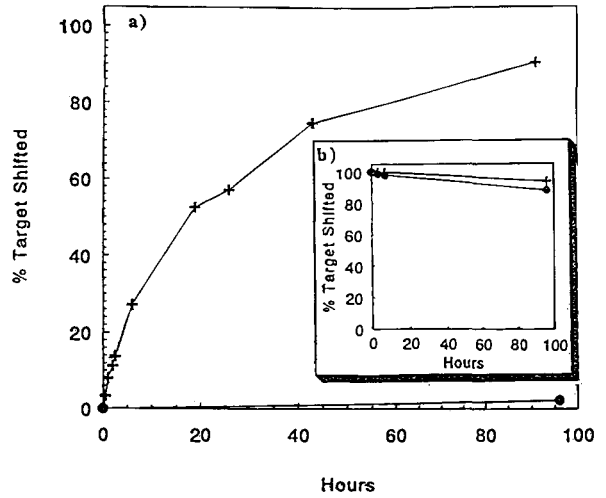

Figure 2. Kinetics of strand invasion by PNAs **1** (+) and **3** (●). (a) Binding: 250 nM PNA was incubated with 50 pM target **B** at 37 °C in the buffer described in Table 1. After hybridization for the indicated time, fraction target hybridized was evaluated via a standard gel mobility assay.[15] (b) Dissociation: invaded complex was preformed by incubation of 250 nM PNA with 50 pM target **B** for 16 h at 37 °C in 10 mM sodium phosphate, pH 7.0, and 0.1 mM EDTA. At $t = 0$, the solution was brought to 100 mM in sodium (charge balance made up by chloride), and 10 μM single-stranded target **D** was added to capture PNA as it dissociated from **B**. Aliquots were removed at the indicated times, and the fraction hybridized was evaluated using a gel mobility assay.[15]

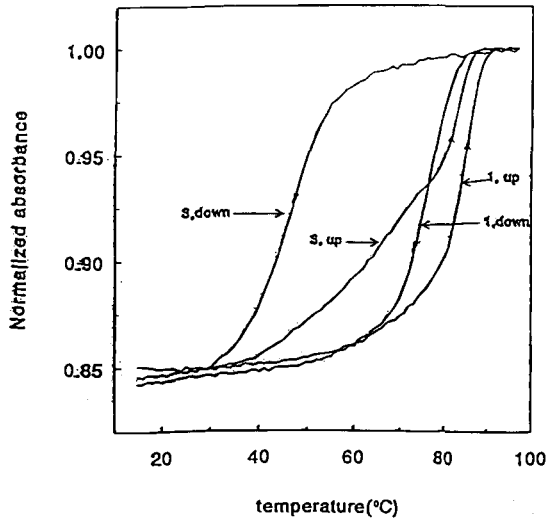

Figure 3. Absorbance vs temperature profiles for bis-PNA **1** and PNA **3** hybridized to DNA complement **D** in 100 mM Na^+, 10 mM phosphate, and 0.1 mM EDTA, pH 7.0. The melting (dissociation) curves for the complexes with **1** and **3** are designated **1 up** and **3 up**, respectively. The cooling (reassociation) curves are similarly labeled as **1 down** and **3 down**. A temperature ramp of 1 °C/min was used.

is consistent with binding of the second PNA strand being rate-limiting for triplex formation.

The enhanced affinity and faster binding observed for ds DNA invasion by the bis PNA **1** might be due to electrostatic interactions of the cationic lysine-based linker with the high negative charge density on the ds target. The single PNA **4**, however, containing the Lys_3/aminohexyl$_2$ linker, showed only a slight increase in binding over the single PNA for both ss and ds DNA. No preference was observed for the linker being oriented toward the 5′ end of the target versus the 3′ end. The binding of ss DNA, unlike that of the ds target, was substantially improved for both bis-PNAs **1** and **2** compared to single PNA **3** (Table 1). Melting data for the complex formed between **1** and the ss target showed more cooperative melting, higher T_m, and smaller hysteresis than observed for the single PNA **3** (Figure 3). For the ss target, results obtained for the PEG-linked bis-PNA were similar to those seen with **1** (data not shown).

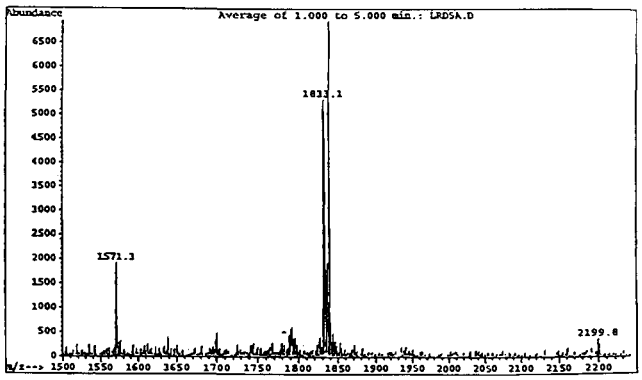

Figure 4. Mass spectrum of the bis-PNA/DNA complex. The molecular weights of the individual components are 6012 and 4991 for **1** and **D**, respectively. The peaks observed at $m/z = 1571$, 1833, and 2199 correspond respectively to the 7-, 6-, and 5-charge states of the 1:1 complex of mass 11 004. Some mono- and disodium adducts of the complex are also observed.

We also examined the stoichiometry of binding of the bis-PNAs to ss DNA in order to assess formation of the desired 1:1 complex. A comparison of the gel shift mobilities of the complexes of **1**–**4** with the ss target showed that **4** was the most retarded, followed by **1**, while the complexes with **2** and **3** moved the fastest and approximately equal distances. When both the size and the charge of each PNA are considered, these results are consistent with the model that single PNAs form 2:1 complexes with target while bis-PNAs form 1:1 complexes. This qualitative analysis was confirmed by electrospray mass spectrometry of the ss DNA complexes of **1** and **3** (Figure 4). The molecular weight observed for the complex of bis-PNA **1** with ss DNA was that of a 1:1 complex, with less than 5% of the 2:1 complex present.[13] Conversely, the molecular weight seen for the single PNA **3** complexed to the ss target was that expected for a 2:1 complex, with only a very minor amount (<5%) of the 1:1 complex present (data not shown).[13]

We have shown that the covalent linkage of two PNAs, particularly via a positively charged lysine-containing linker, significantly increases the rate of invasion of ds DNA as well as the affinity for ss DNA. The stoichiometry of binding of both single and bis-PNAs to ss DNA has also been determined by direct mass spectrometric observation of the complexes. Future work will further investigate the details of PNA/DNA and PNA/RNA hybridization to asses the potential utility of PNAs as antigene and antisense agents.

Acknowledgment. The authors would like to thank Dr. Peter E. Nielsen for helpful discussions.

Supplementary Material Available: Synthesis and characterization of **1**–**4** (7 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

JA942870K

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