Communication

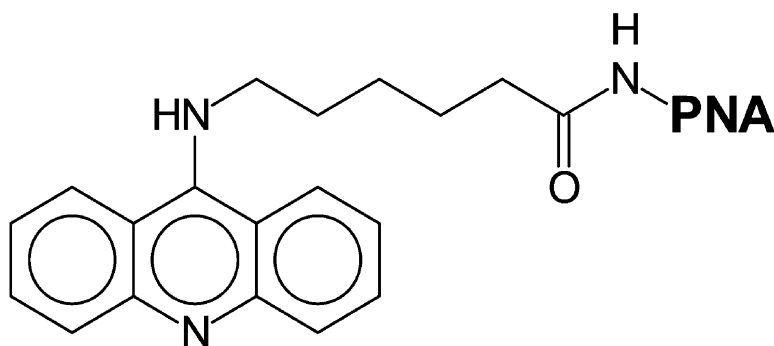# Superior Duplex DNA Strand Invasion by Acridine Conjugated Peptide Nucleic Acids

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Superior Duplex DNA Strand Invasion by Acridine Conjugated Peptide Nucleic Acids

Thomas Bentin and Peter E. Nielsen*

Center for Biomolecular Recognition, IMBG, Department B, The Panum Institute, University of Copenhagen, Blegdamsvej 3c, 2200 Copenhagen N, Denmark

Received December 27, 2002; E-mail: pen@imbg.ku.dk

Homopyrimidine peptide nucleic acid oligomers (PNAs) bind sequence complementary targets in duplex DNA with high affinity and sequence specificity by helix invasion forming triplex P-loop complexes.[1] A range of molecular biology applications have emerged using such triplex invading PNAs,[2] and obviously gene targeting applications for functional genomics and drug discovery are also considered.[3] However, the helix invasion mechanism makes the binding very sensitive to elevation of ionic strength as this stabilizes the target DNA duplex, and simple PNAs cannot effectively bind their target at physiologically relevant ionic conditions. Therefore, novel PNA constructs with improved helix invasion properties at elevated ionic strength are warranted. We now report that simply conjugating a DNA intercalating 9-aminoacridine moiety to the PNA is a major step toward this goal.

It has been shown that positively charged PNAs bind their target in duplex DNA with significantly increased efficiency. Optimally, a bis-PNA having 4−6 positive charges should be used, and such PNAs can indeed bind their target at physiologically relevant ionic strength conditions (140 mM K^+).[4] Likewise, it was recently shown that larger cationic peptide domains have an analogous effect.[5] We argue that the cationic nature of such PNAs provides sequence neutral electrostatic affinity for the DNA and ensures a high local concentration around the anionic DNA, thereby increasing the probability for invasion events during natural DNA "breathing". We reasoned that a similar and expectedly less ionic strength sensitive effect could be attained by using a DNA intercalator.

We therefore synthesized a set of mono- and bis-PNAs conjugated to the DNA intercalator 9-aminoacridine (Figure 1).[6,7] We chose the simplest sequence (T_{10}) that does not contain cytosines as this would allow us to directly compare mono- and bis-PNAs without the added complication for cytosine protonation in the Hoogsteen binding strand. Furthermore, we introduced charges in the backbone of the PNA via lysine modified PNA units.[6b] The DNA binding efficiency[8] of these PNAs was measured using an electrophoretic mobility shift assay[9] at various ionic strength conditions (Figure 2a, Table 1). The results show that the DNA binding of the bis-PNA (1743) with three positive charges is sensitive to increasing concentrations of K^+ (100-fold decrease in binding is observed at 150 mM K^+/Na^+ as compared to 10 mM Na^+) and the binding is reduced further 5-fold upon addition of 2 mM Mg^{2+}.[10] Adding an extra charge to the PNA (2399) has only a marginal effect on this behavior. However, conjugation of the DNA intercalator 9-aminoacridine (PNA1765) significantly reduces the sensitivity to physiological ionic strength. At 150 mM K^+/Na^+, the binding is reduced only 10-fold, and addition of 2 mM Mg^{2+} causes only an extra 2-fold reduction. Thus, the 50% binding percentile at 2 h incubation of this acridine-PNA conjugate is 0.4 μM (Table 1), which is 20−40 times lower than that of the corresponding PNAs without the 9-aminoacridine (PNAs 1743 and 2399). This enhancer effect of the 9-aminoacridine was even more
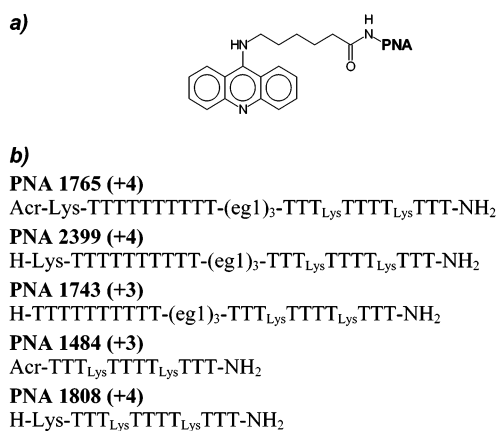

Figure 1. PNAs used in this study. (a) Chemical structure of the acridine moiety. (b) Structure and charge at pH 6.5 of the PNAs. Abbreviations: Acr (9-aminoacridine), eg1 (8-amino-2,6-dioxaoctanoic acid), Lys (lysine), T (thymine), T_{Lys} (thymine monomer having D-lysine instead of glycine in the backbone).[6b] The PNA oligomers were synthesized as described,[6a,b] and the 6-(9-acridinyl)aminohexanoic acid was attached to the amino terminal on the solid support.[6c] The pK_a value of 9-aminoacridine is 9.6 at 37 °C.[7] Acr thus contributes +1 under the experimental conditions used here.

PNA 1765 (+4)
Acr-Lys-TTTTTTTTTT-(eg1)₃-TTT$_{Lys}$TTTT$_{Lys}$TTT-NH₂
PNA 2399 (+4)
H-Lys-TTTTTTTTTT-(eg1)₃-TTT$_{Lys}$TTTT$_{Lys}$TTT-NH₂
PNA 1743 (+3)
H-TTTTTTTTTT-(eg1)₃-TTT$_{Lys}$TTTT$_{Lys}$TTT-NH₂
PNA 1484 (+3)
Acr-TTT$_{Lys}$TTTT$_{Lys}$TTT-NH₂
PNA 1808 (+4)
H-Lys-TTT$_{Lys}$TTTT$_{Lys}$TTT-NH₂

Table 1. C50 Values for Strand Invasion[a]

PNA	10 mM Na⁺	60 mM K⁺/ Na⁺	110 mM K⁺/ Na⁺	150 mM K⁺/ Na⁺	150 mM K⁺/Na⁺ 2 mM Mg²⁺
1765	0.02 ± 0.01	0.03 ± 0.01	0.07 ± 0.02	0.2 ± 0.03	0.4 ± 0.01
2399	0.03 ± 0.003	0.22 ± 0.1	0.7 ± 0.2	3.1 ± 0.9	9.2 ± 1.9
1743	0.03 ± 0.01	0.21 ± 0.04	0.4 ± 0.09	3.2 ± 1.6	17 ± 2.6
1484	0.004 ± 0.0004	0.03 ± 0.01	0.05 ± 0.02	0.1 ± 0.06	0.5 ± 0.2
1808	0.02 ± 0.003	0.21 ± 0.05	6.8 ± 3	15 ± 3	>50

[a]C50 values (μM) at the indicated K^+/Na^+ concentrations[10] as determined by electrophoretic mobility shift analysis. The employed target DNA pT10 was constructed by inserting the oligonucleotides 5′-GATCCAAAAAAAA-AAG/5′-GATCCTTTTTTTTTTTG in the BamHI site of pUC19. The pT10 DNA (10 μg) was digested with EcoRI and PvuII and endlabeled with [α-^{32}P]dATP using the Klenow fragment of DNA polymerase. The 250 bp DNA fragment containing the PNA sequence target was purified using 5% PAGE. Strand invasion was conducted by incubating 1−2 nM DNA fragment in 10 mM sodium phosphate pH 6.5 supplemented with PNA and the indicated amount of KCl in a final volume of 20 μL at 37 °C for 2 h. C50 values were obtained by PNA titrations, electrophoretic mobility shift analysis (10% PAGE, TBE buffered), and phosphorimaging. ImageQuant, Excel, and Prism software were used for analysis of data. The mean of 2−6 experiment repetitions is given ± standard error of the mean. The singly mismatched control plasmid pT9C used in Figure 2 was constructed by insertion of the oligonucleotides 5′-TCGACAAAAAGA-AAAG and 5′-TCGACTTTTCTTTTTG in the SalI site of pUC19. The DNA was HindIII−PvuII restriction digested, ^{32}P-labeled, and the 159 bp fragment containing the mismatch target was purified as for pT10.

pronounced in the case of one-stranded mono-PNAs (PNA 1808 compared to PNA 1484) for which the enhancement was 150-fold at 150 mM K^+/Na^+ and ≫100-fold at 150 mM K^+/Na^+ and 2 mM Mg^{2+}, under which conditions PNA 1808 showed hardly any binding.
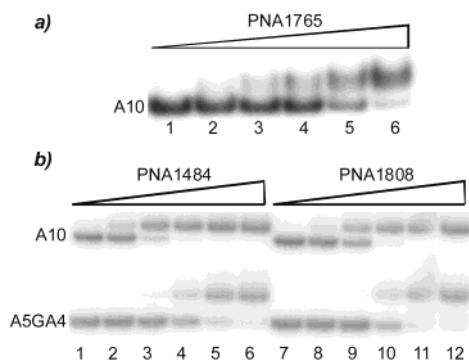
Figure 2. Triplex invasion at physiologically relevant ionic strength and target specificity of acridine-PNAs. Autoradiographs showing PNA binding to the indicated DNA sequence targets as analyzed by the electrophoretic mobility shift assay. (a) PNA 1765 binding to a fully matched A10 sequence target in the presence of 150 mM K^+/Na^+, 2 mM Mg^{2+} (see ref 10). PNA and DNA were incubated as described in the legend to Table 1 using the following PNA concentrations: 0.03 μM (lane 1), 0.06 μM (lane 2), 0.12 μM (lane 3), 0.23 μM (lane 4), 0.5 μM (lane 5), 0.9 μM (lane 6). (b) Binding of the indicated PNAs to a fully complementary (A10) and a singly mismatched (A5GA4) target in 10 mM sodium phosphate. A 1:1 mixture of the DNAs containing fully matched (A10; 250 bp) and singly mismatched (A5GA4; 159 bp) targets was incubated with the following PNA concentrations: 0.0005 μM (lane 1), 0.002 μM (lane 2), 0.005 μM (lane 3), 0.015 μM (lane 4), 0.045 μM (lane 5), 0.13 μM (lane 6), 0.002 μM (lane 7), 0.007 μM (lane 8), 0.02 μM (lane 9), 0.07 μM (lane 10), 0.2 μM (lane 11), and 0.6 μM (lane 12) as described in the legend to Table 1.

Presumably, the acridine, which relies on a combination of hydrophobic (base pair intercalation) and electrostatic forces for DNA binding, and therefore is much less sensitive to ionic strength, ensures a high local concentration of the PNA in close proximity to the DNA helix. This increases the probability of the PNA "catching" the transiently open DNA helix in a "breathing event".

While acridine conjugation thus provides enhanced dsDNA strand invasion, the sequence specificity of the reaction could possibly be compromised. To investigate this, binding was conducted using a 1:1 mixture of two DNA fragments, one containing a fully complementary target (A10) and another containing a single mismatch target (A5GA4) (Figure 2b). The specificity ratio (i.e., the ratio of the C50 values for binding to the singly mismatched and fully complementary targets) was analyzed at 10 mM Na^+ and 60 mM K^+/Na^+ and found to be in the range of 3−20 for all PNAs examined (PNAs 1765, 1743, 1484, and 1808; Figure 2 and data not shown). Moreover, the acridine conjugated PNAs were consistently as specific or superior as compared to the conventional PNAs. Thus, enhancement of dsDNA binding efficiency by acridine conjugation does not occur at the expense of target specificity. However, the moderate specificity ratio reported here is somewhat at odds with the much higher specificities observed previously.[1c,e] This discrepancy warrants further analysis but could be due to differences of experimental strategies including the use of T-lys. In conclusion, we have shown that simply conjugating a DNA intercalator such as 9-aminoacridine to a helix invading PNA dramatically enhances the DNA binding efficiency at physiologically relevant ionic conditions. These results should be a significant step toward the development of helix invading PNAs for in vivo gene targeting.

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(8) Dissociation of P-loops involving decameric triplex invasion PNAs is an extremely slow process ($k_{off} \approx 0.001$ h^{-1} at 37 °C)[4a] (see also: Kosagonov, Y. N.; Stetsenko, D. A.; Lubyako, E. N.; Kvito, N. P.; Lazurkin, Y. S. Nielsen, P. E. *Biochemistry* **2000**, *39*, 11742−11747), and therefore binding is kinetically controlled under most circumstances.[1c] Consequently, a pseudo K_D defined as C50 (the PNA concentration that gives 50% binding in 2 h incubation at 37 °C) was employed for comparing DNA binding of the different PNA oligomers. Preliminarily, a binding rate constant for PNA 1765 at 150 mM K^+/Na^+, 2 mM Mg^{2+} of 10^2 M^{-1} s^{-1} was determined.

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(10) The Na^+ concentration was maintained constant at 10 mM, and the K^+ concentration was varied. When Mg^{2+} was included, a formal concentration of 3 mM was used, but due to the addition of 1 mM EDTA, the effective $MgCl_2$ concentration was 2 mM.

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