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# Kinetic sequence discrimination of cationic bis-PNAs upon targeting of double-stranded DNA

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## ABSTRACT

**Strand displacement binding kinetics of cationic pseudoisocytosine-containing linked homopyrimidine peptide nucleic acids (bis-PNAs) to fully matched and singly mismatched decapurine targets in double-stranded DNA (dsDNA) are reported. PNA–dsDNA complex formation was monitored by gel mobility shift assay and pseudo-first order kinetics of binding was obeyed in all cases studied. The kinetic specificity of PNA binding to dsDNA, defined as the ratio of the initial rates of binding to matched and mismatched targets, increases with increasing ionic strength, whereas the apparent rate constant for bis-PNA–dsDNA complex formation decreases exponentially. Surprisingly, at very low ionic strength two equally charged bis-PNAs which have the same sequence of nucleobases but different linkers and consequently different locations of three positive charges differ in their specificity of binding by one order of magnitude. Under appropriate experimental conditions the kinetic specificity for bis-PNA targeting of dsDNA is as high as 300. Thus multiply charged cationic bis-PNAs containing pseudoisocytosines (J bases) in the Hoogsteen strand combined with enhanced binding affinity also exhibit very high sequence specificity, thereby making such reagents extremely efficient for sequence-specific targeting of duplex DNA.**

## INTRODUCTION

Reagents that can be designed to bind effectively with high sequence specificity to desired targets in double-stranded DNA (dsDNA) are of major interest in medicine as gene therapeutic agents and in molecular biology as ‘genetic tools’ (1). The unique properties of peptide nucleic acids (PNAs) (2,3) has attracted considerable interest in this respect, not least due to the unexpected duplex invasion binding mode that has opened a novel avenue by which to target dsDNA. Thus a further characterization of PNAs and their binding mode(s) are warranted.

Homopyrimidine PNAs (hpyPNAs) bind efficiently and sequence specifically to dsDNA targets by duplex invasion via formation of a strand displacement complex composed of an internal $PNA_2$–DNA triplex and a virtually single-stranded (non-complementary) DNA strand (4–8). Sequence discrimination of dsDNA targets by monomeric hpyPNAs was found to be kinetically controlled, so that both high complex stability and high specificity are achieved (9).

Since two hpyPNA strands are required for these strand displacement complexes, the construction of linked PNAs (bis-PNAs) was a logical consequence (10,11). Not surprisingly, such bis-PNAs, especially when containing several lysine residues to supply positive charges, show superior binding properties in terms of complex stability and binding kinetics (11,12; Nielsen,P.E. and Demidov,V.V., unpublished results). An additional advantage is provided by incorporation of pseudoisocytosines (J bases) instead of cytosines in one PNA strand to obtain strong binding independently of pH (10). However, it is not known whether the increase in binding efficiency is obtained at the expense of sequence discrimination. In fact, one could expect that PNA oligomers with enhanced binding potential due to non-specific electrostatic interactions between PNA and DNA backbones would discriminate poorly between correct and mismatched targets in dsDNA, as is in general observed with other ligands which have enhanced affinity for the corresponding target (13–16).

We demonstrate here that sequence discrimination between correct and mismatched DNA targets by positively charged bis-PNAs can be comparable to that of mono-PNAs, but is sensitive to the location of positive charges. Furthermore, we show that sequence discrimination increases with increasing ionic strength. Hence, such PNA modification, which provides significantly increased affinity, does not compromise the very high specificity of DNA targeting.

## MATERIALS AND METHODS

The PNAs were synthesized by standard methods, purified by reverse phase HPLC and characterized by MALDI-TOF mass spectrometry as described (10,17). The following PNAs were used in this study: PNA 365, H-$Lys_4$-$T_2CT_2CT_4$-$NH_2$ ($5^+$); PNA 522, H-$T_2JT_2JT_4$-$egl_3$-$T_2CT_2CT_2$-$LysNH_2$ ($2^+$); PNA 554, H-$T_2JT_2JT_4$-$(Lys\text{-}aha)_2$-Lys-$T_4CT_2CT_2$-$LysNH_2$ ($5^+$); PNA 655, H-$Lys_3$-$T_2JT_2JT_4$-$egl_3$-$T_4CT_2CT_2$-$LysNH_2$ ($5^+$). The PNAs are written from the N-terminus to the C-terminus using normal peptide conventions: H, free amino group; $NH_2$, terminal carboxamide; Lys, lysine residue; the numbers in parentheses indicate the total charge of PNA oligomers; J denotes pseudoisocytosine (10); aha and eg1

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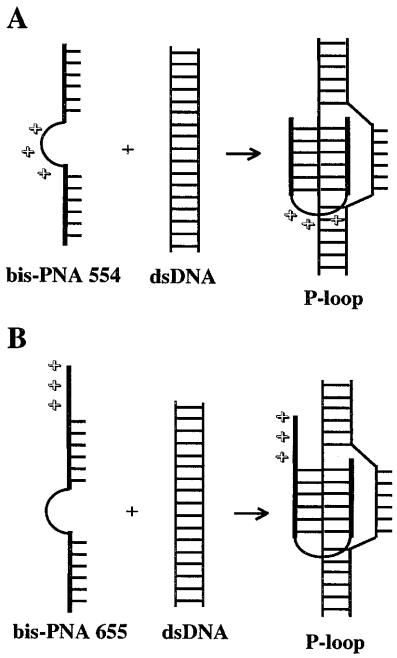


**Figure 1.** Schematic showing P loop formation on binding of bis-PNA 554 (**A**) and of bis-PNA 655 (**B**) to the correct target site in dsDNA. Of the bis-PNAs only the charges due to three additional lysine residues which were distributed at different locations are denoted (+).

denote the linker units 6-aminohexanoic acid and 8-amino-3,6-dioxaoctanoic acid respectively. PNA concentrations were determined spectrophotometrically at 260 nm using the following molar extinction coefficients: 8800 (T), 7300 (C) and 3000 (J) per M/cm.

Plasmids containing the target sequences were constructed by cloning the appropriate oligonucleotides into the polylinker of plasmid pUC19 as previously described (7,18). Plasmid pA8G2 has $T_4CT_2CT_2{\cdot}A_2GA_2GA_4$ cloned into the *Pst*I site and pT9C has $A_5GA_4{\cdot}T_4CT_5$ cloned into the *Sal*I site. $^{32}$P-Labeled 250 bp long DNA fragments were prepared by cutting the plasmids with restriction enzymes *Eco*RI and *Pvu*II and labeling with [α-$^{32}$P]dATP using the Klenow fragment of *Escherichia coli* DNA polymerase. The concentration of radiolabeled DNA target sites in all experiments did not exceed 5 nM, so that the PNA quantities were in large excess in the experimental PNA/DNA mixtures.

PNA binding to dsDNA fragments was performed in presiliconized tubes at 37°C in 10 mM Na phosphate buffer solution, pH 7.0, containing 1 mM EDTA and up to 40 mM NaCl added (the total concentration of $Na^+$ was varied from 10 to 50 mM). The reaction was quenched at desired time points by increasing the NaCl concentration in the reaction aliquots up to 340–370 mM and cooling. The samples were then analyzed by electrophoresis in 10% polyacrylamide gels run in TBE (Tris–borate, EDTA) buffer using a gel mobility shift assay. In this assay the radiolabeled DNA fragments were complexed with PNA and the PNA–DNA complex was separated from free DNA by gel electrophoresis with subsequent autoradiographic quantification of the intensities of both bands. The intensity of the faster migrating band gives the quantity of free DNA, whereas the slower migrating band corresponds to the complex, which retards due to P loop formation (9). By measuring the intensities of these two bands, normalized to their integral intensity, one can follow the kinetics of PNA binding to DNA. Quantitative analysis was performed by scanning autoradiographs

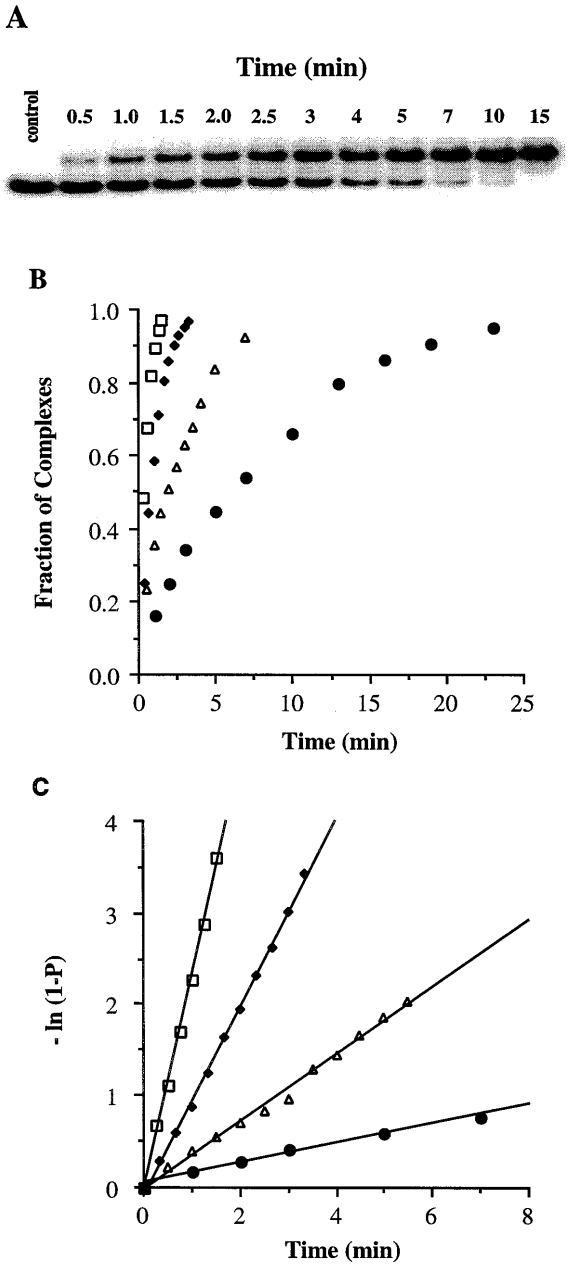


**Figure 2.** Strand displacement PNA binding kinetics with the fully complementary DNA target. (**A**) The $^{32}$P-labeled *Eco*RI–*Pvu*II fragment of plasmid pA8G2 was incubated at 37°C with bis-PNA 554 in the presence of 40 mM $Na^+$ at various times, indicated above each lane. Lane 1 is a control without PNA. The PNA concentration was 1 µM and the dsDNA concentration was 2 nM. (**B**) Plots of fraction of complexes as determined from densitometer analysis of autoradiographs (as exemplified above) as a function of the time of incubation at 20 (open squares), 30 (closed diamonds), 40 (open triangles) or 50 mM $Na^+$ (closed circles). (**C**) Semilogarithmic plots of the kinetics of strand displacement reactions. The initial binding rates were obtained from the slopes of lines in plots of $-\ln(1 - P)$ versus time $t$, where $P$ is the fraction of DNA fragments bound to PNA. Symbols denote various ionic strengths as above.

on a Molecular Dynamics computing densitometer or by scanning exposed storage phosphor screens on a Molecular Dynamics PhosphorImager with the use of ImageQuant software. Parts of the kinetic experiments were done with ethidium bromide staining of the polyacrylamide gels and data quantification with a CCD camera

using the IS-1000 digital imaging system and image analysis software (Alpha Innotech Corp.). All these three analytical methods gave comparable results. The rate constants presented are average values of at least three independent experiments.

## RESULTS

### Strand displacement with the fully complementary DNA target

The time course of bis-PNAs binding to the fully complementary dsDNA target, carrying the insert $T_4CT_2CT_2{\cdot}A_2GA_2GA_4$, was studied at various ionic strengths from 20 to 50 mM $Na^+$ by a gel mobility shift assay (6). Figure 1 shows schematically binding of bis-PNAs 554 and 665, which differ in the location of three lysine residues, to dsDNA. In order to compare the data to the mismatched situation we did not use higher ionic strengths. Correct PNA binding was virtually irreversible (see Fig. 2A and B) and, as was observed previously for mono-PNA and bis-PNA association (9,12,19), the data obeyed pseudo-first order kinetics

$$\ln(1 - P) = -k \times t \qquad \mathbf{1}$$

where $P$ is the fraction of PNA–DNA complexes formed at time $t$ and $k$ is the pseudo-first order rate constant corresponding to the initial PNA binding rate for the non-equilibrium case of PNA binding. In all cases of binding to the fully matched target the experimental data plotted in coordinates $-\ln(1 - P)$ versus $t$ lie on a straight line (as presented in Fig. 2 for bis-PNA 554), indicating that association of the charged bis-PNAs studied here does obey pseudo-first order kinetics.

The initial rate of binding of bis-PNA 554 to the correct DNA target (the $k$ value in equation **1**) is twice as high as the value determined for bis-PNA 655 at all ionic strengths measured (Table 1). At 20 mM $Na^+$ binding of 1 µM bis-PNA 554 to the target was complete within 2 min with $k$ = 2.33/min. With these PNAs an increase in ionic strength led to an exponential decrease in the initial binding rates (Fig. 3). At 50 mM $Na^+$, for instance, strand displacement using bis-PNA 554 was complete within 30 min with $k$ = 0.13/min.

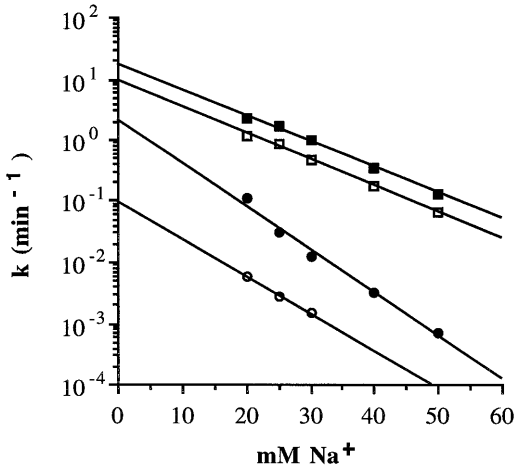


**Figure 3.** Influence of ionic strength on strand displacement reactions. Semilogarithmic plots of initial binding rates of 1 µM bis-PNA 554 (closed symbols) and bis-PNA 655 (open symbols) to the perfect (squares) and single mismatched (circles) targets respectively, as a function of $Na^+$ concentration.

At the same PNA concentration (1 µM) and the same ionic strength (20 mM $Na^+$) the initial rate constant of mono-PNA 365 association to the correct target was found to be >15 times lower than for bis-PNAs 554 or 665. Bis-PNA 522, which contains three lysines less than PNAs 554, 665 or 365 (and therefore carries only two positive charges), binds very slowly to the correct target under the same conditions. The observed rate constant ($k = 1.6 \times 10^{-3}$/min at 20 mM $Na^+$) is more than 700 times smaller than for the bis-PNAs containing five charges. Kinetic experiments of binding to the correct and mismatched targets were also performed at suitable higher concentrations for mono-PNA 365 (5 µM) and bis-PNA 522 (25 µM), since no binding to the mismatched target due to too slow association rates could be detected at 1 µM concentration of these PNAs. At higher PNA concentrations the association rates to the correct target are 5-fold (PNA 365) and 125-fold increased (PNA 522) respectively compared with the rates at 1 µM.

**Table 1.** Strand displacement kinetics of dsDNA by PNAs: initial binding rates ($k$) and specificities of association[a]

| PNA | [PNA] (µM) | [$Na^+$] (mM)[b] | k (per min) Perfect match | Single mismatch | Specificity ($k_{match}/k_{mismatch}$) |
|---|---|---|---|---|---|
| bis-PNA 655, H-$Lys_3$-$(TTJ)_2T_4$-$egl_3$-$T_4(CTT)_2$-$LysNH_2$ | | 20 | 1.18 ± 0.08 | $6.0 \pm 0.4 \times 10^{-3}$ | 197 |
| | | 25 | 0.85 ± 0.06 | $2.8 \pm 0.1 \times 10^{-3}$ | 304 |
| | 1 | 30 | 0.48 ± 0.04 | $1.5 \pm 0.2 \times 10^{-3}$ | 320 |
| | | 40 | 0.17 ± 0.03 | ND[c] | ND |
| | | 50 | 0.064 ± 0.018 | ND | ND |
| bis-PNA 554, H-$(TTJ)_2T_4$-$(Lys\text{-}aha)_2$-Lys-$T_4(CTT)_2$-$LysNH_2$ | | 20 | 2.33 ± 0.11 | 0.11 ± 0.02 | 21 |
| | | 25 | 1.65 ± 0.09 | $3.1 \pm 0.7) \times 10^{-2}$ | 53 |
| | 1 | 30 | 1.02 ± 0.04 | $1.3 \pm 0.2) \times 10^{-2}$ | 78 |
| | | 40 | 0.35 ± 0.02 | $3.2 \pm 0.3) \times 10^{-3}$ | 109 |
| | | 50 | 0.13 ± 0.01 | $6.7 \pm 0.8) \times 10^{-4}$ | 194 |
| mono-PNA 365, H-$Lys_4$-$T_4(CTT)_2$-$NH_2$ | 1 | 20 | $7.7 \pm 0.6 \times 10^{-2}$ | ND | ND |
| | 5 | 20 | 0.42 ± 0.09 | $8.3 \pm 1.4 \times 10^{-4}$ | 506 |
| bis-PNA 522, H-$(TTJ)_2T_4$-$(eg1)_3$-$T_4(CTT)_2$-$LysNH_2$ | 1 | 20 | $1.6 \pm 0.5 \times 10^{-3}$ | ND | ND |
| | 25 | 20 | 0.20 ± 0.03 | $3.6 \pm 0.5 \times 10^{-4}$ | 625 |

[a]All reactions were performed at 37°C in 10 mM Na phosphate buffer solution, pH 7.0.
[b]Total concentration of $Na^+$.
[c]ND, not determined.

### Strand displacement with a DNA target carrying a single mismatch

The strand displacement reaction was performed with dsDNA target carrying the insert $A_5GA_4{\cdot}T_4CT_5$, which corresponds to a single mismatch in the middle of the target. As shown in Figure 4, the reactions do not go to completion, even at long incubation times, but instead are saturated at some stationary level of PNA–DNA complex formation. Control experiments were performed to ensure that it is in fact an equilibrium situation we observed. To rule out 'inactivation' of the bis-PNA at long incubation times due to self-aggregation or non-specific aggregation with dsDNA, in one experiment bis-PNA 554 was preincubated with salmon sperm DNA for 5 h before the mismatched target was added. This experiment gave the same results as the analogous one without preincubation (data not shown). To investigate whether dissociation of mismatched PNA–DNA complexes can be observed a 10-fold excess of an oligonucleotide complementary to bis-PNA 554 was added after overnight incubation of the mismatched target with this bis-PNA to reach equilibrium. Indeed, an off-rate of ~$2.4 \times 10^{-3}$/min could be observed after oligonucleotide addition (data not shown).

The experimental kinetic data for binding of positively charged bis-PNAs to the mismatched DNA target also fit pseudo-first order kinetics. Assuming a simple reversible reaction, the following generalized pseudo-first order kinetic equation could be obtained

$$\ln(1 - P/P_e) = -k \times t/P_e \qquad \mathbf{2}$$

where $P_e$ and $P$ denote the yields of PNA–DNA complexes at equilibrium and at time $t$ respectively and constant $k$ corresponds to the initial PNA binding rate for the equilibrium case of PNA binding. By plotting the experimental data in coordinates $-P_e\ln(1 - P/P_e)$ versus time $t$ we obtained experimental points lying on a straight line (Fig. 4). This proves that the above assumption is a good approximation and gives the initial binding rates for mismatched bis-PNA association as a slope in semilogarithmic coordinates according to equation **2**.

Similar to the results with the correct DNA target, the initial binding rates for bis-PNA association with a mismatched target decrease exponentially with increasing ionic strength (Fig. 3). Additionally, the percentage mismatched strand displacement at equilibrium decreases with increasing salt concentration. Thus in the case of bis-PNA 655 no incorrect binding to dsDNA was observed at 40 mM $Na^+$. At 30 mM $Na^+$ the maximum yield of mismatched strand displacement was 10–15%, with an initial binding rate of $1.5 \times 10^{-3}$/min.

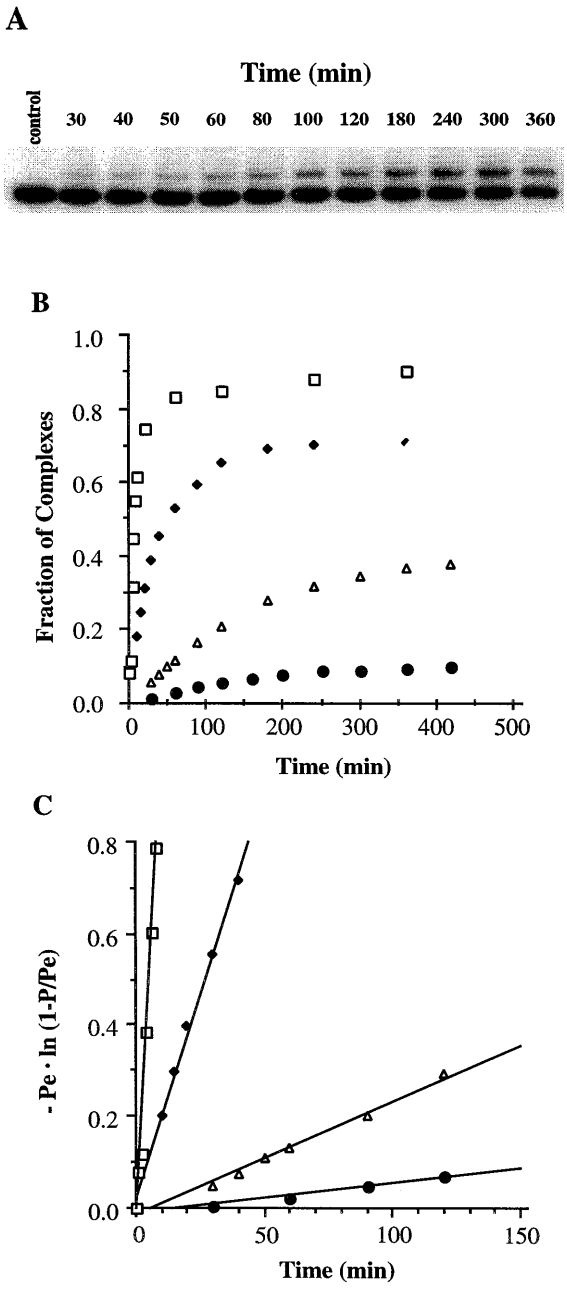


**Figure 4.** Strand displacement binding kinetics of bis-PNA 554 with the single mismatched DNA target. (**A**) The $^{32}$P-labeled *Eco*RI–*Pvu*II fragment of plasmid pT9C was incubated at 37 °C with PNA in the presence of 40 mM $Na^+$ at various times, indicated above each lane. Lane 1 is a control without PNA. The PNA concentration was 1 µM and the dsDNA concentration was 2 nM. (**B**) Plots of fraction of complexes as a function of the time of incubation at 20 (open squares), 30 (closed diamonds), 40 (open triangles) or 50 mM $Na^+$ (closed circles). (**C**) Semilogarithmic plots of the kinetics of strand displacement reactions. The initial binding rates were obtained from the slopes of lines in plots of $-P_e\ln(1 - P/P_e)$ versus time $t$. $P_e$ and $P$ denote the yield of product at equilibrium and at time $t$ respectively. Symbols denote various ionic strengths as above.

### Specificity of bis-PNA binding to dsDNA

Comparison of the data obtained for the initial rates $k$ of bis-PNA binding to matched and mismatched targets (see Table 1) shows that in some cases the difference in initial rates is dramatic, whereas in other cases it is not very significant. We define the kinetic specificity of PNA binding to its target site as the $k_{match}/k_{mismatch}$ ratio. The specificity values thus defined are shown in the last column of Table 1. The most striking result is the dramatic difference in specificity of bis-PNAs 554 and 655, which differ only in the location of positive charges and in the linker units. We believe that the difference in the chemical structure of the linker units should be of minor steric influence, since both linkers are conformationally flexible and are of similar length. Thus we attribute the difference in specificity between bis-PNA 554 and 655 to the different location of the lysines and thereby the charges. At the very low salt concentration of 20 mM $Na^+$ the discrimination is an order of magnitude higher for bis-PNA 655 than for bis-PNA 554. However, at 50 mM $Na^+$ the specificity of binding of bis-PNA 554 is 10-fold better than at

20 mM $Na^+$, reaching the magnitude of discrimination of bis-PNA 655 observed at very low ionic strength.

## DISCUSSION

Sequence discrimination upon binding of T-rich homopyrimidine mono-PNAs to dsDNA targets has previously been found to be predominantly kinetically controlled when comparing a fully matched 10mer target and one containing a centrally positioned mismatch. Furthermore, it has been proposed that discrimination is accomplished via a reversible searching step with formation of a transient PNA–DNA complex followed by an essentially irreversible locking step due to formation of a $PNA_2$–DNA triplex (6,9). Therefore, using bis-PNAs, which have been found to bind faster than the corresponding mono-PNAs (11,12; Nielsen,P.E. and Demidov,V.V., unpublished results), could severely compromise sequence discrimination of binding. It can even be imagined that in the case of binding of bis-PNAs to dsDNA concerted triplex formation occurs instead of a two-step mechanism as proposed for monomeric PNAs (9).

The present results clearly show that poor sequence discrimination is not a hallmark of bis-PNAs. On the contrary, bis-PNAs can discriminate against a base mismatch in the dsDNA target almost as efficiently as a corresponding mono-PNA. Also, at low ionic strength (20 mM $Na^+$; Fig. 4) discrimination is essentially kinetically controlled, since close to complete binding can be achieved to the mismatched target. At higher ionic strength discrimination may contain both a kinetic as well as a thermodynamic equilibrium component, since mismatch binding decreases (at the employed PNA concentration) with increasing ionic strength. The kinetic component though shows a strong positive influence of ionic strength on discrimination (Table 1). This is most dramatically illustrated with bis-PNA 554, which discriminates against a single mismatch quite poorly (20-fold) at 20 mM $Na^+$, but 10 times better at 50 mM $Na^+$. Thus the results are very encouraging from a specificity point of view for the prospect of using bis-PNAs as gene targeting reagents *in vivo* at physiological ionic strength.

With regard to binding efficiencies, here measured as binding rates, the present results support earlier findings that bis-PNAs bind faster to dsDNA targets than do comparable monomeric PNAs and that inclusion of positive charges in bis-PNAs greatly increases binding efficiency (11,19; Nielsen,P.E. and Demidov,V.V., unpublished results). However, quantitatively our results cannot be directly compared with those of Griffith *et al.* (11), since the PNAs of the present study contain pseudoisocytosine in the 'Hoogsteen strand' and are of different sequence (although of identical base composition). These issues will be addressed in a subsequent study.

The large difference in sequence discrimination capacity of bis-PNA 554 compared with bis-PNA 655 is intriguing. These two PNAs differ mainly in the position of the lysine residues and thus of the positive charges, which in the case of PNA 554 are placed in the linker between the two PNA strands, while PNA 655 has terminally positioned positive charges, mainly in the Hoogsteen strand. Naturally we cannot exclude that differences in flexibility and hydrophobicity between the two linkers could also play a role. Nonetheless, a positional effect of the charges, which as expected is leveled out at higher ionic strength, could relate to the binding mechanism of bis-PNA (Fig. 1). The results indicate that bis-PNAs with the charges in the linker reach the 'locked' virtually irreversible complex faster, perhaps by more efficient 'zippering' from the charged linker region, and thereby lose discrimination potency, which is ascribed to reversible searching by intermediary complexes. This is consistent with the observation that bis-PNA 522 (with only two charges), which binds more slowly to its dsDNA target than bis-PNA 655, exhibits better sequence discrimination. However, a more complete understanding of the detailed molecular binding mechanism must await further studies.

Up till now there are very few examples of a design of oligonucleotide and synthetic peptide ligands where binding specificity is not compromised by binding affinity (15,16,20–27). Cationic bis-PNAs exemplify a successful case of such molecular design. This example is doubly remarkable because relatively short PNA oligomers are able to recognize sequences within a linear DNA duplex by strand invasion. Such a mode of duplex DNA recognition by PNAs is favorable for development of innovative methodologies (28–35). All other cases of oligonucleotide–polynucleotide binding via strand displacement described for DNA or RNA (36,37) have some critical intrinsic limitations, such as a requirement for DNA supercoiling or a necessity for long oligomers to be hybridized, otherwise binding will occur only at or near the ends of the DNA duplexes.

## CONCLUSION

Several conclusions can be drawn from the present results. Most importantly, our data show that decameric bis-PNAs can discriminate almost as efficiently as the corresponding mono-PNAs against a single mismatch in a dsDNA target, exhibiting a 200-fold discrimination at 20 mM $Na^+$. Quite surprisingly, however, the linker chemistry appears to be critical, since a bis-PNA with a charge-neutral ethyleneglycol-type linker (and lysines at the N-terminus) discriminates between the correct and mismatched DNA sites at low ionic strength up to 10-fold better than a bis-PNA in which the lysines are built into the linker. Furthermore, a 10-fold increase in discrimination by the latter PNA can be obtained upon increasing the ionic strength from 20 to 50 mM $Na^+$. Finally, a bis-PNA with fewer lysines ($2^+$ charge) was found to discriminate 3-fold better than a more lysine-rich bis-PNA ($5^+$ charge). However, this PNA binds ~1000-fold less efficiently to the dsDNA target.

## ACKNOWLEDGEMENTS

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