Extended DNA-Recognition Repertoire of Peptide Nucleic Acid (PNA): PNA−dsDNA Triplex Formed with Cytosine-Rich Homopyrimidine PNA

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ABSTRACT: Peptide nucleic acid (PNA) is an oligonucleotide mimic in which the backbone of DNA has been replaced by a pseudopeptide. Thymine-rich homopyrimidine PNA oligomers have been found to recognize double-stranded DNA targets by displacement of the pyrimidine DNA strand and forming an internal Watson−Crick−Hoogsteen base-paired PNA(pyr)−DNA(pu)−PNA(pyr) triplex. We here show that cytosine-rich homopyrimidine PNA sequences instead add to double-stranded polynucleotide targets as Hoogsteen strands forming PNA(pyr)−DNA(pu)−DNA(pyr) triplexes. Furthermore, PNA strands with homopurine or alternating thymine-guanine sequences are shown to invade their respective DNA targets by displacing the identical DNA strands of the polynucleotides and forming new PNA−DNA duplexes. These results indicate distinct mechanistic variations as to how PNA interacts with a DNA target depending on choice of nucleobases, which could be of importance for future design of gene-specific diagnostic or therapeutic agents.

Reagents that bind sequence-selectively to double-stranded DNA are of significant interest in molecular biology and medicinal chemistry as they may be developed into gene-targeted agents for diagnostic and therapeutic purposes, and to provide tools for sequence-specific modification of DNA. Oligonucleotides and their close analogs have so far been the main candidates for developing such reagents (Moser & Dervan, 1987; Dervan, 1992; Hélène, 1993; Frank-Kamenetskii & Mirkin, 1995). They are, however, not optimal in terms of biological stability, solubility, cellular uptake properties, or ease of synthesis. Furthermore, a general code for recognition of any base sequence of double-stranded DNA has not yet been revealed, and so far, mainly purine-rich stretches of DNA may be targeted by an oligonucleotide. For these reasons, alternative concepts of oligonucleotide mimics have attracted interest.

PNA (peptide nucleic acid)[1] is a recently developed oligonucleotide mimic in which the entire deoxyribose phosphate backbone of DNA has been replaced by a chemically completely different, but structurally homomorphous, backbone composed of (2-aminoethyl)glycine units (Nielsen et al., 1991; Egholm et al., 1992). Mixed sequences of PNA have been found to be very potent DNA mimics, forming Watson−Crick base-paired duplexes with complementary DNA strands of high specificity and thermal stability (Egholm et al., 1993). Homopyrimidine PNA oligomers form triplex structures with complementary homopurinic sequences and have been found to recognize double-stranded DNA targets by a mechanism that involves displacement of the pyrimidine DNA strand (Cherny et al., 1993; Nielsen et al., 1994; Demidov et al., 1995; Wittung et al., 1996). It has further been concluded, based on chemical probing experiments (Nielsen & Christensen, 1996), that a purine PNA decamer with mixed A and G bases can bind to a single target in a double-stranded DNA by invading the duplex and forming a Watson−Crick base-paired PNA−DNA duplex with the complementary DNA strand. Recently, also a mixed T/G-PNA was observed to bind to its Watson−Crick target sequence by invasion of the double-stranded DNA (Nielsen & Egholm, 1997).

To further elucidate the sequence dependence for PNA recognition of double-stranded DNA, we here study the interaction of different 10-meric PNA oligomers, two homopurine sequences, and an alternating guanine-thymine sequence, as well as some cytosine-rich homopyrimidine sequences (Table 1), with their respective double-stranded target polynucleotides. By using circular and linear dichroism spectroscopy, which reflect, respectively, helical base stacking and orientation of nucleobases, we can monitor the formation of PNA−DNA complexes to assess binding stoichiometry and dependency of PNA binding on variations in temperature, ionic strength, PNA concentration, and pH. We find the cytosine-rich PNA oligomers to add to the double-stranded target polynucleotides as Hoogsteen strands, forming PNA−DNA_2 triplex structures. The homopurine PNAs, as well as the alternating T/G-PNA oligomer, are instead found to invade their DNA targets, by displacing the identical DNA strand, forming new PNA−DNA complexes.

EXPERIMENTAL PROCEDURES

Chemicals. Poly(dA)·poly(dT), poly(dG)·poly(dC), poly(dAdG)·poly(dTdC), and poly(dAdC)·poly(dTdG), all purchased from Pharmacia, were dissolved in 5 mM sodium phosphate buffer, pH 7.0, and dialyzed several times against this buffer. The PNAs, H-TTTTTTTTTT-Lys-NH_2,

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[1] Abbreviations: PNA, peptide nucleic acid; CD, circular dichroism; LD, linear dichroism.

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H-AAAAAAAAAA-Lys-NH_2, H-CCCCCCCCCC-Lys-NH_2, H-TCTCTCTCTC-Lys-NH_2, H-TGTGTGTGTG-Lys-NH_2, H-AGAGAGAGAG-Lys-NH_2, H-JJJJJJJJJ-Lys-NH_2, and H-(TCTCTCTC)-$(eg)_3$-(TJTJTJTJ)-Lys-NH_2 (J = pseudoisocytosine), were synthesized as described elsewhere (Christensen et al., 1995; Egholm et al., 1995).

We determined the concentrations spectrophotometrically by using the molar extinction coefficients (per base): ϵ_{260} = 6000 M^{-1} cm^{-1} for poly(dA)·poly(dT), ϵ_{253} = 7400 M^{-1} cm^{-1} for poly(dG)·poly(dC), ϵ_{258} = 6500 M^{-1} cm^{-1} for poly(dAdG)·poly(dTdC) and poly(dAdC)·poly(dTdG). For the PNAs, we used the following extinction coefficients (per base): ϵ_{260} = 8800 M^{-1} cm^{-1} for PNA-T_{10}, ϵ_{260} = 10 400 M^{-1} cm^{-1} for PNA-A_{10}, ϵ_{260} = 7300 M^{-1} cm^{-1} for PNA-C_{10}, ϵ_{260} = 8050 M^{-1} cm^{-1} for PNA-$(TC)_5$, ϵ_{260} = 13 200 M^{-1} cm^{-1} for PNA-$(AG)_5$, and ϵ_{260} = 9900 M^{-1} cm^{-1} for PNA-$(TG)_5$. The molar extinction coefficient for pseudoisocytosine was determined to be ϵ_{260} = 2800 M^{-1} cm^{-1}.

If not otherwise stated, the experiments were performed in a 5 mM phosphate buffer, pH 7.0, with or without added NaCl. Varied parameters (temperature, type of PNA and polynucleotide, concentration of PNA, NaCl concentration, pH) are as indicated in the table, figure captions, or in the text. The PNA binding reactions were initiated by the addition of a small aliquot PNA to the cuvette containing double-stranded polynucleotide, buffer, and salt. The concentration used was 20 μM base-pairs of polynucleotide, or as indicated.

Methods. Circular dichroism (CD), defined as the differential absorption of left and right circularly polarized light, was measured on a Jasco 720 spectropolarimeter using a 1-cm quartz cell. We collected spectra in the 200−320 nm wavelength range as averages of at least 8 scans. The reaction kinetics were normally monitored around 260 nm (where the largest amplitude of CD difference upon reaction is found). The kinetic data were collected for 20 min with a time-constant of 1 s and a 2-nm band-pass. A thermostated cuvette holder was used to study kinetics at various temperatures.

Linear dichroism (LD) is defined as the differential absorption of linearly polarized light, polarized parallel and perpendicular, repectively, to the flow direction in a Wada-type Couette flow cell (Nordén et al., 1992). LD is affected by factors such as the contour length and flexibility of DNA, temperature, flow gradient, flow symmetry, and solution viscosity. Flow LD was measured on a Jasco 500A spectropolarimeter, converted from CD to LD mode and calibrated as described before (Nordén & Seth, 1985). All LD measurements were performed with a shear rate of 300 s^{-1}, at ambient temperature. The p−p* transitions of the DNA bases in the polynucleotides are responsible for the observed negative LD around 260 nm, due to the perpendicular orientation of the base planes relative to the alignment of the polymer long axis.

Finally, the isotropic absorbance (to determine the concentrations) was measured on a CARY 2300 spectrophotometer.

RESULTS

Figure 1 shows some representative circular dichroism (CD) titrations of the studied PNAs with their matching double-stranded DNAs.

The homopyrimidinic PNAs [PNA-C_{10} and PNA-$(TC)_5$] were found to form 1:1 (PNA bases to DNA base pairs) complexes with the matching dsDNAs (Figure 1a,b). The binding of these PNA is not strong enough to give stoichiometric yields of complexes under our experimental conditions, but the titration curves still suggest 1:1 ratios in both cases. Binding constants could be estimated from the titration curves with values around 2×10^5−10^6 M^{-1} (per base). The CD spectra for the complexes formed between PNA-$(TC)_5$ and PNA-C_{10} and their respective target dsDNA molecules (see Figure 1a,b) are similar in shape to that of an all-DNA poly(dC)−poly(dG)−poly(dC^+) triplex (i.e., a C−G*C^+ triplex, where the asterisk denotes Hoogsteen binding of the third strand and the − dash the Watson−Crick pairing).

Upon titrating PNA-A_{10} oligomers to poly(dA)·poly(dT), saturation is reached at about 1.5 PNA bases per DNA base pair (Figure 1c). The CD spectrum of the resulting complex between PNA-A_{10} and poly(dA)·poly(dT) can, however, be identified as that of a PNA−DNA duplex (Egholm et al., 1993) as it closely resembles the spectrum observed upon mixing poly(dT) with PNA-A_{10}, in which case a duplex structure is formed as concluded from titration experiments (data not shown). The titration curve indicates that the binding of PNA-A_{10} is not fully stoichiometric, and a binding constant of about 10^6 M^{-1} (per base) can be estimated.

As can be concluded from Figure 1d, PNA-$(TG)_5$ forms a 1:1 complex with poly(dAdC)·poly(dTdG) at 40 °C. However, at temperatures below 30 °C, a 3:1 (PNA bases to DNA base pairs) complex forms upon titrating the PNA to the DNA (not shown). Similarly, PNA-$(AG)_5$ was found to form a 2:1 PNA−DNA complex with poly(dAdG)·poly(dTdC) below 50 °C, but a clear 1:1 complex at higher temperatures (not shown). Thus, at lower temperatures, these two PNA−DNA complexes appear to contain additional PNAs that are bound rather loose.

The kinetics of PNA−oligomer binding to polynucleotides were followed as an increase in CD signal at the wavelength of maximum CD amplitude change (in most cases, a wavelength between 250 and 270 nm). Upon varying the PNA concentration in these binding kinetic experiments, we found the reaction rate to depend approximately linearly on the PNA concentration for all PNAs [PNAs C_{10}, $(TC)_5$, A_{10}, $(AG)_5$, and $(TG)_5$] (data not shown). This shows these PNA binding reactions to be different from the reactions between PNA-T_{10} and poly(dA)·poly(dT), where an approximately quadratic dependence on the PNA concentration was found (Wittung et al., 1996).

From the rates of PNA binding monitored at different temperatures, approximating the kinetics as monoexponentials, activation energies were estimated from corresponding Arrhenius plots (Table 1). The activation energies for the PNA binding reactions were found to be large for the homopurine PNAs, PNA-A_{10} and PNA-$(AG)_5$, and also for the alternating PNA-$(TG)_5$ (Table 1), and similar in size to the activation energy previously determined for binding of PNA-T_{10} to poly(dA)·poly(dT) (Wittung et al., 1996). This observation is consistent with the hypothesis that opening of DNA is an important part of the activation step. By contrast, we observed almost no temperature dependence for binding of the cytosine-rich homopyrimidine oligomers PNA-C_{10} and PNA-$(TC)_5$ (Table 1), which indicates a binding mode for these PNAs that does not involve base-pair opening.

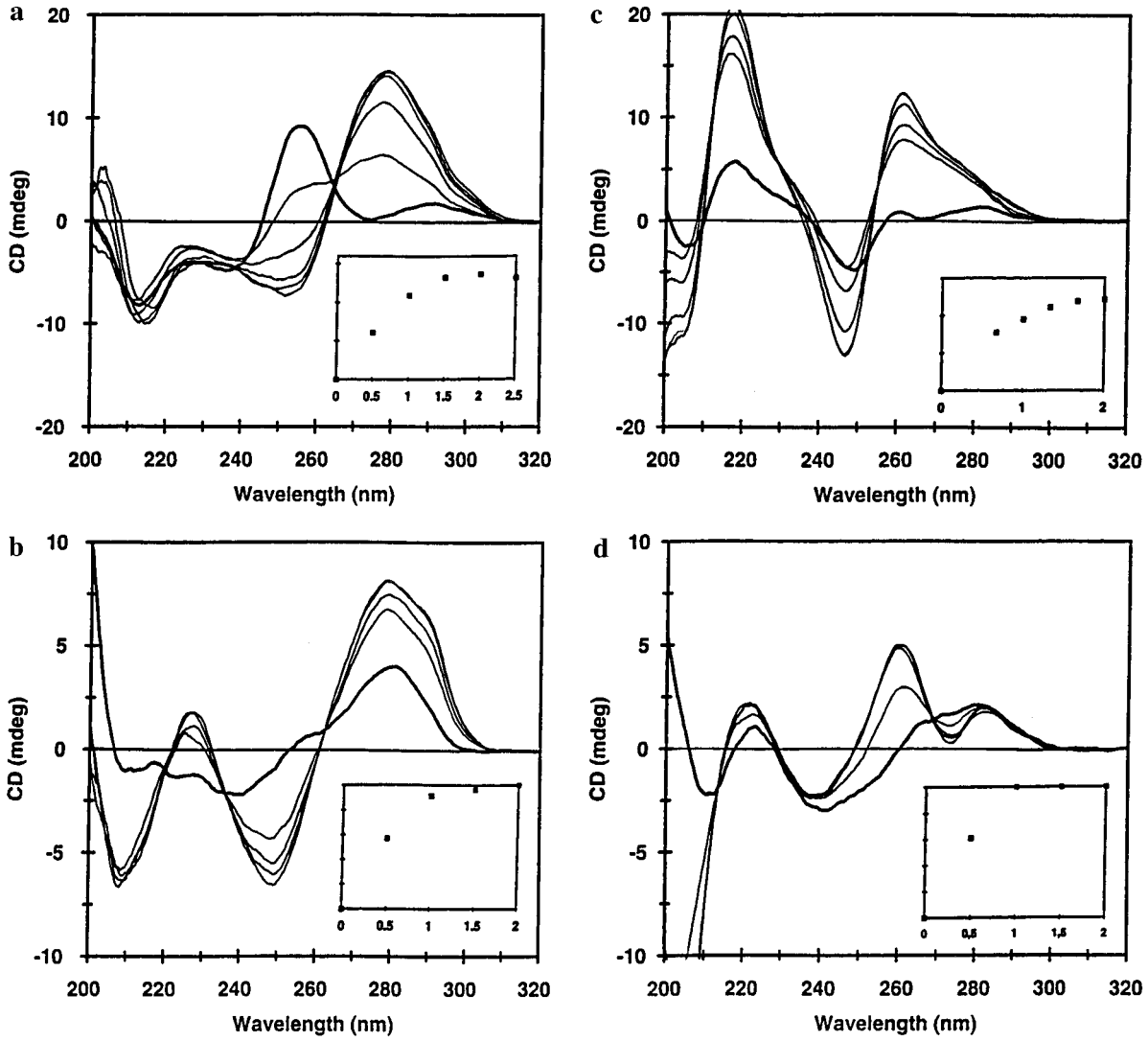

FIGURE 1: CD titrations of PNA to double-stranded DNA in the absence of added NaCl. Boldface lines show CD spectra of the double-stranded polynucleotides in each titration before PNA addition. Inserts show the CD signal as a function of the ratio of PNA bases to DNA basepairs. (a) PNA-C_{10} added to poly(dG)·poly(dC); (b) PNA-$(TC)_5$ added to poly(dAdG)·poly(dTdC); (c) PNA-A_{10} added to poly(dA)·poly(dT); and (d) PNA-$(TG)_5$ added to poly(dAdC)·poly(dTdG) [PNA-$(AG)_5$ added to poly(dAdG)·poly(dTdC) shows very similar behavior]. All experiments were performed with 15−30 min of incubation between the PNA additions, at ambient temperature (ca. 25 °C), except in (d) where a temperature of 40 °C was used.

Table 1: Activation Energies for PNA Binding to dsDNA, Estimated from the Temperature Dependencies of the Reaction Rates Observed by CD

reaction	E_a (kJ/mol)	DNA recognition
poly(dG)·poly(dC) + PNA-C_{10}	not detectable	Hoogsteen triplex
poly(dAdG)·poly(dTdC) + PNA-$(TC)_5$	13 (50 mM NaCl)	Hoogsteen triplex
poly(dG)·poly(dC) + PNA-J_{10}	not determined	Hoogsteen triplex
poly(dAdG)·poly(dTdC) + PNA-$(TC)_4$-$(TJ)_4$	16 (50 mM NaCl)	Hoogsteen triplex
poly(dA)·poly(dT) + PNA-A_{10}	90 (25 mM NaCl)	invasion duplex
poly(dAdG)·poly(dTdC) + PNA-$(AG)_5$	84 (50 mM NaCl)	invasion duplex
poly(dAdC)·poly(dTdG) + PNA-$(TG)_5$	74 (25 mM NaCl)	invasion duplex
poly(dA)·poly(dT) + PNA-T_{10}	60 (50 mM NaCl)	invasion triplex

In Figure 2, the ionic strength dependence for the PNA binding reactions to DNA observed by CD is shown for PNA-$(TG)_5$ (its behavior is also representative for the purine-rich PNA oligomers) as well as for PNA-$(TC)_5$ (representing a cytosine-rich PNA oligomer). The binding rates of the cytosinic PNAs, PNA-C_{10} and PNA-$(TC)_5$ (Figure 2b), are only slightly reduced upon increasing the concentration of NaCl and therefore do not show strong inhibitory effects by salt. The binding of PNA-C_{10} and PNA-$(TC)_5$ to their respective matching target dsDNA is found to proceed to completion at least up to 200 mM NaCl (at pH 7.0).

By contrast, very strong ionic strength dependencies are observed for binding of the purine-rich oligomers [PNA-A_{10} and PNA-$(AG)_5$], as well as PNA-$(TG)_5$, (Figure 2a) to their corresponding DNAs, and at moderate NaCl concentrations (50−100 mM), the reactions are more or less inhibited. For example, the binding of PNA-$(TG)_5$ is completely inhibited at 50 mM NaCl, whereas binding of PNA-$(AG)_5$

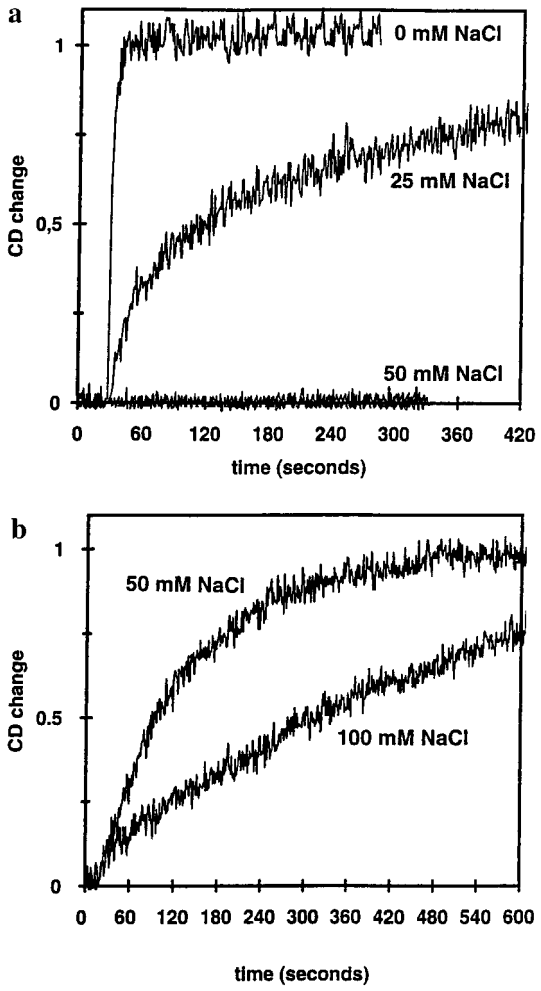

FIGURE 2: PNA binding kinetics at various NaCl concentrations, in 5 mM phosphate buffer, pH 7, monitored by CD (20 μM DNA base pairs and 40 μM PNA bases). (a) PNA-$(TG)_5$ binding to poly(dAdC)·poly(dTdG). CD signal measured at 260 nm, 40 °C. (b) PNA-$(TC)_5$ binding to poly(dAdG)·poly(dTdC). CD signal measured at 280 nm, 20 °C.

is inhibited to about 50% in 100 mM NaCl and to 25% in 50 mM NaCl. The PNA-A_{10} binding proceeds to only 40% completion at 50 mM NaCl. By comparison, PNA-T_{10} binding [to the poly(dA)·poly(dT) duplex] is complete at least up to 150 mM NaCl, however, with a drastically reduced rate (Wittung et al. 1996). [These data all correspond to adding 40 μM PNA (in bases) to 20 μM DNA (in base pairs) in phosphate buffer, pH 7.0.]

Further, the PNA-C_{10} and PNA-$(TC)_5$ binding reactions were found to proceed much faster at pH 5 compared to at pH 7, as shown in Figure 3a,b, indicating protonation of at least some of the cytosines to be required for the binding. Further, at pH 9, in the presence of 50 mM NaCl, the reaction between poly(dG)·poly(dC) and PNA-C_{10} was completely inhibited (when using 40 μM bases PNA and 20 μM base pairs of DNA; see Figure 3b). As seen in the insert of Figure 3b, by omitting NaCl the reaction proceeded to some extent, however slowly. At pH 5, in principle, no NaCl dependence was found for PNA-C_{10} and PNA-$(TC)_5$ binding (comparing rates at 0 and 50 mM NaCl). The binding kinetics of the other PNAs studied (without cytosine bases) showed almost no pH dependence.

By using flow linear dichroism (LD) (Nordén et al., 1992), we could confirm the conclusion from the CD results about

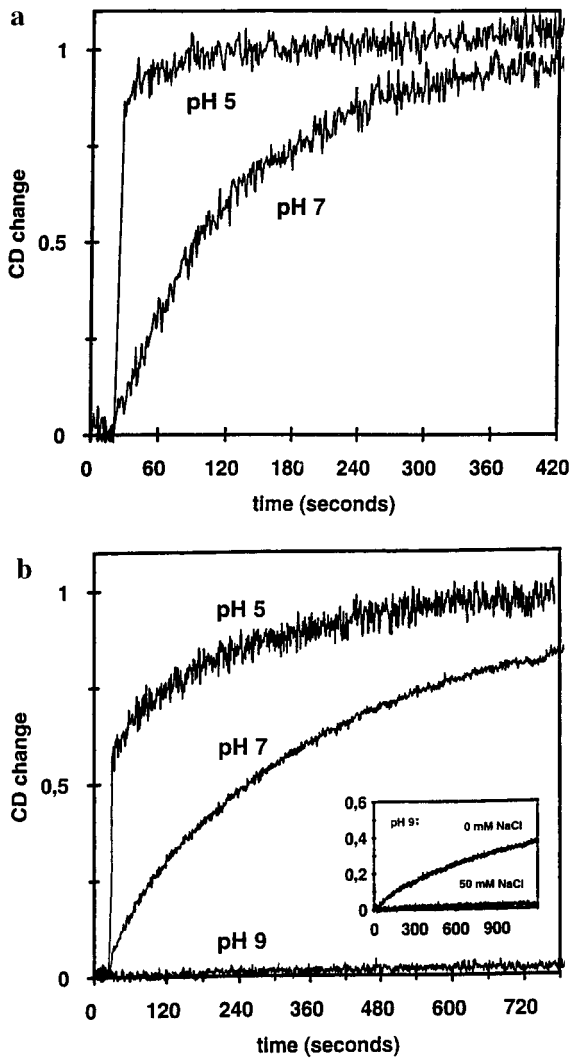

FIGURE 3: (a) Kinetics, measured by CD at 280 nm, of PNA-$(TC)_5$ binding to poly(dAdG)·poly(dTdC) at pH 5 and pH 7 in the presence of 50 mM NaCl, 20 °C (20 μM DNA base pairs and 40 μM PNA bases). (b) Kinetics, measured by CD at 275 nm, of PNA-C_{10} binding to poly(dG)·poly(dC) at pHs 5, 7, and 9, in the presence of 50 mM NaCl, 20 °C (20 μM DNA base pairs and 40 μM PNA bases). Insert: CD kinetics at pH 9 in the presence and absence of 50 mM NaCl.

saturation at 1:1 stoichiometric ratios of PNA bases to DNA base pairs (Figure 4). In the cases of PNA-TG and PNA-AG, which showed stoichiometric PNA:DNA ratios of 2:1, or 3:1, at room temperature as judged from CD, the ratios observed with LD, however, suggest 1:1 complexes. The extra bound PNA strands, detected with CD, are thus probably loosely bound, as already concluded, and only the 1:1 complexes are stable enough to survive the shear flow applied in the LD technique.

Upon titrating the respective DNAs with PNA-C_{10} or PNA-$(TC)_5$, the LD signal increased approximately linearly with the amount of added PNA (see Figure 4a,b), suggesting a direct association of the PNA to the intact DNA duplex, resulting in an increased stiffness of the complex. By contrast, we observe a gradual decrease of the LD signal of DNA upon addition of increasing amounts of homopurinic PNAs, as well as the alternating thymine-guanine PNA, to their matching DNAs, shown in Figure 4a,c. This is expected for an invasion reaction by the PNAs, since the resulting PNA−DNA duplex with one long DNA polymer strand covered with numerous short PNA oligomer fragments

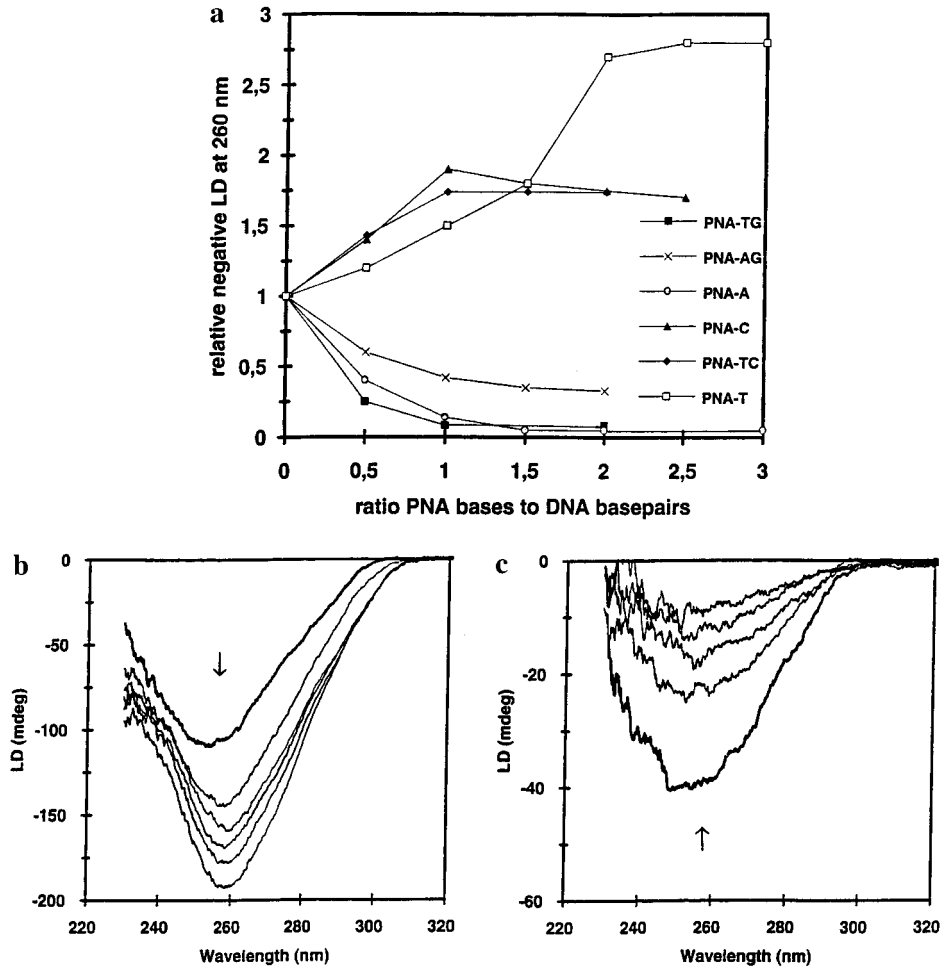

FIGURE 4: (a) Negative flow LD signal at 260 nm as a function of the PNA:DNA base ratio, upon titrating the PNAs into their matching double-stranded polynucleotides. Representative examples of LD spectra from two titration experiments are shown in (b) and (c). Boldface curves show the LD of the double-stranded DNA without added PNA. (b) LD spectra at varying poly(dG)·poly(dC)/PNA-C_{10} ratios. (c) LD spectra at varying poly(dAdC)·poly(dTdG)/PNA-$(TG)_5$ ratios.

is likely to be more flexible than the original DNA duplex, containing two long DNA polymer strands. In the case of PNA-T_{10}, also shown in Figure 4 for comparison, the LD signal increases in a nonlinear way, with the strongest rise close to the triplex stoichiometry of 2 PNA bases per DNA base pair. This nonlinearity has been proposed to be due to the formation of local triplexes on the poly(dA) strand, not resulting in increased stiffness until the whole strand is covered with closely packed PNA (Kim et al., 1995).

We also studied the DNA binding of a 10mer PNA with only pseudoisocytosines (Egholm et al., 1995) (denoted "J"), which do not require low pH for Hoogsteen binding, to poly(dG)·poly(dC). The binding kinetics show almost no NaCl dependence; PNA-J_{10} was found to bind very rapidly to the polynucleotide in the presence of up to 600 mM NaCl. At 1.2 M NaCl, the PNA binding reaction is inhibited to 40% (PNA was added in a 2:1 stoichiometric ratio of PNA bases to DNA base pairs). Titration of poly(dG)·poly(dC) with PNA-J_{10} shows saturation at an approximately 1:1 ratio of PNA bases to DNA base pairs (Figure 5). An LD titration shows the signal to increase linearly upon adding PNA (not shown), suggesting the PNA-J_{10} to bind directly to the intact DNA, as is the case with PNA-C_{10} and PNA-$(TC)_5$. The increase in negative LD is only 30% for PNA-J_{10} binding, in contrast to around 70−80% in the case of PNA-C_{10} and PNA-$(TC)_5$ binding, which is ascribed to the low extinction coefficient for the pseudoisocytosine base at 260 nm. These

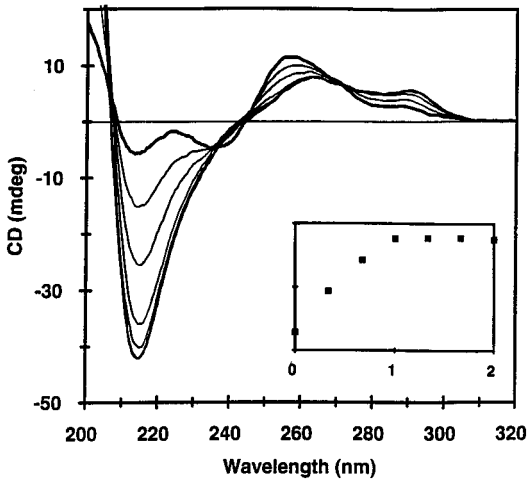

FIGURE 5: CD titration of PNA-J_{10} to poly(dG)·poly(dC). The boldface line shows the double-stranded polynucleotide before PNA addition. The insert shows the CD signal as a function of the ratio of PNA bases to DNA base pairs.

results all suggest that PNA-J_{10} binds as a Hoogsteen third strand to the DNA.

The conclusion that Hoogsteen binding is favored in cases of the cytosine-rich homopyrimidinic PNAs is further supported by an experiment in which a bis-PNA, with one strand of alternating TC bases and the other of TJ bases connected via a flexible linker, was found to add directly to

Table 2: Melting Temperatures for the PNA−DNA Complexes, As Determined from Midpoints of Denaturing Curves Obtained by CD in 5 mM Phosphate Buffer, No NaCl, pH 7.0

complex	T_m (°C)
poly(dG)·poly(dC) + PNA-C	>95
poly(dAdG)·poly(dTdC) + PNA-TC	85−90
poly(dAdG)·poly(dTdC) + PNA-(TC-TJ)	85−90
poly(dAdG)·poly(dTdC) + PNA-AG	95 (50−55)[a]
poly(dAdC)·poly(dTdG) + PNA-TG	85−90 (30−35)[a]
poly(dA)·poly(dT) + PNA-T	>85
poly(dG)·poly(dC)	80
poly(dAdG)·poly(dTdC)	45
poly(dAdC)·poly(dTdG)	60
poly(dA)·poly(dT)	60−65

[a] Melting temperature for the first complex with a PNA−DNA ratio of 2:1, or 3:1, going to a distinct 1:1 PNA−DNA complex.

poly(dAdG)·poly(dTdC) as a Hoogsteen-bound third strand, instead of strand-displacement binding. Upon titrating the polynucleotide with the PNA, we found saturation at a stoichiometry of 1 PNA base per DNA base pair, a low activation energy (see Table 1), and a strong pH dependence for the binding kinetics (much faster rate at pH 5 than at pH 7), all results which suggest that this alternating bis-PNA only associates to the intact duplex DNA as a Hoogsteen strand, at least in our polynucleotide system. By contrast, both single PNAs and bis-PNAs containing thymines and cytosines have earlier been shown to bind by strand invasion to single targets in double-stranded DNA (Egholm et al., 1995; Griffith et al., 1995; Praseuth et al., 1996).

All the PNA−DNA complexes studied have very high thermal stabilities, as deduced from CD melting experiments carried out in 5 mM phosphate buffer without extra added NaCl, showing melting temperatures around 85 °C or even higher (Table 2). The T_ms for the separate duplex DNAs ranged from 45 to 75 °C under the same experimental conditions. This shows that the PNA−DNA complexes, whether concluded to be invasion complexes or Hoogsteen-associated complexes, are all very stable.

DISCUSSION

Contrary to what might have been expected, homopyrimidine PNA oligomers that are rich in cytosines, such as PNA-C_{10} or PNA-$(CT)_5$, do not invade double-stranded polynucleotides by forming triplexes in a way analogous to that observed with thymine-rich PNAs (Wittung et al., 1996), but instead form Hoogsteen-type DNA_2−PNA triplexes (see Table 1, third column). This conclusion is based on the observed 1:1 LD and CD titration ratios (Figure 1 and Figure 4), the linear PNA concentration dependencies, and, finally, the very low activation energy for binding of these PNAs (Table 1). Furthermore, these reactions are significantly pH-dependent (Figure 3), indicating protonation of cytosine to be required (as is the case for Hoogsteen base-pairing). It should be noted that a pH dependence is also observed for the formation of invasion triplexes with cytosine-rich PNAs, since one of the strands in such a complex will bind as a Hoogsteen strand (Nielsen et al., 1994). Finally, LD experiments (Figure 4) show the complexes formed to have markedly larger (negative) LD signals compared to the original DNA double strands. In contrast to PNA−DNA invasion complexes, which by disrupting the order and stacking of the nucleobases are anticipated to show an impaired overall polymer orientation (less negative linear dichroism), the addition of extra stacked nucleobases from PNA to an intact DNA duplex is expected to increase the LD signal, both as a consequence of an increased stiffness of the polymer fiber and due to the increase of the numbers of stacked nucleobases.

A purine PNA decamer, with the sequence AAAAG-GAGAG, has been shown, by chemical probing, to bind a single target in double-stranded DNA by forming an invasion duplex (Nielsen & Christensen, 1996). Our results, obtained with CD and LD spectroscopy, are in agreement with this conclusion. We observe invasion duplexes with the PNA oligomers A_{10} and $(AG)_5$ (see Table 1, third column), as supported by titrations (Figure 1 and Figure 4) showing a 1:1 stoichiometric ratio of PNA bases to DNA base pairs, a linear PNA concentration dependence, a large activation energy consistent with base-pair opening (Table 1), a very strong salt dependence (Figure 2), and, finally, a decrease in LD signal, indicative of a more flexible polymer fiber formed upon PNA binding compared to the original DNA duplex (Figure 4).

A mixed T/G-PNA decamer was recently observed to bind a single Watson−Crick target by invasion into the double-stranded DNA, presumably forming a PNA_2−DNA triplex as evidenced by $KMnO_4$ probing and job plots (Nielsen & Egholm, 1997). Initially, we tested the ability of PNA-$(TG)_5$ to bind to the homopurine target poly(dAdG)·poly(dTdC), i.e., to form a PNA−DNA−DNA triplex with T*A−T and G*G−C base triplets, as has been reported for oligonucleotides (Sun et al., 1991). In this case, however, we did not detect any binding at all of the PNA. Instead, the PNA-$(TG)_5$ was, as expected, found to bind to the Watson−Crick complementary polynucleotide, poly(dAdC)·poly(dTdG), by forming an invasion complex. A 1:1 stoichiometry (i.e., a PNA−DNA duplex) at temperatures above 30 °C was found (see Figure 1).

At present, we can only speculate about the origin of the observed variations in how the different PNAs interact with DNA. Kinetic (Wittung et al., 1996) and structural (Kim et al., 1993) studies show that when forming the PNA(T)−DNA(A)−PNA(T) triplex, the two PNAs coalesce around the DNA in a concerted manner that may be related to dehydration effects of binding the PNA molecules close to each other. By contrast, the easily protonizable cytosine could make Hoogsteen binding to a retained DNA duplex energetically favorable, both as a result of electrostatic attraction to the doubly negative dsDNA and by the less pronounced hydrophobic character of the protonated PNA. This, however, cannot be the sole explanation since the uncharged pseudoisocytosine also binds efficiently as a third Hoogsteen strand. An additional, or alternative, explanation could be intrinsically different secondary structures of the target duplex DNA molecules, i.e., between the homo(dA)·homo(dT) duplex and guanine-containing homopurine−homopyrimidine duplexes. From a thermodynamic point of view, a Hoogsteen−Watson−Crick PNA−DNA−DNA triplex should be less stable than a PNA−DNA−PNA triplex. The reason why we do not observe the formation of the latter is probably a kinetic inertness of the PNA−DNA−DNA triplex (the Watson−Crick DNA−DNA duplex being kinetically stabilized by the presence of PNA). Since the melting temperature of the PNA−DNA−DNA complex is above 95 °C (Table 2), this point cannot simply be tested by heating. When targeting a single site in a large

double-stranded DNA with pyrimidine PNAs containing about 50% cytosines, formation of invasion triplexes has been observed (Praseuth et al., 1996; Vickers et al., 1995), indicating that the binding mode of such PNAs might vary depending on the exact conditions.

As to the behavior of the homopurine−PNA oligomers, as well as the alternating thymine-guanine PNA, this is less surprising; the alternative to "normal" Watson−Crick duplex formation is reversed Hoogsteen binding (Beal & Dervan, 1991; Pilch et al., 1991), giving either PNA(pu)−DNA(pu)−DNA(pyr) or PNA(pu)−PNA(pu)−DNA(pyr). The second of these is anticipated to be more stable than a PNA−DNA Watson−Crick duplex and may be the species observed with CD at low temperature for both PNA-$(AG)_5$ and PNA-$(TG)_5$ (see Results). In this case, presumably PNA*PNA−DNA triplexes with G*G−C and A*A−T or T*T−A base triplets are formed, as was indicated for a mixed T/G-PNA sequence (Nielsen & Egholm, 1997).

CONCLUSION

Although we have here limited ourselves to polynucleotides, and more detailed studies of mixed sequences of isolated PNA targets embedded in longer DNA stretches should ideally be studied before any general conclusions can be drawn, the present results demonstrate an extended repertoire of PNA recognition of nucleic acids. Hence, the recognition properties of PNA, as indicated or confirmed by the present findings, may be described in terms of the following "binding rules": (1) homopyrimidine PNAs invade dsDNA to form PNA_2−DNA triplex structures; (2) purine-rich PNAs can invade dsDNA via PNA−DNA duplexes; and (3) cytosine-rich pyrimidine PNAs can add directly to dsDNA to form PNA−DNA_2 triplexes.

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