Direct Observation of Strand Invasion by Peptide Nucleic Acid (PNA) into Double-Stranded DNA

Pernilla Wittung,[†] Peter Nielsen,[‡] and Bengt Nordén*[,†]

Contribution from the Department of Physical Chemistry, Chalmers University of Technology, S-41296 Gothenburg, Sweden, and the Department of Biochemistry B, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen N, Denmark

Received February 19, 1996[⊗]

Abstract: Homopyrimidine PNA can recognize a purine target of double-stranded DNA by local displacement of the DNA pyrimidine strand and formation of a PNA−DNA−PNA triplex. In order to elucidate the mechanism of this PNA/DNA recognition reaction we have studied the interaction of PNA−T oligomers with poly(dA):poly(dT) as a model system. The PNA binding kinetics can be observed *in situ* by spectroscopy as a change in the circular dichroism spectrum. The overall reaction rate decreases with increasing ionic strength and proceeds in minutes at 50 mM NaCl. From the temperature dependence of the rate constants an activation energy of about 60 kJ/mol (at 50 mM NaCl) was estimated, consistent with the hypothesis that a rate limiting step could be the DNA opening frequency. As expected from the stability of a DNA double helix toward large-scale openings, also the activation energy of the strand displacement increases somewhat with increasing salt concentration. There is a nonlinear dependence of the overall rate constant of triplex formation on the PNA concentration with an exponent of 2 or somewhat higher. With PNA in excess, the CD signal shows an overshoot suggesting the presence of a third species. The CD spectrum of this intermediate indicates an ordered structure. The presence of intercalators ethidium bromide and 9-aminoacridine is found to increase the overall PNA binding rate, whereas minor groove binder DAPI and major groove binder methyl green both decrease the rate. The mechanism of strand invasion of duplex DNA by PNA is discussed in the light of these results.

Introduction

Reagents that bind sequence selectively to single- or double-stranded DNA are of significant interest in molecular biology and medicinal chemistry as they may be developed into gene-targeted drugs for diagnostic and therapeutic applications, and to provide tools for sequence specific modification of DNA.[1,2] Oligonucleotides and their close analogs have so far been the main candidates for developing such reagents.[2−4] They are, however, not optimal in terms of stability against biological degradation, solubility, cellular uptake properties, or ease of synthesis. For these reasons alternative concepts of oligonucleotide mimics have attracted interest.

PNA (peptide nucleic acid) is a recently developed novel 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.[5,6] Mixed sequences of PNA have been found to be very potent DNA mimics, forming Watson−Crick base-paired duplexes with complementary DNA of high specificity and thermal stability,[7] and have consequently been considered promising leads for development of gene therapeutics.[8]

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.[9,10] PNA binds to the complementary DNA strand by forming a very stable local triplex in which one PNA is bound by Watson−Crick base-pairing and the other by Hoogsten base-pairing to the central purinic strand.[11] The non-complementary DNA strand is displaced into a loop structure.[12,13]

For the future development of molecular biological and medicinal applications of PNA, it is essential to understand the molecular mechanism of, and the factors controlling, this type of PNA/DNA reaction. So far, it has not been possible to detect the presence of any intermediate species, such as a PNA−DNA duplex or PNA−DNA_2 triplex, in the reaction between homopyrimidine PNA and DNA. Based on gel-shift studies of plasmid DNA containing a single 10mer purine target, it has been proposed that the PNA first forms a Watson−Crick hybrid with the transiently opened DNA duplex, preceding the final Hoogsten base-pairing of the second PNA.[14]

* Author to whom correspondence should be addressed.
† Chalmers University of Technology.
‡ University of Copenhagen.
⊗ Abstract published in *Advance ACS Abstracts,* July 1, 1996.

(1) Hélène, C.; Toulmé, J.-J. *Biochim. Biophys. Acta* **1990**, *1049*, 99−125.
(2) Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, *90*, 554−584.
(3) Goodchild, J. *Bioconj. Chem.* **1990**, *1*, 165.
(4) Moser, H. E.; Dervan, P. B. *Science* **1987**, *238*, 645−650.
(5) Egholm, M.; Buchardt, O.; Nielsen, P. E.; Berg, R. H. *J. Am. Chem. Soc.* **1992**, *114*, 1895−1897.
(6) Christensen, L.; Fitzpatrick, R.; Gildea, B.; Petersen, K. H.; Hansen, H. F.; Koch, T.; Egholm, M.; Buchardt, O.; Nielsen, P. E.; Coull, J.; Berg, R. H. *J. Peptide Sci.* **1995**, *3*, 175−183.
(7) Egholm, M.; Buchardt, O.; Christensen, L.; Bejrens, C.; Freier, S. M.; Driver, D.; Berg, R. H.; Kim, S. K.; Nordén, B.; Nielsen, P. E. *Nature* **1993**, *365*, 566−568.
(8) Crook, S. T. *Curr. Opin. Biotechnol.* **1992**, *3*, 656−661.
(9) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497−1500.
(10) Cherny, D. Y.; Belotserkovskii, B. P.; Frank-Kamenetskii, M. D.; Egholm, M.; Buchardt, O.; Berg, R. H.; Nielsen, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1667−1670.
(11) Betts, L.; Josey, J. Veal, J.; Jordan, S. *Science*, **1995**, *270*, 1838−1841.
(12) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *AntiCancer Drug Design* **1993**, *8*, 53−63.
(13) Nielsen, P. E.; Egholm, M.; Buchardt, O. *J. Mol. Recognit.* **1994**, *7*, 165−170.
(14) Demidov, V. V.; Yanilovich, M. V.; Belotserkovskii, B. P.; Frank-Kamenetskii, M. D.; Nielsen, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2637−2641.

S0002-7863(96)00521-5 CCC: $12.00

We here use circular dichroism spectroscopy to follow the kinetics of the invasion by homopyrimidine PNA into double-stranded DNA. As a model system we use double-stranded homopolynucleotide poly(dA):poly(dT) and PNA−T oligomers. The dependence of the kinetics of the strand-displacement reaction on ionic strength, temperature, PNA concentration, and length is investigated. The effects on reaction rate and activation characteristics upon interaction of fully matched and singly mismatched PNA oligomers are compared and, finally, the effects of the presence of some DNA−binding ligands have been studied.

Experimental Section

Chemicals. Poly(dA):poly(dT) (length of 5.2 kbp:s), purchased from Pharmacia, was dissolved in 5mM sodium phosphate buffer, pH 7.0, and dialyzed several times against this buffer. The PNAs (H-TTTTTTTTTT-Lys-NH_2, H-TTTTTTTT-Lys-NH_2, H-TTTTTTT-Lys-NH_2, H-TTTTT-Lys-NH_2, H-TTTTTCTTTT-Lys-NH_2, H-TTCT-TCTTTT-Lys-NH_2) were synthesized as described elsewhere.[5,6] Ethidium bromide (EB), methyl green (MG), 9-aminoacridine (9AA), and 9′,6-diamidino-2-phenylindole (DAPI) were purchased from Sigma.

We determined the concentrations spectrophotometrically by using the molar extinction coefficients (per base); $\epsilon_{260} = 6000\ M^{-1}\ cm^{-1}$ for poly(dA):poly(dT), $\epsilon_{260} = 8800\ M^{-1}\ cm^{-1}$ for PNA−T_{10}, PNA−T_8, PNA−T_7, and PNA−T_6, $\epsilon_{260} = 8750\ M^{-1}\ cm^{-1}$ for PNA−T_5CT_4; and $\epsilon_{260} = 8500\ M^{-1}\ cm^{-1}$ for PNA−$T_2CT_2CT_4$. The experiments were normally performed in a 5 mM phosphate buffer, pH 7.0, containing 50 mM NaCl. Varied parameters (temperature, type of PNA, concentration of PNA, NaCl concentration) are as indicated in the tables, figure captions, or text. The reaction studied is the following:

$$\text{poly(dA):poly(dT)} + 2\text{PNA}-\text{T} \rightarrow \text{poly(dA):(PNA}-\text{T)}_2 + \text{poly(dT)}$$

We initiated it by the addition of a small aliquot of PNA to the cuvette containing poly(dA):poly(dT), buffer, and salt. The concentrations used are as indicated in the figure captions (normally 20 μM poly(dA):poly-(dT) in base pairs).

Methods. Circular dichroism (CD), defined as 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 at 255 nm (where the largest amplitude of CD difference upon reaction is found). In an additional set of experiments, we recorded the kinetics at several different wavelengths (see Figure 4). 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. In the CD experiments involving DNA binding drugs we collected data between 200 and 450 nm to observe the induced CD from the bound drugs as well, and in so doing, verify binding.

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

Results

We used duplex poly(dA):poly(dT) and PNA−T oligomers as a model system to study the reaction in which two strands of PNA−oligoT invade the DNA duplex and the DNA−T strand is displaced. A titration of poly(dA):poly(dT) with various amounts of PNA−T_8 shows a saturation at a 2:1 ratio of PNA thymine bases to adenine bases (or A:T base pairs) of DNA in the CD spectrum (Figure 1). Upon reaction of PNA−T with poly(dA):poly(dT), the overall CD spectrum becomes more positive and the final shape of the CD curve is practically identical to that observed for a PNA_2−poly(dA) triplex,[15] after subtraction of the (weak) CD of a single poly(dT) strand. The final 2:1 stoichiometry was also confirmed by a corresponding flow linear dichroism (LD) titration. Moreover, the amplitude and shape of the reduced linear dichroism (LD^r) at saturation (results not shown) were found to be consistent with the presence of a stiff (easy flow-orientable) (PNA−T)$_2$−poly(dA) triplex[15] and a single, free (unorientable) poly(dT) strand. Thus a triplex is formed on the poly(dA) strand by two PNAs and the poly-(dT) strand is displaced into solution.

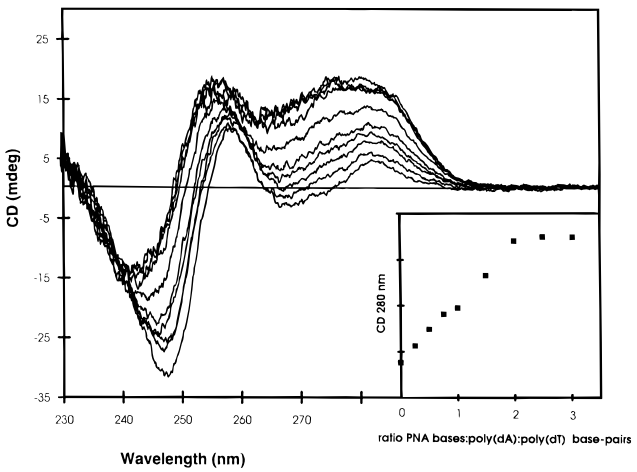

Figure 1. CD titration of PNA−T_8 (0−300 μM bases) to 50 μM base pairs of poly(dA):poly(dT). Insert shows the corresponding CD signals at 280 nm, measured at equilibrium, versus the ratio of PNA bases to DNA base pairs. Saturation is reached at 2 PNA bases per DNA base pair.

The strand displacement binding of PNA to a single purinic target in DNA has been observed to be salt dependent and to be inhibited at high salt concentrations.[13] Furthermore, we confirmed in our system that the inclusion of 500 mM NaCl completely inhibits detectable strand displacement binding (notably no change in CD signal was observed showing that a PNA−poly(dA):poly(dT) triple helix did not form either), whereas the binding was completed within a few seconds in 5 mM salt (not shown). An NaCl concentration of 50 mM gave reaction rates which are convenient for kinetic measurements and, unless otherwise stated, the experiments refer to this ionic medium.

The kinetics of the strand displacement of poly(dA):poly-(dT) by the PNA−T oligomers was followed by the increase in the CD signal at 255 nm, where the largest CD amplitude change occurs upon triplex formation. Figure 2 shows representative recordings of the rate of strand displacement by PNA octamers (PNA−T_8,) at three different temperatures. Approximating the kinetics as monoexponential, we estimated activation energies from appropriate Arrhenius plots (Table 1). In 50 mM NaCl, the PNA−T_8 and PNA−T_{10} showed similar temperature dependencies, giving an activation energy E_a of approximately 60 kJ/mol (Table 1). Increasing the salt concentration in the solution retarded the reaction kinetics as expected (Table 1). Upon increasing the NaCl concentration to 150 mM for PNA−T_8 E_a was significantly increased, as well as for PNA−T_{10} (125 mM NaCl). This indicates that the activation energy increases with ionic strength. A 10mer PNA with the sequence T_5CT_4, *i.e.* having one mismatch per 10 PNA bases, showed a 60-fold reduction in the rate of binding to poly-(dA):poly(dT) at 20 °C compared with the fully complementary oligomer PNA−T_{10}. A considerably higher activation energy was also found for this reaction (Table 1). In addition, a 10mer PNA with two mismatches ($T_2CT_2CT_4$) did not show any detectable strand invasion at 20 °C.

(15) Kim, S. K.; Nielsen, P. E.; Egholm, M.; Buchardt, O.; Berg, R. H.; Nordén, B. *J. Am. Chem. Soc.* **1993**, *115*, 6477−6481.

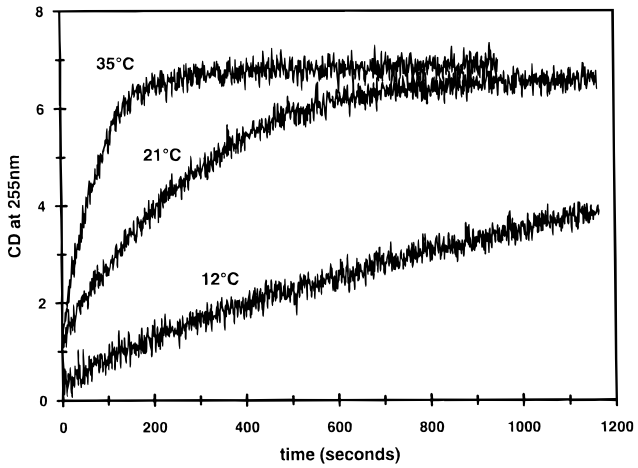

Figure 2. Kinetics followed by CD at 255 nm upon mixing 80 μM bases of PNA$-T_8$ with 20 μM base pairs of poly(dA):poly(dT) performed at 12, 21, and 31 °C.

Table 1. Estimated Activation Energies of PNA Binding to Poly(dA):Poly(dT)

PNA	condition	E_a (kJ/mol)
PNA$-T_{10}$	50 mM NaCl	58.4 ± 3
PNA$-T_{10}$	125 mM NaCl	68.8 ± 4
PNA$-T_8$	50 mM NaCl	60.1 ± 3
PNA$-T_8$	150 mM NaCl	73 ± 3
PNA$-T_5CT_4$	50 mM NaCl	79 ± 4
PNA$-T_8$	2 μM EB	39 ± 3
PNA$-T_8$	1 μM DAPI	71.4 ± 5

Table 2. Effects on the Strand Invasion Rate by Length of PNA and Presence of Drugs Prebound to Poly(dA):Poly(dT)[a]

PNA	PNA concn, μM	drug	drug concn, μM	$t_{1/2}$, s
T_6	80			600
T_7	80			340
T_8	80			200
T_{10}	80			35
T_{10}	40			130
T_{10}	40	DAPI	1	420
T_{10}	40	DAPI	4	no reaction
T_{10}	40	MG	2	410
T_{10}	40	MG	4	940
T_{10}	40	EB	4	58
T_8	80	EB	2	120
T_8	80	EB	4	48
T_8	80	EB	6	38
T_8	80	EB	8	24
T_{10}	80	9AA[b]	4	11
T_{10}	80	9AA[b]	8	8

[a] CD kinetics followed at 255 nm; 20 μM (basepairs) poly(dA):poly(dT) in 50 mM NaCl. [b] CD kinetics showed an overshoot similar to that found at high PNA concentration.

PNA oligomers of varying lengths (6, 7, 8, and 10 bases long) were studied to compare their rates of binding to poly(dA):poly(dT) at one single temperature (20 °C). As seen in Table 2 the longer the PNA strand the faster the reaction proceeds, consistent with the expectation that fewer nucleations per poly(dA):poly(dT) molecule need to occur for the longer PNA strands, provided this step is the rate-limiting one.

The dependence of the strand displacement rate on the PNA concentration was studied for PNA$-T_{10}$ and PNA$-T_8$. The poly(dA):poly(dT) concentration was kept constant (20 μM in base pairs) whereas the PNA concentration was varied between 40 and 200 μM bases (*i.e.* up to 5-fold PNA excess). The rate is strongly dependent on the PNA concentration, as seen in Figure 3 (dotted lines). The concentration dependence of the

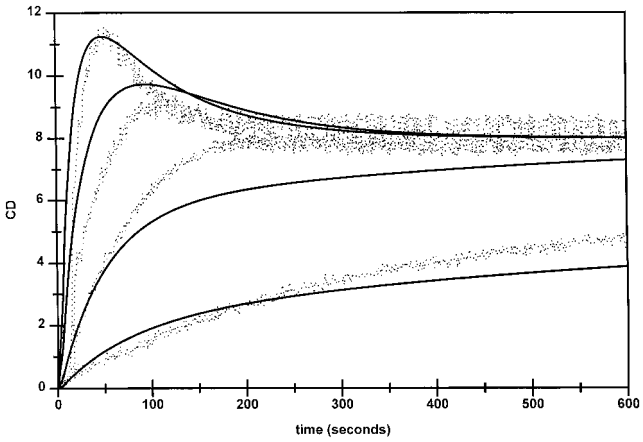

Figure 3. Dotted line curves: Kinetics followed by CD at 255 nm upon mixing PNA$-T_{10}$ with 20 μM base pairs poly(dA):poly(dT). The curves correspond to different PNA concentrations: 35, 70, 140 and 210 μM bases, respectively. As seen, for the two highest concentrations, the CD signals go through overshoots before reaching a plateau value. Solid line curves: Simulated kinetics for the PNA interaction with DNA based on a mechanism

$$xA + B \rightarrow A_xB \rightarrow C$$

where A denotes PNA and B is DNA. The main features of the experimental CD curves (dotted curves) can be reproduced by taking $x = 2$ and the CD intensity of the intermediate, A_2B, to be 1.5 times that of the final product and the ratio between the first and second rate constants equal to 15.

pseudo-first-order rate constants (k) can be approximated to follow a power law,

$$k \propto [\text{PNA}]^{\gamma} \quad (1)$$

with an exponential factor, γ, of about 2−2.5 which is in agreement with the concentration dependence reported for PNA invasion into plasmid DNA.[14] We estimated this exponential factor both from initial rate constants of fitted monoexponentials and from logaritmic plots of reaction halftimes (see also Discussion).

With an excess of PNA (140 or 210 μM, *i.e.* 3- or 5-fold excess) evidence for an intermediate species was obtained, detectable within the first 10−20 s, as the CD signal went through an overshoot before it reached its final value as shown for PNA$-T_{10}$ in Figure 3. By following the CD reaction kinetics at various wavelengths with PNA in excess, an "intermediate CD spectrum" could be constructed from the observed transient CD signals at their respective turning points (Figure 4). This intermediate CD spectrum resembles more a (PNA$-$T)$_2$/poly(dA) triplex spectrum than a duplex PNA−DNA spectrum.[7] It was verified that the dependence of the strand displacement rate on the PNA concentration could be described by an exponential factor of 2−3 for the T_5CT_4 sequence as well. However, with this mismatched PNA no evidence for any intermediate was observed.

The effects on the strand displacement rate by the presence of various DNA−binding ligands, which were added to poly(dA):poly(dT) prior to the incubation with PNA−T, were also investigated (Table 2). The dicationic dye 6-diamidino-2-phenylindole (DAPI), known to bind in the minor groove of AT regions of DNA duplexes, ethidium bromid (EB), and 9-aminoacridine (9AA), both of which intercalate between the base pairs, and also the major groove binder methyl green (MG) were studied. The binding modes of these drugs with respect to poly(dA):poly(dT) have been verified in terms of binding geometric characterizations, using linear dichroism spectroscopy.[16,17]

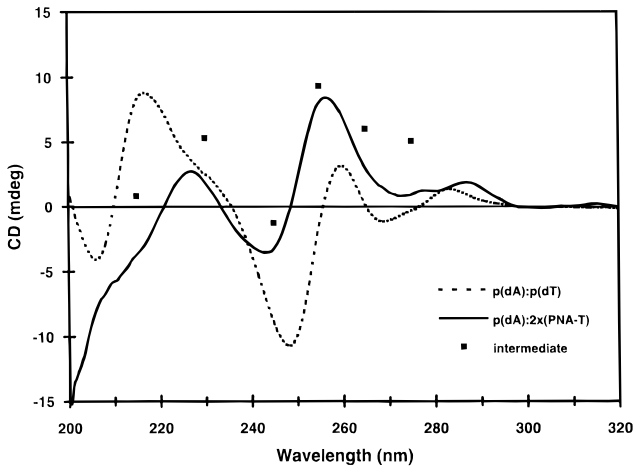

Figure 4. CD spectra of poly(dA):poly(dT), of the final poly(dA)−$(PNA-T)_2$ triplex, and of the intermediate structure estimated from observed transient CD signals at various wavelengths at their respective turning points.

It was found that the reaction rate between PNA−T_8 and poly(dA):poly(dT)−DAPI was significantly slower than the corresponding rate without DAPI and an increase in E_a was found (Tables 1 and 2). At binding ratios $r > 0.1$ (DAPI versus poly(dA):poly(dT) bases) no detectable PNA invasion of the duplex occurred. In addition, the presence of the major groove binding drug MG was found to decrease the rate of PNA binding to poly(dA):poly(dT) in a concentration-dependent manner (Table 2). By contrast, when the intercalator ethidium bromide was present we found the strand displacement rate between PNA−T_8 and poly(dA):poly(dT):EB considerably faster than in the absence of drug. In Table 2 the dependence of the PNA binding rate on the EB concentration is shown. The presence of EB decreased the E_a for the strand displacement reaction, an example shown for $r = 0.05$ EB per DNA base in Table 1. Likewise, the presence of intercalator 9AA prebound to poly(dA):poly(dT) was also found to increase the PNA binding rate (Table 2). The mechanism, however, by which the overall reaction rate is enhanced by 9AA may be different from that of EB enhancement, since when 9AA is present the CD shows the same kind of overshoot as at very high PNA concentrations (results not shown). With the exception of DAPI, which exhibits a certain affinity for PNA:DNA:PNA T:A:T triplex[18] and for which an induced CD indicated some binding, none of the drugs remained bound in the final product as evidenced by the respective drug spectroscopic signals (fluorescence of EB, induced CD of 9-AA and MG, results not shown). As shown previously intercalators, such as EB, do not bind to PNA−DNA duplexes.[18]

Discussion

The first important conclusion concerns the applicability of our polynucleotide model system to the strand invasion experiments with PNA−pyrimidines at a single purine target in a plasmid DNA as previously reported.[14] Upon addition of PNA−T oligomers to double-stranded DNA poly(dA):poly(dT) the reaction leading to the formation of a triplex can be monitored by CD in real time. Both the observed salt dependence and the PNA concentration dependence (vide infra) of the reaction rate are similar to what was found for the plasmid system, justifying the application of our model system. Furthermore, an activation energy comparable to those observed here has been found for the plasmid system too.[19]

Our discussion of potential reaction mechanisms will be based on the tentative scheme presented in Figure 5. The second-order (or higher) PNA concentration dependence suggests the importance of some reaction step(s) in which two, or more, PNA molecules are involved. A first logical step is a precursory "outer-sphere" association of one or several PNA molecule(s) to the intact poly(dA):poly(dT) duplex (starting species S), in the scheme denoted as species **1** or **1′**. This species could be in principle a Hoogsteen-like triple-helical complex but the PNA may as well be nonspecifically (hydrophobically) associated as indicated by non base pairing in the scheme. Such a species is probable although its thermodynamic stability may be small, as indicated by the fact that we have not been able to detect it at increased salt concentration at which the final product is not formed. A nonspecific association step is expected to be diffusion controlled and therefore rather fast; a low stability then requires an even faster dissociation.

As a potential next step one may envisage the opening of the Watson−Crick DNA duplex (species **2**) followed by the entering of one of the "outer-sphere" associated PNAs into a Watson−Crick base-paired position (species **3**). The formation of species **2**, possibly together with species **3** and **4**, probably constitutes the rate-limiting steps. Indeed, the observed values of activation energies are comparable to the activation energy deduced for base-pair opening of a few base pairs of normal DNA[20] suggesting that the activation energy for transferring the PNA from the outer to the inner position is comparatively small. The hypothesis that the rate-limiting process is the opening of the DNA should be discussed also in the light of the observed salt dependence. High salt will stabilize the DNA duplex as the phosphate−phosphate repulsion is decreased *via* the shielding by the counterions. While small-scale DNA openings (single base pair) have not been found associated with any salt-dependent activation energy,[20] a large-scale opening is expected to have an activation energy that increases with ionic strength. The observed slight increase in activation energy may thus suggest that the bulge indicated in the drawing of species **2** represents a major opening of DNA.

The association of the Hoogsten PNA strand, leading to the completion of a PNA−DNA−PNA triplex (species **F**, for final), is very fast as indicated by the observation of a practically immediate reaction of PNA with a single poly(dA) strand (results not shown, cf. also ref 15). Back reactions from the triplex are extremely slow so the reaction could be considered as "irreversible".

It is clear both from the present results as well as from previous findings[14] that the reaction order with respect to PNA is more than unity (2 or more) which excludes that the main transition state is simply species **2** or **3**. A second PNA molecule must be involved in the rate-limiting step, or the steps preceeding it. Here several possibilities bringing the two PNA:s into position can be imagined. For example, one of them may enter from the bulk solution, if the rate limiting step is a later one, as indicated in the formation of species **4**. For energetic reasons it is very unlikely that the activation barrier to formation of species **4** should be higher than for species **2**.

In order to explain a reaction order >2, and the observation of an overshoot in the CD, we have in Figure 5 introduced a reaction line involving a larger number of PNA molecules. Here,

(16) Nordén, B. Kubista, M.; Kurucsev T. *Q. Rev. Biophys.* **1992**, *25*, 51−170.
(17) Tuite, E.; Nordén, B. *Bioorg. Med. Chem.* **1995**, *3*, 701−711.
(18) Wittung, P.; Kim, S.; Buchardt, O.; Nielsen, P.; Nordén, B. *Nucleic Acid Res.* **1994**, *22*, 5371−5377.
(19) Bentin, T.; Nielsen, P. *Biochemistry* In press.
(20) (a) Leroy, J. L.; Kockoyan, M.; Huynh-Dinh, T.; Guéron, M. *J. Mol. Biol.* **1988**, *200*, 223. (b) Braunlin, W. H.; Bloomfield, V. A. *Biochemistry* **1988**, *27*, 1184−1191.

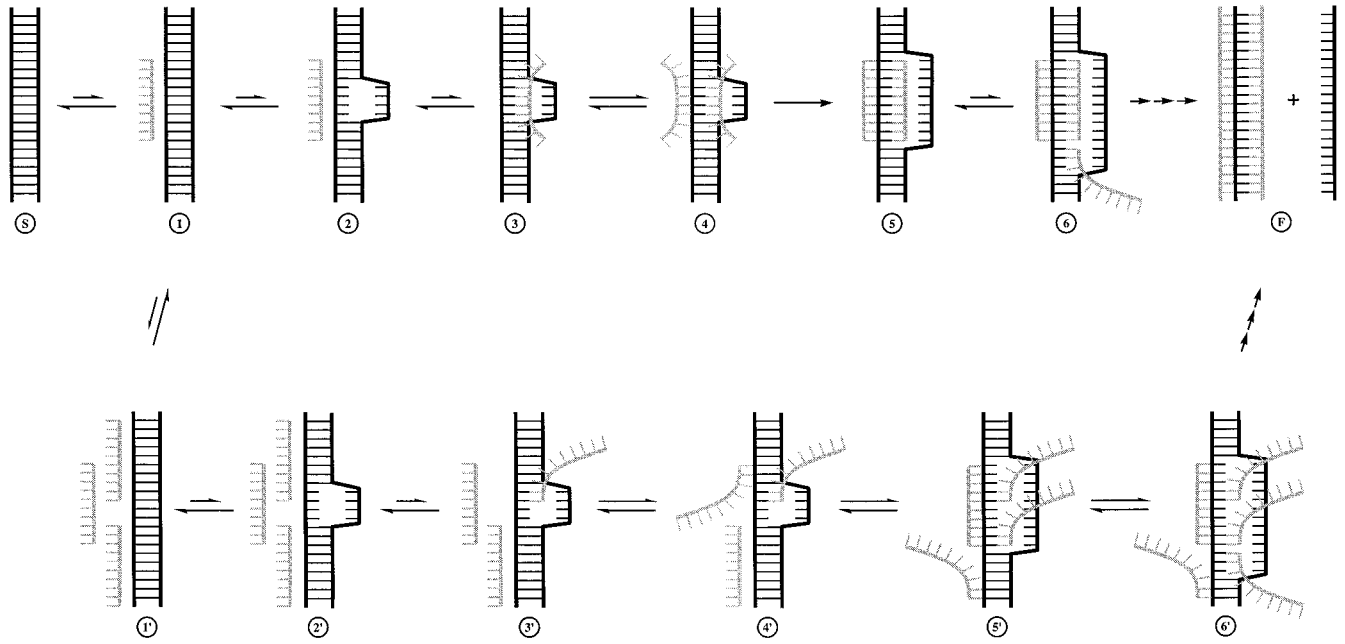

Figure 5. Proposed mechanisms of PNA strand invasion into a double-stranded DNA (see text).

species **5′** and **6′** schematically represent metastable structures in which several bound PNA:s are blocking each other, leading to slow rearrangement toward the final product. Such a mechanism would explain the observation of an intermediate species at higher PNA concentration (Figure 3). Another possibility, which we cannot yet completely exclude, is that species **1′** is the observed long-living intermediate. However, there is no reason why this complex should not be formed at high salt conditions at which strand invasion does not occur. Our failure to detect **1′** at high salt, therefore, makes the latter possibility less probable.

The CD signal in Figure 4, recorded at a few wavelengths at the overshoot, indicates a spectrum consistent with the intermediate having an ordered helical structure with three or more base-stacked strands, such as for example species **5′**−**6′** with excess of bound PNA (or species **1′**, with outside stacked PNA).

The gross features of the PNA concentration dependence can be explained by a model reaction of the type:

$$xA + B \rightarrow A_xB \rightarrow C \qquad (2)$$

where A = PNA, B = DNA, A_xB represents the intermediate species, C is the final product, and x is the number of PNAs involved in the intermediate structure (in Figure 3, solid lines show a fit corresponding to $x = 2$). As is further seen from the parameters chosen to model the experimental data in Figure 3 (see figure caption), the specific CD amplitude for the intermediate must be considerably larger (50%) than the maximum in the overshoot indicates. Again, this indicates that the intermediate species is an ordered structure, the high amplitude suggesting a structure containing even more than the three strands of the final triplex, in accord with the proposed mechanism. Varying the PNA dependence in the model (the value of x) gave reasonable resemblance to the experimental kinetics in the range 2−2.5, but not with 1.5 or 3.0.

As to the important central steps of the strand invasion, it is interesting to address the effects of the length of PNA and of mismatches as well as the influence of interfering drugs. The increased reaction rate upon increasing the length of PNA may be regarded as an effect of fewer nucleations per poly(dA):poly(dT) molecule. Upon introducing one mismatch in the PNA (an inserted C) the reaction rate decreases dramatically and the apparent activation energy increases significantly. It is expected that an impaired association property in the Watson−Crick base pairing of PNA (i.e. faster runoff rate of PNA) will also slow down the invasion rate. The increased activation energy indicates that the nucleation complex includes the mismatch position that is ≥5 bases.

In our model, the rate determining step including the opening of duplex DNA, ligands that stabilize DNA are also expected to slow down the strand invasion rate of PNA. Our findings that the groove binders DAPI and MG, which both stabilize duplex DNA, decrease the invasion rate are thus not surprising (MG renatures solvent-denatured DNA[21] and DAPI increases the melting temperature of poly(dA):poly(dT) by approximately 30 °C[22]). Also, it can be anticipated that the presence of a groove-binding ligand will interfere with the putative precursory outer-sphere PNA−DNA complexes. The minor groove-binding DAPI is known to be able to bind to a PNA−DNA−PNA T:A:T triplex to some extent (with an orientation consistent with minor groove binding).[15,18] The dye MG is believed to bind in the major groove[23] of duplex DNA but shows no significant binding to triplex PNA−DNA−PNA (unpublished results). The fact that MG is less efficient than DAPI in inhibiting the strand exchange (Table 2) is consistent with its lower binding constant versus DNA.[24]

In contrast to the groove binders, the intercalating ligands studied instead promote the PNA strand displacement process. One may only speculate about the origin of the enhancing effects of intercalators on the PNA binding rate. A contributing effect might be the unwinding of DNA by intercalators, making the grooves wider and the DNA more accessible to PNA in the entering steps; also, the unwinding could have a topological effect, favoring the formation of bulges. However, intercalators are known to stabilize the double helix. A possible explanation is that one of the precursory or intermediate PNA complexes, by having a higher affinity for the intercalator, may decrease the activation energy. A Watson−Crick DNA−PNA intermediate is a poor candidate as intermediate in such a mechanism

(21) Nordén, B.; Seth, S.; Tjerneld, F. *Biopolymers* **1978**, *17*, 523−525.
(22) Kim, H.; Kim, J.; Kim, S.; Rodger, A.; Nordén, B. *Biochemistry* in press.
(23) Kim, S.; Nordén, B. *FEBS Lett.* **1993**, *315*, 61−64.
(24) Nordén, B.; Tjerneld, F.; Palm, E. *Biophys. Chem.* **1978**, *8*, 1−15.

since we know that such a duplex has little affinity for intercalators.[18] However, if the externally bound PNA intermediates are stabilized by an intercalator bound to the DNA duplex, this would increase the overall reaction rate. A decreased activation energy would then require, though, that the rate-limiting step be 2′ which is very unlikely. The differences observed between the kinetics of interaction of PNA in the presence of EB and 9-AA, however, indicate that the processes are complex and that subtle differences in modes of ligand binding may cause shifts between alternative reaction paths, such as those suggested by the upper and lower lines in Figure 5.

Conclusions

A number of important conclusions about the strand-displacement mechanism may be drawn from the present results:

1. CD allows *in situ* study of the strand displacement by a PNA−T oligomer in duplex poly(dA):poly(dT) as a model system and the reaction can be attenuated by the presence of salt to suitable rates.

2. Activation energies are of a magnitude similar to that observed for DNA base-pair opening, suggesting this to be a substantial part of the activated state. It is suggested that the nucleation step contains a PNA_2−DNA invasion triplex of approximately 5 bases.

3. The dependence of the overall rate constant on the PNA concentration may be described by an exponent of 2−2.5, indicating that at least two PNA molecules are involved in the rate-limiting step(s).

4. An overshoot in the CD trajectory at high PNA concentration indicates the presence of an intermediate species, possibly due to DNA complexes that are locally oversaturated with PNA.

5. Salt as well as groove-binding duplex−DNA ligands decrease the rate of PNA strand invasion, whereas intercalating ligands enhance the rate.

Acknowledgment. We are grateful to Dr. Per Lincoln for valuable discussion regarding the kinetic mechanisms and help with the simulation and to Tommi Ratilainen for help with computer drawings. The Danish National Research Foundation and the Swedish Natural Science Research Council are acknowledged for financial support.

JA960521F