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Effect of Temperature and Ionic Strength on the Dissociation Kinetics and Lifetime of PNA−DNA Triplexes[†]

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ABSTRACT: Dissociation kinetics of triplexes formed by molecules of peptide nucleic acid (PNA) and DNA have been studied. The complexes consisted of oligomeric PNA containing 10 thymine bases and the dA_{10} target incorporated in single-stranded (ssDNA) or double-stranded DNA (dsDNA). Their dissociation was followed by means of the gel mobility shift assay at various temperatures and sodium ion concentrations. In all experiments, the dissociation kinetics of triplexes were exponential; the effective lifetime of a triplex, τ, depended on temperature in accordance with the Arrhenius law. The τ values for T_{10} PNA complexes with ss- and dsDNA were equal within the accuracy of experiments. The activation energy, U, value for T_{10} PNA−DNA complexes did not change when the NaCl concentration was increased from 50 to 200 or 600 mM. Conversely, the τ values decreased with the increase in NaCl concentration. The equal lifetimes of the T_{10} PNA−DNA triplexes containing ss- and dsDNA suggest that the loop formed in dsDNA does not noticeably affect the triplex structure. The decrease in the triplex lifetime τ with an increase in ionic strength was accounted for by the fact that the PNA backbone is neutral. The lack of relationship between the activation energy of dissociation and salt concentration suggests that the dissociation enthalpy does not depend on the ionic strength. Thus, the effect of ionic strength on the lifetime is entropic by its nature. Contrary to this, for complexes of ssDNA with bis-PNA 1743, which also consists of 10 thymine bases but contains 2 additional positive charges inside the sequence in 1 of the PNA arms, an increase of the dissociation enthalpy at low salt concentration was observed. We suggest that this effect is a result of a direct electrostatic interaction of the positive charges of the PNA with the DNA backbone. Finally, our results allow an estimate of the lifetime of a 10-mer triplex invasion complex in dsDNA at 37 °C in excess of several hundred days.

Peptide nucleic acid (PNA)[1] is a very potent DNA mimic in which the negatively charged sugar−phosphate backbone is substituted with a charge neutral pseudopeptide chain. Hybridization complexes between PNA and DNA or RNA (*1*, *2*) exhibit high stability and sequence specificity, and these properties combined with high biostability have attracted attention to PNA as a promising gene therapeutic agent (*2−5*). In addition to the possible use in medicine, PNA has a number of other interesting applications, e.g., in DNA mapping (*6*), isolation, and purification (*7*); for developing artificial rare-cutting "restrictases" (*8−11*); and as gene-specific diagnostic agents (e.g., *12−14*), etc.

Homopyrimidine PNAs interact sequence-specifically with both single-stranded (ssDNA) and double-stranded DNA (dsDNA). They bind to complementary DNA targets by forming triplexes consisting of a homopurine DNA segment and two PNA molecules, which form Watson−Crick and Hoogsteen bonds with DNA, respectively (*15−16*). In the case of dsDNA, the second (homopyrimidine) DNA strand is displaced to form a single-stranded loop (*1*, *15−17*). In some cases, cytosine-rich PNAs have been shown to form conventional PNA−DNA_2 triplexes without local displacement of a DNA strand (*18*).

Extremely high stability and sequence specificity is a hallmark of DNA/$(PNA)_2$ triplexes. Hence, a more detailed understanding of the thermodynamic stability and complex lifetime as a function of ionic strength and temperature is essential for any application of PNA. Typically, the relative stability of the triplexes is estimated from their thermal melting profile determined spectrophotometrically under standard conditions. This is, however, only a (very rough) guideline for the thermodynamic properties. For instance, the following data on the melting temperature of triplexes formed by bis-PNA 1743 (see Materials and Methods) with ssDNA were obtained by a spectrophotometric method. The equilibrium values of T_m depended on the concentration of complex and at $[Na^+] = 200$ mM varied from 75.8 to 81.9 °C in the range of molar concentations from 0.45×10^{-6} to

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[1] Abbreviations: PNA, peptide nucleic acid; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

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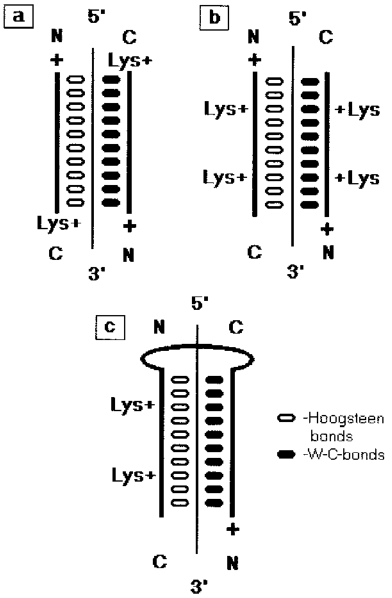

FIGURE 1: Schematic sketch of the complexes of PNA T_{10} (a), PNA 1460 (b), and PNA 1743 (c) with single-stranded DNA. Open circles denote Hoogsteen bonds; closed circles, Watson−Crick bonds. The letters N and C denote N- and C-termini of PNA, respectively.

18.2×10^{-6} M (unpublished data). To obtain more detailed data on triplex stability under various conditions, we have studied the effects of temperature and salt concentration on the dissociation kinetics of triplexes formed by homopyrimidine PNA with ss- and dsDNAs. Using a polyacrylamide gel electrophoresis mobility shift assay, we measured the effective lifetime (τ) of DNA/$(PNA)_2$ triplexes at various temperatures and ionic strengths. In the case of T_{10} PNA, τ was essentially identical for ss- and dsDNA complexes, which surprisingly suggests that the P-loop does not affect the PNA dissociation rate. We determined the activation energy of triplex dissociation and found a decrease in triplex stability with an increase in ionic strength. It was found that, as the triplex lifetime decreased with an increase in the NaCl concentration, the Arrhenius plots of τ versus $1/T$ remained parallel. This indicates that the dissociation enthalpy is independent of ionic strength.

MATERIALS AND METHODS

Two types of PNA thymine decamers, which differed from each other in the number of positive charges attached to the molecule, were used. One PNA species, H-T_{10}-$LysNH_2$ (designated hereinafter as T_{10} PNA), had positive charges only at the N- and C-termini. The other species (PNA 1460) had, in addition to the N-terminal positive charge, two positively charged lysine-derived PNA units *(19)*. The third species was bis-PNA (PNA 1743) which contained two positively charged lysine-derived PNA units in the middle of only one of the arms.

Schematic representation of T_{10} PNA, PNA 1460, and PNA 1743 is given in Figure 1. For clarity, triplexes formed by these PNAs and a single-stranded A_{10} target are shown. The PNAs were obtained by manual synthesis as described previously *(20−24)*.

The concentrations of the decamers were estimated based on the molar extinction coefficient for a thymine monomer (8800) and the molecular weights of T_{10} PNA and PNA 1460. The dsDNA was a 65 bp fragment of the *Eco*RI/*Hin*dIII polylinker of the pUC 19 plasmid containing a $dA_{10}{\cdot}dT_{10}$ insertion (the target for PNA) at the *Bam*HI restriction site *(1)*. Following the restriction, radioactive labels were incorporated into both ends of the restriction fragment with the use of Klenow fragment in the presence of [α-^{32}P]ATP and the other three (unlabeled) NTPs. As the final stage of purification, the fragment was centrifuged through a Sephadex G50 microcolumn. The dsDNA concentration was estimated spectrophotometrically assuming 1 unit of light absorbancy at 260 nm to correspond to 50 μg/mL or 1.16×10^{-6} M of the 65 bp molecules.

The single-stranded nucleotide target, 5′-GGATCA$_{10}$-GACTG-3′, was ^{32}P radiolabeled at the 5′ end. The molar concentration of ssDNA was estimated assuming 1 unit of light absorbancy at 260 nm to correspond to 35 μg/mL or 5.3×10^{-6} M of the oligonucleotide.

PNA−DNA complexes were formed at a low ionic strength (10 mM Na^+, 10 mM Tris HCl, pH 7.5) by incubating $(7-9) \times 10^{-5}$ M DNA for 1 h at 37−47 °C with the PNA. (Within this interval, formation and the subsequent behavior of the complex did not depend on the incubation temperature.)

The formation of PNA−dsDNA complexes was carried out under the conditions of a PNA molar excess of 2500. In the case of ssDNA, the PNA excess was 10−20-fold. The optimal PNA excess was determined preliminarily by titration.

Following PNA−dsDNA complex formation, free PNA was removed by passing the mixture through Sephadex G50. Subsequently, the ionic strength was either increased to the desired value by adding a concentrated NaCl solution or decreased by dilution with the buffer solution. The PNA−ssDNA complexes were diluted approximately 10-fold and brought to the desired ionic strength.

The dissociation of the complexes followed first-order kinetics. The data obtained were compared with the exponential curves generated by the Graph Pad Prism computer program, which were used to determine the dissociation rate constant, k, and the characteristic lifetime of the complex, $\tau = 1/k$, i.e., the time required for the concentration of the complex to decrease by e times.

The dissociation kinetics of PNA−DNA complexes were studied as follows. At a chosen temperature, solution aliquots were sampled at specified time intervals, quickly cooled, and frozen at −70 °C. Afterward, all samples were simultaneously analyzed by means of mobility shift assay in 5% (dsDNA) or 12% (ssDNA) polyacrylamide gel *(17)*. After the electrophoresis, the gel was dried, an autoradiograph was obtained, and the positions of the bands carrying the radioactive label were determined. The corresponding areas of the gel were cut out, and their radioactivity was measured (in a Mark III scintillation counter) to determine the amount of DNA in each band. The concentration of the complex and the degree of its dissociation in relation to time were determined from the ratio between the radioactivity of the band corresponding to the complex and the total activity of the bands corresponding to the complex and free DNA.

RESULTS

To observe exclusively the dissociation of PNA−DNA complexes, the reverse reaction, i.e., reassociation, must be suppressed during the experiment. In the case of dsDNA, removing the unbound PNA was enough to prevent reassociation. Running to completion of the dissociation reaction was the criterion for the absence of reassociation. In the case of ssDNA, reassociation was suppressed by adding a 600–3000-fold molar excess of the same but unlabeled ("cold") oligonucleotide, which served as a scavenger of free PNA molecules. Variation in the concentration of the cold oligonucleotide within the aforementioned limits did not affect the results of the dissociation experiments; therefore, the only role of the cold oligonucleotide was the binding of free PNA. The complex dissociated completely unless the excess of cold DNA was below 100-fold. As expected, in the case of dsDNA, addition of cold oligonucleotide did not change the dissociation kinetics if the excess PNA had been removed from the solution.

Autoradiographs representing the dissociation kinetics were obtained for all of the complexes studied. The dissociation was followed at various temperatures from 55 to 72.5 °C. The upper temperature of this interval is approaching the T_m of the complexes, and the lower value is limited by the lifetime of complexes so as to obtain experimentally reasonable dissociation times.

Processing of experimental data according to the procedure described under Materials and Methods revealed that the complexes always underwent an exponential decay. In some experiments, the concentration of the complex initially dropped by approximately 20–40%, within a time considerably shorter than 1 min, which was followed by the major, exponential decrease. The initial drop is most likely caused by the dissociation of the imperfect, less robust PNA–DNA complexes that had formed at 37 or 47 °C and were quite stable at these temperatures. Probably, these were the complexes in which only 9 or 8 of 10 links of the PNA chain bound the target A_{10} DNA. At higher temperatures, such complexes dissociate much faster than perfect ones. This initial stage is followed by a slower exponential dissociation of the perfect complexes. When processing the experimental data, we excluded the initial rapid dissociation. Figure 2a and 2b show a set of dissociation curves for T_{10} PNA–ssDNA and PNA 1460–ssDNA triplexes at various temperatures.

Similar sets of curves were obtained for triplexes of T_{10} PNA with both ss- and dsDNA templates at different concentrations of NaCl. Similar experiments were performed with ssDNA and PNA 1460 as well as with PNA 1743. Figure 3 shows the relationship between ln τ and the reciprocal of temperature for triplexes of T_{10} PNA with ss- and dsDNAs at a Na^+ concentration of 50 mM. As is seen from the figure, the plot is linear within experimental error, thus obeying Arrhenius's law. Values originating from single- and double-stranded templates fit the same linear plot, yielding an activation energy, U, of 75 ± 5 kcal/mol.

Similar linear Arrhenius plots were obtained for T_{10} PNA triplexes at salt concentrations of 200 and 600 mM (Figure 1s in Supporting Information). The straight lines are parallel at all salt concentrations. In most experiments, we used PNA–ssDNA complexes. Control experiments with dsDNA confirmed that the τ values for complexes of PNA with ssDNA and dsDNA were equal also at 200 and 600 mM salt.

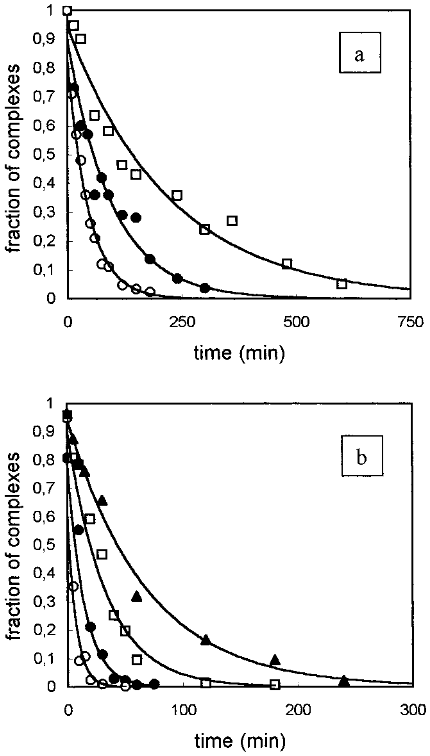

FIGURE 2: Kinetics of $(PNA)_2$/ssDNA triplex dissociation. (a) Plots of fraction of (PNA $T_{10})_2$/ssDNA complexes as a function of incubation time in the presence of 200 mM Na^+ at various temperatures: 70 °C (open circles), 67.5 °C (closed circles), and 65 °C (open squares). (b) Plots of fraction of (PNA $1460)_2$/ssDNA complexes as a function of incubation time in the presence of 600 mM Na^+ at various temperatures: 70 °C (open circles), 67.5 °C (closed circles), 65 °C (open squares), and 62.5 °C (closed triangles). In panels a and b, points show experimental data, and exponential curves were obtained with the use of the Graph Pad Prism program.

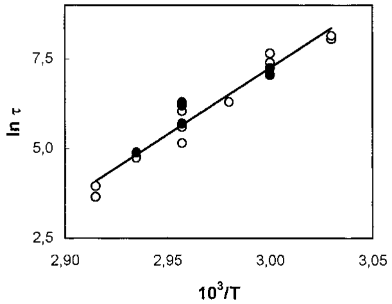

FIGURE 3: Effect of temperature on the lifetime of (PNA $T_{10})_2$/DNA triplexes. Semilogarithmic plot of lifetime τ (minutes) as a function of $10^3/T$ at 50 mM Na^+. Open circles, ssDNA; closed circles, dsDNA.

The activation energies are 78 ± 5 and 76 ± 5 kcal/mol at Na^+ concentrations of 200 and 600 mM, respectively. Thus, as the ionic strength increases, the lifetime (τ) decreases, while the activation energy of dissociation of complexes remains almost the same.

We found a similar relationship for PNA 1460–ssDNA triplexes (Figure 2s in Supporting Information). In this case, the Arrhenius's plots were also parallel to one another, and the activation energy varied within a narrow range from 74 ± 5 to 77 ± 5 kcal/mol.

The relationship between the lifetime (τ) and the ionic strength is presented in Figure 4 for T_{10} PNA–DNA

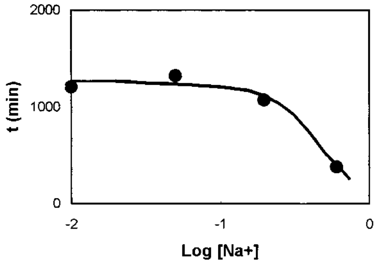

FIGURE 4: Effect of Na^+ concentration (M) on the lifetime τ of (PNA $T_{10})_2$/ssDNA triplexes at 60 °C. Points represent the mean of 3−5 separate experiments.

complexes at 60 °C. At other temperatures, this relationship had similar patterns.

DISCUSSION

The present results show that the T_{10} PNA triplexes with ss- and dsDNA have identical lifetimes (τ) within the accuracy of experiments. This is true for the whole range of temperatures and salt concentrations studied. Surprisingly, these results imply that the complementary DNA strand does not influence the dissociation rate. A question may arise whether this is due to the fact that at the high temperatures used in our experiments (60−70 °C) the P-loop DNA segment was melted and, hence, its renaturation was energetically disadvantageous, the more so as this segment is comprised of the low-melting AT pairs. However, this is unlikely to be the case. Assuming that the borders of the P-loop are fixed, the melting point of a DNA segment consisting of n AT pairs may be approximately estimated as $T_m \cong T_0(1 + 1/n)$, where T_0 is the melting point of an infinitely long DNA with the nucleotide sequence dA•dT. At a NaCl concentration of 0.15 M, $T_0 = 68$ °C $= 341$ K (*25*). Therefore, $T_m \cong 375$ K $= 102$ °C. If [NaCl] $= 0.6$ M, the T_m value will additionally be 10 °C higher, i.e., 112 °C. Even if we assume the loop to be longer than 10 nucleotides (e.g., 15 nucleotides), the melting point will be 101 °C in a 0.6 M NaCl solution.

Thus, under the experimental conditions, the temperature is about 20−40 °C lower than the P-loop segment of the dsDNA melting point. Hence, the P-loop closing is by itself energetically advantageous. Nevertheless, this does not decrease the stability of the triplex; therefore, the interaction between the P-loop and the triplex (PNA $T_{10})_2$/dsDNA can be neglected, and this indicates that the displaced DNA strand is irrelevant in the rate-determining step for dissociation.[2]

In the further discussion of the experimental results, it is assumed that the activation energy of dissociation of triplexes PNA_2/DNA does not depend on the melting entropy but only on the melting enthalpy. Very likely the validity of this contention does not depend on a specific mechanism of dissociation. Let us consider two possible limiting dissociation mechanisms. The process of gradual "diffusion" unwinding of oligonucleotide duplexes from the ends was theoretically studied in ref *26*. Equality between the activation energy and the dissociation enthalpy follows from this study. The activation energy does not depend on the melting entropy and is somewhat smaller than the melting enthalpy. It can be suggested that a similar relationship holds true for slow dissociation of triplexes as well. If the dissociation follows the "all-or-none" principle, i.e., the chains separate in a concerted fashion due to a large thermal fluctuation, the activation energy of dissociation should be equal to the melting enthalpy.

Table 1: Activation Energy of Dissociation of PNA_2/DNA Complexes

PNA	target DNA	$[Na^+]$ (mM)	U (kcal/mol)
T_{10}	ss and ds	50	74 ± 5
		200	76 ± 5
		600	77 ± 5
1460	ss	50	74 ± 5
		200	76 ± 5
		600	77 ± 5
1743	ss	50	95 ± 5
1743	ss	600	69 ± 5

Thus, in both cases the activation energy of dissociation does not depend on the entropy and is determined only by melting enthalpy.

As for the mechanism of dissociation, our experiments demonstrated that the activation energy of dissociation of bis-PNA−DNA triplexes is considerably smaller than their melting enthalpy (data not shown). This argues for the gradual mechanism of dissociation (see above).

As the salt concentration increases, the triplex lifetime decreases (Figure 4). This is in accordance with the behavior of PNA/DNA duplexes. For example, an increase in Na^+ concentration from 10 to 100 mM decreases the T_m of the PNA/DNA duplex by ∼8 °C *(2)*. This effect is opposite to that exerted by Na^+ on the stability of DNA/DNA duplexes and is accounted for by the electric neutrality of the backbone of the PNA molecule *(27)*. The little impact of Na^+ on τ (a decrease by a factor of only 3−4 in response to $[Na^+]$ increase from 50 to 600 mM) agrees with a similarly little effect of the salt on T_m.

As the Arrhenius curves are parallel for $(PNA_{10})_2$/DNA triplexes at different salt concentrations, the activation energy is independent of the salt concentration (see Table 1). Thus, the enthalpy of dissociation does not depend on ionic strength. Therefore the influence of ionic strength on the stability of PNA−DNA triplexes is of entropic nature. This conclusion is in good agreement with the results from *(27)* on the PNA−DNA duplexes.

As the ends of the T_{10} PNA molecule used here bear positive charges (Figure 1), electrostatic attraction between PNA and the phosphate groups of the DNA backbone could be expected to make some contribution to the increase of the activation energy at low salt concentration. However, as the activation energy does not depend on the ionic strength, the contribution of the direct electrostatic interaction is negligible. This result is also in agreement with that obtained in *(27)*.

PNA 1460, which has two lysine residues inside the backbone, carries a larger positive charge than PNA T_{10}.

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[2] The equality of lifetimes of triplexes formed by PNA T_{10} with ss- and dsDNA is not necessarily the case for PNA−DNA triplexes in general. We have found the lifetime of triplexes formed by bis-PNA 1743 with dsDNA to be at $[Na^+]$ values of 50 and 200 mM but not at 600 mM, considerably above the lifetime of triplexes formed by the same bis-PNA with ssDNA. The effect is of electrostatic nature and stems probably from the interaction of the positive charges located in the middle of one of the bis-PNA arms with the displaced negatively charged P-loop of DNA (O. V. Krupnik, N. P. Kvitko, P. E. Nielsen, and Yu. S. Lazurkin, unpublished experiments).

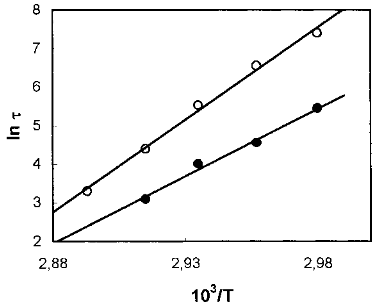

FIGURE 5: Effect of temperature and ionic strength on the lifetime τ (minutes) for (PNA 1743)$_2$/ssDNA triplexes. Semilogarithmic plot of the lifetime τ as a function of $10^3/T$ at 50 mM Na^+ (open circles) and 600 mM Na^+ (closed circles).

Thus, a stronger effect of direct electrostatic interaction could be expected. However, the stability of the triplex of this PNA and DNA was even lower than that of the (PNA T_{10})$_2$/DNA triplex (the lifetime was 2.5−3 times shorter) under all experimental conditions. This result appeared to be in fair agreement with the data on the melting temperatures of triplexes, formed by PNAs T_{10} and 1460 with ssDNA (72 and 66 °C, respectively, under standard conditions).

The enthalpy of dissociation of the (PNA 1460)$_2$/DNA triplex proved to be independent of the ionic strength (Table 1). In this case, the decreased stability and the absence of the contribution of PNA−DNA Coulombic interaction to the dissociation enthalpy may be accounted for by the compensation for the PNA−DNA attraction due to electrostatic repulsion between PNA 1460 chains.

To support this suggestion, we carried out experiments with bis-PNA 1743 which contained lysines in only one of the arms (Figure 1). In this case, there is no repulsion between different chains of PNA. The results of the experiments are shown in Figure 5 and Table 1. The enthalpy of dissociation at 50 mM Na^+ was 20−25 kcal/mol larger as compared to 600 mM Na^+. At the high salt concentration, it was very similar to the value for other PNAs. These results can be explained by an additional contribution of the direct electrostatic interaction of the positive charges on the PNA with the negatively charged DNA backbone in the dissociation enthalpy of the triplexes. At the high ionic strength, this interaction diminishes and its contribution to the dissociation enthalpy becomes negligible.

These results confirm our conclusion that the absence of the increase of the dissociation enthalpy in the case of PNA 1460 which carries a significant positive charge is due to the compensation of its attraction to the DNA backbone and repulsion of the PNA chains.

Thus, the influence of the positive charge of the PNA molecule on the dissociation enthalpy of the PNA−DNA triplexes has a complicated nature. The PNA charge can either increase the dissociation enthalpy of the triplexes or leave it unchanged as in the case of single charges at the ends of the PNA molecule (for example, PNA T_{10}; see Figure 1). Thus, it is possible to rationally tune the stability of PNA−DNA triplexes by changing the number and localization of the positive charges on the PNA molecules.

Finally, the results allow us to estimate by extrapolation a lifetime of a 10-mer PNA triplex invasion complex in a duplex DNA target under physiological conditions (37 °C, 140 mM salt) of several hundred days. Therefore, such complexes would behave close to permanent modifications of the DNA.

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SUPPORTING INFORMATION AVAILABLE

Arrhenius plots for PNA T_{10}−ssDNA and PNA 1460−ssDNA triplexes at salt concentrations of 50, 200, and 600 mM (2 pages). This material is available free of charge via the Internet at http://pubs.asc.org.

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