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DNA unwinding upon strand-displacement binding of a thymine-substituted polyamide to double-stranded DNA

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ABSTRACT **It was recently found that polyamide nucleic acid (PNA) analogues consisting of thymines attached to an aminoethylglycine backbone bind strongly and sequence-selectively to adenine sequences of oligonucleotides and double-stranded DNA [Nielsen, P. E., Egholm, M., Berg, R. H. & Buchardt, O. (1991) *Science* 254, 1497–1500]. It was concluded that the binding to double-stranded DNA was accomplished via strand displacement, in which the PNA bound to the Watson–Crick complementary adenine-containing strand, whereas the thymine-containing strand was extruded in a virtually single-stranded conformation. This model may provide a general way in which to obtain sequence-specific recognition of any sequence in double-stranded DNA by Watson–Crick hydrogen-bonding base-pair recognition, and it is thus paramount to rigorously establish this binding mode for synthetic DNA-binding ligands. We now report such results from electron microscopy. Furthermore, we show that binding of PNA to closed circular DNA results in unwinding of the double helix corresponding to approximately one turn of the double helix per 10 base pairs. The DNA·PNA complex, which is formed at low salt concentration (only a small portion of DNA molecules show complex formation at NaCl concentration higher than 40 mM), is exceptionally kinetically stable and cannot be dissociated by increasing salt concentration up to 500 mM.**

Synthetic reagents that bind sequence selectively to single- and, especially, double-stranded nucleic acids are of great interest in molecular biology and medicinal chemistry, since such reagents may provide the tools for developing gene-targeted drugs and other sequence-specific gene modulators. Until now oligonucleotides and their close analogues have appeared to be the best candidates for such reagents (1, 2).

However, it was recently found that polyamide ("peptide") nucleic acid (PNA) analogues consisting of thymines attached to an aminoethylglycine oligomer backbone bind strongly and sequence-selectively to adenine sequences of oligonucleotides and double-stranded DNA (3, 4). Based on enzymatic and chemical probing experiments, it was concluded that the binding to double-stranded DNA took place by strand displacement, in which the PNA bound to the Watson–Crick complementary adenine-containing (A) strand, whereas the thymine-containing (T) strand was extruded in a virtually single-stranded conformation (3). Since this model may provide a general way in which to obtain sequence-specific recognition of any sequence in double-stranded DNA by Watson–Crick hydrogen-bonding base-pair recognition, it is paramount to rigorously establish this binding mode for synthetic DNA-binding ligands. We now report such results from electron microscopy and show that binding of PNA to closed circular DNA results in unwinding of the double helix corresponding to approximately one turn of the double helix per 10 base pairs (bp).

MATERIALS AND METHODS

PNA. PNA H-T_{10}-LysNH_2 (PNA-1 in ref. 3) was synthesized as described (3, 4). Its structure may be written as follows.

H-{$NHCH_2CH_2N[C(O)CH_2$-Thy$]CH_2C(O)$}$_{10}$-Lys-NH_2

Plasmid. The pA98 plasmid containing the $(dA)_{98}$·$(dT)_{98}$ insert incorporated into the *Pst* I site of the polylinker of the pUC19 plasmid was taken from the collection of plasmids with different inserts in the Moscow laboratory.

Electron Microscopy. One-tenth microgram of the pA98 plasmid linearized by the *Cfr*10I restriction enzyme was incubated with 0.15 μg of PNA H-T_{10}-LysNH_2 at 37°C for 3 hr in 10 μl of TE buffer (10 mM Tris·HCl/1 mM Na_3EDTA, pH 8.0). The complex was diluted with 10 mM Tris·HCl/10 mM NaCl, pH 7.5, to a final DNA concentration of 0.2–0.5 μg/ml and absorbed to the surface of a glow-discharged carbon grid for 2 min as described (5). The grid was stained in aqueous 0.1–0.5% uranyl acetate for 10–15 sec and dried. The sample was shadowed with Pt/C (95:5, wt/wt) and studied in a Philips 400 electron microscope at an accelerating voltage of 40 kV. The lengths of the DNA molecules were measured and the histogram was plotted as described (6). The length of unwound region was measured by its thicker strand, for which the number of base pairs per unit length was assumed to be the same as for the DNA duplex. During preparation of the histogram the images of the molecules were oriented in such a manner that the left boundary of the loop was closer to the end of the molecule.

Relaxed Circular DNA (rcDNA). Ordinary supercoiled plasmid DNA was treated with an extract containing DNA topoisomerase I, as described (7). DNA·PNA complexes were obtained by incubation of 1–2 μg of DNA in 5–20 μl of TE buffer with PNA at 0.4 OD_{260}/ml for 4 hr at 20–22°C. This corresponded to about 10 times molar excess of PNA to its potential binding sites. Agarose (1.5%) gel electrophoresis was performed in TAE buffer (40 mM Tris acetate/2 mM EDTA, pH 8.0) with chloroquine (1 μg/ml) at 10°C for 15 hr at 1.5 V/cm.

Two-Dimensional Gel Electrophoresis. Electrophoresis in the first direction (from top to bottom) was performed in TAE buffer. Electrophoresis in the second direction (from left to right) was performed in the same buffer with addition of chloroquine (1 μg/ml).

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Abbreviations: PNA, polyamide ("peptide") nucleic acid; rcDNA, relaxed circular DNA.

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RESULTS

Electron Microscopy of PNA·DNA Complex. We used a $(dA)_{98}\cdot(dT)_{98}$ target contained within a supercoiled plasmid, pA98, linearized with the *Cfr*10I restriction enzyme, and challenged this with PNA H-T_{10}-LysNH_2. Consequently, full occupancy of the target would result in a strand-displacement loop of 90–100 bases, which is detectable within the resolution of electron microscopy.

Fig. 1 shows that this is indeed the case. The DNA molecules carried an open region in the form of an "eye," equivalent to D-loop formation (8). In all cases one of the two strands in the open region was thicker than the other one and had the same thickness as the normal DNA duplex. We believe that the thicker strand corresponds to the A strand covered by PNA, while the thinner strand corresponds to the displaced T strand.

The positions of two branch points of the loops were 47.1% and 51.2%, which, within the error of electron microscopy measurements (0.5%), coincided with the ends of the $(dA)_{98}\cdot(dT)_{98}$ insert (47.5% and 51.0%). The average size of the loop was 4.1%—i.e., 114 bp, which also agreed well with the length of the insert (the standard error corresponds to 17 bp). In control experiments, carried out in the same way but without PNA, we never observed the eye-like structures.

Only a small portion of the DNA molecules we studied carried the eye-like structures. This does not necessarily mean that only a small fraction of the DNA molecules were unwound while complexing with PNA but could rather be the result of sample preparation for electron microscopy. Indeed, the complex could have dissociated while DNA molecules were absorbed on the carbon grid [such dissociation has been observed for unfixed DNA–protein complexes (9, 10)] or in the process of drying of the grid accompanied by a sharp increase of salt and uranyl acetate concentration. Because the length of the unwound regions we detected was close to the resolution limit of the electron microscopy method, we

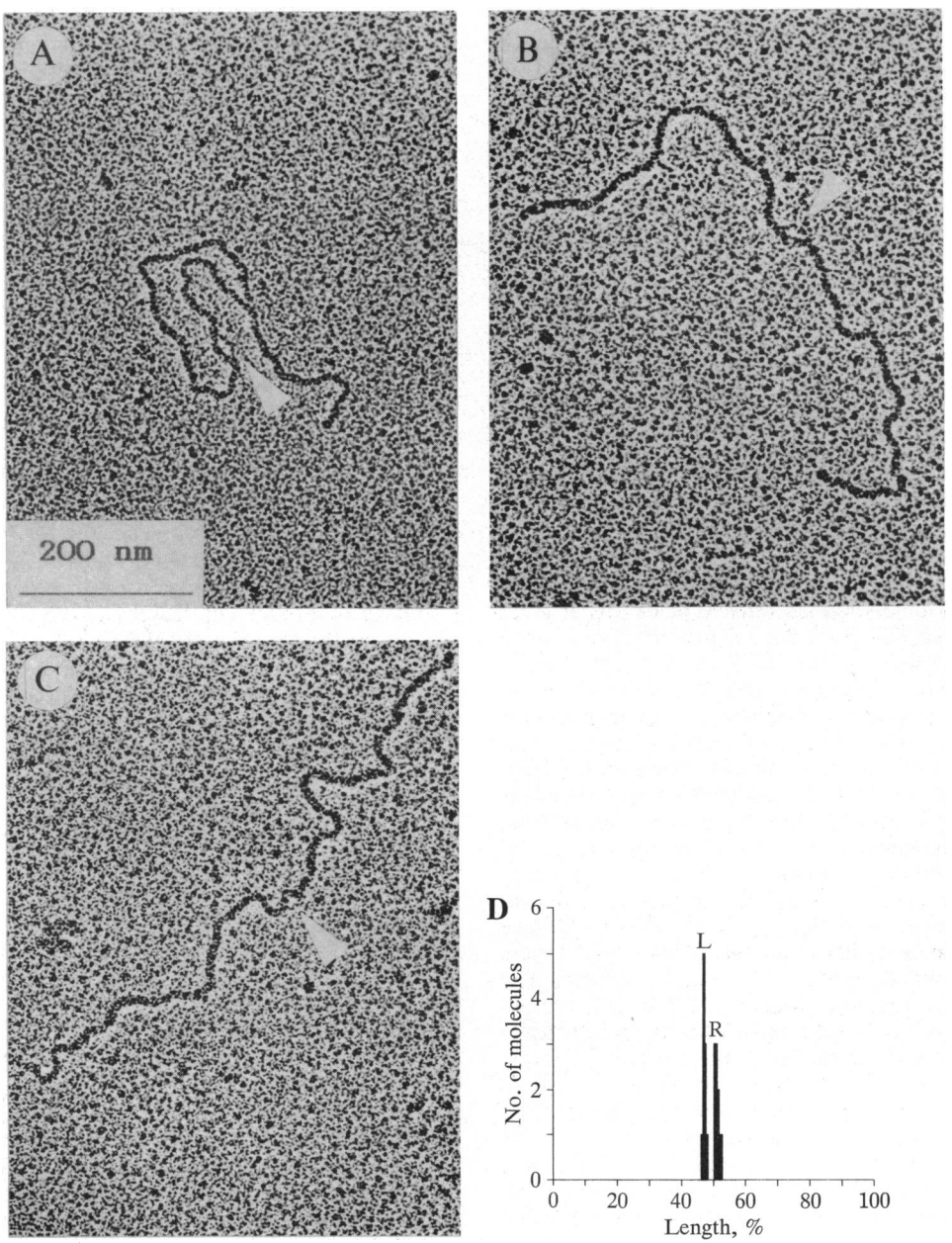

FIG. 1. Electron microscopy visualization of DNA unwinding by PNA. (*A–C*) Microphotographs of DNA molecules carrying unwound regions. Arrowheads point to the unwound regions. (*D*) Histogram of the distribution of the left (L) and right (R) boundaries of the unwound regions.

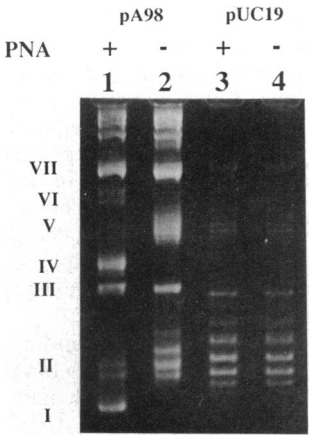

FIG. 2. DNA unwinding of the pA98 plasmid due to the T-strand displacement by PNA H-T_{10}-LysNH_2. rcDNA was used as substrate. pA98 was used in lanes 1 and 2, whereas pUC19 as a control was used in lanes 3 and 4. The samples of lanes 1 and 3 contained PNA, whereas the samples of lanes 2 and 4 did not. The groups of bands represent: strongly positively supercoiled monomer plasmids (I), equilibrium set of relaxed closed circular monomer molecules (II), nicked monomers (III), the same as I, but for the dimeric plasmid (IV), the same as II for the dimeric plasmid; (V), highly positively supercoiled trimer (VI), and the same as III for the dimeric plasmid (VII).

could observe the regions only if they were properly oriented and shadowed.

Therefore, to examine how efficiently PNA H-T_{10}-LysNH_2 incorporates into the DNA duplex by displacing the DNA T strand, we studied the unwinding of DNA by PNA in solution.

Unwinding of Closed Circular DNA by PNA. We prepared our pA98 plasmid in the form of relaxed circles (rcDNA). We expected that complexing with PNA would unwind about 10 turns of the DNA duplex. Because of topological constraints (the two strands in rcDNA were closed and therefore topologically linked), this unwinding would make the rcDNA molecules behave as if they were positively supercoiled by 10 superturns. This would manifest itself in an increase of electrophoretic mobility of the complex as compared with control rcDNA preincubated in the same buffer without PNA.

Fig. 2 shows that after incubation with PNA in the low-salt TE buffer for 4 hr at 20°C, virtually all rcDNA molecules significantly increased their mobility and moved as if they were highly supercoiled. Two-dimensional gel electrophoresis experiments (Fig. 3) showed that these molecules actually behaved as positively supercoiled ones. We additionally exploited the power of two-dimensional gel electrophoresis to estimate the degree of unwinding. This technique resolves individual topoisomers, and by using a sample with a wide distribution over topoisomers as reference, it is clearly seen (Fig. 3) that on the average 8–10 turns of the double helix are released upon binding of PNA. In making this estimate we assume that positively and negatively supercoiled molecules with the same absolute number of supercoils have the same

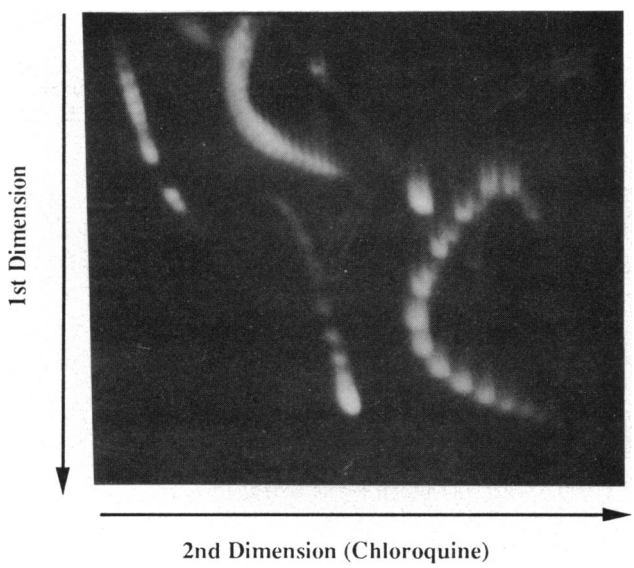

FIG. 3. Two-dimensional gel electrophoresis. Lower part of the pattern corresponds to monomeric plasmids, whereas the upper part corresponds to dimeric ones. The left patterns correspond to experimental complexes of the pA98 plasmids with PNA. The right patterns correspond to a specially prepared control sample of closed circular DNA with a wide distribution over topoisomers.

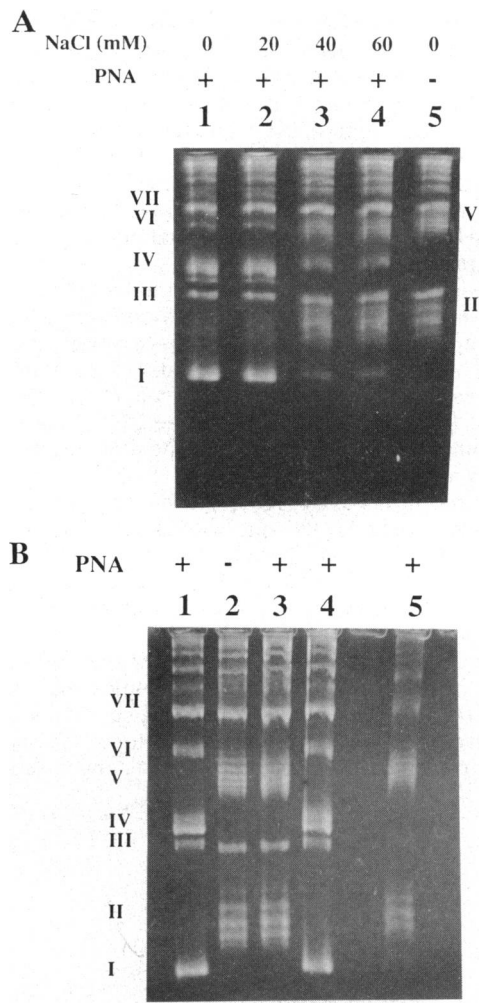

FIG. 4. Sensitivity of DNA·PNA complex to the salt concentration. The DNA species are labeled as in Fig. 2. (*A*) Incubations were performed in TE buffer with the indicated addition of NaCl. (*B*) Lane 1, the same experiment as in lane 1 of Fig. 2, but incubation was at 37°C for 3 hr; lane 2, as in lane 1 but without PNA; lane 3, the same experiment as in lane 1, but the incubation with PNA was performed in the presence of 500 mM NaCl; lane 4, as in lane 1 but after the incubation with PNA in TE buffer, NaCl was added to a final concentration of 500 mM and the sample was incubated for 1 hr at 37°C; lane 5, to dissociate the complex, to 1 volume of the sample containing standardly prepared DNA·PNA complex in TE buffer, 0.6 volume of 0.2 M NaOH/1% SDS was added and incubated for 1 min, after which the mixture was neutralized with 0.45 volume of 3 M NaOAc (pH 5.2) and subjected to electrophoresis.

electrophoretic mobility. The above results indicate that in the TE buffer, PNA occupied all available binding sites on the DNA duplex, thereby efficiently displacing the DNA T strand in the $(dA)_{98}$·$(dT)_{98}$ insert.

When NaCl was added to the incubation buffer, the degree of conversion of rcDNA into rapidly moving species decreased dramatically within a narrow range between 20 and 40 mM NaCl (Fig. 4*A*). This emphasizes a strong sensitivity of PNA incorporation to salt concentration. However, after being formed at low salt, the complex showed remarkable stability and tolerated increasing salt concentrations up to at least 500 mM NaCl (Fig. 4*B*, lane 4). The complex could be dissociated by alkali treatment (Fig. 4*B*, lane 5).

DISCUSSION

Our interpretation of the above data is as follows. Although PNA form remarkably stable complexes with corresponding single-stranded DNA (3, 4), the incorporation into the duplex is associated with overcoming a significant potential barrier: several base pairs need to be opened to provide a single-stranded region long enough to nucleate the complex. The barrier may be lowered by subjecting DNA to conditions favoring DNA melting. At fixed temperature, this can be done by decreasing the salt concentration. However, once the barrier is overcome the complex becomes kinetically trapped and remains stable even under conditions which completely block the complex formation—i.e., at high salt concentration.

This kinetic trapping stems from the fact that PNA·DNA complexes have much higher stability constants than normal DNA duplexes. As a result the dissociation time for the PNA·DNA complex in which PNA displaces m nucleotides may be estimated as $\tau = \tau_0 (s_p/s_d)^m$, where s_p is the stability constant (per one nucleotide) for the PNA/single-stranded DNA complex, s_d is the stability constant of the DNA duplex, and τ_0 is about 10^{-7} sec (11).

Note that in our case, although the number of displaced nucleotides is about 100, one should substitute $m = 10$ to roughly estimate the dissociation time because the detachment of any PNA-T_{10} molecule is an irreversible event under electrophoresis conditions (as well as on the carbon grid in the electron microscopy experiments).

Since the PNA backbone (except for the terminal amino groups) is neutral, stability of its complex with the single DNA strand (i.e., the s_p value) should be salt-independent. By contrast, the stability of the DNA duplex (the s_d value) is known to increase with increasing salt concentration. As a result, the τ value should decrease with increasing salt concentration, and one could expect that the τ value will become lower, at some point, than the characteristic time of the experiment (duration of electrophoresis or the time of drying in the course of sample preparation for electron microscopy). However, the salt-independent stability constant, s_p, proved to be so large that in our electrophoresis experiments we could not reach the point when PNA was displaced from the DNA. Moreover, the data in Figs. 2–4 show that even significant positive supercoiling is unable to squeeze PNA out of DNA. This emphasizes the remarkable strength of PNA·DNA interaction.

We have previously shown that binding of PNA H-T_{10}-LysNH_2 to $(dA)_{10}$ involves two PNAs binding to one oligonucleotide (4, 12). Furthermore, we have suggested and presented evidence that both Watson–Crick and Hoogsteen base pairing are involved in the PNA·DNA recognition (12). The remarkable stability of such a PNA·DNA·PNA triplex as compared with the triplex formed by DNA strands may be explained solely in terms of the electrostatic repulsion of phosphates in the case of the DNA triplex, which is completely absent in the case of the PNA·DNA·PNA triplex.

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1. Helene, C. & Toulme, J. J. (1990) *Biochim. Biophys. Acta* **1049**, 99–125.
2. Uhlmann, E. & Peyman, A. (1990) *Chem. Rev.* **90**, 544–584.
3. Nielsen, P. E., Egholm, M., Berg, R. H. & Buchardt, O. (1991) *Science* **254**, 1497–1500.
4. Egholm, M., Buchardt, O., Nielsen, P. E. & Berg, R. H. (1992) *J. Am. Chem. Soc.* **114**, 1895–1897.
5. Dubochet, J., Ducommun, M., Zollinger, M. & Kellenberger, E. (1971) *J. Ultrastruct. Res.* **35**, 147–167.
6. Kurakin, A. V., Zaritskaya, L. S., Metliskaya, A. Z., Volodin, A. A. & Cherny, D. I. (1991) *Micron. Microsc. Acta* **22**, 213–221.
7. Lyamichev, V. I., Mirkin, S. M. & Frank-Kamenetskii, M. D. (1985) *J. Biomol. Struct. Dyn.* **3**, 327–338.
8. Kasamatsu, H., Robberson, D. L. & Vinograd, J. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 2252–2257.
9. Kadesch, T. R., Williams, R. C. & Chamberlin, M. J. (1980) *J. Mol. Biol.* **136**, 65–78.
10. Koller, T., Kubles, O., Portmann, R. & Sogo, J. M. (1978) *J. Mol. Biol.* **120**, 121–131.
11. Anshelevich, V. V., Vologodskii, A. V., Lukashin, A. V. & Frank-Kamenetskii, M. D. (1984) *Biopolymers* **23**, 39–58.
12. Egholm, M., Nielsen, P. E., Buchardt, O. & Berg, R. H. (1992) *J. Am. Chem. Soc.* **114**, 9671–9678.