Sequence specific inhibition of DNA restriction enzyme cleavage by PNA

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ABSTRACT

Plasmids containing double-stranded 10-mer PNA (peptide nucleic acid chimera) targets proximally flanked by two restriction enzyme sites were challenged with the complementary PNA or PNAs having one or two mismatches, and the effect on the restriction enzyme cleavage of the flanking sites was assayed. The following PNAs were used: T₁₀-LysNH₂, T₅CT₄-LysNH₂ and T₂CT₂CT₄-LysNH₂ and the corresponding targets cloned into pUC 19 were flanked by *Bam*H1, *Sal*I or *Pst*I sites, respectively. In all cases it was found that complete inhibition of restriction enzyme cleavage was obtained with the complementary PNA, a significantly reduced effect was seen with a PNA having one mismatch, and no effect was seen with a PNA having two mismatches. These results show that PNA can be used as sequence specific blockers of DNA recognizing proteins.

INTRODUCTION

Reagents that bind sequence selectively to double stranded DNA are of significant interest in medicinal chemistry and molecular biology since they may provide the tools for sequence specific modification of DNA and for gene targeted drugs (1,2).

Oligonucleotides and their analogues capable of forming triple helices are at present the prime candidates for developing such reagents. It has been shown that properly designed oligonucleotide conjugates cleave or alkylate DNA sequence specifically *via* triple helix formation (3–23), and it was also shown that triple helix forming oligonucleotides interfere sequence specifically with DNA recognizing proteins, such as restriction methylases (24,25), restriction endonucleases (26,27), transcription factors (28) and RNA polymerases (29).

We have recently found that PNA (peptide nucleic acids chimera), *i.e.*, DNA analogues in which the deoxyribose phosphate backbone has been exchanged for a peptide backbone consisting of (2-aminoethyl)glycine units (Figure 1) have retained the hybridization properties of DNA. In fact, we have shown that PNA binds more strongly to complementary oligonucleotides than DNA itself (30). Furthermore, we found that PNA can also bind sequence specifically to double stranded DNA. This binding

takes place by strand displacement rather than by triple helix formation (31). PNA is thus a very interesting alternative to traditional DNA and DNA analogues for the development of gene modulating drugs and reagents.

In the present study we have examined the interference of PNA with DNA recognizing/modifying proteins in the form of restriction endonucleases, and we find that PNA bound proximal to a restriction endonuclease cleavage site causes complete inhibition of cleavage at this site.

MATERIALS AND METHODS

The PNAs T₁₀-LysNH₂, T₅CT₄-LysNH₂ and T₂CT₂CT₄-LysNH₂ were synthesized as described (30,32). (PNAs are written from the amino to the carboxyterminal. LysNH₂ designates that a lysine amide is attached to the PNA. This group suppresses aggregation of the PNA).

Plasmids containing the target sequences were obtained by cloning of the appropriate oligonucleotides into the vector pUC19. To obtain pT10 16-mers 5'-GATCCT₁₀G and 5'-GATCCA₁₀G were cloned into the *Bam*H1 site. pT9C was obtained by cloning 5'-TCGACT₅G and 5'-TCGACACA₅GA₄G into the *Sal*I site and pA8G2 was obtained by cloning 5'-GT₄CT₂CT₂CTGCA and 5'-GA₂GA₂GA₄CTGCA into the *Pst*I site. *E. coli* JM103 was used as host in all cases, and transformations, isolation of clones and characterization of plasmids by DNA dideoxy sequencing were done by standard techniques (33). Plasmids were purified by boyant density centrifugation in CsCl gradients. A typical restriction enzyme cleavage experiment was performed by incubating 0.5 µg of plasmid DNA with the desired amount of PNA in 10 µl 10 mM Tris-HCl (pH 7.4), 1 mM EDTA for 60 min at 37°C. Subsequently 1 µl of 10× concentrated enzyme buffer (500 mM Tris-HCl, pH 7.4, 100 mM MgCl₂, 1 M NaCl, 10 mM DDT) was added together with 1–5 units of the desired restriction enzymes. Incubation was continued for 30 min at 37°C and the DNA in the samples was precipitated by addition of 25 µl 2% KOAc in 96% ethanol (–20°C, 16 hrs). The samples were subsequently taken up in 10 µl TBE (90 mM Tris-borate, 1 mM EDTA, pH 8.3) buffer, 10% glycerol, and analyzed by electrophoresis in 6% polyacrylamide (0.2% bisacrylamide) gels run in TBE buffer and stained with ethidium bromide.

Figure 1. (a) Chemical structure of PNA (R^1 is lysine amide in the PNAs used in this study) compared to DNA, and (b) DNA sequences of the polylinker region of the *pUC19* constructs pT10, pT9C and pA8G2.

RESULTS AND DISCUSSION

Since the target sequence for PNA T_{10} -LysNH₂ was cloned into the *Bam*H1 site of pUC 19, it is flanked by two *Bam*H1 sites (Figure 1b). Thus binding of PNA to this target could result in inhibition of cleavage at both sites. In order to rule out the possibility that PNA could inhibit enzymes in general by direct binding to these, cleavage with *Pvu*II was used as an internal control. Therefore three DNA fragments from pT10 of 2364 bp, 248 bp and 90 bp in length (Figure 2a) would be expected without inhibition of *Bam*H1, and in case of specific inhibition of *Bam*H1, a fourth fragment of 338 bp is predicted with simultaneous decrease in the amounts of the 248 bp and 90 bp fragments. As shown in Figure 2a, this is exactly what is observed with the addition of PNA- T_{10} -LysNH₂. In fact, at a concentration of PNA of $\sim 200 \mu\text{M}$ corresponding to a PNA/target ratio of ~ 15 , 50% inhibition of the *Bam*H1 cleavage is seen (by interpolation of the results presented in Figure 2a) and total inhibition is achieved at a ratio of 60. Furthermore, the migration of the *Pvu*II-*Pvu*II 338 bp fragment is slightly retarded in the samples containing PNA and we assign this band to the PNA/dsDNA complex. We have previously found that PNA-dsDNA complexes have a half-life exceeding 24 h and are stable at 70°C (34). It is thus not surprising that they apparently survive the ethanol precipitation step involved in the experimental procedure.

Essentially identical results were obtained with PNA T_5CT_4 -LysNH₂ and *Sal*I (Figure 2b) and PNA $T_2CT_2CT_4$ -LysNH₂ and *Pst*I (Figure 2c), thereby extending the results to other restriction enzymes and to PNA with mixed T-C sequences and thus mixed A-G sequences in double stranded DNA targets.

In order to examine the sequence discrimination of the DNA recognition by PNA, we performed an experiment in which all three targets were challenged with either of the three complementary PNAs (Figure 3). With the A_{10} target it is seen that at a concentration of PNA T_{10} -LysNH₂ that causes 100%

Figure 2. Inhibition of restriction enzyme cleavage by PNA. The following concentrations of PNA were used (the PNA to target ratio is given in parenthesis): Lanes 1–6: 0, 8 (0.6), 25 (2), 80 (6), 250 (20), 800 (60) μM . The sample of lane 7 contained no PNA and was not treated with the second restriction enzyme (*Bam*H1, *Sal*I or *Pst*I). a: Plasmid pT10, PNA T_{10} -LysNH₂ and restriction enzyme *Bam*H1 were used. b: pT9C, PNA T_5CT_4 -LysNH₂ and *Sal*I were used. c: pA8G2, PNA $T_2CT_2CT_4$ -LysNH₂ and *Pst*I were used.

Figure 3. Sequence discrimination of PNA binding to targets flanked by restriction enzyme recognition sites. The following PNAs were used: Lane 1: no PNA; lanes 2–4: PNA T_{10} -LysNH₂; lanes 5–7: PNA T_5CT_4 -LysNH₂; lanes 8–10: PNA $T_2CT_2CT_4$ -LysNH₂. The concentrations of PNA were: 250 μ M (lanes 1, 5 & 8), 800 μ M (lanes 2, 6 & 9) or 2.5 mM (lanes 3, 7 & 10). **a:** Plasmid pT10 and restriction enzyme. *Bam*H1 was used, **b:** pT9C and *Sal*I were used, **c:** pA8G2 and *Pst*I were used.

inhibition of *Bam*H1 cleavage (Figure 3a, lane 4) only a very slight inhibitory effect (<10%) is observed with PNA T_5CT_4 -LysNH₂ (lane 7), and no effect is seen with PNA $T_2CT_2CT_4$ -LysNH₂ (lane 10). A similarly high degree of

Figure 4. Schematic model for the formation of the (PNA)₂/dsDNA strand displacement complex. In brief, a rather unstable strand displacement complex is first formed with only one PNA molecule bound to the target by Watson–Crick hydrogen bonding, and this is subsequently trapped by the binding of a second PNA molecule via Hoogsteen hydrogen bonding.

Figure 5. Effect of salt on PNA binding. Plasmid pT10 (3 μ g) was incubated for 60 min at 37°C with PNA T_{10} -LysNH₂ (250 μ M) in TE containing the concentration of added cation (as chloride) as indicated (lanes 1–8). In lane 9 no Na⁺ was present but the sample was incubated in the presence of 1 mM Mg²⁺. Concentrated enzyme buffer was added and the samples were treated with *Bam*H1 and *Pvu*II. Lane 1 is a control without PNA.

discrimination is observed for the $A_2GA_2GA_4$ -target for PNA $T_2CT_2CT_4$ -LysNH₂ (Figure 3c, lanes 9, 10). The A_5GA_4 -target likewise exhibits a clear preference for binding the complementary PNA- T_5CT_4 -LysNH₂ (Figure 3b, lanes 6, 7), but at elevated concentrations PNA- T_{10} -LysNH₂ also binds to this target (lane 4). These results clearly show that total discrimination is obtained with two mismatches out of ten, while one mismatch out of ten results in a ca. ten fold reduction in binding relative to the fully complementary PNA as judged by restriction enzyme inhibition. Using these three PNAs, similar results have been obtained by probing with nuclease *S*₁ (33).

Such a high sequence discrimination of the binding of PNA to double stranded DNA may seem surprising in view of the high thermal stability of the complexes between PNA and oligonucleotides. For instance the $(T_{10}$ -LysNH₂)₂/d(A_{10}) complex has a T_m of 72°C while the introduction of a mismatch only lowers the T_m by approximately 13°C (30,32). Furthermore, we have found that binding of PNA to double stranded DNA also involves two PNA molecules (34). Thus even decamer complexes containing one mismatch should be stable

at 37°C. Therefore, the sequence discrimination cannot be thermodynamically controlled. Rather, we ascribe the sequence discrimination to a kinetic effect, which is determined by the stability of the PNA/DNA-duplex intermediate and thus of the population of this complex, which in turn is trapped by the binding of a second PNA molecule (Figure 4).

We note that in the presence of PNA, additional DNA bands are seen in the gel apart from the four fragments expected from the restriction enzyme cleavage. The origin of these bands is not clear at present. They could be specifically retarded forms of the *PvuII*-*PvuII* fragment. However, this is an unlikely explanation since a corresponding decrease in the amount of this fragment should occur which is not the case. Alternatively, the binding of PNA to non-perfect targets could induce enzyme cleavage at these sites. Experiments to clarify this issue are now in progress.

It is of interest to examine how far from a PNA binding site the effect is felt by the DNA recognizing enzyme, this reflecting both the binding region of the enzyme and the propagation of the distortion induced by strand displacement. As a preliminary attempt to address this question, we analyzed the cleavage of the pT10/PNA T₁₀-LysNH₂ complex by restriction enzymes further away from the cloning site. These results showed (data not presented) that restriction enzymes with recognition sites further away (5–10 base pairs) from the PNA binding target are not affected by the binding of PNA to this target. Thus a more systematic study regarding the propagation of the distortion is required before any firm conclusions can be drawn.

Since it was initially observed that PNA did not inhibit restriction enzyme cleavage when PNA was just added to the DNA in the buffer used for the cleavage reaction, the salt dependence of the binding of PNA to dsDNA was studied. As seen from the results presented in Figure 5, Na⁺ concentrations exceeding 80–100 mM are strongly inhibitory for PNA binding, while 1 mM Mg²⁺ does not have a significant influence. Thus all experiments presented in this paper involving PNA binding to dsDNA were performed by preforming the complex in low salt (TE buffer) with subsequent addition of the buffer required for enzyme activity, since the (PNA)₂/dsDNA complexes once formed are resistant to elevated ionic strength. Binding of PNA to ssDNA (or ssRNA) is not affected by salt (30). This inhibitory effect of salt is of minor importance if PNA is to be used as a molecular biology tool analogously to triple helix forming oligonucleotides (3, 24–26), but does, of course, constitute a severe obstacle which must be overcome if PNA is to be developed into gene targeted drugs.

CONCLUSION

The present results show that conditions can be obtained which result in 100% occupancy of a dsDNA target by PNA. Furthermore, binding of PNA to a restriction enzyme target completely inhibits the action of the corresponding enzyme. We are confident that this result can be extrapolated to other DNA binding proteins such as transcription factors, RNA polymerases and DNA methylases, which indicates that PNA should have the same *in vitro* use profile as triple helix forming oligonucleotides (24–29). However, contrary to oligonucleotides strong binding with high sequence specificity is obtained with only decamer PNA. Furthermore, the strand displacement binding mode suggests that it should be possible to extend the recognition to mixed purine/pyrimidine targets using PNA.

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