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In Vitro Stability of α-Helical Peptide Nucleic Acids (αPNAs)

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Abstract—α-Helical peptide nucleic acids (αPNAs) are synthetic molecules that merge the α-helix secondary structure of peptides with the codified Watson–Crick base pairing capability of nucleic acids. It is now demonstrated that αPNAs made up of either L- or D-amino acids are resistant to degradation by the proteases present in human serum. The increased stability of αPNAs towards proteases may be attributable to the presence of unnatural nucleoamino acid residues [–NHCH(CH_2OCH_2B)CO–, where B = thymine or cytosine] since the replacement of these amino acids by serine yields a control peptide that does break down in human serum. The stability of αPNAs towards proteases makes them attractive candidates for further development as antisense agents.

α-Helical peptide nucleic acids (αPNAs) are novel synthetic constructs that merge the α-helix secondary structure of peptides with the codified base pairing capability of nucleic acids (Fig. 1).[1] Cationic αPNAs are largely disordered in aqueous solution, assuming their characteristic helical secondary structure in a cooperative fashion upon binding to their nucleic acid targets. This behavior is reminiscent of peptides corresponding to the basic region of DNA-binding proteins.[2] Because they rely on peptide secondary structure to achieve the correct nucleobase spacing for hybridization to their nucleic acid complement, αPNAs represent a conceptual departure in terms of peptide-based nucleic acid surrogate design. The αPNA structure differs from Nielsen's polyamide nucleic acids (PNAs)[3] in that the αPNA backbone consists entirely of readily available α-amino acids and that the nucleobases are attached to regularly spaced Ser residues via a hemiaminal linkage. This unique merger of peptide and nucleic acid structural features allows one to modify the ancillary amino acid residues of αPNAs without compromising their primary molecular recognition capabilities. The ability to introduce structural diversity can be used to enhance specific properties of αPNAs or add function to these molecules.

We have shown that αPNAs hybridize with high affinity and sequence-specificity to their complementary ssDNA targets.[4] The binding data on complexes with varying G·C and A·T content as well as mismatched bases is consistent with a Watson–Crick base-pairing model. The rate of annealing could be increased by the introduction of multiple lysine residues into the peptide backbone. Both the affinity and orientational specificity (N/5′ vs N/3′) can be enhanced by the introduction of hydrophobic N-caps.[5] Preliminary studies also suggest that αPNAs can hybridize with complementary RNA targets.[6] Since we propose to develop a new approach to

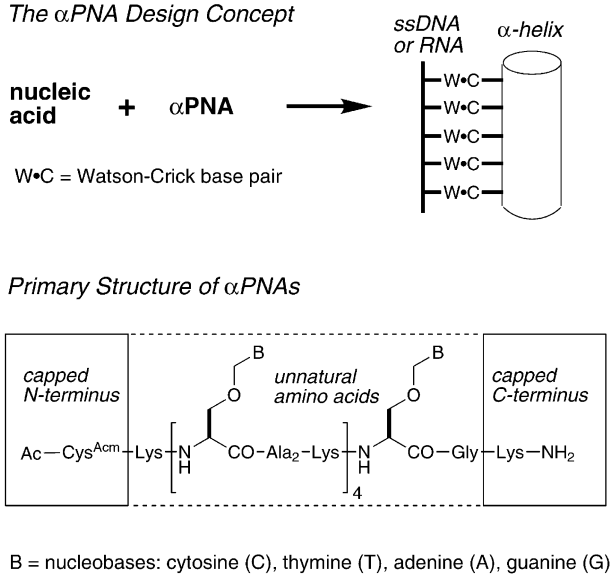

Figure 1. Nucleic acid recognition by α-helical peptide nucleic acids and general molecular structure of cationic αPNA module.

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antisense therapeutics based on αPNAs, it is important to show that these molecules will not be degraded by proteases prior to reaching their nucleic acid targets. Such degradation would, of course, undermine the therapeutic potential of our αPNAs. To address this concern, we examined the susceptibility of αPNA to protease degradation by comparing the lifetime of αPNA with that of a control peptide using human serum as a protease source. We chose to conduct the experiment in this manner since measurement of in vitro peptide stability towards human serum is a convenient way to estimate in vivo peptide stability.[7] Powell and co-workers have used human serum as a protease source to access the in vitro stability of small peptide major histocompatibility complex (MHC) antagonists.[8] Demidov et al. performed a similar study on Nielsen's PNAs and concluded that they were relatively stable towards human serum.[9] Thus, our experimental data on αPNAs can be directly compared with those reported for Nielsen's PNAs.

Materials and Methods

Two αPNAs were used in this study: Ac-Cys^{Acm}-Lys-Ser^{C}-Ala$_2$-Lys-Ser^{T}-Ala$_2$-Lys-Ser^{C}-Ala$_2$-Lys-Ser^{C}-Ala$_2$-Lys-Ser^{T}-Gly-Lys-NH$_2$ (hereafter referred to as L-CTCCT)[10] as well as its antipode Ac-cys^{Acm}-lys-ser^{C}-ala$_2$-lys-ser^{T}-ala$_2$-lys-ser^{C}-ala$_2$-lys-ser^{C}-ala$_2$-lys-ser^{T}-gly-lys-NH$_2$ (hereafter referred to as D-CTCCT) made up of all D-amino acids. A control peptide, Ac-Trp-Cys^{Acm}-Lys-Ser-(Ala$_2$-Lys-Ser)$_4$-Gly-Lys-NH$_2$, was also synthesized with the expectation that it would be degraded and thus validate the experiment. In this control peptide, the Ser^{B} residues were replaced by unmodified Ser and a Trp residue was inserted at the N-terminus. The latter modification was made to facilitate UV detection in the absence of nucleobase chromophores. αPNA/peptide synthesis was performed on a Rink amide MBHA resin support (Novabiochem, 0.54 mmolar scale) by following our published procedure.[4] Acetylation and cleavage from the resin were performed as described previously. The crude αPNAs/peptides were purified by reverse-phase HPLC (XTerra^{TM} RP$_{18}$ 7 μm, 19×150 mm column using a linear AB solvent gradient, with 0.56 and 0.73% B/min for αPNAs and peptides, respectively. A = 0.1% trifluoroacetic acid (TFA) in water, B = 0.1% TFA in MeCN; λ = 254 and 220/280 nm for αPNAs and peptides, respectively; flow rate = 15 mL/min). Collections were made at 25.4, 23.2, and 13.0 min for D-CTCCT, L-CTCCT, and the control peptide, respectively. Electrospray mass data (Micromass Quattro II triple-quadruple ESI mass spectrometer) for the purified αPNAs/peptides: L-CTCCT, calcd [M] 2708.97, obsd 2708.35 ± 0.36 (from ref 3); D-CTCCT, calcd [M] 2708.97 obsd 2708.27 ± 0.12; control peptide calcd [M] 2206.20 obsd 2206.73 ± 0.18.

Blood was collected from a healthy male donor, spun for 20 min, and the serum was removed. A 25% (by volume) solution of fresh (no freeze/thaw) human serum in RPMI-1640 medium (Sigma) was prepared and incubated at 37 °C for 15 min. A solution of αPNA or peptide was prepared and added to the reaction mixture to give an initial peptide concentration of 36.3 μM. The mixture was incubated at 37 °C and at known time intervals 20 μL aliquots were removed and quenched with 20 μL of 10% trichloroacetic acid (TCA) solution. The resulting samples were cooled at 4 °C for 15 min, then spun at 4 °C and 13 000*g* for 15 min. The supernatant was removed and stored at 4 °C until analyzed. Degradation was monitored by following the changes in the concentration of the αPNA and peptide substrates over time by reverse-phase HPLC (XTerra^{TM} RP$_{18}$ 3.5 μm, 4.6×50 mm column with a linear AB gradient 0.56% B/min and 0.73% B/min for αPNA and peptide, respectively; A = 0.1% TFA in water, B = 0.1% TFA in MeCN; λ = 254 and 220/280 nm for αPNA and peptide, respectively; flow rate = 1 mL/min). Concentrations of αPNA solutions were determined by UV absorbance at 254 nm using the nearest neighbor approximation,[11] assuming the extinction coefficients of nucleobases in αPNAs are the same as in DNA. The $t = 0$ values for the αPNAs were obtained using heat denatured serum. The concentration of the control peptide solution was determined by its UV absorbance in a 3 M guanidine–HCl solution at 280 nm using extinction coefficient published for Trp.[12] A 5 μL sample of Boc-L-tyrosine solution was added to each sample prior to analysis to serve as an internal standard for peak quantification.

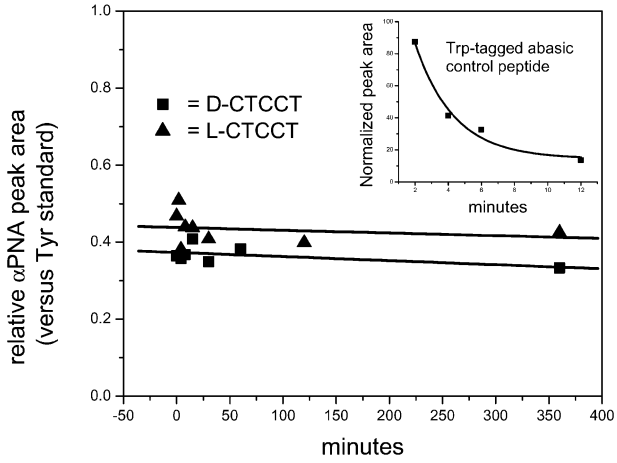

Figure 2. Relative stability of αPNAs to human serum.

Results and Discussion

Upon exposure to freshly prepared solutions of human serum, changes in the relative concentrations of the αPNAs as well as the control peptide were followed by analyzing their HPLC profiles over time. These data are presented graphically in Figure 2 and lead to the following observations. First, it should be noted that the concentration of the control peptide decreased exponentially reaching a baseline value after about 12 min (half-life estimated to be about 4 min). This experiment demonstrated that our human serum had not been deactivated during its processing and contained active protease. Perhaps not unexpectedly, the unnaturally configured D-CTCCT (made up of all D-amino acids) showed insignificant degradation after exposure to

human serum for 6 h. Most surprising, however, was the fact that no degradation was observed when the 'naturally-configured' L-CTCCT was exposed to active human serum over this same period of time. In these experiments, we did not detect the formation of any αPNA derived products. As discussed below, these experimental results could have important implications for the future development of αPNA-based drugs.

A major hurdle to the development of peptide-based pharmaceuticals is their rapid clearance from the bloodstream.[13] This process typically begins with enzyme-mediated peptide metabolism via the combined action of proteases that cleave either the C-terminal amino acids (carboxypeptidases) or N-terminal amino acids (aminopeptidases) as well as proteases that cleave internal amide bonds at specific sites. Therefore, one approach to preventing proteolytic peptide degradation involves modifying their structure so that they are no longer substrates for proteases. Tactics that have been employed to enhance peptide stability include (1) N-terminal acylation, (2) C-terminal amidation, (3) incorporation of unnatural amino acids including D-amino acids, (4) restriction of peptide conformation, and (5) modification/replacement of the peptide backbone. The versatility of our αPNA scaffold readily lends itself to all but the last of these stabilizing modifications, thus offering multiple avenues to render αPNAs protease resistant.

The preliminary data suggests that αPNAs, by their very nature, may be inherently stable towards degradation by proteases. While the *N*-acetyl group likely protects the N-terminus from the action of aminopeptidases (tactic 1) and the carboxamide moiety protects the C-terminus from carboxypeptidases (tactic 2), these features alone do not prevent degradation of the control peptide. The stability of αPNAs towards proteases is independent of the absolute configuration of the backbone amino acids (tactic 3). The αPNAs used in this study are disordered in the absence of nucleic acid complements (ref 5—supporting information), so enforced helicity (tactic 4) cannot be a factor in their enhanced metabolic stability.[14] Thus, it appears that the unnatural nucleoamino acid residues (another expression of tactic 3) are primarily responsible for the enhanced stability of L-configured αPNAs towards degradation by proteases in human serum. While it is tempting to ascribe this behavior to the inability of the enzyme to accommodate the nucleobase containing amino acid side chain, other possible explanations cannot yet be rigorously excluded (enzyme inactivation by αPNA, for example). In any case, we are now poised to begin addressing the issue of cellular uptake of αPNAs as well as their *intracellular* distribution and activity.

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10. Amino acid abbreviations: Cyc^{Acm} = *S*-acetamidomethyl-L-cysteine, Gly = glycine, Lys = L-lysine, Ser = L-serine, Ser^{T} = 1-(Ser)methyl]thymine, Ser^{C} = 1-(Ser)methyl]cytosine, Trp = L-tryptophan
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