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Construction of Peptide Conjugates with Peptide Nucleic Acids Containing an Anthracene Probe and Their Interactions with DNA

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Abstract—We designed and synthesized the peptide nucleic acid (PNA)–peptide conjugates having anthracene chromophores and investigated their interactions with calf thymus DNA, $[d(AT)_{10}]_2$, $[d(GC)_{10}]_2$, and $[d(AT)_{10}dA_6]_2$. Considering the synthesis compatibility and expecting that a novel DNA analogue, PNA, can improve DNA binding properties of α-helix peptides, we attempted to attach thymine PNA oligomers at the C-terminus of a 14 amino acid α-helix peptide that contained a pair of artificial intercalators, anthracene, as a probe, and to examine their interactions with DNA using anthracene UV, fluorescence and circular dichroism properties. The results observed in this study showed that the designed peptide folded in an α-helix structure in the presence of calf thymus DNA, $[d(AT)_{10}]_2$, and $[d(AT)_{10}dA_6]_2$ with the chromophores at the side-chain being fixed with a left-handed chiral-sense orientation. The α-helix and the anthracene signals were not observed for $[d(GC)_{10}]_2$. Incorporation of thymine PNA oligomers into the designed α-helix peptide increased the DNA binding ability to $[d(AT)_{10}dA_6]_2$ with increasing the length of the PNA without changing the conformations of the peptide backbone and the anthracene side-chains.

Introduction

The design of synthetic molecules that bind sequence-specifically to the unique site of DNA and mimic the action of proteins may have major implications for regulation of cellular reactions mostly mediated by the specific interaction between proteins and DNA.[1] Peptides with an α-helical conformation play an important role in many biological systems, as this is a very important protein secondary structure. For example, motifs such as helix-turn-helix, helix-loop-helix, basic leucine-zipper, and zinc fingers use α-helix structures in the framework of DNA major groove interaction as most of the contacts between amino acids and DNA bases are made within such recognition helices.[2] On the other hand, peptide nucleic acid (PNA), a well established DNA mimic developed by Nielsen et al., in which the entire sugar-phosphate backbone has been replaced with a peptide-like backbone, has attracted much attention in nucleic acid science since it was developed.[3–5] PNAs form double helices with complementary PNA, DNA, and RNA with the Watson–Crick base pairings.[6–8] It has been shown that PNA molecules have a potential as therapeutic agents, tools in diagnostics and probes in molecular biology.[9–12] Although many DNA analogues have been studied extensively, PNA exhibits better advantages such as stability to degradation by enzymes, and hybridization property to complementary sequences with higher affinity and specificity. Furthermore, from the synthetic point of view, PNA oligomer synthesis is compatible to standard solid-phase peptide synthesis and that makes the synthesis easier either by manual or by machine. Since PNA molecules are achiral, conjugation with chiral and structured peptides will lead to a new property of PNA.

In the design of small DNA binding molecules, many DNA binding agents interact noncovalently with DNA through intercalative association in which a planar, hetero-aromatic moiety slides between base pairs.[13] Intercalation binding of small molecules with DNA has been investigated at the molecular level, while intercalators are important as drugs against several diseases.[14] In general, intercalators are planar, and planarity is

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proposed to be one of the important features for effective intercalation into the DNA helix. So far, a variety of anthracene derivatives have shown significant antitumor activity in animal models and many of them have been used for clinical testing.[15] It has been also demonstrated that anthracene ring system should have particularly good stacking interactions with DNA base pairs.[16] The strong absorption and fluorescence property of the anthracene group provide a sensitive spectroscopic handle to study its intercalation with DNA. Changes in the intensities can be used to find the nature and the strength of the interactions between molecules and DNA.[17]

In this work, we expected that the linkage of peptide and PNA might provide a novel mechanism to gauge the interaction between peptide and DNA. For this purpose, to combine the excellent properties of PNA and α-helix peptides in the framework of peptide–DNA interactions, we synthesized a 14 amino acid α-helix peptide that conjugated with a series of PNA thymine oligomers at the C-terminus and that employed two anthracenes as functional chromophores on two lysine residues at 6th and 9th positions (Fig. 1). Thymine PNA was chosen because it can participate with no synthetic obstacle for this purpose. In this study we used anthracene as a probe to detect the chiral orientation of chromophores and to adopt the intercalation properties to the side-chains of the α-helix peptide. We investigated interaction of the anthracene peptide–thymine PNA conjugates with calf thymus DNA, $[d(AT)_{10}]_2$, $[d(GC)_{10}]_2$, and $[d(AT)_{10}dA_6]_2$ using anthracene UV, fluorescence and circular dichroism (CD) properties.

Results and Discussion

Design and synthesis

The 14 peptide was designed to take an amphiphilic α-helix structure upon binding to DNA as shown in Figure 1. The amino acids of this peptide sequence are selected for their helical propensity.[18] Four positively charged lysine residues were employed for the interaction with negatively charged DNA phosphate groups. A pair of anthracene groups were introduced on two lysine residues at the 6th and 9th positions to give the intercalation function in the α-helix peptide and to examine how

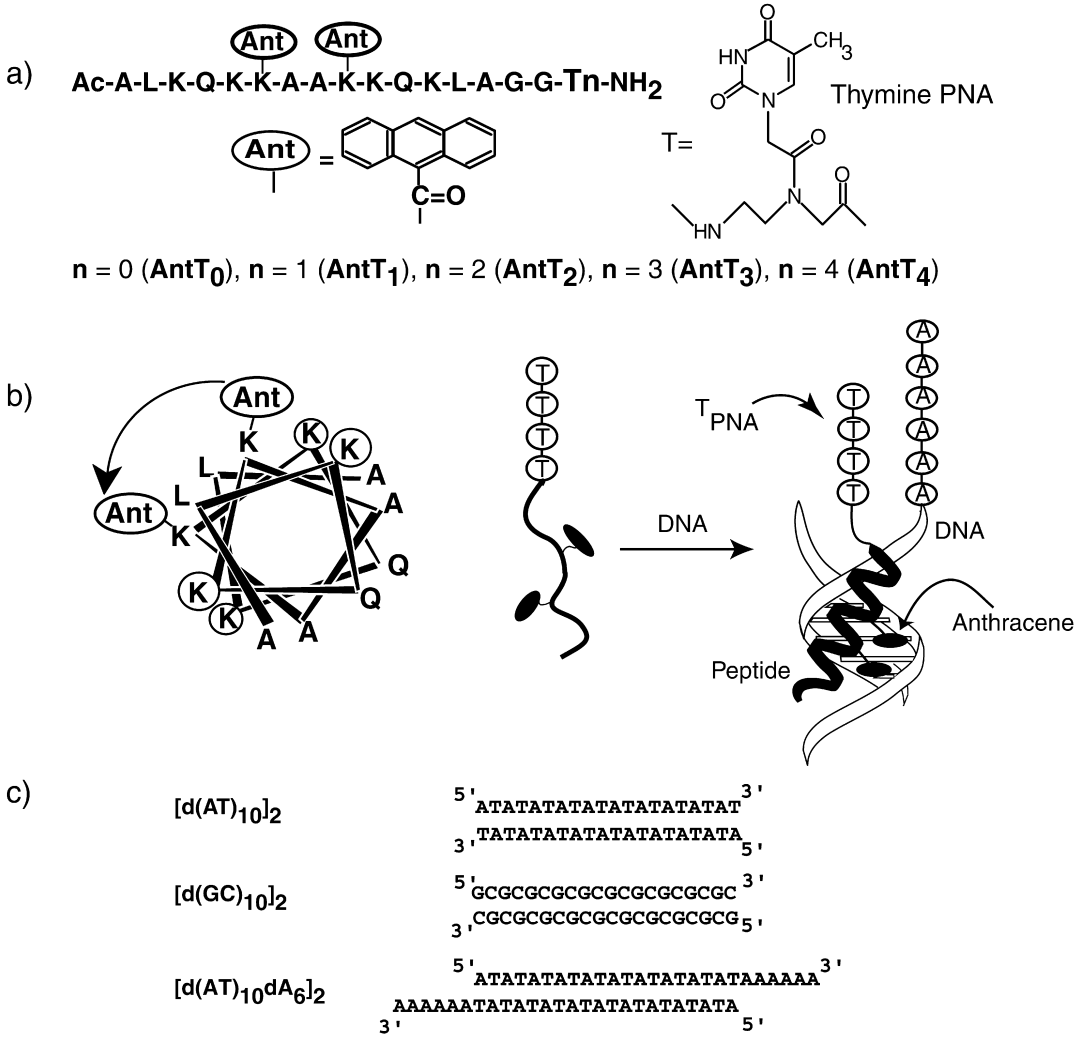

Figure 1. Amino acid sequences of anthracene peptide–thymine PNA conjugates and chemical structures of anthracene moiety and thymine PNA (a). α-Helical wheel and schematic illustration of their interactions with DNA (b). DNAs used in this study (c).

the chromophores behave via DNA binding. Earlier, we used similar α-helix peptide sequences with two anthracene chromophores at different positions and investigated their DNA binding properties.[19] The study revealed that the presence of anthracene chromophores on lysine residues at the 6th and 9th positions exhibited a left-handed chiral-sense orientation and anthracene chromophores at the 6th and 10th positions showed a right-handed chiral-sense orientation upon DNA binding. Moreover, the study showed that the peptide with the left-handed chiral-sense of anthracenes displayed a higher DNA binding ability for an AT sequence compared to the right-handed chiral-sense peptide and the peptide with a single chromophore. In order to evaluate the influence of introduction of PNA on DNA binding of peptides, we have synthesized a series of peptides, $AntT_0$–$AntT_4$, containing various lengths of thymine-PNA at the C-terminus of the anthracene peptide. Two glycine residues were used between the peptide and PNA as a spacer (Fig. 1).

The peptide–PNA conjugates were synthesized by the solid-phase method using standard Fmoc strategy. In our experiences, elongation of thymine PNA oligomers to the amino terminus of this same α-helix sequence by solid-phase synthesis encountered with coupling problems, probably due to the unwanted side reaction of *N*-acyl transfer.[20] However, this experiment showed that thymine PNA oligomerisation at the carboxyl terminus was achieved with single coupling. The synthesized products were treated with trifluoroacetic acid (TFA) to remove the peptide from resin. Anthracene groups were attached on the lysine residues at the 6th and 9th positions with 9-anthracene carboxylic acid using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/1-hydroxybenzotriazole (HOBt). After confirming the anthracene coupling reactions, the 2-chlorobenzyloxycarbonyl (ClZ) protection groups of other lysine residues were removed by trimethylsilyl trifluoromethanesulphonate (TMSOTf)/TFA treatment.[21] The final products were purified by semi-preparative reversed-phase HPLC (RP-HPLC) to give high purity (>98% on analytical RP-HPLC). The conjugates were identified by matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS).

CD study of the peptide–PNA conjugates with DNA

To obtain the conformational information, we performed CD spectroscopy. In general, intercalators bound to DNA exhibit induced circular dichroism spectra due to their asymmetric environment. From this one can easily evaluate the relative orientation of the chromophore with respect to the DNA helix.[22–24] In this experiment, the CD study revealed that the peptides ($AntT_0$–$AntT_4$) were almost in a random structure in aqueous buffer (pH 7.4) with 40 mM NaCl and 2 mM $MgCl_2$. On the contrary, in trifluoroethanol (TFE), an α-helix forming solvent, the peptides showed an α-helix CD pattern (Fig. 2a). The excitation coupling was remarkably observed at the anthracene region (254 nm wavelength) displaying the negative and positive splitting from the longer wavelength. This suggests that the two anthracene groups were oriented in a chiral-sense to the left-handed arrangement as expected.[25]

Next, we studied the CD analysis with calf thymus DNA, $[d(AT)_{10}]_2$, $[d(GC)_{10}]_2$. In addition to these DNAs we used $[d(AT)_{10}dA_6]_2$, expecting that the thymine PNA part of the helix sequence may have any binding influence. All these measurements were carried out when the peptides were completely bound to DNA. As shown in the Figure 2b–d, at the amide region, an α-helix structure was strongly induced via binding to calf thymus DNA, $[d(AT)_{10}]_2$, and $[d(AT)_{10}dA_6]_2$. The positive and negative splitting was observed at the anthracene region showing the left-handed chiral-sense orientation. These chiral signals also indicate the α-helix formation of the peptides as a result of interaction with DNA. Peptides with $[d(GC)_{10}]_2$ did not induce any CD pattern at both the amide region and the anthracene region (Fig. 2c). The peptide without the anthracene groups showed also the α-helix induction by the binding with AT-rich DNA.[19] These results indicate that the α-helix and anthracene signals are AT selective. However, though the conjugates exhibited a similar CD pattern with a particular DNA, they showed different ratios in negative and positive peaks. $[d(AT)_{10}]_2$ and $[d(AT)_{10}dA_6]_2$ showed almost equal area of negative and positive peaks, whereas with calf thymus DNA the positive peak was found to be greater than the negative peak. These changes in peak ratios are probably due to slightly different interaction modes with different DNAs. Overall, the CD patterns at the amide region and the anthracene region showed that there occurred no conformational changes by the presence of thymine PNA oligomers, indicating that all anthracene peptide–PNA conjugates ($AntT_0$–$AntT_4$) possess a similar binding mode to each DNA. These results demonstrated that the peptides fold in an α-helix structure and the chromophore at the side chain being fixed in a coincidental manner as they are deployed, when they interact with AT part of DNA.

UV study of the peptide–PNA conjugates with DNA

Prior to investigating the anthracene peptide–DNA interactions by fluorescence spectroscopy, we performed UV measurements of the peptides with DNA. Figure 3 depicts the UV spectral changes that occur when $d[(AT)_{10}dA_6]_2$ was added to a solution of $AntT_4$ peptide. By the addition of $[d(AT)_{10}]_2$, $[d(GC)_{10}]_2$, calf-thymus, and $d[(AT)_{10}dA_6]_2$, the absorbance of anthracene groups were decreased and shifted to a longer wavelength range, indicating the interaction between the aromatic chromophore and the DNA bases. Isosobestic points were observed in all DNA cases, suggesting that the free and bound ligands are spectroscopically distinguishable. Furthermore, the observed large hypochromism shows a sign of close proximity of the anthracene chromophore to the DNA bases due to a strong interaction between the electronic states of the intercalating chromophore and those of the DNA bases.[26–29] Virtually, these UV patterns indicate that the anthracene chromophore has a property of intercalating to the all selected DNAs. Further insight about the binding ability is discussed in the subsequent fluorescence study.

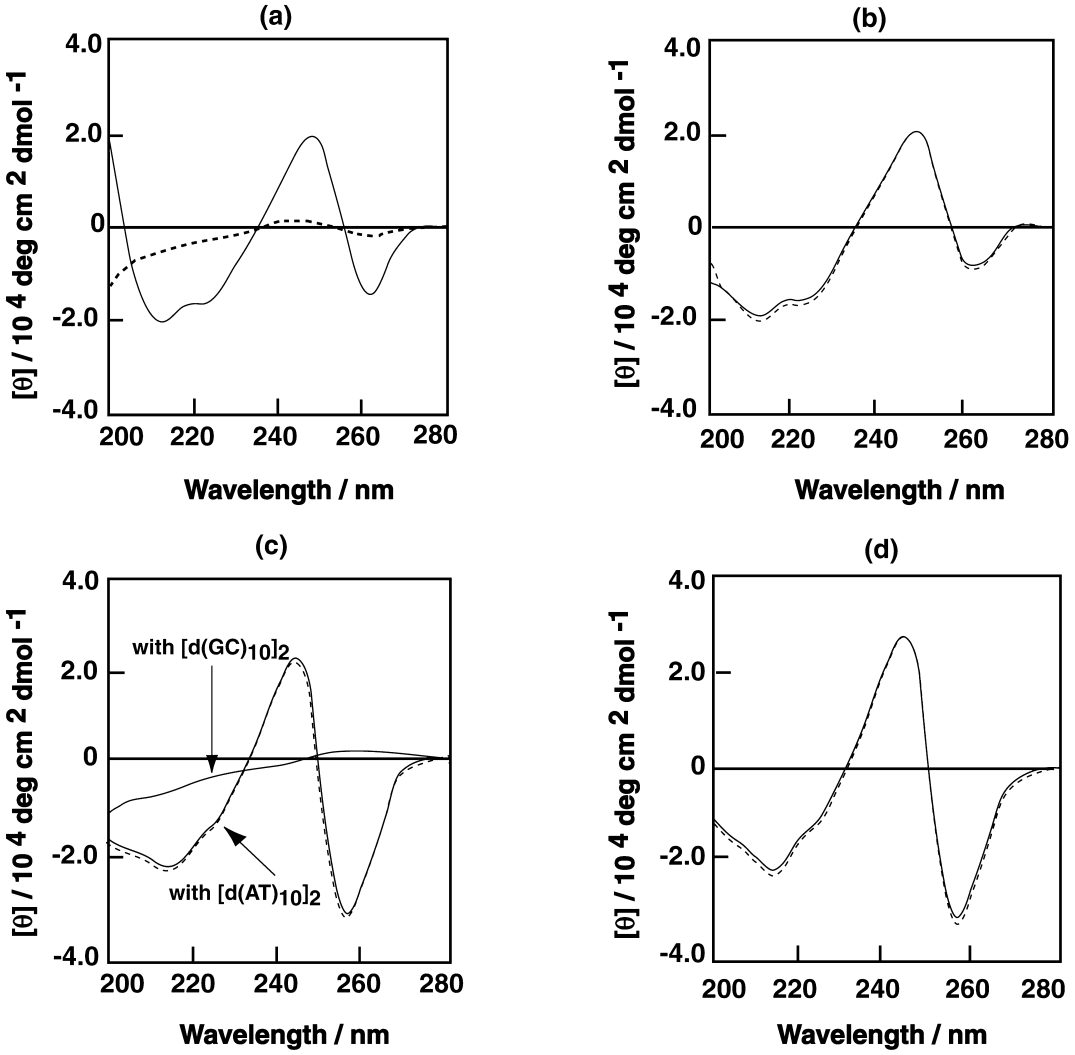

Figure 2. CD spectra of conjugates in TFE (solid line) and 20 mM Tris·HCl (broken line) (a), with calf-thymus DNA (b), with $[d(AT)_{10}]_2$, and $[d(GC)_{10}]_2$ (c), with $[d(AT)_{10}dA_6]_2$ (d). From (b) to (d) CD patterns of $AntT_0$ (broken line) and $AntT_4$ (solid line) are shown. Spectral measurements were carried out at 25 °C in 20 mM Tris·HCl buffer (pH 7.4) containing 40 mM NaCl and 2 mM $MgCl_2$. [Peptide] = 5.0 μM, [DNA nucleotide] = 300 μM.

Fluorescence study of the peptide–PNA conjugates with DNA

Next we investigated the interactions of the anthracene peptide–PNA conjugates with DNA quantitatively by fluorescence measurements. Both the previous study[19] and this study showed that a similar α-helix peptide exhibited a binding preference with AT base pairs rather than GC base pairs. In this study, in addition to $[d(AT)_{10}]_2$, $[d(GC)_{10}]_2$, and calf-thymus DNA, we used $[d(AT)_{10}dA_6]_2$, expecting that the thymine PNA part of the helix peptide may have any binding influence. Figure 4 depicts the fluorescence titration for $AntT_4$ peptide with $[d(AT)_{10}dA_6]_2$. In all DNA cases, except $[d(GC)_{10}]_2$, the addition of DNA to the conjugates increased the fluorescence intensity. The higher DNA concentration part of the curve corresponds to the saturation limit of fluorescence. However, unlike other DNAs, the titration with $[d(GC)_{10}]_2$ showed decreases in the fluorescence intensity due to the quenching by G.[17] To characterize the interaction quantitatively, the binding constants were determined by making a curve-fitting analysis of $\Delta I/I_0$ as a function of DNA concentration (Fig. 5). The previous study[19] showed that the same anthracene peptide displayed the 1:4 stoichiometry with base pairs of $[d(AT)_{10}]_2$, $[d(GC)_{10}]_2$, and calf-thymus DNA based on the site exclusion equation of McGhee and Von Hippel.[30] In this experiment, since $[d(AT)_{10}dA_6]_2$ contains both the single strand and the double strand, we used the nucleotide concentration instead of base pair concentration for all DNAs uniformly. Coincidently, the fluorescence results showed that for the DNAs, the best curve-fitting was achieved with a 1:8 stoichiometry using eq. 1 in the experimental section.

Table 1 shows the binding constants calculated by the fluorescence titration for various DNAs. Interaction with calf thymus DNA gave the binding constant value of $7.2\times10^5\,M^{-1}$ for $AntT_0$ peptide. Although the spectral measurements with calf thymus DNA explained the binding properties of all peptides, there was not any

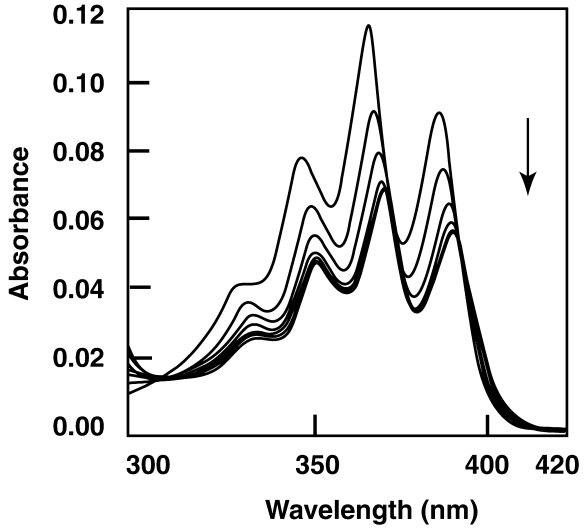

Figure 3. UV spectra of $AntT_4$ peptide in the presence of various concentrations of $[d(AT)_{10}dA_6]_2$. From top to bottom the nucleotide concentrations were 0, 35, 70, 105, 140, 175, 210 μM. (Peptide) = 5.0 μM, 25 °C.

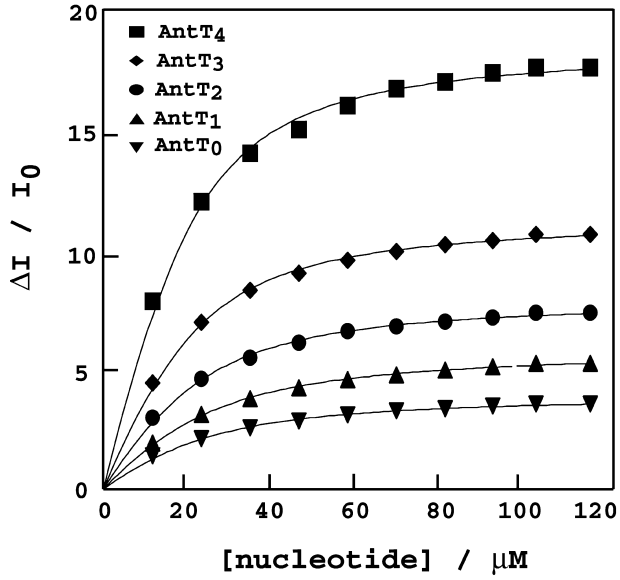

Figure 5. Curve-fitting analysis for the fluorescence intensities of conjugates with $[d(AT)_{10}dA_6]_2$ at 424 nm.

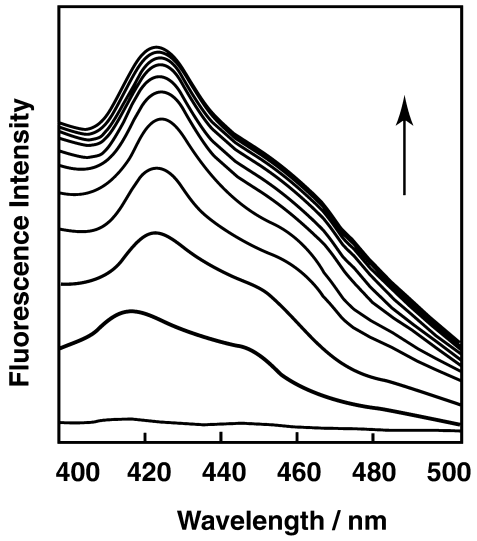

Figure 4. Fluorescence spectra of $AntT_4$ peptide in the presence of various concentrations of $d[(AT)_{10}dA_6]_2$. From bottom to top the nucleotide concentrations were 0, 9, 18, 27, 36, 45, 54, 63, 72, 81, 90 μM. (Peptide) = 2.0 μM, λex = 389 nm, 25 °C.

observed DNA binding influence by the addition of thymine PNA. Interaction of $AntT_0$ with $[d(AT)_{10}]_2$ showed a binding constant value of $11.8\times10^5\,M^{-1}$. However, the results of $AntT_1$ through $AntT_4$ displayed similar binding constants at the maximum of $13\times10^5\,M^{-1}$. The lowest binding constants were observed with $[d(GC)_{10}]_2$, indicating that the binding ability of this anthracene peptide to the AT sequence is two times higher than that of the GC sequence. These results showed that the presence of thymine PNA does not discriminate the DNA binding ability in these cases of double strand DNAs.

Interestingly, there were noticeable variations in binding constants with $[d(AT)_{10}dA_6]_2$ by the increasing number of thymine PNA. Figure 5 shows the curve-fitting analysis performed for conjugates with $[d(AT)_{10}dA_6]_2$. As shown in Figure 5 and Table 1, both the $\Delta I/I_0$ value and binding constant were increased from $AntT_0$ to $AntT_4$ ($11.0\times10^5\,M^{-1}$ to $16.5\times10^5\,M^{-1}$). These results suggest that the thymine PNA can interact with the single strand dA_6 of $[d(AT)_{10}dA_6]_2$ and this interaction is enhanced by increasing the number of thymine PNA (Fig. 6). Although, the addition of single thymine PNA is not effective, further addition of thymine PNA remarkably increased the binding ability of the conjugates to the DNA.

In order to verify the influence of thymine PNA part of $AntT_4$ peptide with the single strand part of $[d(AT)_{10}dA_6]_2$, the fluorescence inhibition test was performed in the presence of excess amount of Ac-$(T_4)_{PNA}$-Gly-NH_2. The measurement was carried out by titrating $AntT_4$ peptide (2.0 μM) with $[d(AT)_{10}dA_6]_2$ in the presence of Ac-$(T_4)_{PNA}$-Gly-NH_2 (100 μM) (data not shown). A binding constant value of $8.1\times10^5\,M^{-1}$ was observed, lower than the other anthracene peptide–PNA conjugates with $[d(AT)_{10}dA_6]_2$ (Fig. 6). This suggests that the Ac-$(T_4)_{PNA}$-Gly-NH_2 binds to the dA_6 part of the $[d(AT)_{10}dA_6]_2$ and thereby decreases the overall binding ability of $AntT_4$ peptide with the DNA. This inhibition experiment confirms the influence of thymine PNA part of AntT4 peptide with $[d(AT)_{10}dA_6]_2$. Additionally, Ac-$(T_4)_{PNA}$-Gly-NH_2 might affect the peptide binding to the double strand part of the DNA to some extent so that the binding constant decreased to a lower level than $AntT_0$. All these fluorescence results reveals that PNA thymine oligomersation with the helix peptide has no significant influence with calf thymus DNA, $[d(AT)_{10}]_2$, and $[d(GC)_{10}]_2$, whereas increase in the binding ability was observed only with $[d(AT)_{10}dA_6]_2$.

Table 1. Binding constants of anthracene peptide-PNA conjugates with DNA estimated from fluorescence studies

	K_b ($\times 10^5\ M^{-1}$)				
	$AntT_0$	$AntT_1$	$AntT_2$	$AntT_3$	$AntT_4$
Calf thymus	7.2 ± 0.1	6.4 ± 0.1	7.4 ± 0.1	7.2 ± 0.3	ND[a]
$[d(AT)_{10}]$	11.8 ± 0.5	11.0 ± 0.6	12.0 ± 0.6	10.5 ± 0.5	13.0 ± 0.2
$[d(GC)_{10}]$	5.2 ± 0.2	5.5 ± 0.6	5.7 ± 0.2	5.9 ± 0.3	5.0 ± 0.1
$[d(AT)_{10}\ dA_6]_2$	11.0 ± 0.06	10.8 ± 0.1	12.8 ± 0.1	13.2 ± 0.1	16.5 ± 0.1

[a]Not determined due to the formation of aggregates.

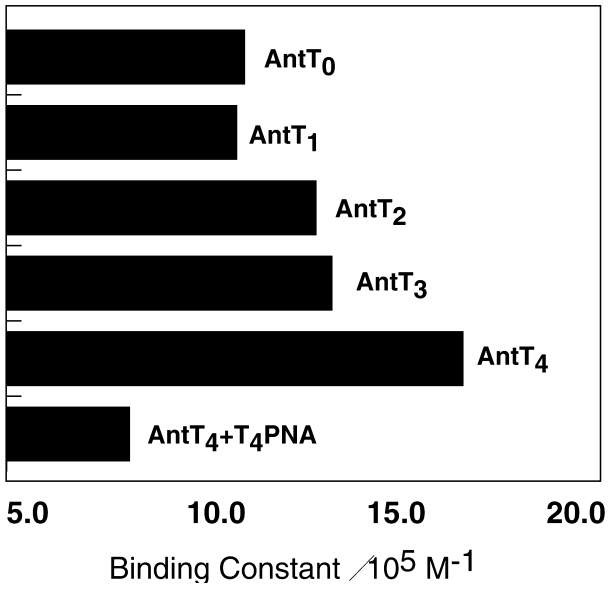

Figure 6. Variation of binding constants derived from fluorescence measurements of conjugates with $[d(AT)_{10}dA_6]_2$.

Conclusion

A new type of peptidyl molecule was designed and synthesized which bound to DNA. In the α-helix peptide, the anthracene chromophores were fixed to arrange via binding to DNA so as to interact with each other. These interactions of anthracene groups enable the detection of the conformational changes of the peptides by the induced CD with DNAs. Incorporation of thymine PNA oligomers at the carboxyl terminus of the helix peptide showed an increased binding ability with $[d(AT)_{10}dA_6]_2$ by the increase of the thymine PNA length without changing the conformational property of the helix peptide at both the anthracene and amide regions. This study demonstrates the feasibility of our method in utilizing anthracene as a probe and functionality for DNA binding and the possible use of PNA in the framework of peptide–DNA interaction. Furthermore, the strategy involved in this study can be readily adopted to use other natural peptides with PNA oligomers to modify the DNA/RNA binding properties.[31]

Experimental

General methods

All chemicals and solvents used for synthesis and analysis were of reagent grade or HPLC grade and used without further purification. Fmoc amino acid derivatives, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin (Rink amide resin),[32] 1-hydroxybenzotriazole (HOBt), water soluble carbodiimide (EDC) were purchased from Watanabe Chemical (Hiroshima, Japan). Benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) was purchased from Novabiochem. 9-Anthracene carboxylic acid was purchased from Aldrich. Boc protected thymine PNA monomer was prepared according to the published method.[33] This was converted to the Fmoc derivative using *N*-(9-fluorenylmethoxycarbonyl succinimide) (Fmoc-OSu). The synthesis of PNA–peptide conjugates was carried out manually according to the method available for standard solid-phase peptide synthesis.[34] Completion of the each acylation steps was monitored by the Kaiser test.[35] Recoupling was performed if the couplings were incomplete. Capping of the unreacted amino groups was achieved using acetic anhydride. The following amino acid derivatives were used in this experiment: Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Lys(ClZ)-OH, Fmoc-Lys(Boc)-OH, and Fmoc-Gln(Trt)-OH. The lysines engaged in anthracene introduction were incorporated as Fmoc-Lys(Boc)-OH and other lysines were protected with ClZ groups. All amino acids and thymine PNA couplings were performed with BOP and HOBT. After addition of the last amino acid (Ala) and removal of the Fmoc group, the products were acetylated with acetic anhydride. The protecting groups (except ClZ) and resin were removed by TFA/m-cresol treatment. The coupling with 9-anthracene carboxylic acid was achieved with EDC/HOBT in DMF for 24 h. Finally all ClZ groups were removed by treating with TMSOTf/TFA/thioanisole mixture.[21] TOFMS was performed on a Shimadzu MALDI III mass spectrometer by using sinnapinic acid as a matrix. Final purification was carried out by RP-HPLC on a YMC-Pack C18 A-323 column (10×250 mm) to give the products with a single peak on analytical HPLC [Wakosil 5C18 (4.6×150 mm)] with a linear gradient of acetonitrile/0.1% TFA. MALDI-TOFMS found *m/z* (calcd $[M+H]^+$); $AntT_0$, 2116.8 (2117.7); $AntT_1$, 2382.2 (2383.9); $AntT_2$, 2649.5 (2650.1); $AntT_3$, 2916.7 (2916.3); $AntT_4$, 3180.8 (3182.5).

Spectroscopic measurements

All spectroscopic measurements were carried out at 25 °C at pH 7.4 using 20 mM Tris·HCl buffer containing 40 mM NaCl and 2 mM $MgCl_2$. Calf thymus DNA was purchased from Pharmacia Biotech. Calf thymus DNA (4 mg/mL) was dissolved by slowly strirring for 24 h in

20 mM Tris·HCl buffer (0.15 M NaCl; pH 7.4). The solution was sonicated for 30 min under Ar gas and filtered. Concentration of the DNA was determined spectroscopically using molar extinction coefficient per base pair with the extinction coefficient of $\epsilon_{260} = 6600$ at 260 nm. Synthetic DNA oligomers were purchased from Sawady Technology, and concentration of each of them was determined using the manufacturer's extinction coefficients. UV spectra were recorded using Shimadzu UV-3100 spectrophotometer using a quartz cell with 1 cm path-length. Fluorescence measurements were carried out on a Shimadzu RF-5300PC fluorescence spectrophotometer using a 1 cm path-length quartz cell with excited wavelength at 389 nm. The fluorescence intensity was monitored at 424 nm. Binding constants of anthracene peptide–PNA conjugates with DNA were calculated by the following equation by making a fitting-curve to the $\Delta I/I_0$ as a function of nucleotide concentration with a 1:8 stoichiometry using Kaleidagraph (Synergy Software);

$$\Delta I/I_0 = \frac{(\Delta I_{max}/I_0)\{[P_0] + [8D_0] + 1/K_b - (([P_0] + [8D_0] + 1/K_b)^2 - 8[P_0][8D_0]))^{0.5}\}}{2[P_0]} \quad (1)$$

$\Delta I = I - I_0$, where I and I_0 are the fluorescence intensities of the conjugates in the presence and the absence of DNA, respectively. $\Delta I_{max} = I_{max} - I_0$, where I_{max} is the fluorescence intensity when peptide bound to DNA. $[P_0]$ is the total peptide concentration and $[D_0]$ is the total DNA nucleotide concentration. K_b is the binding constant of the conjugates for DNA nucleotide. CD measurements were recorded on a JASCO-720WI spectropolarimeter using a 0.1 cm quartz cell. All spectral data were collected in the 200 nm to 280 nm wavelength range as the average of 10 scans only when the anthracene peptides completely bound to DNA.

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