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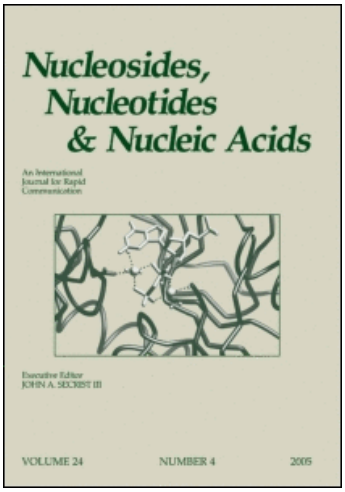

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RECOGNITION OF A FLIPPED BASE IN A HAIRPINLOOP DNA BY A SMALL PEPTIDE

Junji Kawakami,[1,2] Shinji Okabe,[2] Yoshiatsu Tanabe,[2] and Naoki Sugimoto[1,2]

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□ *Two tiny hairpin DNAs, CORE (dAGGCTTCGGCCT) and AP2 (dAGGCTXCGGCCT; X: abasic nucleotide), fold into almost the same tetraloop hairpin structure with one exception, that is, the sixth thymine (T6) of CORE is exposed to the solvent water (Kawakami, J. et al.,* Chem. Lett. ***2001****, 258–259). In the present study, we selected small peptides that bind to CORE or AP2 from a combinatorial pentapeptide library with* 2.5×10^6 *variants. On the basis of the structural information, the selected peptide sequences should indicate the essential qualifications for recognition of the hairpin loop DNA with and without a flipped base. In the selected DNA binding peptides, aromatic amino acids such as histidine for CORE and glutamine/aspartic acid for AP2 were found to be abundant amino acids. This amino acid preference suggests that CORE-binding peptides use* π–π *stacking to recognize the target while hydrogen bonding is dominant for AP2-binding peptides. To investigate the binding properties of the selected peptide to the target, surface plasmon resonance was used. The binding constant of the interaction between CORE and a CORE-binding peptide (HWHHE) was about* 1.1×10^6 M^{-1} *at 25°C and the resulting binding free energy change at 25°C* (ΔG°_{25}) *was* -8.2 *kcal* mol^{-1}. *The binding of the peptide to AP2 was also analyzed and the resulting binding constant and* ΔG°_{25} *were about* 4.2×10^4 M^{-1} *and* -6.3 *kcal* mol^{-1}, *respectively. The difference in the binding free energy changes* ($\Delta\Delta G^\circ_{25}$) *of 1.9 kcal* mol^{-1} *was comparable to the values reported in other systems and was considered a consequence of the loss of* π–π *stacking. Moreover, the stabilization effect by stacking affected the dissociation step as well as the association step. Our results suggest that the existence of an aromatic ring (T6 base) produces new dominant interactions between peptides and nucleic acids, although hydrogen bonding is the preferable mode of interaction in the absence of the flipping base. These findings regarding CORE and AP2 recognition are expected to give useful information in the design of novel artificial DNA binding peptides.*

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Keywords Hairpin loop DNA; Base recognition; DNA binding peptide; Binding model; π–π stacking

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INTRODUCTION

Many specific protein–nucleic acid interactions have been identified and investigated as key events of living organisms. In many of the double stranded DNA binding proteins with helix-turn-helix, leucine zipper, Zn finger, or other minor motifs,[1–3] the principal determinants of specificity are hydrogen bonds involving functional groups of bases at the grooves in the helical DNA structure. On the other hand, for binding to RNA, proteins recognize their target RNAs via various recognition modes. Proteins can recognize many RNA structures, for example, bulges, internal loops, hairpin loops, pseudoknots, and other structures with unpaired nucleotides. Indeed, in many cases, proteins bind to such unpaired regions of RNA.[4–6] From the analysis of many naturally occurring examples, the roles and importance of local interactions, for example, salt bridges, charged or neutral hydrogen bonds, weakly polar and aromatic π–π interactions, precise apposition of hydrophobic surfaces, have become clear.[7–9] Based on this information gleaned from nature, many tiny model systems of protein–nucleic acid recognition have been constructed.[10–13] Specificity and rigidity would obviously be useful features of the models for applications and analyses.

Tight binding should contain many local interactions between a protein and the cognate target to stabilize the complex, however these local interactions should not participate in binding to noncognate pseudotargets for specific binding. This statement indicates the difficulty of designing specific and tight binding in the recognition or discrimination of targets such as nucleic acids with similar structural motifs and similar functional groups. By referring to nature, short-range interactions such as hydrogen bonding networks and π–π stacking (dispersion interactions) are particularly important for the acquisition of specificity among local interactions.[14–16] In particular, π–π stacking may be useful for designing a model system. In the case of the N–boxB complex, a tryptophan (Trp, W) lies over the top of the induced GNRA (N refers to any nucleotide and R refers to purine bases, G or A) as occurs in RNA folding and the indole ring of the Trp interacts with a base at the loop region.[17,18] Of possible amino acid substitutions, only aromatic amino acids, Tyr and Phe, permit efficient binding of the mutant N proteins to boxB RNA with high affinity in vitro.[19,20] This finding suggests that a single π–π contact plays a critical role in target recognition and is sufficient to distinguish the cognate target from noncognate ones.

The aim of the present study is construction of a DNA–protein interaction model system in which π–π stacking is involved. One of the candidate

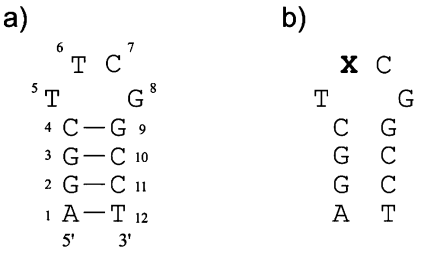

FIGURE 1 Secondary structure of DNA hairpin loops, CORE (a) and AP2 (b), used in this study. X in AP2 indicates an abasic nucleotide. Bases are numbered from the 5′ end.

DNA structures for the model system should contain a flipped base with an accessible aromatic surface.[21–23] Previously, we developed two stable hairpin loop model DNAs with (CORE) and without (AP2) a flipped out base as shown in Figure 1.[24] Thermodynamic parameters and circular dichroism spectra indicate that these two hairpin loop DNAs fold into almost the same ordered structure and clearly bear out the existence of a flipping base (sixth thymine, T6) in CORE. These hairpin loop DNAs should be one of the most useful tiny model DNAs for studying the function of base flipping. Moreover, these hairpin loop structures are highly stable and thus they should be suitable for a specific recognition system.[25,26] We have carried out a selection experiment with a combinatorial pentapeptide library to evaluate the difference between CORE and AP2 recognition by small peptides. As a result, many aromatic amino acids were observed in the selected CORE-binding peptides, indicating an interaction mode that involves π–π stacking, while the AP2-binding peptides bound to AP2 by hydrogen bonds. The binding analysis demonstrated that a representative CORE-binding peptide (HWHHE) distinguished the cognate and noncognate target DNAs.

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EXPERIMENTAL SECTION

Oligonucleotides

Two DNAs, dAGGCTTCGGCCT (CORE) and dAGGCTXCGGCCT (AP2; X denotes an abasic nucleotide), were synthesized on solid support with the phosphoramidite method on an Applied Biosystems model 391 DNA/RNA synthesizer.[27] For preparation of biotinylated CORE and AP2, biotin phosphoramidite (GLEN Research, Sterling, VA, USA) was coupled to the 5′ ends. The synthesized DNA oligomers were removed from the solid support and deblocked by treatment with concentrated 25% ammonia at 55°C for 3 hours. After drying in vacuo, the DNA oligonucleotides were deblocked at their 5′ ends and purified by poly-Pak DNA purification cartridges (GLEN Research). After deblocking operations, further purification

of the DNA oligonucleotides was carried out by HPLC with a TSKgel Oligo DNA RP (reverse phase) column (4.6 mm × 15 cm, TOSOH, Tokyo, Japan) by a linear gradient of 0–50% methanol/H_2O containing 0.1 M triethylammonium acetate buffer (pH 7.0). Single strand extinction coefficients at 260 nm were calculated from mononucleotide and dinucleotide data by using a nearest-neighbor approximation.[28] It was assumed that the extinction coefficient of a DNA containing an abasic nucleotide was the sum of the two regions separated by the abasic site. The calculated extinction coefficients ($\times 10^{-4}$ cm^{-1} M^{-1}) at 260 nm are 10.64 and 9.83 for CORE and AP2, respectively.

Peptides

Peptides were synthesized with the Fmoc strategy on a solid support as described previously.[29] A solid support, Fmoc–NH–SAL resin (N-α-9-fluorenylmethoxycarbonyl super acid labile polystyrene resin, Watanabe Chem., Hiroshima, Japan) producing an amido at the carboxyl terminus, was treated with piperidine to remove the Fmoc group for the coupling. Protected Fmoc-amino acids (Watanabe Chem.) were activated at their carboxyl group by three molar amounts of BOP [benzotriazole-1-yloxytris (dimethylamino) phosphonium hexafluorophosphate], three molar amounts of HOBt (1-hydroxybenzotriazole), and six molar amounts of DIPEA (N,N-diisopropylethylamine), and coupled to the elongating peptide on the resin. After the last amino acid was coupled, the resin was treated with acetic anhydride to cap the amino terminus. Cleavage of the synthesized peptides from the resin and removal of their protecting groups was performed simultaneously by TFA (trifluoroacetic acid) with *m*-cresol, 1,2-ethanedithiol, and thioanisole. The deblocked peptides were quantitated by the excitation coefficient of Trp in these peptides.[30]

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Combinatorial Chemistry

A random synthetic pentapeptide library was constructed by a split synthesis approach with the standard solid phase peptide synthesis method using Fmoc chemistry as described previously.[31,32] Poly(ethylene glycol)-grafted polystyrene (PEG–PS), which has a hydrophilic PEG linker with a substitution of 0.19 mmol/g and a diameter of approximately 90 μm, was chosen as the solid phase support. Nineteen natural L-amino acids except for cysteine were used for the library construction. Therefore, the library theoretically contained $19^5 = 2.5 \times 10^6$ pentapeptide sequences. The pentapeptide attached library beads (1 g, ca. 3.8×10^6 beads) and the target hairpin loop DNA (final concentration of 120 μM) were mixed in a 200 μL phosphate buffer containing 100 mM NaCl and 1 mM Na_2HPO_4 (pH 7.0), and shaken gently for 16 hours at room temperature. After removing the

DNA solution, beads were washed three times by 2 mL of the same buffer used for the DNA incubation containing 100 mM NaCl. DNAs on the beads were visualized by ethidium bromide (EtBr) staining. After washing, the DNA treated beads were incubated in a buffer containing 12.7 μM EtBr for 1 hour and then washed twice by the buffer (2 mL). EtBr bound to the DNA on the beads was excited by violet light (414 nm) to identify beads with peptides bound to the DNA. After the selection procedure, sequences of the pentapeptides on the selected beads were determined by the pulse liquid-phase method with a Procise 491 protein sequencer (Applied Biosystems, Foster City, CA, USA).

UV Measurements

Absorbance versus temperature curves were measured at 260 nm with an U–3210 spectrophotometer (Hitachi High-Tech, Tokyo, Japan) connected to an SPR–10 thermo-programmer (Hitachi High-Tech) using a 0.1 cm path length cuvette as described previously.[33] Measurements were carried out in a buffer containing 10 mM Na_2HPO_4 (pH 7.0) and 0.1 mM Na_2EDTA at a heating rate of 0.5 or 1°C/minutes. Water condensation on the cuvette exterior at the low temperature range was avoided by flushing with a constant stream of dry N_2 gas. Oligonucleotide concentrations (12 μM) were determined from the absorbance measured at 90°C.

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Fluorescence Measurements

Fluorescence spectra of peptide–nucleic acid complexes were measured by a fluorescence spectrophotometer F–3010 (Hitachi High-Tech) under a constant cell temperature (5°C) controlled by a thermostatic circulator. Each peptide concentration was determined from the UV absorption of the Trp residue at 278 nm. Water condensation on the cuvette exterior at the low temperature range was avoided by flushing with a constant stream of dry N_2 gas. All measurements were done in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl and 0.1 mM Na_2EDTA with an excitation wavelength of 280 nm. Fluorescence spectra were obtained every 1 nm at a scan rate of 50 nm min^{-1}. Fluorescence quenching of peptides with addition of oligonucleotides was measured with a fluorescence intensity change at 360 nm. The concentration of both the hairpin DNAs and the peptide was 20 μM in the measurements.

Surface Plasmon Resonance (SPR) Measurements

Peptide–DNA interactions were also examined using an SPR system with a BIAcore 1000 (GE Healthcare, Amersham, UK) by measuring a function of the mass change on the matrix surface. For DNA immobilization on

the SPR sensorchip, a 5′-biotinylated DNA solution was injected onto a streptavidin attached carboxymethylated dextran matrix coating on the gold sensor surface. Binding of the peptides to the immobilized DNA hairpin loop was monitored by passing the peptide solution in 10 mM phosphate buffer containing 100 mM NaCl and 0.1 mM Na_2EDTA (pH 7.0) across the sensor chip at a constant flow rate of 5.0 μL min^{-1} at 25°C. SPR signals were collected every 1.0 second for 300 seconds. Bound peptides to the immobilized DNA tetraloop were removed by a flash flow of 0.05% sodium dodecyl sulfate (SDS) solution to regenerate the surface.

The association and dissociation rate constants (k_a and k_d) for the DNA–peptide interaction were determined from the association phase of the sensorgram with the following equations:

$$-\mathrm{d}RU/\mathrm{d}t = k_{obs} \cdot RU + k_a \cdot RU_{max} \quad (1)$$

$$k_{obs} = k_a \cdot [\text{Peptide}] + k_d \quad (2)$$

where [Peptide] is the molecular concentration of injected peptide sample and RU (RU_{max}) is the response unit (and its maximum value). The observed rate constant, k_{obs}, was obtained as the slope of the $-\mathrm{d}RU/\mathrm{d}t$ versus RU plot according to Equation (1). The rate constants, k_a and k_d, were obtained as the slope and the intercept of the k_{obs} vs [Peptide] plots with Equation (2). Binding constants K and free energy changes during the binding ΔG°_{25} were calculated from the resulting rate constants, k_a and k_d:

$$K = k_a / k_d \quad (3)$$

$$\Delta G^\circ_{25} = -298R \cdot \ln K \quad (4)$$

where R is the gas constant. The transition free energy change $\Delta G^\ddagger_{25}$ was also calculated from the rate constants:

$$\Delta G^\ddagger_{25} = -298R \cdot \ln(kh/298k_B) \quad (5)$$

where k represents k_a in the association step and k_d in the dissociation step, respectively, h is Planck's constant, and k_B is the Boltzmann constant.

RESULTS AND DISCUSSION

The construction of tiny and novel model systems to study protein–DNA interactions was carried out by using ordered structured small DNAs and a random pentapeptide library. As reported in the previous study, CORE and AP2 fold into an almost identical hairpin loop structure with a B-form stem and a tetraloop having nucleobases in the same ordered orientation

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except for the second nucleotide.[24] UV melting curves and CD spectra of CORE and AP2 overlap each other. Thermodynamic parameters for the two hairpin loop formations measured in a buffer without NaCl (10 mM Na_2HPO_4, 0.1 mM Na_2EDTA, pH 7.0) also exhibit great similarities. Enthalpic, entropic, and free energy changes at 37°C (ΔH°, ΔS°, and ΔG°_{37}) for CORE and AP2 folding are −52.4 and −53.3 kcal mol^{-1}, −155 and −157 cal mol^{-1} K^{-1}, and −4.3 and −4.6 kcal mol^{-1}, respectively. In particular, it should be noted that the ΔS° for the structural change of CORE from a random coil to a hairpin loop (−155 cal mol^{-1} K^{-1}) is almost the same as that of AP2 (−157 cal mol^{-1} K^{-1}). This clearly indicates that the environment surrounding the T6 base, for example, the solvation of water to the base, does not change during the loop formation. Thus, comparison of the selected peptides should reflect the recognition elements of the second T in the loop of CORE exposed to solvent water.

Screening of Peptides that Bind to the Tetraloops from the Pentapeptide Library

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Tetraloop binding peptides were screened from the combinatorial synthetic pentapeptide library with CORE or AP2 as a target. In this study, about 2.5×10^6 pentapeptide sequences were used for the experiments to find hairpin loop DNA binding peptides. The weight of a bead was estimated to be 2.6×10^{-7} g from catalog specifications and thus the library of 1 g should contain about 3.8×10^6 beads. During the screening of tetraloop binding peptides with CORE or AP2, several hundreds of beads exhibited emission of EtBr and six beads with significant fluorescence were found for both CORE and AP2 (supplemental Figure S1). Table 1 lists the peptide sequences on the selected beads for CORE and AP2. As listed in the tables, histidine (His), threonine (Thr), glutamic acid (Glu), glutamine (Gln), and aspartic acid (Asp) were found to be abundant amino acids in the selected peptides for CORE and AP2, respectively. All the frequently occurring amino acids are able to make hydrogen bonds and thus hydrogen bonding is suspected to be fundamentally indispensable for the hairpin loop–peptide interactions. In the six CORE-binding pentapeptides, ten His, five Thr, and five Glu residues were observed. The frequencies of amino acids in the selected peptides are summarized in Figure 2. Arg and Lys were not observed in the selected peptides, although many RNA and DNA binding peptides have some Arg and Lys to recognize nucleic acids via electrostatic interactions.[34,35] Indeed, peptide selection experiments with an mRNA display system gave highly basic peptides as tight binders to nucleic acids.[36,37] Basic amino acids should lead to faster association and a larger binding constant while these largely do not affect the dissociation phase.[38] Our experimental conditions were tailored to select for specific binders with slow dissociation kinetics and to prevent condensation of non-

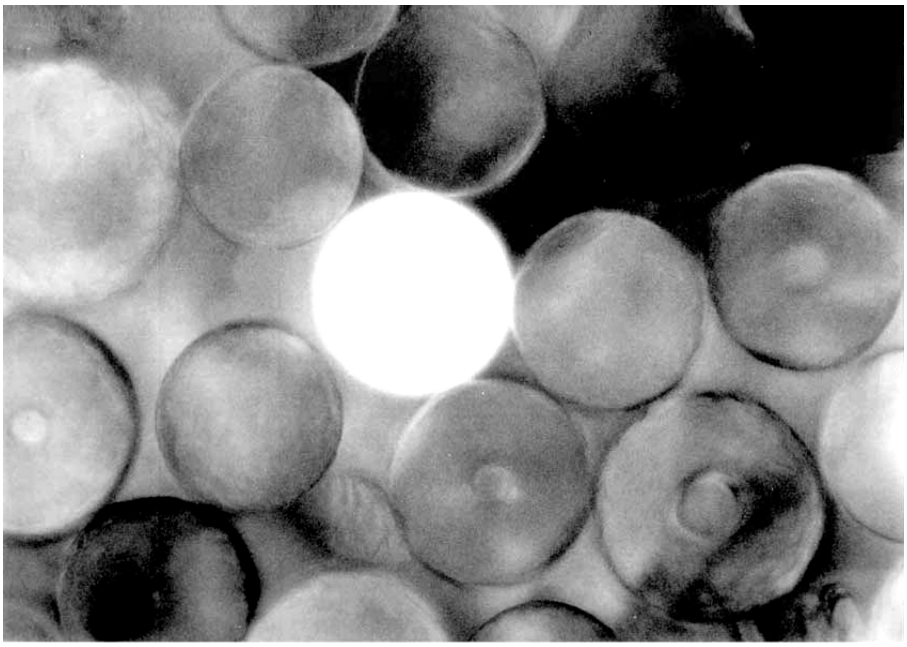

FIGURE S1 A bead with significant positive signal after the selection procedure. The peptide on this bead is one of the six representative CORE-binding peptides sequenced in this study.

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specific binders by washing the beads three times with salt containing buffer. Therefore, binding of our selected peptides to CORE and AP2 should not be due mainly to electrostatic interactions. In addition, hydrophobic amino acids such as valine (Val), isoleucine (Ile), leucine (Leu), and alanine

TABLE 1 Amino acids found in the pentapeptide library screened against hairpin DNAs[a]

		Position from N terminus				
Target DNA	Sequence #	1	2	3	4	5
CORE	1	**His**	Trp	**His**	**His**	Glu
	2	**His**	Ser	**His**	**His**	Glu
	3	Thr	Tyr	Thr	**His**	Glu
	4	Glu	Thr	Thr	**His**	Glu
	5	Gly	Ser	Thr	Ser	**His**
	6	Met	Tyr	**His**	Gly	Gln
AP2	1	**Gln**	Pro	Pro	Asn	**Asp**
	2	**Gln**	**Asp**	Pro	Gly	**Asp**
	3	**Gln**	Gly	**Asp**	Ser	**Asp**
	4	**Gln**	Gly	**Gln**	**Asp**	Ser
	5	**Gln**	Leu	Glu	**Asp**	Ser
	6	**Gln**	His	Gly	Tyr	Glu

[a]Amino acids are represented in three-letter abbreviation. Bold face letters indicate abundant amino acids. Amino acids with aromatic side chains were underlined.

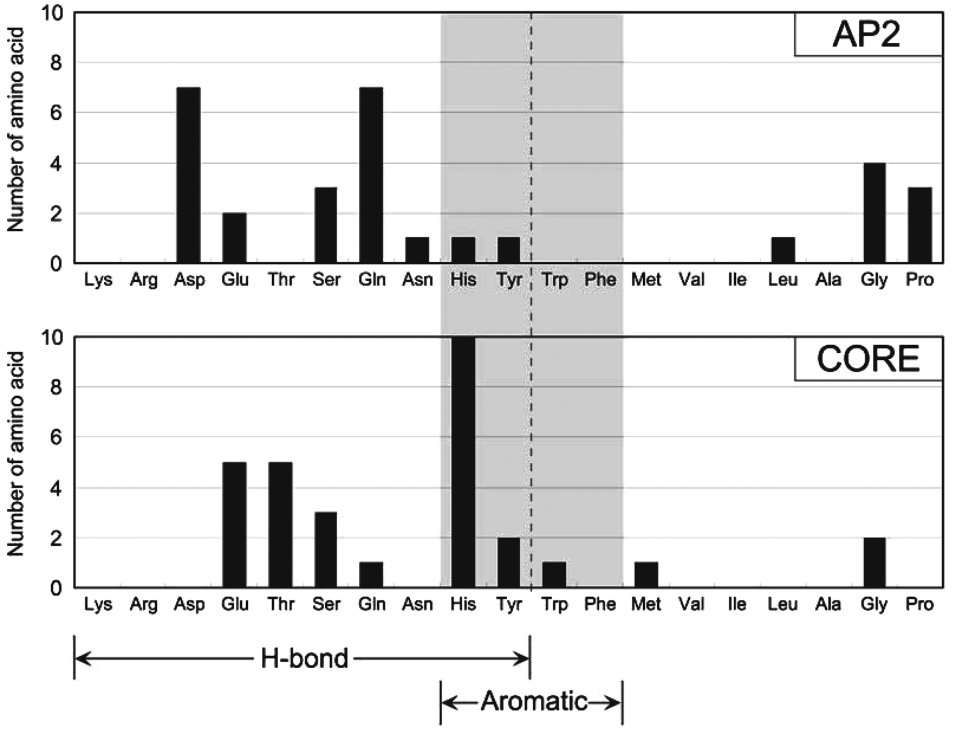

FIGURE 2 Composition of amino acids in the selected peptide libraries. Number of amino acids found in the peptides screened with CORE and AP2 are shown as black bars. Amino acids in the "H-bond" group on the left side of the dashed line have side residues that possibly make hydrogen bonds. "Aromatic" indicates amino acids that have aromatic side chains in the gray-colored area. Amino acids are represented by their three-letter abbreviations.

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(Ala) were not observed. This phenomenon indicates that hydrophobic interactions are not important intrinsically for the small hairpin loop DNA recognition mechanism. On the other hand, several glycine (Gly) residues were observed in both CORE and AP2-binding peptides. Due to its lack of sidechain and flexibility, Gly can assist in fitting the conformation of a peptide to its target and also has many specific recognition points (hydrogen bonding network).

AP2-Binding Peptides

From the results of the amino acid sequencing as listed in Table 1, almost all the AP2-binding peptides contained Gln and Asp residues. Both amino acids have polar side chains and are able to form hydrogen bonds to functional groups with proton donors (Gln and Asp) or acceptors (Gln). Some tendencies can be seen in terms of positioning of the amino acids, that is, Gln and Asp are located at the N- and C-terminal regions, respectively. Gln in particular was seen as the first (N-terminus) amino acid in all the

AP2-binding peptides. This tendency clearly indicates the existence of a certain binding mode between AP2 and the selected peptides. Gln has an amide residue with both C=O and N–H at the end of the side chain and "bidentate" hydrogen bonding at these functional groups with nucleic acids is abundantly seen in nature.[39,40] For example, the interaction between Gln and an unpaired uracil base serves a critical role in determining the specificity of protein–nucleic acid interactions in the aminoacyl-tRNA synthetase–tRNA recognition system.[41,42] Also, in an artificial system, Frankel et al. selected highly conserved peptide sequences with Asp at the N-terminal and Gln at the C-terminal end as a hairpin loop RNA recognition motif.[37] Although the position of Asp and Gln is the opposite in their case, it is suggested that Asp and Gln at the ends of peptides may play an important role for binding to hairpin loop nucleic acids. Although the details about the interactions between AP2 and AP2-binding peptides are still unclear, the first Gln can interact with a base pair in the stem or with T5 at the boundary of the stem-loop,[39,43] and this interaction may have a significant role as a hook. On the other hand, Asp is sometimes reported as a residue which forms water-mediated hydrogen bonds to unpaired regions of nucleic acids, leading to specificity.[43] The C-terminus Asp may also bind to another region of AP2 via a water molecule, most likely the loop.

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CORE-Binding Peptides

His, Thr, and Glu are found to be the most abundant amino acids in the CORE-binding peptides. All three amino acids also have polar side chains that can form hydrogen bonds as seen in the AP2-binding peptides. Nevertheless, a new feature can be seen: His has an aromatic side residue. As shown in Figure 2, the content of some other aromatic amino acids also increased in the CORE-binding peptides relative to the AP2-binding peptides. This suggests that the aromatic ring might participate only in the case of the CORE–peptide interaction. In contrast, the number of Gln and Asp residues, the most abundant amino acids in the AP2-binding peptides, drastically decreased in CORE-binding peptide sequences. These results strongly suggest that CORE-binding peptides interact with CORE by a mechanism different from the interaction between AP2 and AP2-binding peptides. As mentioned above, CORE and AP2 have the same structure and stability with only one difference: a flipped out T6 base of CORE which exposes its nonpolar surface area to the solvent.[24] In other words, the flipped base of T6 creates a novel interaction in the hairpin loop–peptide interaction. Therefore, π–π contacts are the most appropriate explanation for the interaction between CORE and CORE-binding peptides.

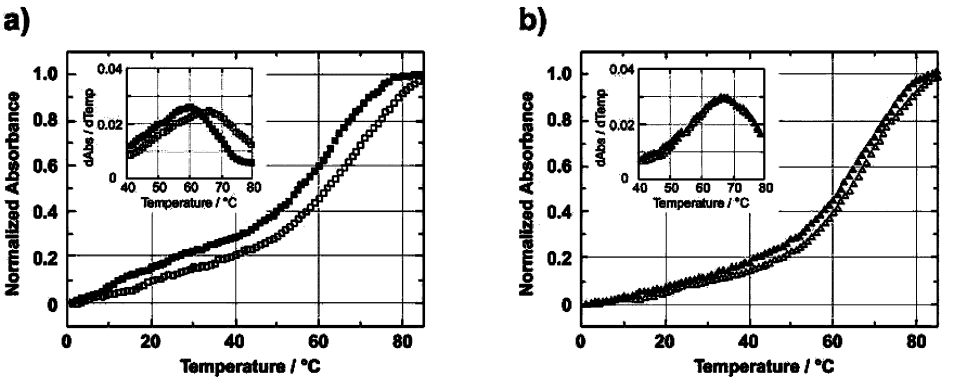

FIGURE 3 UV melting curves for the hairpin DNAs, CORE (a) and AP2 (b), at 260 nm in 10 mM phosphate buffer (pH 7.0). Open symbols show the melting curves of 12 μM of CORE (squares) and AP2 (triangles). The melting curves of the hairpin DNAs in the presence of 12 μM HWHHE peptide are shown by the closed symbols. Inset: Derivative plot of absorbance with respect to temperature.

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Importance of π–π Stacking in the CORE/CORE-Binding Peptide Interaction

π–π Stacking plays an important role in many RNA–protein and DNA–protein interaction systems.[18,25,35,44] To investigate the importance of π–π stacking for the interaction between CORE and CORE-binding peptides, UV melting analysis and fluorescence quenching experiments[45,46] were carried out with a representative CORE-binding peptide. Pentapeptide His–Trp–His–His–Glu (HWHHE) was used for the experiments because the bead with this peptide showed maximum emission of ethidium bromide among all the beads in the selection procedure. As shown in Figures 3a and 3b, both CORE and AP2 (open squares and open triangles, respectively) melted at around 65°C with almost the same melting curves as reported previously.[24] However, in the presence of HWHHE, the melting temperature (T_m) of CORE decreased more than 5°C (closed squares in Figures 3a) probably due to interactions at the stem or well-stacked loop that accompanied HWHHE binding,[47] while T_m of AP2 was not affected by the peptide (closed triangles in Figures 3b). Although the details of the decrease in T_m of CORE are unclear, this phenomenon is clearly sequence dependent, indicating a specific interaction of the peptide to CORE. The sole difference between CORE and AP2 is the exposed T6 and thus the peptide should interact with the flipped T6 base preferentially by π–π stacking. To confirm the participation of π–π stacking, fluorescence quenching experiments were carried out. As shown in Figure 4, emission of Trp was quenched 16.1% with CORE while quenching of only 5.4% was observed with AP2. The quenching of 5.4% was similar to the quenching value (4.0%) of a random RNA library at the same concentration and therefore it may be considered

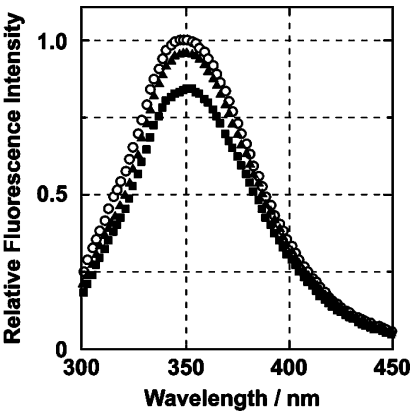

FIGURE 4 Fluorescence quenching of Trp in HWHHE by the hairpin DNAs. The spectrum drawn by open circles is that of 20 μM HWHHE in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl at 5°C. Closed squares and closed triangles show the spectra of the peptide in the presence of 20 μM CORE and AP2, respectively.

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as background (data not shown). This result also suggests that a π–π contact between aromatic ring(s) in the peptide and CORE, probably the base of T6, was involved in the interaction between CORE and CORE-binding peptides. It is clear that π–π contacts make a significant contribution to the HWHHE–CORE interaction, however, the selected peptide also contains various polar amino acids. Therefore, additional hydrogen bonding by His, Glu, Thr, and Ser may exist and contribute to the specificity.

Kinetic and Thermodynamic Parameters of the Peptide–Tetraloop Interaction

To investigate the binding constant of the peptide–DNA hairpin loop complex, SPR measurements were carried out in 1 mM phosphate buffer (pH 7.0) containing 100 mM NaCl at 25°C. The SPR profile was obtained by injection of several concentrations of HWHHE to the immobilized hairpin loop DNA. All the kinetic traces measured were saturated with various maximum response unit (RU) values on the experimental time scale. All the kinetic traces followed ideal exponential curves as mentioned in the experimental section, indicating that this reaction was a simple binding reaction with only two states of 1:1 interaction (data not shown). The observed rate constants k_{obs} were calculated for each peptide concentration. The rate constants k_a, k_d, and the binding constant K were determined from the plots of k_{obs} versus the concentrations of HWHHE as shown in Figure 5. As a result, k_a and k_d for the HWHHE–CORE interaction at 25°C were determined to be 5.5×10^4 M^{-1} s^{-1} and 5.1×10^{-2} s^{-1}, respectively. Thus the binding constant was calculated as 1.1×10^6 M^{-1} and the resulting binding free energy change at 25°C (ΔG°_{25}) was −8.2 kcal mol^{-1}. For the

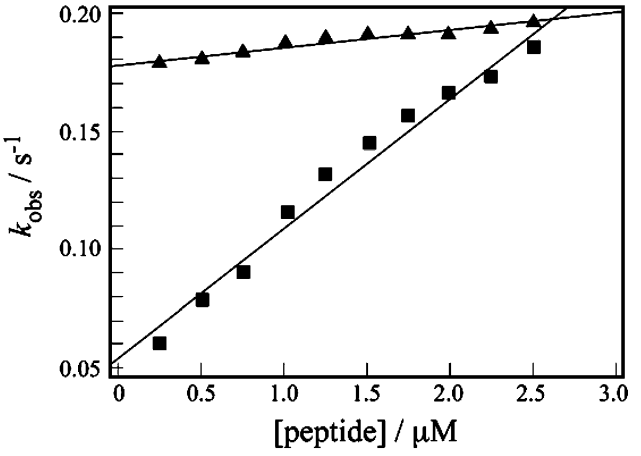

FIGURE 5 Determination of rate and binding constants of HWHHE interaction to hairpin DNAs. The observed rate constants for binding to CORE (closed squares) and AP2 (closed triangles) were plotted against the concentration of HWHHE, indicating k_a as the slope and k_d as the intercept. All experiments were carried out in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl at 25°C.

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HWHHE–AP2 interaction, k_a, k_d, binding constants, and ΔG°_{25} were $7.6 \times 10^3\ M^{-1}\ s^{-1}$, $1.8 \times 10^{-1}\ s^{-1}$, $4.2 \times 10^4\ M^{-1}$, and -6.3 kcal mol^{-1}, respectively. These parameters are summarized in Tables 2 and 3.

The k_a with $10^4\ M^{-1}\ s^{-1}$ order for our HWHHE–CORE system is slightly larger than that of the order of $10^3\ M^{-1}\ s^{-1}$ for the actinomycin D–hairpin loop DNA interaction at 20°C, but this k_a is sufficient for such a small peptide–DNA interaction.[48] Although electrostatic interactions between basic amino acids and the phosphate backbone should enhance the k_a and binding constant,[38,49,50] the lack of basic amino acids in our selected peptides clearly indicates that hydrogen bonding or π–π stacking is more favorable than electrostatic interactions in our system, as described previously. This feature would be suitable for specific recognition because basic peptides can bind not only to the cognate target but also to noncognate nucleic acids, though the basicity is very important for tight binding. Basic amino acids were not selected preferentially in our system, in order to remove nonspecific fast binding. In our small systems, the charges of peptides and DNAs would be easily accessible to the 100 mM NaCl water

TABLE 2 Rate and binding constants for HWHHE binding to hairpin DNAs[a]

hairpin DNA	$10^{-3} \times k_a/M^{-1}\ s^{-1}$	$10^2 \times k_d/s^{-1}$	$10^{-4} \times K_a/M^{-1}$
CORE	54.7 ± 1.3	5.11 ± 0.25	107 ± 6
AP2	7.55 ± 0.48	17.8 ± 1.1	4.24 ± 0.38

[a]All experiments were carried out at 25°C in a phosphate buffer containing 100 mM NaCl, pH 7.0.

TABLE 3 Thermodynamic parameters in kcal mol^{-1} for HWHHE binding to hairpin DNAs[a]

hairpin DNA	ΔG°_{25}	$\Delta\Delta G^{\circ}_{25}$[b]	$\Delta G^{\ddagger}_{25,a}$[c]	$\Delta\Delta G^{\ddagger}_{25,a}$[bc]	$-\Delta G^{\ddagger}_{25,d}$[c]	$-\Delta\Delta G^{\ddagger}_{25,d}$[bc]
CORE	-8.22 ± 0.33	-1.89	11.0 ± 0.1	-1.2	-19.2 ± 0.3	-0.7
AP2	-6.31 ± 0.53		12.2 ± 0.4		-18.5 ± 0.3	

[a]All parameters were calculated from the rate constants listed in Table 2. Parameters were shown as smaller value is more favorable for the binding.

[b]$\Delta\Delta G$ values were determined as follows, $\Delta\Delta G = \Delta G_{\mathrm{CORE}} - \Delta G_{\mathrm{AP2}}$.

[c]The subscript a and d indicate the association (forward) phase and the dissociation (reverse) phase, respectively.

solution which has high permittivity, thus the charges would be neutralized. This explanation suggests the superiority of π–π stacking in small systems.

Energetic Consideration of the Role of π–π Stacking

The hairpin loop structure of CORE which has an aromatic ring exposed to polar solvent is potentially not stable, even if a large stabilization effect was observed in the free energy change during the hairpin folding process. Therefore, π–π stacking in the water accessible environment may significantly contribute to stabilization of the complex. Several proteins bind to nucleic acids by using π–π contacts between aromatic side residues near the protein surface and nucleobases. However, the energetic contribution of π–π stacking is thought to be rather weak (-1 to -2 kcal mol^{-1}) in sequence specific protein–nucleic acid interactions.[51,52] On the other hand, in some reports, more drastic destabilization was observed by elimination of only one aromatic ring in a protein.[8,44] This discrepancy could be caused by the complexity of the aromatic ring function, that is, stacking interactions in cooperation with accompanying hydrogen bonding can be major contributors to the stability of a protein–nucleic acid complex.[8] This may explain why His and Tyr were selected preferentially for the π–π contact to the T6 base in this study rather than Trp which has the largest aromatic ring system.[53]

By comparison to the HWHHE–AP2 interaction, differences in the free energy changes during the binding are about 1.9 kcal mol^{-1} and therefore, stacking of Trp to T6 will cause a nearly -2 kcal mol^{-1} stabilization (30 times larger binding constant) in the case of the HWHHE–CORE interaction. Our results show other important information about the mechanism of peptide–nucleic acid interactions. Activation free energy can be calculated from the rate constants using Equation (5) in the experimental section. As listed in Table 3, $\Delta G^{\ddagger}_{25}$ for the association phase of HWHHE–CORE and HWHHE–AP2 was 11.0 and 12.2 kcal mol^{-1}, respectively, with a difference of 1.2 kcal mol^{-1}. Similarly, $-\Delta G^{\ddagger}_{25}$ for the dissociation phase of HWHHE–CORE and HWHHE–AP2 were calculated to be 19.2

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and 18.5 kcal mol^{-1}, respectively, with a difference of 0.7 kcal mol^{-1}. To summarize, the destabilization effect of eliminating the flipped base (1.9 kcal mol^{-1}) depends on both the association step (1.2 kcal mol^{-1}) and the dissociation step (0.7 kcal mol^{-1}), indicating the difference caused by the effect of a charge: a mutation of a charged residue will mainly affect the association phase.[39] This finding, that a stacking of aromatic functional groups can achieve faster association and slower dissociation simultaneously, is indispensable for future work in designing new functional molecules.

The drastic conversion of the binding mechanism by addition of a nucleobase provides us with another very useful information. The existence of only one small aromatic ring produces a new dominant interaction between peptides and nucleic acids, although hydrogen bonding is still the preferred interaction in the absence of an aromatic ring. In both cases, hydrogen bonding by polar side residues of amino acids is quite important in stabilizing our small model peptide–DNA interactions. The precise features of CORE-binding peptides represent a general motif in artificial peptide–DNA interaction model systems using an aromatic ring as an antenna for recognition.

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