Single-Stranded DNA Recognition of a 24-mer Peptide Derived from RecA Protein

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A 24-mer peptide with L2 and helix G amino acids of *E. coli* RecA protein (Ac–Ile–Arg–Met–Lys–Ile–Gly–Val–Met–Phe–Gly–Asn–Pro–Glu–Thr–Thr–Thr–Gly–Gly–Asn–Ala–Leu–Lys–Phe–Tyr–NH_2) showed a single-stranded DNA (ssDNA) binding property with a more than 1000 times affinity difference than for a double-stranded DNA. However, a truncated 15-mer peptide (sequence) showed no ssDNA binding activity. In the ssDNA binding, the 24-mer peptide changed its conformation with the perturbation of an α-helix structure. The ssDNA binding and the DNA discrimination property of this peptide were due to almost all L2 and helix G amino acids, respectively. This result useful to design synthetic peptides as functional materials for DNA recognition.

RecA protein is one of the ssDNA (single-stranded DNA) binding proteins catalyzing a homologous DNA recombination and an SOS responsible DNA repair in prokaryotes.[1—3] The X-ray structure of *E. coli* RecA protein in the absence of DNA was reported by Steitz and co-workers; it shows two disordered regions of L1 (157-164) and L2 (195-209) projecting toward where the DNA strands must lie, so they are supposed to be a dsDNA (double-stranded DNA) and an ssDNA binding site, respectively.[4,5] Model peptides composed of L2 amino acids, wtw (196-210) and FECO peptide (193-212), were reported to bind to both ssDNA and dsDNA.[6,7] Helix G (210-218), lying next to L2, is also composed of evolutionarily conserved amino acids, especially two absolutely invariant Gly residues (211 and 212) not only for bacterial RecA proteins but also for T4 UvsX, yeast Dmc 1, M. jannaschii Rad A, and mouse Rad 51 proteins.[8,9] Amino acids in helix G are also supposed to be related to the DNA recombination activity of the RecA protein and with the assembly of RecA filaments on the DNA strand.[10,11] We have reported that a 24-mer peptide (195-218 of L2 and helix G amino acids) of *E. coli* RecA protein (Ac–Ile–Arg–Met–Lys–Ile–Gly–Val–Met–Phe–Gly–Asn–Pro–Glu–Thr–Thr–Thr–Gly–Gly–Asn–Ala–Leu–Lys–Phe–Tyr–NH_2) (**1**) showed an ssDNA binding property independent of DNA sequence, but no dsDNA binding one.[12] Here, we have investigated in detail the ssDNA binding properties of **1**, and compared them with newly synthesized 9-mer (Ac–Thr–Gly–Gly–Asn–Ala–Leu–Lys–Phe–Tyr–NH_2) (**2**), and 15-mer (Ac–Gly–Asn–Pro–Glu–Thr–Thr–Thr–Gly–Gly–Asn–Ala–Leu–Lys–Phe–Tyr–NH_2) (**3**) peptides by CD (circular dichroism), fluorescence, and SPR (surface plasmon resonance) analyses. The DNA binding properties of **1**, wtw, and FECO peptides as shown in Fig. 1 are compared and the unique ssDNA binding properties of **1** are reported.

Experimental

Materials. Peptides (**1**—**3**) were synthesized with a Fmoc strategy on a solid support synthesis as described elsewhere.[12,13] A solid support of Fmoc–NH–SAL resin (*N*-α-9-fluorenylmethoxycarbonyl-super acid labile polystylene resin), producing an amino terminus at the carboxy end, was used in this work, and it was treated with piperidine to remove the Fmoc group for the coupling. Protected Fmoc–amino acids purchased from 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 coupling, the resin was treated with acetic anhydride to cap the amino terminus. The synthesized peptides were cleaved from the resin and also their protecting groups were removed by TFA (trifluoroacetic acid) with *m*-cresol, 1,2-ethanedithiol, thioanisole, and TMSBr (trimethylsilyl bromide). The deblocked peptides were purified by the reversed phase HPLC. The amounts of peptides were measured by the excitation coefficient of Tyr in these peptides.[14]

The oligonucleotides used here were synthesized chemically on a solid support using phosphoramidite procedures with a 391 DNA synthesizer (Applied Biosystems) and purified with HPLC after the deblocking operations.[15,16] These oligomers were further purified and desalted with a C-18 Sep–Pak cartridge column. Final purity of the oligomers was confirmed to be greater than 98% by HPLC. Single-strand concentrations of the oligonucleotides were measured by the absorbance at 260 nm at a high temperature, and single strand extinction coefficients were calculated with mononucleotide and dinucleotide data using a nearest-neighbor approximation.[17] Poly(dT) with average 221 nucleotide length and poly(dA–dT) were purchased from Pharmacia Biotech, and their extinction coefficient was ε_{260} = 8100 and ε_{262} = 6600 mol of nucleotide $dm^{-3}\,cm^{-1}$, respectively.[18]

CD Measurements. CD spectra were obtained on a JASCO J-600 spectropolarimeter with a temperature controller and inter-

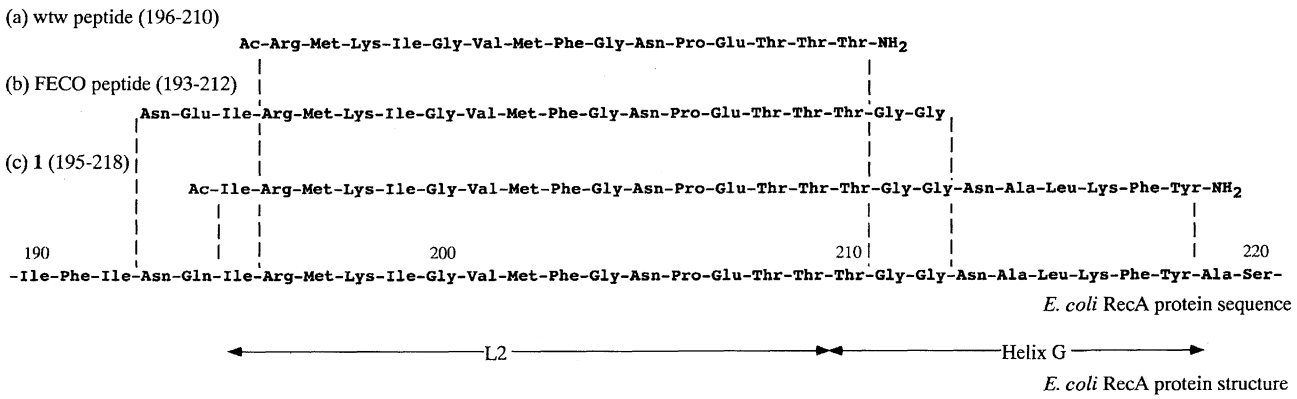

Fig. 1. Sequence alignment of wtw, FECO, and **1** peptide compared with that of *E. coli*. RecA protein. Structure of the RecA protein is obtained from the reported X-ray crystal structure.

faced to a NEC PC-9801 computer. Measurements were done in the range of 200—320 nm in a 1 mm quartz cell, and the experimental temperature was controlled by a thermostatic circulator. The cuvette-holding chamber was flushed with a constant stream of dry N_2 gas. The calculated sum of the signals from separate components was regarded as the signal measured at 0 min after sample mixing. The buffer contained 100 mM NaCl, 10 mM Na_2HPO_4, and 1 mM Na_2EDTA (pH 7.0).

SPR Measurements. The BIA core instrument (Pharmacia Biosensor) was used for SPR experiments of macromolecular interaction by measuring a function of the mass change on the matrix surface.[19] In the immobilization of ssDNA on the SPR sensorchip, biotinated d(CTCCTCCTCTCC) was tightly bound to streptavidine covalently attached to the carboxymethylated dextran matrix coating on the gold sensor surface. Binding of the peptides to the immobilized ssDNA was monitored in passing portions of 25 μL (1 L = 10^{-3} m^3) in 100 mM NaCl–phosphate buffer across the sensor chip at a constant flow of 5 μL min^{-1} at 5 °C. After 90 s from the start of the measurements, the sample injection was started and continued for 300 s. The running buffer includes 100 mM NaCl, 10 mM Na_2HPO_4, and 1 mM Na_2EDTA (pH 7.0), which constantly flowed at 5 μL min^{-1} before and after the sample injection. Peptide bound to the immobilized ssDNA was taken off by a flash 0.05% SDS (sodium dodecyl sulfate) flow to regenerate the surface.

Fluorescence Measurements. Fluorescence spectra of Tyr were measured on a fluorescence spectrophotometer F-3010 (Hitachi) with controlling a cell temperature at 20 °C by a thermostatic circulator. Excitation wavelength of Tyr was 225 nm. Measurements were done in 100 mM NaCl, 10 mM Na_2HPO_4, and 1 mM Na_2EDTA (pH 7.0) buffer. Association constant (K_a) of **1** with polynucleotides was estimated by the curve fitting procedure with the following equation:[20]

$$\Delta F = \Delta F_{max}\{1 + K_a\cdot[E]_0 + K_a\cdot[S]_0 - [\Delta F^2_{max}(1 + K_a\cdot[E]_0 + K_a\cdot[S]_0)^2 - 4\cdot K_a^2\cdot[E]_0\cdot[S]_0\cdot\Delta F^2_{max}]^{1/2}\}/2\cdot K_a\cdot[E]_0,$$

where ΔF is an observed fluorescence change and ΔF_{max} is a maximum fluorescence change. $[E]_0$ and $[S]_0$ are total peptide and added nucleotide concentrations, respectively. Supposing the peptide was binding to poly(dT) in a non-cooperative manner, the number of the nucleotides occupied by the peptide (n) was estimated by the correction of the nucleotide concentration term. The theoretical curve was defined from the non-linear least-squares fitting procedure.

Results

Secondary Structure of the 24-mer Peptide. The structure of **1** in 100 mM NaCl–phosphate buffer without DNA were analyzed by CD spectra. Figure 2a shows that a negative peak at 219 nm (−4.56 mdeg) was observed for **1** at 5 °C. Since a negative CD peak around there is attributed to a β-structural conformation,[7,19] **1** formed predominantly a β-structure in the buffer. But the negative intensity is small for the β-structural signal, suggesting all amino acids of **1** may not contribute to the β-structure formation. Since the signal of the negative peak was less affected by changing the peptide concentration (from 20 to 120 μM), aggregation of the peptide is unlikely to have occurred (data not shown). Furthermore, the β-structure was retained even at 62 °C, though the intensity of the peak slightly decreased to −2.36 mdeg (data not shown). This means that the β-structure of **1** is quite stable.

Truncated peptides of **2** and **3** were also investigated to discover their secondary structure. Peptide **2** contains only helix G amino acids (9-mer), and peptide **3** has half of the L2 amino acids and helix G amino acids (15-mer). Figure 2a shows CD spectra of these truncated peptides compared with that of **1**. Both spectra of **2** and **3** showed a large negative signal at wavelengths shorter than 210 nm, which was dissimilar to that of **1**. The spectrum of **2** agreed with a completely disordered structure as observed for **1** in 6 M GdmHCl (guanidinium hydrochloride) solution, which is a denaturant reagent for proteins, however, the spectrum of **3** showed some ordered structure formation was possible for **3** compared with **2**. Therefore, the differential spectra were analyzed to discover the origins of these structural differences. Two minimum peaks at 206 and 219 nm analogous to that of a canonical α-helical conformation (those at 208 and 222 nm)[21] were observed for the differential spectrum between **2** and **3**, while a negative peak at 220 nm analogous to the β-structural signal was seen for the difference of **1** from **3** (Figs. 2b and 2c). These peaks were slightly shifted compared to widely known CD spectra for canonical α- and β-structural peptides,[22] which would be due to changes of the energy gaps of $\pi\rightarrow\pi^*$ and $n\rightarrow\pi^*$ transitions by the presence of other structure in **1**. Therefore, it is concluded that the 24-mer peptide includes both β- and α-structural components.

Binding Property of the Truncated Peptide. The binding properties of **1** and **3** were directly measured by the SPR

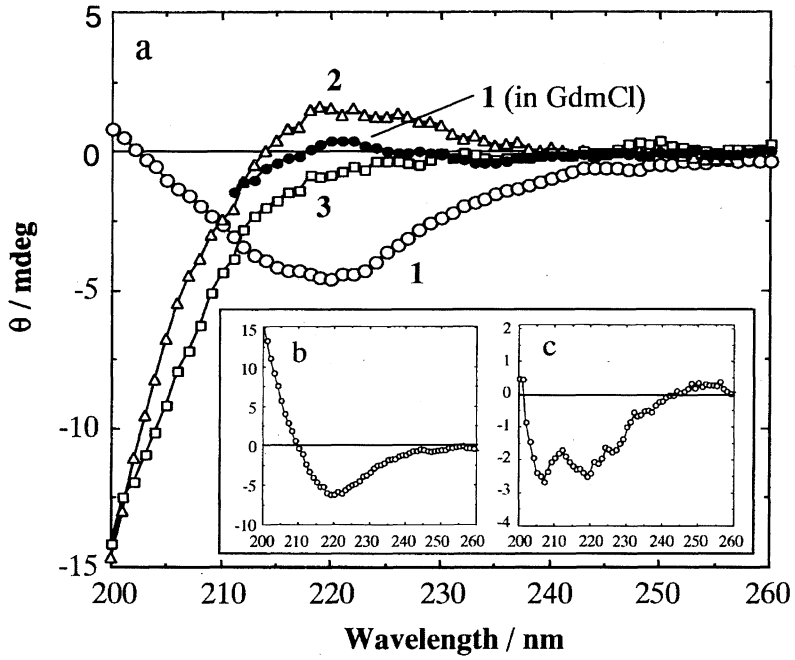

Fig. 2. (a) CD spectra of peptides **1**, **2**, and **3**. (b) A differential spectrum between **1** and **3**. (c) A differential spectrum between **2** and **3**.

apparatus. Figure 3 shows SPR signals for 200 μM of peptides **1** and **3** flowed over the ssDNA, d(CTCCTCCTCTCC) immobilized on the sensorchip at 20 °C. When the substrate binds to the ligand immobilized on the sensorchip, the observed response is increased by the changes of the refractive index as a resonance unit (RU) in real time.[9] When **1** and **3** were flowed over the ssDNA immobilized sensorchip, larger responses (850 RU) were observed for **1**, but only 150 RU was registered for **3** when 200 μM peptides were flowed for 300 s. The observation indicates that the 24-mer peptide binds effectively to the ssDNA, but the truncated peptide **3** binds less strongly to the ssDNA. When the experiment changing the peptide **3** binds less strongly to the ssDNA. When the experiment changing the peptide concentration was examined, the ssDNA binding property was observed for **1** but not for the truncated peptide **3**. The SPR kinetics traces (data not shown) suggested that the binding between **1** and the ssDNA was unlikely to be a simple process.

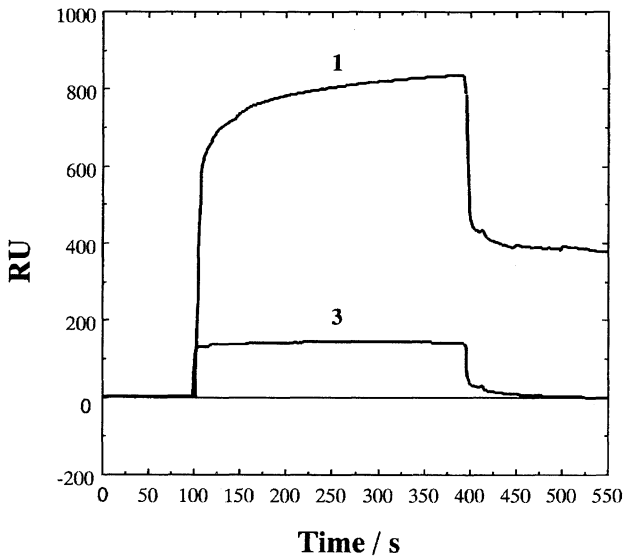

Fig. 3. SPR signals for 200 μM of peptides **1** and **3** flowed over the ssDNA of d(CTCCTCCTCTCC) immobilized on the sensorchip at 20 °C. After 90 s from the start of the measurements, the sample injection was conducted and continued for 300 s.

Conformational Changes of the 24-mer Peptide in the Binding. We have reported briefly about the binding of **1** with octanucleotides of ssDNA, ssRNA, unnatural nucleotides (2′-5′ linked U_8 and ethoxy-dT_8), and dsDNA. **1** was able to bind with an ssDNA efficiently, with an ssRNA slightly, but less with unnatural nucleotides or a dsDNA.[12,13] CD spectra in the binding between **1** and an ssDNA, d(GTCAGGAATCTG) showed a decrement of a negative signal around 220 nm, though the positive peak around 275 nm did not change in the interaction as shown in Fig. 4a. This positive peak reflects the nucleobase conformation of DNA,[23] while the negative peak around 220 nm is attributed to both the DNA and peptide backbone conformation. However, it is needless to consider the conformational change of nucleotides, because the rigid nucleotide structure cannot change its backbone structure without disrupting the nucleobase conformation.[23] Thus, the observed change of the CD spectra around 220 nm is mostly due to the peptide rather than the DNA strand in this case. Actually, CD spectra altered by the interaction between poly(dT) and **1** showed a similar extent of decrement of the signal around 220 nm as shown in Fig. 4a, although the CD spectra of poly(dT) itself showed a small positive signal around 220 nm (unpublished results).

To understand the conformation altered by the interaction, the differential spectra between the signal at 30, 60, or 120

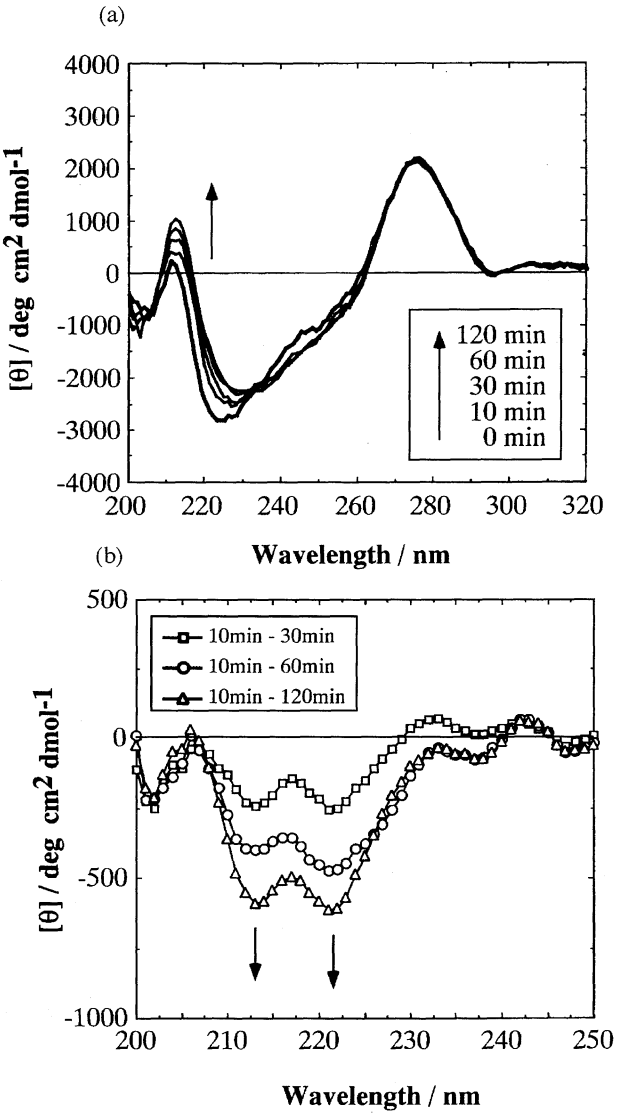

Fig. 4. (a) CD spectra of **1** with an ssDNA of d(GTCAGGAATCTG) at 0, 10, 30, 60, and 120 min after the mixing at 5 °C. Peptide concentration was 40 μM, and the nucleotide concentration was 480 μM. (b) Differential CD spectra of **1** between the signal at 10 min and that at 30, 60, or 120 min after the mixing.

min and that at 10 min after the mixing were analyzed. Figure 4b shows two minimum CD peaks at 212 and 221 nm analogous to α-helical signals. This indicates the perturbation mostly of the α-helical conformation of **1** by interacting with the ssDNA. On the other hand, the CD spectrum of **3** was less affected by adding the ssDNA (data not shown), so that the conformational change of **1** is induced by its binding with the ssDNA.

Energetics of the 24-mer Peptide and ssDNA Complex. Figure 5 shows the changes of the emission at 302 nm due to Tyr of **1** upon the addition of poly(dT) or poly(dA–dT). Since **1** includes fluorescence inactive residues except for Tyr, these fluorescence spectra reflect the emission of Tyr at the carboxy terminus of **1**. Quenching of **1** by adding poly(dT) is very significant compared with the case of poly(dA–dT). The free energy change in the binding of **1** with poly(dT) and the number of the nucleotides excluded by one peptide were calculated from the results of these fluorescence quenching by the curve fitting procedure. Summarized complex stability and n values are listed in Table 1. With the poly(dT) strand, free energy changes at 20 °C (ΔG°_{20}) were -7.8 kcal mol^{-1} and n was 4.3 in 100 mM NaCl–phosphate buffer. The melting temperature (T_m) of the alternating copolymer of poly(dA–dT) was 61 °C in 100 mM sodium citrate buffer,[18] so that it formed a dsDNA structure under the experimental conditions examined here. Though poly(dA–dT) forms a dsDNA structure in 100 mM NaCl buffer at 20 °C,[16] the production of ssDNA regions such as looped and dangled nucleotides are inevitable, so that, this possibility accounted for the analysis in the case of poly(dA–dT). ΔG°_{20} for **1** with a poly(dA–dT) duplex was -3.7 kcal mol^{-1} and n is 7.1. The association constant at 20 °C ($K_{a,\,20}$) between **1** and poly(dT) was more than 1000 times larger than that of **1** with poly(dA–dT). Therefore, it is concluded that **1** discriminates strictly between ssDNA and dsDNA by their secondary structure.

Discussion

Structural Consideration About DNA Binding Activity of 1. Here, we investigated the ssDNA binding activity of the 24-mer peptide **1** derived from *E. coli* RecA protein. Though the apparent CD spectrum of **1** was analogous to a β-structural signal, this peptide also includes an α-helical component suggested by the comparison with truncated peptides **2** and **3** (Figs. 2a, 2b, and 2c) and the altered spectra in the binding with ssDNA (Fig. 4b). Thus, it is likely that the secondary structure of **1** includes both a stable β-structure and an α-helical structure.

It was reported that FECO peptide derived from *E. coli* RecA protein (193-212 amino acid region at L2 with a few conserved amino acids at both termini) was able to bind to both ssDNA and dsDNA with equal binding affinities,[7] though it lacks helix G amino acids except for two glycine residues at 211 and 212. When FECO peptide binds to an ssDNA, its conformation was observed to change from disordered to predominant β-sheet structure by adding an ssDNA,[7] which is similar to the case of **1** as shown in Fig. 2a. Since the predominant β-structure spectrum observed for **1** is equivalent to the spectrum observed for FECO peptide in the presence of a ssDNA, L2 amino acids have potential activity to form the β-structure. Since the differential spectra between **1** and **3**, and **2** and **3** showed β-structural and α-

Table 1. Stability and n Values in the Binding of **1** with Poly(dT) and Poly(dT–dT) at 20 °C[a]

Nucleotide	ΔG°_{20}/kcal mol^{-1}	$K_{a,\,20}/10^3$ M	n
poly(dT)	−7.8	650	4.3
poly(dA–dT)	−3.7	0.57	7.1

a) These values were estimated by the curve fitting procedure as described in the text. The buffer includes 100 mM NaCl, 10 mM Na_2HPO_4, and 1 mM Na_2EDTA (pH 7.0).

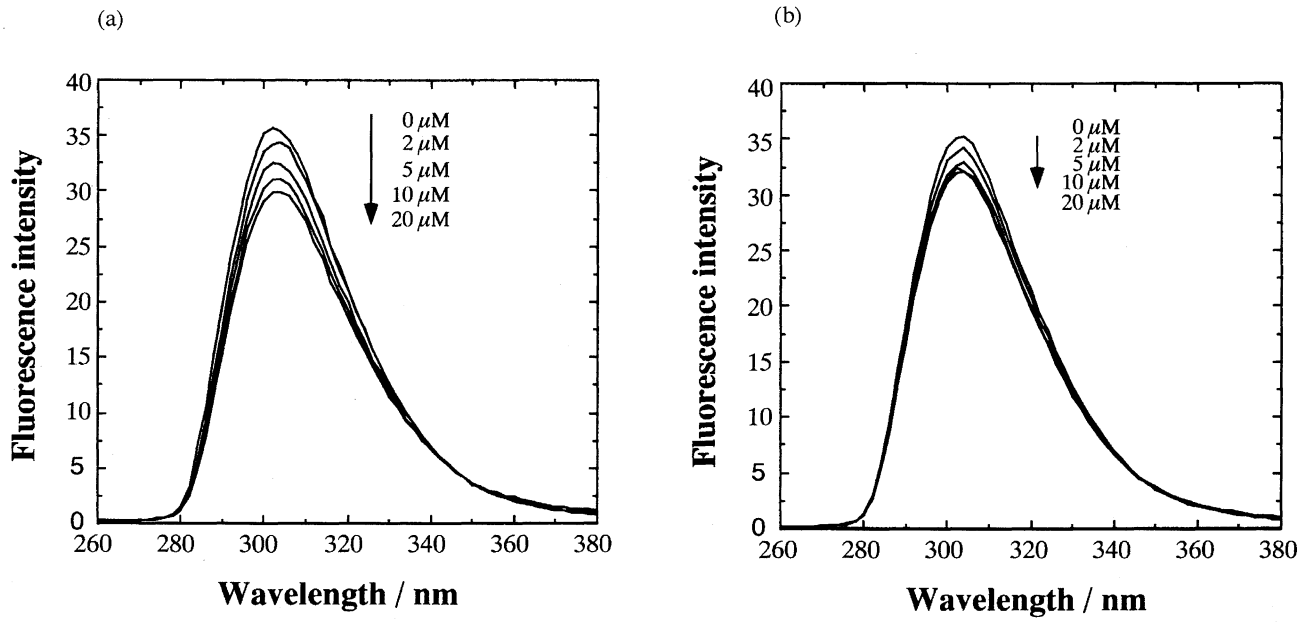

Fig. 5. Observed fluorescence changes of Tyr at the carboxy terminus of **1** by (a) poly(dT) and (b) poly(dA–dT) addition. Concentration of peptide was 20 μM and those of nucleotide were indicated in the figure. Measurements were done with an excitation wavelength at 225 nm in 100 mM NaCl–phosphate buffer at 20 °C.

helical signals, respectively, as shown in Figs. 2b and 2c, it is suggested that the β-structural component of **1** is provided by the full-length of L2 amino acids and its α-helical component is provided by helix G amino acids with several L2 amino acids. Considering these results, it is possible to say that the full-length of L2 and helix G amino acids is necessary for **1** to form the β-structure and an α-helical structure, respectively, and carboxy terminus amino acids, that is, mostly helix G amino acids, are needed to form the α-helical structure of **1**.

In the ssDNA binding, the secondary structure of **1** was altered as shown in Fig. 4a. The CD spectrum from 10 min after the binding showed a perturbation of the α-helix of **1** (Fig. 4b). Thus, the α-helical structure of **1** plays a role in the ssDNA binding. The structural alteration based on 0 min did not show such α-helical signals, so that it is likely that the conformational change of the DNA strand was finished within 10 min. Only the perturbation of the α-helix would be observed in Fig. 4b, which would be the reason for the deviated kinetics traces with a simple binding process in Fig. 3.

Discrimination Activity of the Peptides for Nucleic Acid Structure. The 24-mer peptide of **1** bound to poly(dT) with the stability of -7.8 kcal mol^{-1}, while that with poly(dA–dT) was only -3.7 kcal mol^{-1}, indicating that the peptide has the ability to discriminate between ssDNA and dsDNA. On the other hand, a 24-mer peptide with the change of the Phe residue at the center of **1** to Trp was also investigated to discover its ssDNA binding properties by monitoring the Trp fluorescence, which is thought to be an aromatic side chain directly interacting with nucleobases.[7,24] This peptide bound to poly(dT) with -8.3 kcal mol^{-1} for ΔG°_{20} and 5.8 for n, indicating that the conformational change at carboxy terminus of **1** is well correlated with its ssDNA binding property.

An peptide derived from *E. coli* RecA protein, wtw peptide (196-210 region at L2 amino acids and lacking two invariant Gly residues at 211 and 212) was also shown to interact with single-stranded polynucleotides by fluorescence titration experiments.[6] This peptide was estimated to bind to ssDNAs with about -4 kcal mol^{-1} at 25 °C. On the other hand, the calculated stability of FECO peptide bound with 53-mer ssDNA was about -7 kcal mol^{-1} at room temperature.[7] Sequence alignments of these peptides and **1** are summarized in Fig. 1. According to the fact of similar complex stabilities of an ssDNA with **1** and FECO peptide, difference of the stabilities between **1** and wtw peptide (about -4 kcal mol^{-1}) is unlikely to be due to the positively charged Lys residue (Lys216) in helix G amino acids of **1**. The excess binding energy of **1** and FECO peptide compared with wtw peptide is rather due to Gly residue(s) located at 211 and 212, highly conserved for bacterial RecA proteins and some recombination proteins,[8,9] which may interact with nucleotide backbones analogous to dinucleotide binding proteins.[4,25] Similar stability between **1** and FECO peptide means that most helix G amino acids have little part in the complex stabilization except for the invariant Gly residues.

Peptide **3**, lacking L2 amino acids, showed less binding activity with the ssDNA (Fig. 3), indicating almost all of the L2 amino acids were needed for the ssDNA binding property. Considering that FECO peptide was also able to bind to an ssDNA, it is clear that L2 amino acids are important for the ssDNA binding. Moreover, since FECO peptide showed a dsDNA binding activity as well as to ssDNA,[7] it is also clear that helix G amino acids have a role in the discrimination between ssDNA and dsDNA. The discrimination activity of **1** for ssDNA of poly(dT) and dsDNA of poly(dA–dT) was more than 1000 times, which is not observed for FECO peptide lacking helix G amino acids except for Gly211 and

Gly212.

These results are also supported by the structural results. Figures 4a and 4b show the perturbation of α-helical structure of **1** in the interaction with ssDNA of d(GTCAGGAATCTG). Moreover, fluorescence quenching of the Tyr residue at the carboxy end of **1** was observed, indicating the structural alteration of **1** at its carboxy terminus around the helix G amino acids. Hence, helix G amino acids are affected by the ssDNA binding. And, helix G amino acids play a role in the recognition of ssDNA rather than the complex stabilization except for the conserved Gly residues. Therefore, it is considered that almost all L2 amino acids are needed for the ssDNA binding property and helix G amino acids play a role mostly for the discrimination of ssDNA from dsDNA. These findings are useful for the design of synthetic peptides as functional materials for DNA recognition.

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