PERKIN 2

Association of α-helix peptides that have γ-cyclodextrin and pyrene units in their side chain, and induction of dissociation of the association dimer by external stimulant molecules

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α-Helix peptides bearing one unit of γ-cyclodextrin (γ-CD) and one or two units of pyrene in their side chain have been designed and synthesized as a novel system of peptide dimerization. The dimer was formed based on inclusion of two pyrene units in the γ-cyclodextrin cavity, and the dissociation of the peptide dimer was induced by external stimulant molecules (guests). Circular dichroism studies showed that the cyclodextrin–peptide hybrids (CD–peptides) maintain relatively rich α-helix content (61 to 81%), which was not affected by the guest inclusion into the cyclodextrin cavity. Fluorescence studies revealed that these CD–peptides form stable association dimers, which exhibit excimer emission. The intensity of the pyrene excimer emission decreased upon addition of the guest molecules, indicating dissociation of the CD–peptide dimers to the monomer CD–peptides. These CD–peptide hybrids bind structurally similar steroidal compounds with remarkable discrimination. These results demonstrate that this molecule-assembly system, based on host–guest chemistry, could be applicable to the development of molecule-responsive materials or a molecule-sensing system.

Introduction

There are an enormous number of molecules that play important roles in nature and most of them do not work alone but cooperatively or as assemblies. Especially, association and dissociation of molecules are obviously important in nature as seen in immune systems, biological membranes and protein clusters, like enzymes. And also, in many cases, an external molecule plays a key role in either the association or dissociation of the molecular assemblies. Establishment of well organized molecular assemblies might be useful for the design of novel devices or materials. Here, we wish to report a novel system of peptide dimerization driven by host–guest interaction. In this system, peptide dimerization and dissociation can be controlled by the binding of external stimulant molecules into the cavity of γ-cyclodextrin, which is located in the side chain of the peptides.

Recent development in synthetic peptide chemistry enables us to construct tertiary structures of peptides.[1] And also, a way to construct artificial peptide assemblies has been developed.[2] Since it is not easy to construct a molecule binding site using only small peptides, it is desirable to incorporate a binding site like cyclodextrin into the peptide system. Cyclodextrins are cyclic oligomers having six (α), seven (β), eight (γ) or more glucopyranose units. When cyclodextrins exist in water, their inside cavity is considerably hydrophobic, and they are capable of binding a lipophilic molecule within it. Due to this remarkable characteristic, the chemistry of cyclodextrins and modified cyclodextrins has been studied extensively.[3] On this basis, it might be expected that the combination of cyclodextrin chemistry and peptide chemistry may lead to a novel field of chemistry. When both guest molecule and cyclodextrin were conjugated in the side chain of the α-helix peptide, it was possible to form intramolecular and intermolecular cyclodextrin–guest inclusion complexes.[4] The former may stabilize the α-helix structure of the peptide. The latter may form the association dimer of the peptide. The major aim of this work is the construction of a molecule-assembling system, which is responsive to external stimulants, based on host–guest chemistry. To this end, three cyclodextrin–peptide hybrids (CD–peptides), having one cyclodextrin and one or two pyrene moieties, have been designed and synthesized. Since pyrene is capable of exhibiting excimer fluorescence when pyrene–pyrene interaction exists, it is expected that the pyrene moiety works not only as an internal guest molecule for the cyclodextrin host but also works as an indicator to monitor the association of the peptides. γPR17 and γPL17 are CD–peptides composed of 17 amino acids, bearing one unit of γ-cyclodextrin, with a pyrene moiety located to the right and left side of the γ-cyclodextrin moiety, respectively, when the *N*-terminus side of the peptide is drawn in the left side (Fig. 1). γPP17 is a CD–peptide also composed of 17 amino acids bearing one unit of γ-cyclodextrin and one pyrene moiety in either side of the γ-cyclodextrin in the peptide sequence.

Results and discussion

Design and synthesis

In the design of γPL17, γPR17, and γPP17, alanine was chosen[5] as the main component of the peptide because of its α-helix stabilizing capability. In addition, in order to stabilize a helical structure, three pairs of intramolecular salt bridges[6] (Glu2-Lys6, Glu7-Lys11, and Glu12-Lys16), were introduced into the peptides. All the sequences of the peptides and their schematic illustrations are shown in Fig. 1. The peptides were synthesized by the stepwise elongation of Fmoc-protected amino acids on a rink-amide resin.[7] The lysine side chains (Lys5 in γPL17, Lys13 in γPR17, and Lys5 and Lys13 in γPP17) which are to be coupled with pyrene-1-butyrate, were protected by a *tert*-butyloxycarbonyl (Boc) group while the side chain

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carboxy group of glutamate (Glu9), which is to be coupled with 6-monodeoxy-6-amino-γ-cyclodextrin was protected by a *tert*-butyl (Bu^t) group. The side chains of other Lys and Glu residues were protected with 2-chlorobenzyloxycarbonyl (ClZ) and benzyl (Bn) groups, respectively. To stabilize the α-helix structure of the CD–peptides, *N*-terminal amine and *C*-terminal carbonyl were acetylated and amidated, respectively. Then the synthesized peptides were cleaved from the resin by trifluoroacetic acid (TFA). At this stage, Boc was removed from Lys5 and Lys13 for γPL17 and γPR17, respectively, and from Lys5 and Lys13 for γPP17, while Bu^t was removed from Glu9 for all these peptides. Then, a pyrene-1-butyrate group was introduced into the deprotected side chain of the Lys5 in γPL17 and Lys13 in γPR17, and into the side chains of Lys5 and Lys13 in γPP17. 6-Monodeoxy-6-amino-γ-cyclodextrin was prepared as previously reported[8] and coupled with the side chain of the deprotected Glu9. Finally, remaining protection groups (ClZ and Bn for Lys and Glu, respectively) were removed by treatment with 1 mol dm^{-3} trimethylsilyl-trifluoromethane sulfonate (TMSOTf) in TFA solution.[9] The products, cyclodextrin and pyrene bearing peptides, were purified by HPLC equipped with an ODS column and identified by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS) and amino acid analysis.

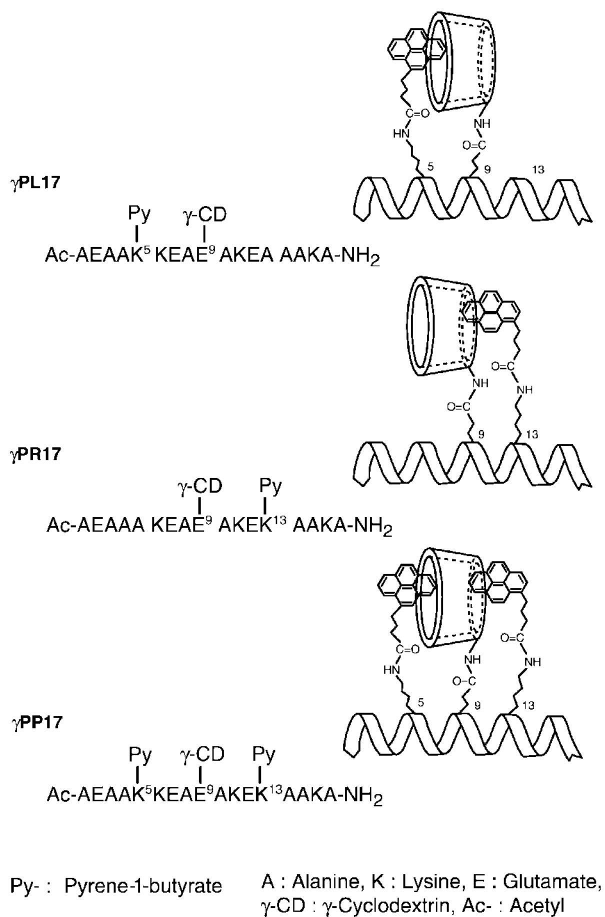

Fig. 1 Peptide sequences and their schematic illustrations.

Circular dichroism spectra of the CD–peptides

For the elucidation of the structural features of the cyclodextrin–peptide hybrids (CD–peptides) and side chain configuration, circular dichroism spectra were measured. All the experiments were carried out in a buffer solution (20 mM, Tris-HCl, pH 7.5) at 25 °C, and the CD–peptide concentrations were 10 μmol dm^{-3} and 30 μmol dm^{-3} for measurement in the amide bond region (200–250) and pyrene absorption region (200–250 nm), respectively. Fig. 2 (A–C) shows the circular dichroism spectra (intensity unit: mean residual weight ellipticity) of the amide bond region (200–250 nm) for the CD–peptides, γPL17, γPR17 and γPP17. All of them exhibited circular dichroism spectra of a typical α-helical pattern. The pyrene-attached peptides exhibit circular dichroism in the pyrene absorption region with much smaller molar ellipticities than those of the amide region.[10] The α-helix contents of γPL17, γPR17, and γPP17 were estimated from the values of their mean residual weight ellipticities at 222 nm, $[\theta]_{222}$.[11] The values

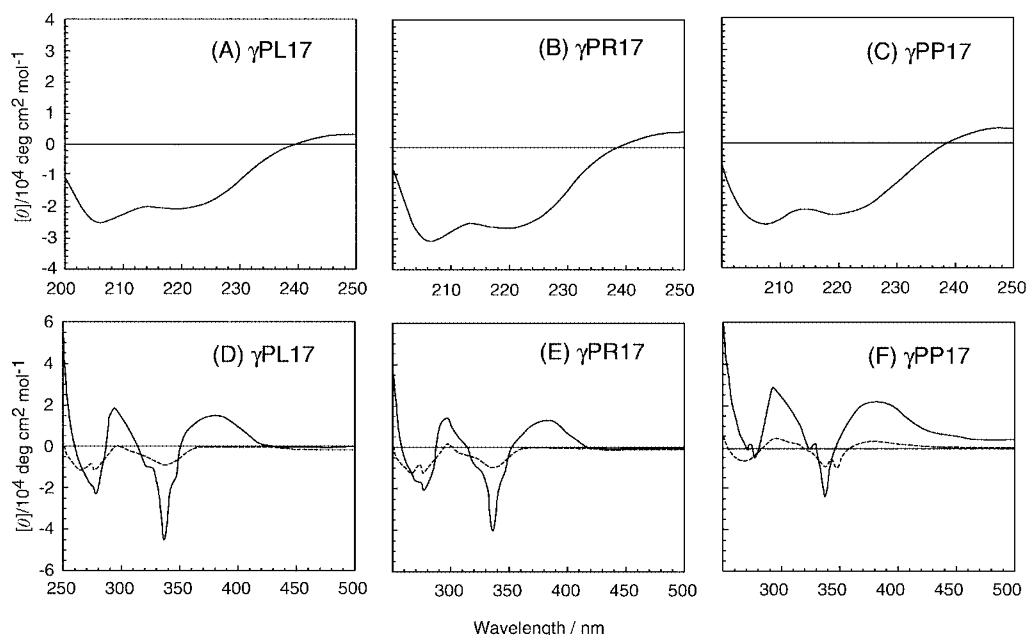

Fig. 2 Circular dichroism spectra in the amide (A–C) and the pyrene (D–F) absorption regions at 25 °C. [θ] denotes mean residual weight ellipticity for A, B and C while [θ] denotes molar ellipticity for D, E and F. For D–F (---) is spectrum in the presence of hyodeoxycholic acid (800 μmol dm^{-3}).

were 61%, 81% and 68% for γPL17, γPR17 and γPP17, respectively. These results show that the helix contents of the CD–peptides are enough to maintain the appropriate location and proximity of γ-cyclodextrin and pyrene moieties, thus enabling the two moieties to form an intramolecular inclusion complex. Upon the addition of hyodeoxycholic acid as a guest molecule, no significant change in the amide region (200–250 nm) of the circular dichroism spectra was observed for any of the CD–peptides. These results suggest that the inclusion of the guest molecules into the cyclodextrin cavity does not cause a significant change in the α-helicity of the CD–peptides. Fig. 2 (D–F) shows the circular dichroism spectra (intensity unit : molar ellipticity) in the pyrene absorption region (250–500 nm) of the CD–peptides in the presence and absence of hyodeoxycholic acid (800 μmol dm^{-3}). The circular dichroism intensities diminished upon the addition of the guest and finally very small signals were observed in the presence of an infinite concentration of hyodeoxycholic acid. These observations suggest that the circular dichroism signals of the CD–peptides in this region are generated by the formation of an inclusion complex between pyrene and γ-cyclodextrin on the α-helix peptide and exclusion of the pyrene group from the γ-cyclodextrin cavity results in the diminishment of the circular dichroism intensities.[12]

Fluorescence spectra of the peptides

Fig. 3 shows the fluorescence spectra of the CD–peptides measured by excitation at 338 nm at 25 °C. All the CD–peptides exhibit not only normal (monomer) fluorescence with a peak at 376 nm and 397 nm, but also remarkable excimer fluorescence at 476 nm. It is interesting that γPL17 and γPR17 exhibit significant excimer emission in spite of the fact that they have only a single pyrene moiety. This result indicates that these two CD–peptides form an association dimer and the association dimer generates pyrene excimer emission.[12] This argument was confirmed by the fact that the intensity ratio of the excimer emission and the monomer emission (I_{476}/I_{376}) for γPL17 and γPR17 increases with increasing concentration of the peptides (Fig. 4). This result suggests that the two CD–peptides are able to form an association dimer depending on their concentrations. Association constants of the dimerization were determined by the non-linear least square curve fitting analysis of the plots of the fluorescence intensity *versus* CD–peptide concentration, using the following eqn. (1) with the assumption

$$r = \Delta r\,(1 - (8K_a[\text{CD–peptide}]_0 + 1)^{1/2})/4 \qquad (1)$$

of 1 : 1 stoichiometry for the CD–peptide association, where r is the intensity ratio of the excimer emission and the monomer emission (I_{476}/I_{376}), Δr is a constant, K_a is the association constant, and $[\text{CD–peptide}]_0$ is the initial CD–peptide concentration. Fig. 4 shows these plots, and the solid lines represent the calculated curves obtained by this analysis. The analysis gave the association constants, 1.8×10^7 and 2.8×10^7 dm^3 mol^{-1} for γPL17 and γPR17, respectively.

Since γPP17 has two pyrene units in the CD–peptide side chain, it is possible for γPP17 to exhibit excimer fluorescence even in dilute solution, in which dimerization of the CD–peptide hardly occurs. The concentration dependence of the excimer emission of γPP17 was also examined, to ascertain whether or not the excimer emission is generated by γPP17 alone. If the excimer fluorescence of γPP17 was solely derived from the monomer form of the CD–peptide, no concentration dependence of the ratio (I_{476}/I_{376}) should be observed. However, concentration dependence on the value of I_{476}/I_{376} was observed. This result suggests that γPP17 is capable of forming an association dimer, depending on its concentration. Although γPP17 forms an intramolecular excimer and the association constant of γPP17 could not be determined accurately by the same method as that used for γPL17 and γPR17, the association constant was estimated to be approximately 2.5×10^7 dm^3 mol^{-1} for γPP17. As described above, all the CD–peptides prepared here successfully formed association dimers with similar magnitudes of the association constants. The schematic representation of the peptide dimerization and dissociation is shown in Fig. 5.

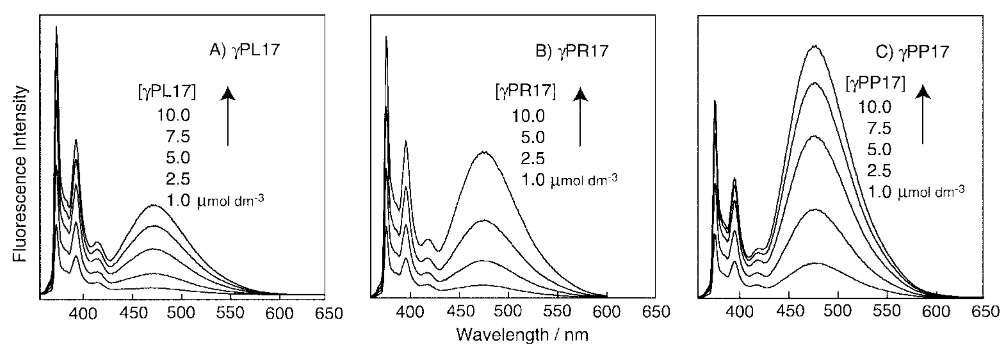

Fig. 3 Fluorescence emission spectra of various concentrations of the CD–peptides at 25 °C.

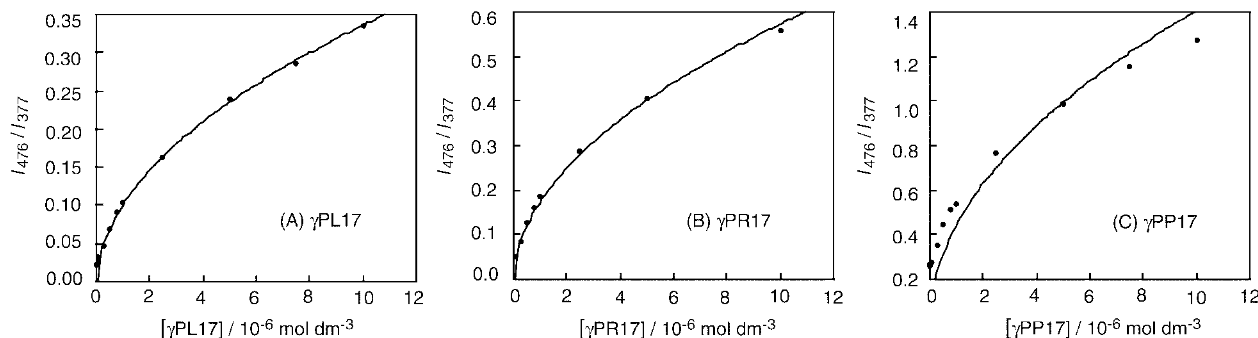

Fig. 4 The ratio of excimer and monomer emission intensities (I_{476}/I_{376}) as a function of the concentration of each CD–peptide and curve fitting analysis of the CD–peptide dimerization.

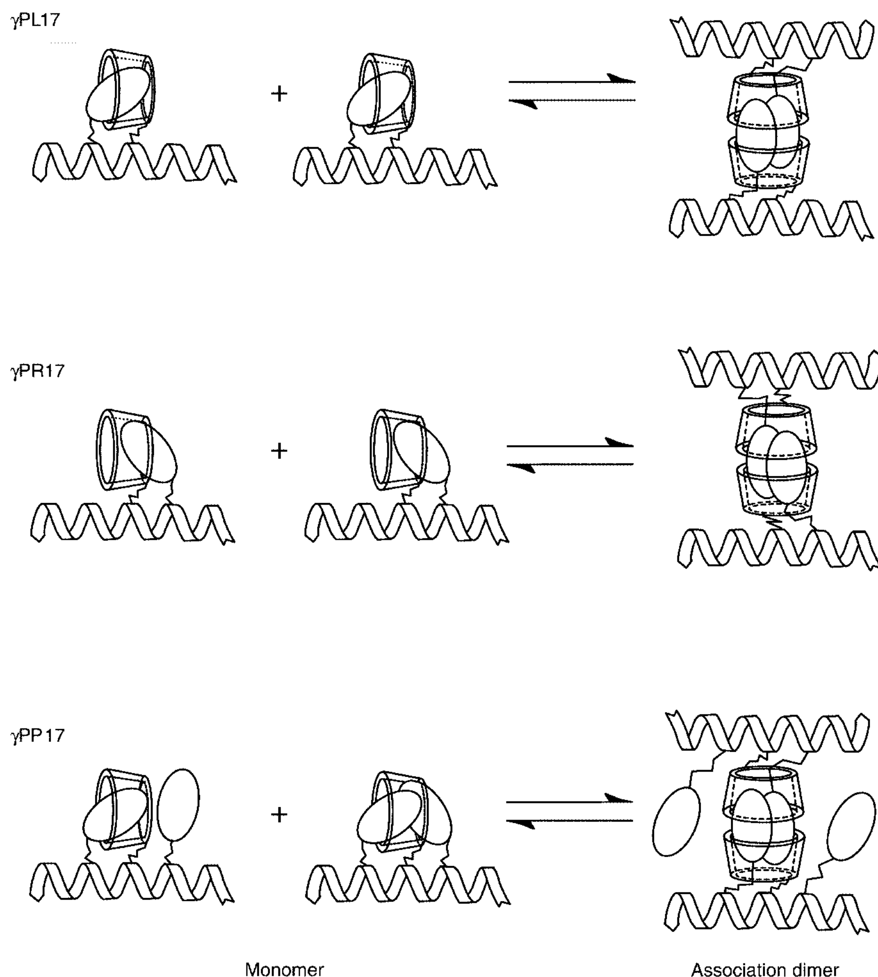

Fig. 5 Schematic representation of the peptide dimerization equilibrium.

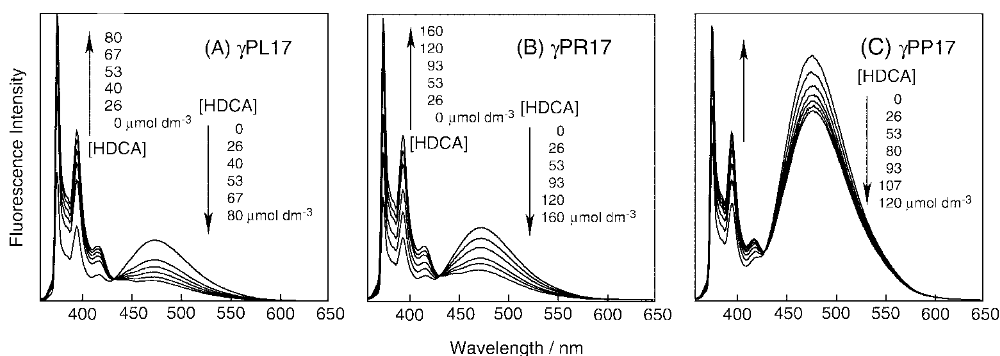

Fig. 6 Fluorescence spectra of the CD–peptides in the absence and presence of hyodeoxycholic acid as a guest at 25 °C. [CD–peptide] = 10 μmol dm^{-3}; Excitation wavelength is 338 nm.

Dissociation of the CD–peptide dimer induced by an external stimulant molecule

The excimer emission of the CD–peptides markedly diminished, but normal emission increased, upon the addition of hyodeoxycholic acid as a guest (Fig. 6). This observation also indicates that γ-cyclodextrin in the side chain of the CD–peptide forms an intermolecular inclusion complex with the guest. This complexation causes exclusion of the pyrene moiety from the γ-cyclodextrin cavity and subsequently causes dissociation of the CD–peptide dimer, generating the CD–peptide monomer. Guest-induced dissociation of the CD–peptide dimers is schematically illustrated in Fig. 7.

Binding affinities of the CD–peptides with four structurally related steroidal compounds

As described above, the fluorescence emission of the CD–peptides changes upon guest addition. It is interesting to determine binding constants of the CD–peptides for various guest molecules. In order to avoid CD–peptide dimerization, the CD–peptide solutions were diluted to 5 nmol dm^{-3}. At this concentration, γPL17 and γPR17 do not exhibit any excimer emission, evidence that they exist as monomers at this concentration. Although γPP17 did show excimer emission in this concentration, it is likely to exist as monomer in this concentration and exhibit excimer fluorescence due to the intramolecular

pyrene–pyrene interaction. The four cholic acid derivatives deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), hyodeoxycholic acid (HDCA), and ursodeoxycholic acid (UDCA) were chosen as guests (Fig. 8). These are all isomers of each other, having identical molecular weight. All the CD–peptides diminished their fluorescence intensity upon addition of the guest molecules (Fig. 9), and the binding constants were determined by the curve fitting analysis of the plots based on the following eqn. (2)[13] assuming 1:1 stoichiometry, where

$$i = i_0 + \Delta i([\text{CD–peptide}]_0 + [\text{guest}]_0 + 1/k_b - (([\text{CD–peptide}]_0 + [\text{guest}]_0 + 1/k_b)^2 - 4[\text{CD–peptide}]_0[\text{guest}]_0)^{1/2})/2 \quad (2)$$

i and i_0 are the fluorescence intensities of the CD–peptide in the presence and the absence of the guest, respectively. Δi is $i - i_\infty$ where i_∞ is the fluorescence intensity in the presence of an infinite concentration of the guest. K_b is the binding constant of the CD–peptides for various guest molecules. Fig. 10, 11 and 12 show the fluorescence intensity at 376 nm of those CD–peptides as a function of guest concentration. The solid curves were obtained by the curve fitting analysis as described above. Binding constants (K_b) of γPP17, γPL17 and γPR17 for various guest molecules are summarized in Table 1. The error limits of the data in Table 1 are within 10%. Of the three CD–peptides, γPR17 showed the smallest binding constants for each of the cholic acid derivatives. This result indicates that γPR17 maintains the most stable intramolecular inclusion complex between the γ-cyclodextrin and pyrene moieties. This argument is consistent with the extremely high helix content of this CD–peptide (81%). In contrast, γPL17 binds all the guest compounds approximately twice as strongly as γPR17. The remarkable difference between γPL17 and γPR17 in guest binding is related to the location of the pyrene unit along the α-helix peptide. Namely, placement of the pyrene group at the *C*-terminus side of the CD–peptide is more favorable for the formation of an intramolecular inclusion complex between the pyrene and the cyclodextrin units than at the *N*-terminus side, and consequently the tight intramolecular complex formation stabilizes α-helix formation of the CD–peptide. These CD–peptide hybrids bind structurally similar cholic acid derivatives with remarkably different binding constants, even if the differences between those cholic acid derivatives reside in only the position of a single hydroxy group (Fig. 8). γPL17, for example, binds hyodeoxycholic acid 3.4 times and 1.4 times stronger than it does deoxycholic acid and ursodeoxycholic acid, respectively. γPP17 could discriminate those cholic acid derivatives more

Fig. 7 Schematic representation of the CD–peptide association dimers and their guest-induced dissociation.

Fig. 8 Four steroidal compounds having identical molecular weights.

Fig. 9 Fluorescence spectra of the CD–peptides (5 nmol dm^{-3}) in the absence and presence of hyodeoxycholic acid as a guest at 25 °C.

Table 1 Binding constants of the peptides with four steroidal compounds ($dm^3 mol^{-1}$)

	Cheno-deoxycholic acid	Deoxy-cholic acid	Hyodeoxy-cholic acid	Ursodeoxy-cholic acid
γPL17	24 600	9 050	30 900	21 400
γPR17	9 300	5 750	13 100	10 600
γPP17	40 200	5 700	21 000	28 300

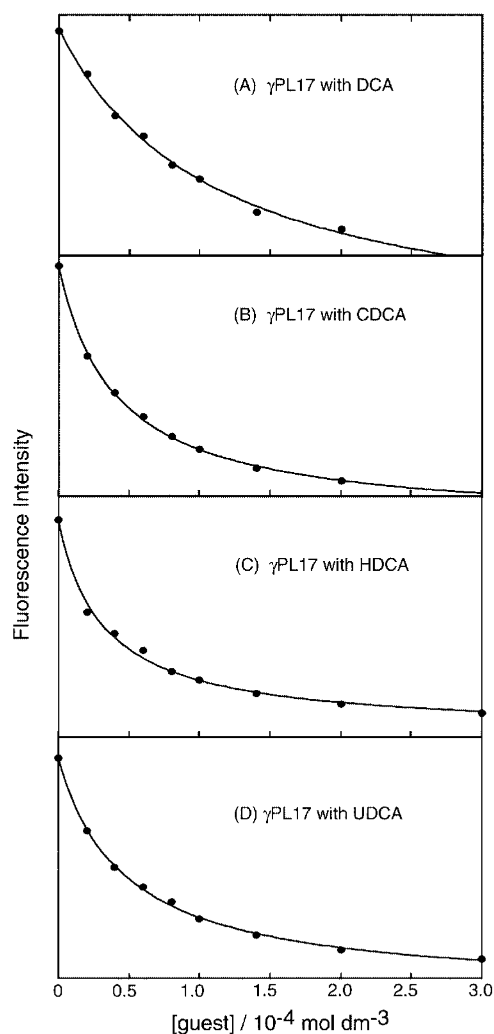

Fig. 10 Fluorescence intensity at 376 nm of γPL17 (5 nmol dm^{-3}) as a function of the guest concentration. (A) Deoxycholic acid (DCA); (B) chenodeoxycholic acid (CDCA); (C) hyodeoxycholic acid (HDCA); (D) ursodeoxycholic acid (UDCA).

clearly than the other two CD–peptides. For example, the binding constant (K_b) of γPP17 for CDCA is 7.1 times higher than that for DCA, while K_b of γPL17 for CDCA is 2.7 times higher than that for DCA and K_b of γPR17 for CDCA is 1.6 times higher than that for DCA. These results suggest that double labeling of CD–peptide with pyrene is favorable for molecular discrimination. These results demonstrate that this system is applicable to a molecule-sensing system.

Conclusion

The CD–peptides γPP17, γPR17 and γPL17 successfully formed association dimers based on cyclodextrin host–guest chemistry and exhibited remarkable pyrene excimer emission. Of the three CD–peptides, γPR17 showed the highest α-helix content. This result suggests that the location of the side chain functional groups is a factor in maintaining the α-helix conformation of these CD–peptides. These CD–peptide dimer systems were responsive to external stimulant molecules and their excimer fluorescence diminished markedly, while their monomer fluorescence intensity increased. This phenomenon was explained as guest responsive dissociation of the CD–peptide dimer. These results demonstrate the applicability of this CD–peptide dimer system to the construction of external stimulant responsive devices or materials.

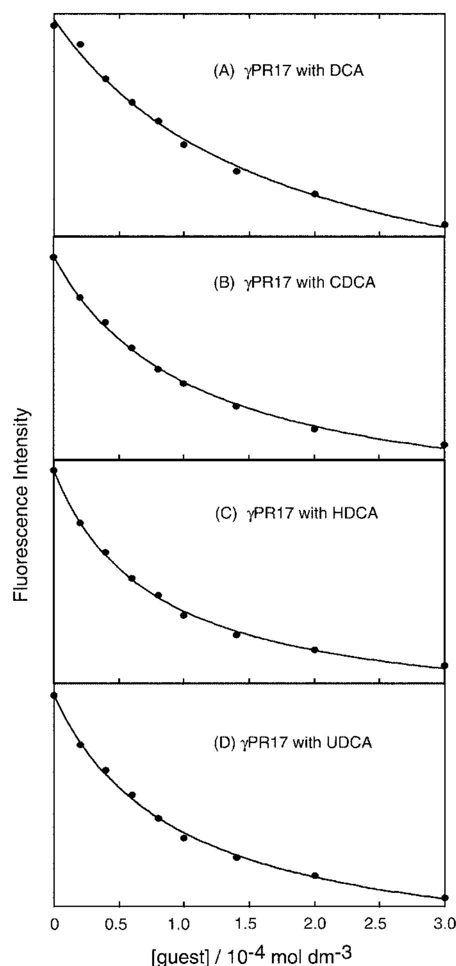

Fig. 11 Fluorescence intensity at 376 nm of γPR17 (5 nmol dm^{-3}) as a function of the guest concentration. (A) Deoxycholic acid (DCA); (B) chenodeoxycholic acid (CDCA); (C) hyodeoxycholic acid (HDCA); (D) ursodeoxycholic acid (UDCA).

Experimental

Materials

Rink amide resin for the solid phase peptide synthesis was purchased from either Advanced Chemtech or Novabiochem. Amino acid derivatives and the reagents for the peptide synthesis were purchased from either Novabiochem or Watanabe Chemical Co. All other chemicals and solvents for the synthesis or HPLC were of the highest purity available.

Syntheses

Synthesis of γPP17. Fmoc-Ala-Glu(Bn)-Ala-Ala-Lys(Boc)-Lys(ClZ)-Glu(Bn)-Ala-Glu(Bu^t)-Ala-Lys(ClZ)-Glu(Bn)-Lys-(Boc)-Ala-Ala-Lys(ClZ)-Ala Rink amide resin was synthesized by stepwise elongation of Fmoc-amino acids (3 equiv.) on 4-(2,4-dimethoxyphenylfluorenylaminomethyl)phenoxy resin (Rink amide resin) with tribenzotriazole-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP, 3 equiv.),

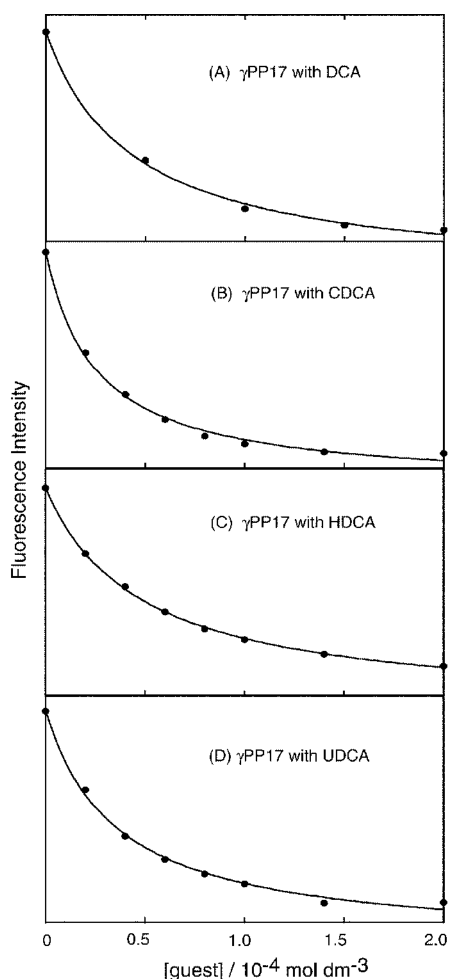

Fig. 12 Fluorescence intensity at 376 nm of γPP17 (5 nmol dm^{-3}) as a function of the guest concentration. (A) Deoxycholic acid (DCA); (B) chenodeoxycholic acid (CDCA); (C) hyodeoxycholic acid (HDCA); (D) ursodeoxycholic acid (UDCA).

1-hydroxybenzotriazole hydrate ($HOBT \cdot H_2O$, 3 equiv.) and *N*,*N*-diisopropylethylamine (DIEA, 6 equiv.) as coupling reagents in *N*-methylpyrrolidone (NMP). The Fmoc group was removed by treatment with 20% piperidine in NMP solution for 15 minutes. The terminal amino group of the peptide was acetylated by treatment with acetic anhydride in NMP for 20 minutes. Then the Ac-peptide was cleaved from the resin and partially deprotected with trifluoroacetic acid (TFA) in the presence of *m*-cresol, by stirring for 1.5 h at room temperature. At this stage, Boc and Bu^t were removed from Lys and Glu, respectively. The crude peptide was analyzed by HPLC and identified by MALDI-TOFMS (*m*/*z* 2562.4 $[(M+H)^+]$, calcd. 2562.4). Ac-peptide (100 mg, *ca.* 39 μmol) and pyrene-1-butyrate succinimide ester (16 mg, *ca.* 120 μmol) were dissolved in DMF in the presence of DIEA, (6 equiv.), and the reaction mixture was stirred at room temperature for 24 h. Then the product was purified by gel filtration method using a sephadex LH-20 column with DMF as eluent. 6-Monodeoxy-6-amino-γ-cyclodextrin (66 mg, *ca.* 50 μmol) and the pyrene labeled peptide (40 mg, *ca.* 17 μmol) were dissolved in NMP in the presence of BOP (3 equiv.), HOBT (3 equiv.) and DIEA (6 equiv.) and the solution was stirred for 20 h. The product was purified by HPLC, then identified by MALDI-TOFMS (*m*/*z* 3605.9 $[(M + H)^+]$, calcd. 3604.3). The remaining protecting groups (ClZ and Bn) were removed by TMSOTf in TFA solution. The final product, γPP17, was purified by HPLC and identified by MALDI-TOFMS and amino acid analysis (7 mg, 19 μmol, yield 11%).

Synthesis of γPL17. This compound was synthesized and purified in an identical way to γPP17, except for the introduction of Ala13 instead of Lys13. MALDI-TOFMS (*m*/*z* 3278.6 $[(M + H)^+]$, calcd. 3277.2) (8 mg, 24 μmol, yield 7%).

Synthesis of γPR17. This compound was synthesized and purified in an identical way to γPP17, except for the introduction of Ala5 instead of Lys5. MALDI-TOFMS (*m*/*z* 3278.2 $[(M + H)^+]$, calcd. 3277.2) (4 mg, *ca.* 12 μmol, yield, 2.5%).

Measurements

The peptides were purified by reversed-phase HPLC (RP-HPLC) on a YMC-Pack C4 A-823 column (10 × 250 mm) (YMC Co.) with a linear gradient of acetonitrile (0.1% TFA)–water (0.1% TFA). All the spectroscopic measurements were carried out in 20 mM Tris-HCl buffer solution (pH 7.5). UV-visible spectra were measured on a Shimadzu UV-1300 or Shimadzu Biospec-1600 in a quartz cuvett with a path length of 1 cm. Fluorescence measurements were carried out on a Shimadzu RF-5300PC equipped with a thermal regulator HAAKE F3, in a quartz cuvett with a path length of 1 cm. Circular dichroism spectra were recorded on a JASCO J-720WI equipped with a thermal regulator JASCO PTC348WI in a quartz cuvett with a path length of 0.1 cm. Mass spectra were measured on a Shimadzu KRATOS KOMPACT MALDI II Spectrometer.

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