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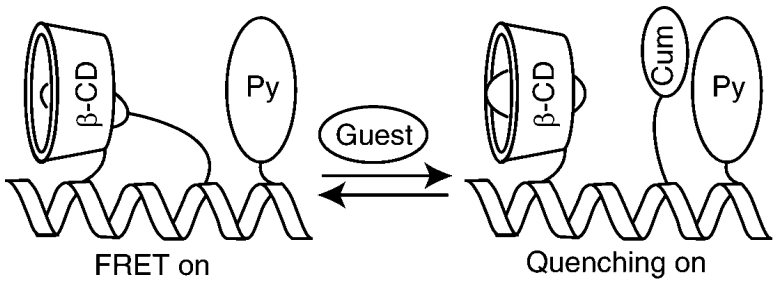

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Novel Peptides Bearing Pyrene and Coumarin Units with or without β-Cyclodextrin in Their Side Chains Exhibit Intramolecular Fluorescence Resonance Energy Transfer

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Recently, various fluorescent probes have been developed to study biological phenomena in living cells where fluorescence resonance energy transfer (FRET) has been used in many cases.[1] In assays of various enzymes, peptides bearing fluorescent dyes have widely been used.[2] For biological applications, FRET peptide probes are much more useful than single dye labeled probes, because the FRET system is independent of the concentration of a single dye as, in the FRET system, one can measure the ratio of the fluorescence intensities at two wavelengths. The ratiometric measurement thus helps to reduce the influence of various factors, such as the localization of a single probe, changes in the environment around a probe (e.g., pH, polarity, etc.), and changes in the excitation intensity. Therefore, this technique allows more precise measurements with the detection of interacting species even at nanomolar concentrations. However, it is not easy to create such FRET peptides, because a peptide in aqueous solution usually forms such a helix conformation that the donor and acceptor moieties are in very close proximity, and therefore the emissions of the fluorophores are quenched.[3] For a practical use of the FRET peptide probe, it is very important to solve this fluorescence-quenching problem. There are some reports that fluorescence quenching can be overcome by using conformationally constrained oligopeptides such as proline-containing oligopeptides[4] as linkers. It has also been shown that it is possible to observe FRET if close contact of the two fluorophores can be prevented either by restricting the flexibility of the linker or by using a hydrophobic pocket for the donor/acceptor on the ethylene glycol linker.[5] However, there are few successful examples of FRET peptide probes with conformationally flexible oligopeptides as linkers.[6] Therefore, we intend to develop such a peptide-based FRET probe usable in an aqueous environment without the quenching of the two dyes. We thought an appropriate host molecule such as cyclodextrin (CD) could prevent the close approach of the fluorophores by accommodating one of the fluorophores in its central hydrophobic cavity. On the other hand, such a CD-peptide probe could be used as a molecule-responsive device in a wide range throughout the molecular world. Keeping this idea in mind, we have designed and successfully synthesized a CD-peptide conjugate, **1**, bearing a pyrene donor and coumarin acceptor in the side chains (Figure 1). To explain the validity of the concept of fluorescence quenching in the FRET peptide probe, we also have synthesized a reference peptide, **2**, which contains no CD in the side chain (Figure 1). The unique feature of these FRET peptide probes is that, unlike existing probes, the FRET in **1** can be regulated by an exogenous guest (HDCA) and in **2** by an exogenous host (β-CD).

The peptides were synthesized by stepwise elongation of 9-fluorenylmethoxycarbonyl amino acids (Fmoc AAs) on the rink amide resin using a similar synthetic procedure as we had reported previously[7] with some modifications (Supporting Information). The final products were purified by reverse phase HPLC and identified by amino acid analysis and MALDI-TOFMS (**1**, *m*/*z* 3266.4 [(M + H)$^+$], calcd. 3266; **2**, *m*/*z* 2148.6 [(M + H)$^+$], calcd. 2148).

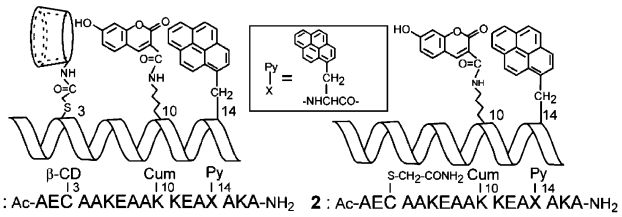

Figure 1. The structures of the peptides bearing pyrene and coumarin with (**1**) and without β-CD (**2**), X (Py): L-α-pyrenylalanine.

CDs are torous-shaped oligosaccharides that form inclusion complexes with various organic molecules in aqueous solution.[8] There are three widely used CDs, among which we have chosen β-CD to cap the coumarin moiety because it has been reported to be a suitable host for coumarin derivatives.[9] It has also been confirmed that coumarin can be fit into the β-CD cavity by means of a molecular modeling calculation.[5b] On the other hand, in this work, pyrene/coumarin has been chosen as the FRET pair, which is yet to be explored as a usable tool for cellular imaging studies. The most fascinating feature of the pyrene/coumarin pair is that the presence of donor affects neither the relative fluorescence nor the wavelength maximum of the acceptor.

The fluorescence excitation spectra of **1** with the emission wavelength of coumarin at 448 nm in 20 mM Tris-HCl buffer at 25 °C (pH 7.4) are shown in Figure 2A. In addition to a peak of coumarin at around 410 nm, we have observed a strong peak at around 340 nm, which corresponds to the absorption wavelength of pyrene. This observation implies that energy transfer occurs from pyrene to the coumarin unit. We have found a constant value of the ratio of fluorescence intensity of the coumarin to that of pyrene (I_{coum}/I_{pyr}) from the excitation spectra of **1** at various concentrations that indicates the concentration independence of I_{coum}/I_{pyr} in the range of 100 nM−100 μM. Figure 2B shows the fluorescence emission spectra of **1** (1.0 μM) in the absence and presence of hyodeoxycholic acid (HDCA, Figure 2Ca). The spectra were obtained by irradiating the solutions at 340 nm, which is the excitation wavelength of pyrene. In the absence of HDCA, we have observed high fluorescence emission of the acceptor coumarin at around 448 nm, indicating that energy transfer occurs from pyrene to coumarin in **1**. The intensity of both the acceptor coumarin and the donor pyrene decreases with an increase in the concentration of HDCA in the solution. This diminishment in the fluorescence intensity is thought to be associated with the exclusion of the coumarin unit from inside to outside of the β-CD cavity by accommodating a HDCA molecule. Being excluded from the CD cavity, coumarin may come into very close contact with the pyrene unit, resulting in the fluorescence quenching. Using the fluorescence variation of coumarin, we have determined the binding constant

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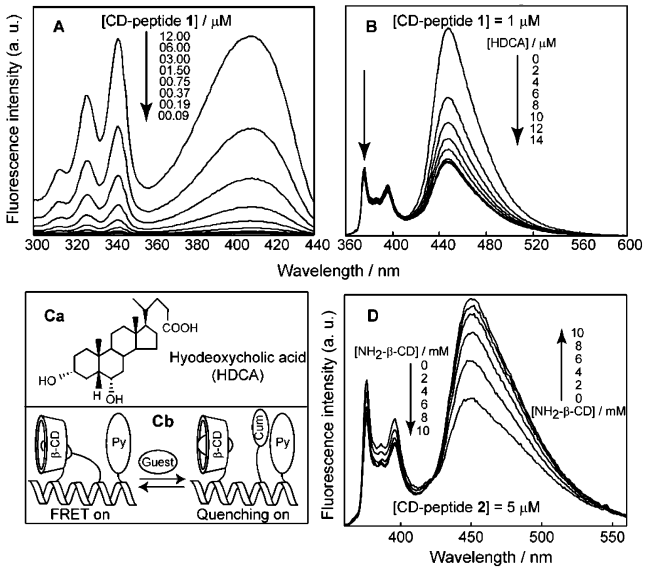

Figure 2. (A) Fluorescence excitation spectra of CD-peptide **1** at various concentrations with the emission wavelength at 448 nm. (B) Fluorescence emission spectra of **1** in the absence and presence of HDCA. (Ca) The structure of hyodeoxycholic acid. (Cb) Schematic representation for the guest-induced structural change in the side chain of **1**. (D) Fluorescence emission spectra of **2** in the absence and presence of NH_2-β-CD. The excitation wavelength is 340 nm.

value of **1** for HDCA (470 700 M^{-1}), which is almost 4.5 times higher than that of the CD-peptide[7a] we had reported previously. The guest-induced diminishment in FRET is schematically shown in Figure 2Cb.

Figure 2D shows the fluorescence emission spectra of **2** (5.0 μM) in 20 mM Tris-HCl buffer at 25 °C (pH 7.4) in the absence and presence of β-CD. Without β-CD, both the donor (at 376 nm) and the acceptor (at 448 nm) fluorescence were quenched, reflecting close contact between the donor and acceptor moieties in aqueous solutions. However, with an increase in the concentration of β-CD, the intensity of the acceptor emission was markedly enhanced, whereas the intensity of the donor emission was decreased. This result is consistent with the result reported by Nagano et al.,[5b] where they have studied the coumarin−fluorescein FRET pair attached with a flexible ethylene glycol linker. These results demonstrate that β-CD includes the coumarin moiety in its hydrophobic cavity, and therefore the energy transfer from the pyrene moiety to coumarin moiety can proceed efficiently after the addition of β-CD. On the other hand, instead of β-CD, when we have added HDCA into the aqueous solution, no change in the fluorescence spectra was observed. The peptide **2** has no endogenous β-CD in the side chain, and, therefore, it is reasonable that HDCA will cause no change in the environment of coumarin.

To study the conformation of the polypeptide chain, circular dichroism spectra in the peptide absorption region (195−250 nm) were measured. Both **1** and **2** showed a typical α-helix pattern (Supporting Information). The helix content of **1** increased from 34% to 41% upon addition of 80 μM of HDCA, whereas no change in the helicity of **2** (42%) was observed. This result demonstrates that HDCA excludes the coumarin moiety from inside to outside of the CD cavity in **1**, resulting in a stable helix formation because of the coumarin−pyrene close interaction at the side chain, the same as in **2**. Because the CD is composed of chiral glucose units, circular dichroism is expected to be induced at the absorption bands of chromophores, which are included in the cavity of chiral β-CD.[7] We thus have investigated the induced circular dichroism (ICD) spectra of both **1** and **2** (100 μM each) at 250−600 nm, in the absence and presence of HDCA (Supporting Information). Positive and negative ICD bands were observed in **1** at around 410 and 360 nm, indicating that coumarin is included in the CD cavity in the absence of the guest.

In conclusion, we have succeeded in constructing two novel FRET peptide probes where FRET can be controlled either by an exogenous guest or by an exogenous host molecule. Introducing a FRET technique with a CD-peptide conjugate will open a new field in the CD-based sensory system to investigate host−guest interactions. Because we have observed CD-peptide **1** to have very high sensitivity and affinity for a steroidal compound (HDCA), we expect that this new FRET peptide probe would be a promising candidate for studying and sensing cholesterol or other steroidal derivatives, which are very important metabolites in the human body. In addition, this FRET system could be applied as a ratiometric probe for monitoring the activity of proteolytic enzymes (Supporting Information). Again, larger separations or orientations in probes may lead them to have a potential application in biophysics, such as in conformational studies.

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Supporting Information Available: Details for synthesis, purification of the peptides, and circular dichroism data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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