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Fluorescence Resonance Energy Transfer in a Novel Cyclodextrin–Peptide Conjugate for Detecting Steroid Molecules

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This article is dedicated to Professor Akihiko Ueno who passed away on March 23, 2003

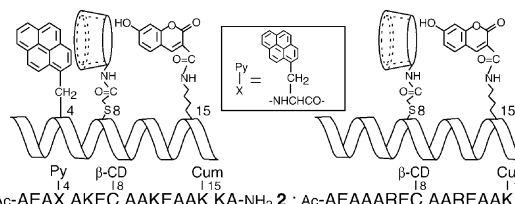
Abstract—A novel cyclodextrin conjugated peptide, **1**, having two different fluorophores, coumarin and pyrene, in the side chains has been designed and synthesized. The circular dichroism study reveals that **1** shows typical α -helix pattern, and forms intramolecular inclusion complex with coumarin. The fluorescence emission study shows that the peptide exhibits intramolecular fluorescence resonance energy transfer (FRET) without quenching of two fluorophores. We have determined the binding constants of **1** for various biologically important steroid molecules as guests using the guest-responsive variation in the fluorescence emission intensity of coumarin.

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Fluorescence resonance energy transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule without emission of a photon. It is a very efficient, sensitive and popular technique for studying biological phenomena in living cells.¹ Much effort has been devoted so far to develop FRET systems using flexible helix peptide as linker of two dyes.² The problem in constructing such a FRET peptide probe is that the fluorophores are quenched because of their dye-to-dye close contact.³ Recently, we have succeeded in constructing a peptide-based FRET probe, and we have reported that the peptide bearing pyrene and coumarin with β -cyclodextrin (β -CD) moiety in the side chains exhibits FRET without quenching of two dyes.⁴ However, in the present study, we intend to study molecular recognition property of such a CD conjugated peptide (CD-peptide) using the advantage of FRET technique.

CDs (α -, β - or γ -) are cone-shaped cyclic oligosaccharides that form inclusion complexes with various organic compounds in aqueous solution.⁵ Because of their high solubility in water, CDs are very popular materials in medicine for encapsulating insoluble drug components.

Based on the unique molecular architecture of CD, various chromophore-modified CDs have been constructed and shown to act as molecular sensors or enzymes.⁶ So far reported, the chromophore-modified CDs acting as chemosensors have single fluorophore or two same fluorophores. However, in this study, we have designed and synthesized a novel β -CD-peptide, **1**, which has two different fluorophores, pyrene donor and coumarin acceptor, on the peptide scaffold (Fig. 1). We report here, for the first time, how it works as a chemosensor when two different fluorophores are present in a single CD-peptide molecule. The idea of sensing a molecule using the FRET technique is that, the coumarin moiety being included into the CD cavity is close to the pyrene moiety so that pyrene can easily donate energy to the coumarin moiety upon photoirradiation, and thus FRET occurs. This state can be regarded as 'FRET on' state. Addition of an exogenous guest will



1 : Ac-AEAX AKEC AAKEAAK KA-NH₂ **2** : Ac-AEAAAAREC AAREAAK RA-NH₂

Figure 1. Structures of the peptides bearing cyclodextrin and coumarin with (**1**) or without pyrene (**2**); X (Py): L- α -pyrnylalanine.

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cause displacement of the endogenous guest (coumarin) from inside of the CD cavity to outside. Since excluded coumarin moiety is apart from the pyrene unit, it might be less effective for coumarin to accept energy from pyrene, resulting in diminishment in the FRET. This can be regarded as the 'FRET off' state. We can monitor this guest-induced 'on-off' switch of FRET process by measuring the fluorescence intensity of the CD-peptide in aqueous solution. A reference peptide, **2**, having no pyrene in the side chain, has also been synthesized for studying guest-induced conformational change in the side chain.

The peptides were synthesized by stepwise elongation of 9-fluorenylmethoxycarbonyl amino acids (Fmoc AAs) on the rink amide resin using the same synthetic procedure as we have reported previously.⁴ The final products were purified by reverse-phase HPLC and identified by amino acid analysis and matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOFMS) **1**: m/z 3266.8 [(M+H)⁺], calcd 3266; **2**: m/z 3152.6 [(M+H)⁺], calcd 3150.0).

In order to study the conformation of the polypeptide chain, circular dichroism spectra in the amide bond absorption region (200–250 nm) were measured. Both **1**

and **2** (10 μ M) showed typical α -helix pattern (Fig. 2). The helix content calculated from the mean residual weight ellipticity at 222 nm $[\theta]_{222}^7$ was found to be 41% and 58% for **1** and **2**, respectively. We have added 80 μ M of hydoxycholeic acid (HDCA) into the aqueous solution of each **1** and **2**. The helix content of **1** decreased from 41 to 28%, and that of **2** decreased from 58 to 47%, indicating that coumarin forms intramolecular inclusion complex with β -CD and HDCA excludes the coumarin moiety from inside to outside of the CD cavity resulting in the destabilized helix formation. This result is consistent with the results previously reported where it has been shown that coumarin can be fit into the β -CD cavity while pyrene remains outside because of its less flexibility in the L- α -form and large size to be accommodated in the β -CD cavity.^{4,6a,8}

The circular dichroism of the achiral coumarin moiety is expected to be induced at the coumarin absorption region if it forms inclusion complex with chiral β -CD.^{4,6,9} Therefore, we have measured induced circular dichroism (ICD) of the CD-peptide **1** (100 μ M) at the pyrene and coumarin absorption region (250–600 nm) to confirm whether coumarin forms intramolecular inclusion complex with β -CD or not (Fig. 3). We have

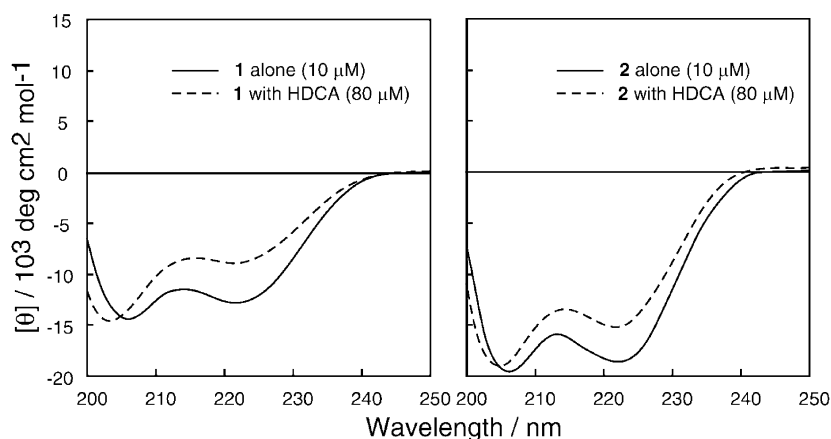


Figure 2. Circular dichroism spectra of the CD-peptide **1**, and its reference **2**, alone and in the presence of HDCA at the amide bond absorption region (200–250 nm) in Tris–HCl buffer (20 mM, pH 7.5) at 25°C.

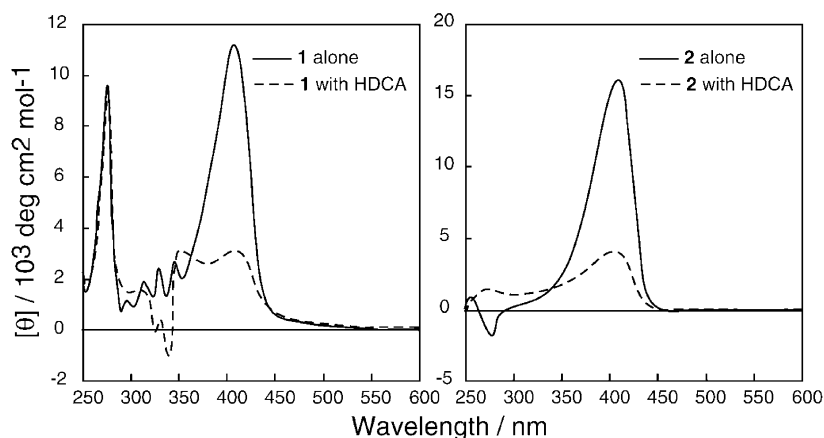


Figure 3. Induced circular dichroism (ICD) spectra of the peptides, **1** and **2**, alone and in the presence of HDCA at the pyrene and coumarin absorption region (250–600 nm) in Tris–HCl buffer (20 mM, pH 7.5) at 25°C; [Peptides] = 100 μ M, [HDCA] = 320 μ M.

observed sharp positive ICD signals in the above region. The magnitude and sign of the ICD effect are directly related to the orientation of the electric dipole transition moment relative to the axis of the CD. According to the rule of Harata,¹⁰ which applies to chromophores immersed in the CD cavity, co-conformations with a parallel alignment produce a positive ICD signal, while those with an orthogonal alignment produce a negative ICD signal. However, addition of HDCA (320 μM) into the aqueous solution of CD-peptide **1** caused drastic diminishment in the ICD signal at around 410 nm, which corresponds to the absorption wavelength of coumarin. The diminishment in the ICD signal is thought to be allied with the exclusion of coumarin moiety from inside to outside of the CD cavity. To confirm the fact that the positive ICD signal in the CD-peptide **1** at around 410 nm emerged solely from the coumarin moiety, we have investigated the ICD of the reference peptide **2** (100 μM) that has no pyrene in the side chain, and observed a similar positive ICD band at the coumarin absorption region, which was dramatically diminished upon addition of HDCA (320 μM) (Fig. 3). This finding is an unequivocal proof for the intramolecular host–guest complexation between coumarin and β -CD.

Figure 4A shows the fluorescence emission spectra of CD-peptide **1** (1 μM) in 20 mM Tris–HCl buffer at 25 °C. We have photo irradiated the peptide at the absorption wavelength of pyrene (340 nm) and observed a strong peak at the emission wavelength of coumarin (448 nm). On the other hand, two other weak peaks

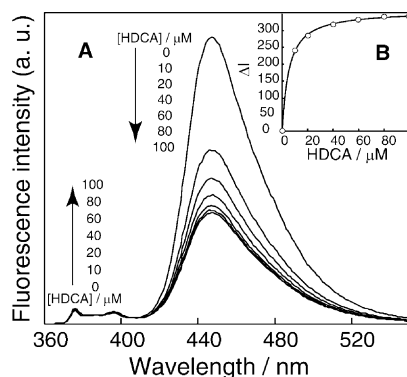


Figure 4. (A) Fluorescence emission spectra of **1** (1 μM) in the absence and presence of HDCA in Tris–HCl buffer (20 mM, pH 7.5) at 25 °C; (B) The plot of ΔI as a function of HDCA concentration. The excitation wavelength is 340 nm.

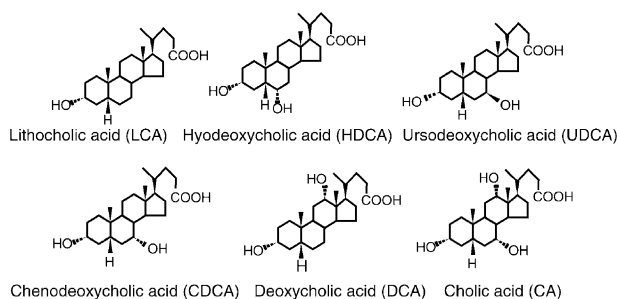


Figure 5. Structures of the steroid guests.

were found at the emission wavelengths of pyrene (376 and 396 nm). This result implies that FRET occurs effectively in the CD-peptide **1**. However, with increasing the concentration of HDCA, the fluorescence intensity of the acceptor coumarin was markedly decreased, whereas the intensity of the donor (pyrene) emission was increased. These results demonstrate that coumarin being included into the hydrophobic cavity of β -CD is near to the pyrene, and therefore the energy transfer from the pyrene moiety to coumarin moiety can proceed efficiently in the absence of HDCA. On the other hand, addition of HDCA caused coumarin to be excluded from the CD cavity, and thus the distance between pyrene and coumarin is increased, resulting in less efficient energy transfer. Figure 4B shows the plot of ΔI ($\Delta I = I - I^0$ when I^0 and I denote the fluorescence intensities of coumarin in the absence and presence of HDCA, respectively) against HDCA concentration. We have determined the binding constants (K_b) of CD-peptide **1** for a variety of steroid compounds (Fig. 5) by the curve-fitting analysis based on the equation of 1:1 stoichiometry and the results are shown in Table 1. The K_b values of **1** for the steroid guests examined are in the order of $\text{LCA} > \text{HDCA} > \text{UDCA} > \text{CDCA} > \text{DCA} > \text{CA}$. When six important steroidal compounds, LCA, HDCA, UDCA, CDCA, DCA and CA, were used as guests, marked differences in the binding constants have been observed in spite of the fact that all of them have the same steroid framework as a major part of their structures. LCA is less polar compound since it contains only one hydroxyl group in the steroid nucleus and we have found that it binds to β -CD very strongly, compared to other analogues. This observation suggests that the polarity of the guest molecules, in addition to size, is an important factor that governs the binding affinities to **1**. Another possibility is that since LCA has only one hydroxyl group while others have two or three, it experiences less steric hindrance while forming inclusion complex with β -CD. On the other hand, in spite of the fact that HDCA, UDCA, CDCA and DCA are four geometrical isomers with one hydroxyl group at opposite orientation (hydroxyl group at C-7 for both UDCA and CDCA) or different position (hydroxyl group at C-6 and C-12 for HDCA and DCA, respectively), they showed significant difference in terms of binding ability to β -CD. This observation suggests that the stereochemistry of one hydroxyl group, in addition to size and polarity of the guest, is also an important factor for determining the binding affinity of the guest to the host molecule. The sensitivity factors of the CD-peptide **1** (1 μM) for various guests were evaluated using fluores-

Table 1. Binding constants and sensitivity values of **1** for various guests at 25 °C

Guests	Binding constants (K_b/M^{-1})	Sensitivity values ($\Delta I/I^0$)
LCA	$438,800 \pm 17,000$	0.450
HDCA	$210,000 \pm 6200$	0.400
UDCA	$173,400 \pm 4100$	0.400
CDCA	$33,300 \pm 800$	0.160
DCA	1100 ± 90	0.010
CA	900 ± 460	0.008

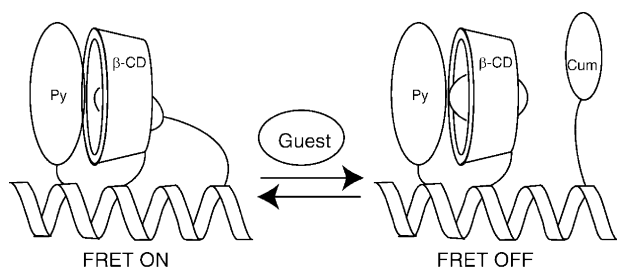


Figure 6. Schematic representation of the guest-responsive structural change in the side chain of the CD-peptide **1**.

cence-intensity variation ($\Delta I/I^0$) at a guest concentration of 10 μM (Table 1). The CD-peptide is sensitive to the guests in the order of $\text{LCA} > \text{HDCA} = \text{UDCA} > \text{CDCA} > \text{DCA} > \text{CA}$, which is almost correlated with the binding constants. The guest-induced diminishment in the FRET is schematically illustrated in Figure 6.

Considering the results, we can conclude that the guest-induced locational change of the appending coumarin moiety in **1** affects FRET process and hence, the system works as a chemosensor for steroid molecule detection. This FRET peptide could be used as a potent sensor for studying cholesterol or their derivatives that are important steroid metabolites in the human blood components. In addition, the idea of constructing such CD-peptide would be useful for designing FRET peptide probe with specific amino acid sequence that would act as a ratiometric probe for studying proteolysis.

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