Anal. Chem. **2001,** *73,* 939−942

Intramolecular Fluorescence Resonance Energy Transfer System with Coumarin Donor Included in β-Cyclodextrin

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In aqueous solutions, the fluorescence of the intramolecular fluorescence resonance energy-transfer (FRET) system 1 was strongly quenched, because of close contact between the donor and acceptor moieties. FRET occurred, and the acceptor fluorescence was increased, by adding β-cyclodextrin (β-CD) to aqueous solutions of 1. Spectral analysis supported the idea that the FRET enhancement was due to the formation of an inclusion complex of the coumarin moiety in β-CD, resulting in separation of the fluorophores. On the basis of this result, we propose that covalent binding of coumarin to β-CD will provide a FRET cassette molecule. So, compound 2 bearing β-CD covalently was designed and synthesized. Fluorescence intensity of 2 was enhanced markedly compared to the intensity of 3. Applying this FRET system, various FRET probes that will be useful for ratio imaging and also the high-throughput screening will be provided.

In recent years, many fluorescent probes[1] have been developed to study biological phenomena in living cells. A fluorescence resonance energy-transfer (FRET) technique was used in some fluorescent probes. FRET is an interaction between the electronic excited states of two fluorophores, in which excitation energy is transferred from a donor to an acceptor without emission of a photon. The FRET technique has been applied to probe biological systems and also for high-throughtput screening of combinatorial libraries,[2] by means of ratiometric measurements. Ratiometric measurements are methods that observe the changes in the ratio of the fluorescence intensities at two wavelengths. Using ratiometric measurements, it is possible to reduce the influence of many artifacts due to the change of the probe concentration and excitation intensity. Therefore, this technique allows more precise measurements, and with some probes, quantitative detection is possible. Recently, Tsien and co-workers reported several indicators using FRET for the detection of adenosine 3′,5′-cyclic monophosphate (cAMP),[3] calcium cation,[4] and β-lactamase[2] activity, and they employed these probes for assays at physiological concentrations and for imaging activity changes in living cells. Peptides bearing fluorescent dyes have been widely used in the protease assay.[5] FRET peptide probes are superior to single dye-labeled probes for this biological application, because we can observe the ratio of the fluorescence. However, it is difficult to obtain such FRET peptide probes, because peptides in aqueous solutions take conformations such that the donor and acceptor moieties are in close proximity, and the emissions of the fluorophores are quenched.[6] This quenching mechanism can be explained in terms of ground-state complex formation.[7] It has been reported that the fluorescence quenching of the ground-state dye-to-dye complex formation is observed in various fluorophore pairs.[6] In general, if the fluorophores have the hydrophobic characteristics, they would form dye-to-dye close contact in aqueous environment and the fluorescence should be quenched.[8,10] For practical use of peptide-based FRET probes, it is necessary to employ conformationally constrained oligopeptides such as pro-

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10.1021/ac001016a CCC: $20.00
Published on Web 01/26/2001

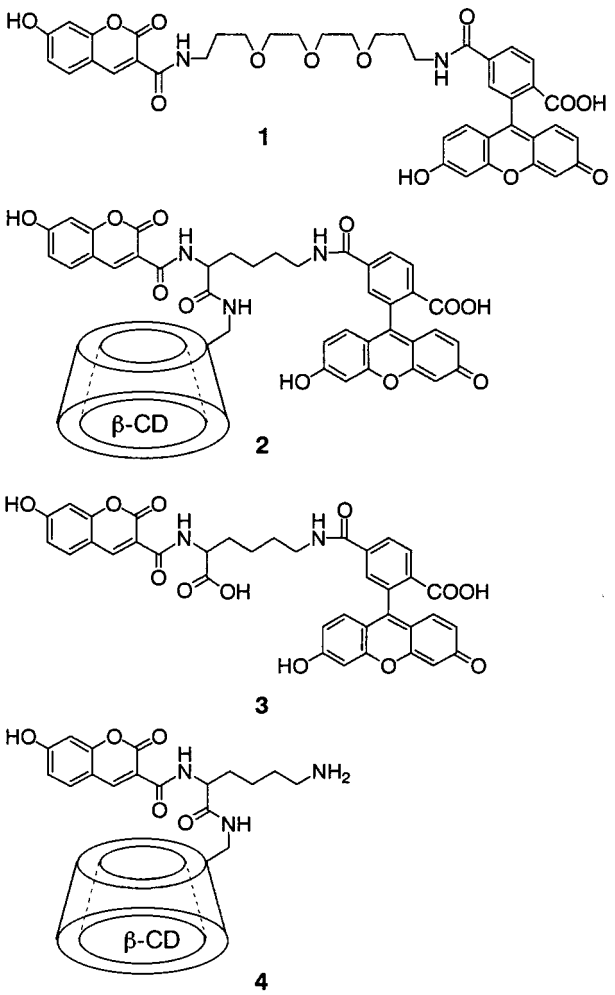

Figure 1. Structures of compounds **1**−**4**.

line-containing oligopeptides as linkers of the donor and acceptor dyes.[9] It is also shown that it is possible to observe the emission of the acceptor if the structure of the probe is such as to prevent close contact of the two fluorophores by restricting the flexibility of the linker.[10] In other words, there are no successful examples of FRET peptide probes with conformationally flexible oligopeptides as linkers. If such FRET systems could be developed, they would be useful for assay of a wide range of proteolytic activities. This study was intended to develop a conformationally flexible FRET system usable in an aqueous environment without quenching of the two dyes.

First, we designed and synthesized the intramolecular FRET compound **1** bearing the coumarin donor and the fluorescein acceptor (Figure 1). Ethylene glycol was employed as a flexible linker. In this system, the excitation energy of the coumarin donor at 408 nm would be transferred to the fluorescein acceptor, which would emit green light. As we expected, the fluorescence emission of **1** was strongly quenched in an aqueous environment, whereas FRET was observed when **1** was dissolved in methanol. It appears that these fluorophores did not come into close proximity in methanol because of its apolar environment compared to water. We thought that if we could prevent close approach of the fluorophores by surrounding one of the fluorophores with an appropriate host molecule, we could obtain a FRET system. So, in the present study, we added β-CD to aqueous solutions of **1**; under this condition, FRET occurred, resulting in the appearance of acceptor emission.

CDs are torus-shaped cyclic oligosaccharides composed of six, seven, or eight D-glucopyranose units (α,β,γ-CD, respectively). A variety of organic compounds can be included in their central cavities in aqueous solution.[11] It was reported that β-CD formed inclusion complexes with coumarin derivatives[12] as well as with naphthalene[13] and dansyl[14] derivatives. In this report, we describe the spectroscopic analysis of the β-CD inclusion complex with **1** in an aqueous environment. On the basis of the results of the titration experiment of β-CD, we propose that covalent binding of coumarin to β-CD will provide a FRET cassette molecule. To demonstrate the validity of this concept, we have designed and synthesized **2** bearing β-CD covalently (Figure 1).

EXPERIMENTAL SECTION

Synthetic details are described in Supporting Information.

Fluorometric Analysis. A fluorescence spectrophotometer (F4500, Hitachi, Tokyo, Japan) was used. The slit width was 2.5 nm for both excitation and emission. The photomultiplier voltage was 950 V. Compound **1** was dissolved in DMSO as a 10 mM stock solution and then diluted to the corresponding concentration for measurement.

Circular Dichroism Analysis. A spectropolarimeter (J-600, Jasco) was used. All samples were prepared from 10 mM stock solutions in DMSO. The following conditions were used: bandwidth, 1.0 nm; slit width, 1.0 nm; autosensitivity, 10 mdeg; time constant, 1.0 s; step resolution, 0.2 nm; scan speed, 20 nm/min; number of scans, 3.

Absorption Analysis. A spectrometer (UV-1600, Hitachi) was used. All samples were prepared from 10 mM stock solutions in DMSO.

RESULTS AND DISCUSSION

Enhancement of the Fluorescence of 1 Caused by the Addition of β-CD. Compound **1** was obtained according to the reaction scheme described in the Supporting Information. The fluorescence emission spectra of **1** in aqueous solutions containing β-CD at various concentrations are shown in Figure 2 a). The spectra were obtained by irradiating the solutions at 408 nm, which is the excitation wavelength of the coumarin fluorophore. Without β-CD, both the donor fluorescence around 445 nm and the acceptor fluorescence around 515 nm were strongly quenched, reflecting close contact between the donor and acceptor moieties in aqueous solutions. However, with increasing concentration of β-CD, the intensity of the acceptor emission was markedly enhanced. These results demonstrate that the energy transfer from the coumarin moiety to the fluorescein moiety can proceed efficiently after the addition of β-CD.

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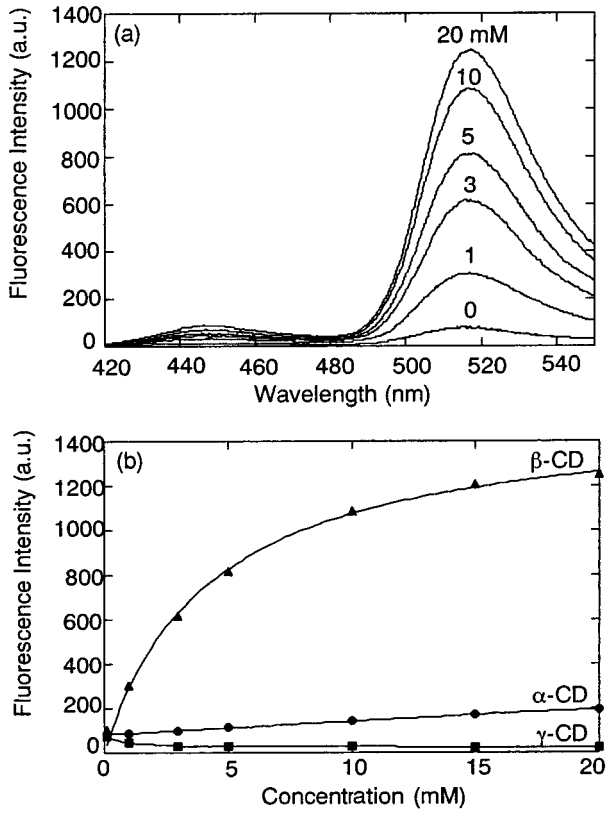

Figure 2. Increase of fluorescence caused by the addition of β-CD (25 °C, sodium phosphate buffer, pH 9.0): (a) fluorescence spectra of **1** (1.0 μM) at various concentrations of β-CD (Ex 408 nm); (b) dependence of fluorescence intensity on the concentration of α,β, and γ-CD (Ex 408 nm; Em 515 nm).

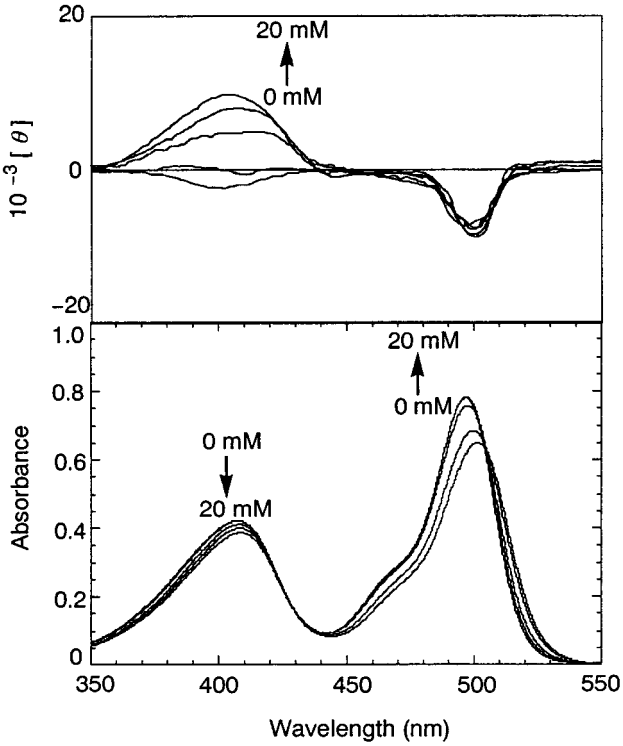

Figure 3. Induced circular dichroism spectra and absorption spectra of **1** (50 and 10 μM, respectively) at various concentrations of β-CD (0, 1.0, 5.0, 10, 20 mM) (25 °C, sodium phosphate buffer, pH 9.0).

In titration experiments with solutions of α-CD and γ-CD, the emission intensity of the acceptor fluorescence was not changed (Figure 2b). These observations show that the enhanced fluorescence in the presence of β-CD is not due to a change in the polarity of the solution caused by the addition of CDs. Considering the size of the CD cavity, that is, α-CD small, β-CD intermediate, and γ-CD large, the size of β-CD should be sufficient to accommodate one coumarin moiety. So, the enhanced fluorescence should be due to inclusion complex formation between β-CD and the coumarin moiety. The dissociation constant of β-CD and **1** was calculated to be 4.2 mM.

Spectral Analysis of the Inclusion Complex between Coumarin and β-CD. Since the cyclodextrins are composed of chiral glucose units, circular dichroism is expected to be induced at the absorption bands of guest molecules which are included in the cavity of chiral β-CD.[15] The absorption and induced circular dichroism (ICD) spectra of **1** in aqueous solutions without and with various concentrations of β-CD are shown in Figure 3. In the absence of β-CD, the absorption spectrum exhibits two peaks at 408 nm (donor absorption) and 501 (acceptor absorption). The coumarin absorption intensity was decreased dose-dependently by the addition of β-CD to a 10 μM aqueous solution of **1**, while the absorption of fluorescein was increased. There are two isosbestic points at 429 and 504 nm. These observations suggest that the addition of β-CD caused an environmental change around the donor−acceptor moieties. The reason for the changes in absorption spectra is described in the Supporting Information.

There were two peaks around 400 and 500 nm in the ICD spectrum of **1** alone. With increasing β-CD concentration, the positive ICD signal around 400 nm due to coumarin was increased dose-dependently, whereas the signal around 500 nm due to fluorescein showed no change. These results suggest that the coumarin moiety is included in β-CD and the fluorescein moiety is not. We also confirmed that coumarin can be fit into the β-CD cavity by means of a molecular modeling calculation using Spartan (version 5.0).[16] There was nonzero CD signal of **1** in the absence of β-CD. We measured the CD spectrum of fluorescein (50 μM) in aqueous solution and there was a negative signal around 500 nm. Because fluorescein (50 μM) had no signal in methanol, the negative signal in aqueous solution would be due to the intermolecular close contact. And we measured the CD spectrum of **1** (50 μM) in methanol and there was no signal. From these observations, the nonzero CD signal of **1** in the absence of β-CD in aqueous solution was also due to the dye-to-dye contact.

Evidence of 1:1 host−guest complex formation (m/z 1923.7 (β-CD + **1** + Na)$^+$, theoretical m/z 1923.6) was also obtained by ESI MASS spectrometry. A minor signal of a 2:1 host−guest complex (m/z 1530.2 (2 β-CD + **1** + H + Na)$^{2+}$, theoretical m/z 1530.0) was observed at lower β-CD concentrations, and the signal was enhanced by the addition of excess β-CD. Thus, the stoichiometry of host−guest complex formation is dependent on the concentration of β-CD.

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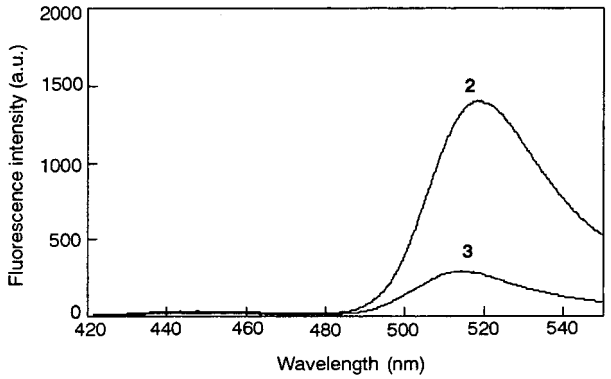

Figure 4. Fluorescence spectra of **2** and **3** (25 °C, sodium phosphate buffer, pH 9.0; Ex 408 nm).

The above experimental observations demonstrate that β-CD inclusion complex formation with the coumarin moiety of **1** results in disruption of close contact between the coumarin and fluorescein moieties in aqueous solution. Consequently, FRET can take place.

Fluorometric Analysis of 2. Compound **2** bearing β-CD covalently and a reference compound **3** (Figure 1) were synthesized according to the reaction scheme in the Supporting Information. The fluorescence spectra of **2** and **3** are shown in Figure 4. The fluorescence intensity of **2** was enhanced markedly compared to the intensity of **3**. This fluorescence enhancement was considered to be due to the formation of an inclusion complex between coumarin and β-CD. To estimate the fraction of coumarin that is actually bound within the cavity of β-CD of **2**, we added β-CD to the solution of **2** and observed the changes in the fluorescence spectra (Supporting Information). In the titration experiment, it was shown that the fluorescence spectra of **2** were not changed by the addition of β-CD. If there was a coumarin molecule out of the cavity of intramolecular β-CD due to the linker length, the fluorescence will be enhanced by the addition of β-CD. So, it was suggested that all the coumarin moiety that can be included in β-CD was in β-CD binding pocket of **2**.

CONCLUSION

From these results, we suggest that covalent binding of coumarin to β-CD facilitates disruption of close contact between the coumarin and fluorescein moieties and thereby enhances the fluorescence intensity. In an analytical format, this FRET system will be applied as ratiometric probes for the hydrolytic enzymes. To obtain such probes, β-CD should be incorporated at the ends of the substrate peptides by following strategy. The FRET donor cassette **4** (Figure 1) should be conjugated at the C-terminus of the peptides and the carboxyfluorescein should be conjugated at N-terminus by the peptide bonding. In such probes, FRET can take place and the acceptor fluorescence can be observed before the hydrolysis by an enzyme. After the hydrolysis, the excitation energy of the donor cannot be transferred to the acceptor intermolecularly and the donor fluorescence will be observed. So, by detecting the ratio of the donor and acceptor fluorescence intensities, the activities of the hydrolytic enzymes can be measured.

ACKNOWLEDGMENT

We thank Dr. Kentaro Yamaguchi and Mr. Shigeru Sakamoto for mass spectrometry. This work was supported in part by the Ministry of Education, Science, Sports and Culture of Japan (Grant 11794026, 12470475, and 12557217 for T.N. and 11771467 and 12045218 for K.K.), by the Mitsubishi Foundation and by the Research Foundation for Opt-Science and Technology. K.K. is thankful for financial support from the Nissan Science Foundation, Shorai Foundation of Science and Technology, and Uehara Memorial Foundation.

SUPPORTING INFORMATION AVAILABLE

Synthetic details, a discussion concerning the absorption spectra of **1**, and the titration experiment of β-CD to an aqueous solution of **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review August 24, 2000. Accepted December 18, 2000.

AC001016A