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# Detection of Bimodal Inclusion Complexes of Heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin with Naphthalene Derivatives Using Fluorescence Spectroscopy

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Simultaneous formation of two types of 1:1 complexes of 6-(4-methylphenylamino)-2-naphthalenesulfonate (6,2-TNS) with heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TMCD) in solution was detected by fluorescence spectroscopy. Fluorescence titration supported the formation of 1:1 complexes. However, the observed fluorescence spectrum was resolved into two components by time-resolved spectroscopy.

Cyclodextrins (CDs) and its derivatives have been widely used as model compounds for enzymes.$^{1,2)}$ The great interests toward CDs as artificial enzymes come from the ability of CDs to include guest molecules in aqueous solution into their hydrophobic cavity. A lot of efforts in spectroscopic studies have been devoted to determine the structure of the inclusion complexes in solution.$^{3)}$ Among many spectroscopic methods fluorescence spectroscopy is unique in its high time resolution, although it gives poor information on the molecular structure. Though fluorescence spectroscopy has been used in many investigations on the inclusion complexes of CDs with fluorescent molecules,$^{4)}$ however, few time-resolved measurement has been done yet. Recently time-resolved fluorescence spectroscopy has succeeded in analysing dynamic behavior of fluorescent probes bound to enzymes, other proteins, or membranes.$^{5)}$ Then we have applied this method to the study of inclusion complexes.

Complex formation of 6,2-TNS with CDs was studied using flurescence spectroscopy by Kondo et al.$^{6)}$ α-CD forms 1:1 complex with 6,2-TNS. However, β- and γ-CD form 2 hosts: 1 guest complex besides 1:1 complex. In this communication complex formation of TMCD$^{7)}$ with 6,2-TNS was studied. Fluorescence spectra of the solutions containing 6,2-TNS and TMCD have the peak at an identical wavelength (443 nm) even when the total concentration of TMCD is changed from 0.4 to 20 mM. A plot of fluorescence intensity versus total concentration of TMCD was fitted very well to a Benesi-Hildebrand-type equation derived on the assumption of 1:1 complex formation (Fig. 1). From this plot binding constant of 2180 $M^{-1}$ was obtained. These observations strongly support the formation of only 1:1 complex(es).

TMCD concentration in solutions used in routine measurement is 10 mM. At this concentration mole ratio of the free probe to the total probe is calculated at 0.025. The ratio of the intensity of the fluorescence of the free probe to that of the total fluorescence is about 3 x $10^{-4}$. Therefore fluorescence of the free probe is almost negligible.

Although sample solutions contain only 1:1 complex(es), observed fluorescence spectra have the characteristics which are expected for multi-component systems. First, maximum wavelength of fluorescence emission was dependent on excitation wavelength. Excitation at the edge of absorption band resulted in the shift of emission band to longer wavelength. Secondly, nanosecond time-resolved fluorescence spectra showed the temporal change in the band shape (Fig. 2). The transient spectra obtained at the earlier phase are expanded over broader range of wavelength. The decay curves detected at a specific wavelength could not be fitted to any single exponential function, but the curves were fitted well to a linear combination of two exponential functions. At any wavelength two decaying components were dominant. Two lifetimes and the quantum yields for each component were calculated at several pairs of excitation and emission wavelengths. The results are summarized in Table 1. The lifetimes and the ratio of the quantum yields which were obtained for the two different solutions containing 0.3 and 10 mM TMCD, respectively, coincided within experimental error. This result also confirms that the two species are both 1:1 complexes simultaneously formed in the solution.

The ratio of the quantum yields for the two components varied on changing emission wavelength. The quantum yields at each wavelength is essentially proportional to the weight of contribution of each component to the steady-state fluorescence intensity at the wavelength. Thus, on the basis of

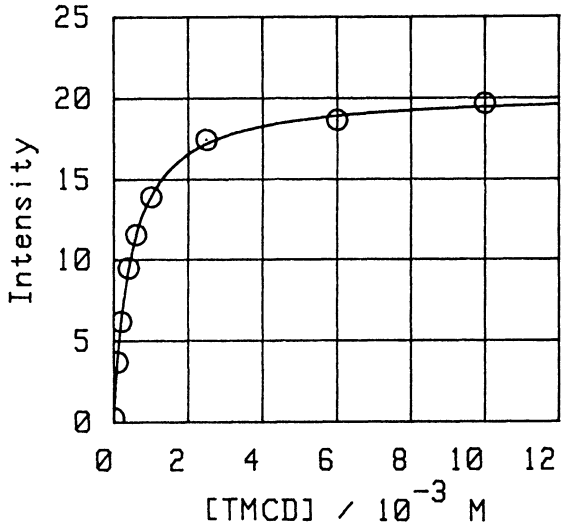

Fig. 1. Dependence of fluorescence intensity of 6,2-TNS on total TMCD concentration in solution. Total concentration of 6,2-TNS is 3 x $10^{-5}$ M; Excitation wavelength, 360 nm; Measured at 25 °C, pH 7.0, I = 0.1. A theoretical equation for the case of 1:1 complex formation was fitted to the observed data, and the curve for the equation was drawn in the figure.

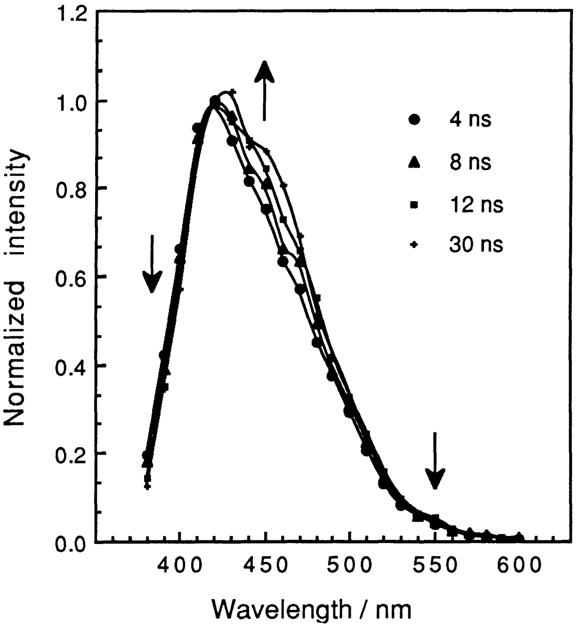

Fig. 2. Nanosecond time-resolved fluorescence spectra of 6,2-TNS in TMCD solution. Spectral distortion by the characteristics of the spectrometer is not corrected here. [6,2-TNS] = 3 x $10^{-5}$ M, [TMCD] = 10 mM; Measured at 25 °C, pH 7.0, I = 0.1.

Table 1. Two-exponential analysis of 6,2-TNS fluorescence decay in TMCD solution[a)]

| Excitation wavelength / nm | Emission wavelength / nm | Lifetime / ns ( Quantum yield / % ) $\tau_1$ ( $\Phi_1$ ) | $\tau_2$ ( $\Phi_2$ ) |
|---|---|---|---|
| 320 | 400 | 3.2 ( 54 ) | 9.6 ( 46 ) |
| | 440 | 3.5 ( 55 ) | 10.0 ( 45 ) |
| | 480 | 4.2 ( 62 ) | 10.5 ( 38 ) |
| | 540 | 4.6 ( 72 ) | 10.5 ( 28 ) |
| | 600 | 4.8 ( 81 ) | 11.4 ( 19 ) |
| 280 | 440 | 3.6 ( 54 ) | 10.0 ( 46 ) |
| 320 | | 3.5 ( 55 ) | 10.0 ( 45 ) |
| 360 | | 3.7 ( 64 ) | 9.9 ( 36 ) |
| 380 | | 3.6 ( 63 ) | 9.9 ( 37 ) |
| 400 | | 3.6 ( 64 ) | 9.9 ( 36 ) |
| 410 | | 3.6 ( 61 ) | 9.8 ( 39 ) |

a) Decay curves were fitted to the equation: $I(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$. Relative quantum yield was calculated by multiplying each by A-factor, and was normalized: $\Phi_i = A_i\tau_i / (A_1\tau_1 + A_2\tau_2)$. [6,2-TNS] = $3 \times 10^{-5}$ M, [TMCD] = 10 mM; Measured at 25 °C, pH 7.0, I = 0.1.

these weights, steady-state spectrum was resolved into two pure components (Fig. 3). By the resolved pure spectra we can estimate the micro-environment of each species.[8)] One, which emits fluorescence of shorter lifetime and of broader spectrum, resides in more polar environment, and the other, which emits fluorescence of longer lifetime and of narrower band, lies in less polar environment.

There are at least four possible ways of inclusion of 6,2-TNS with TMCD, because 6,2-TNS has two aromatic rings and TMCD has two open ends. Preliminary experiments on inhibition of TNS binding by adding complex-forming inhibitors[9)] supported two among these four possibilities. The prausible two are the ways putting TNS into only one of the two ends of TMCD cavity from

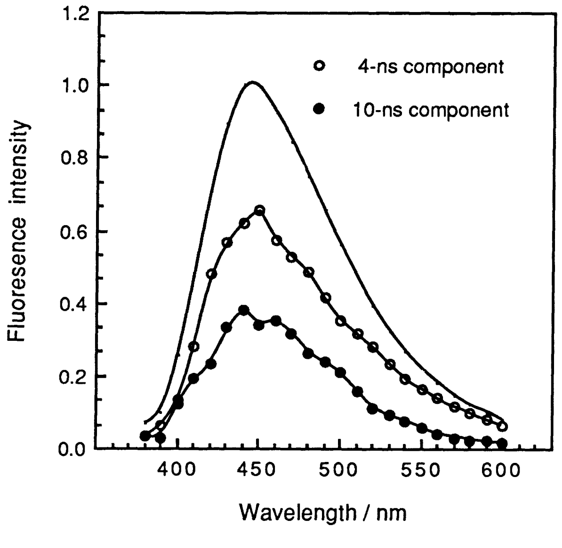

Fig. 3. Resolution of corrected steady-state fluorescence spectrum of 6,2-TNS in TMCD solution into two components. The ratio of the two components was calculated on the basis of the two-exponential decay curves obtained at each wavelength (Table 1).

either of two ends of TNS. Heterogeneity of the fluorescence was also observed for complex of TMCD with 8,1-ANS (8-phenylamino-1-naphthalenesulfonate).

Fluorescence decay for 6,2-TNS in α-CD solution followed a three-exponential curve (0.8, 3.7, and 10 ns). In the case of β-CD with 6,2-TNS, two components of different lifetimes (1.5 ns and 4 ns) were observed at the β-CD concentration of 2 mM. At a higher concentration of β-CD (10 mM), however, fluorescence of a 2:1 complex (lifetime of 11 ns) was added. Thus there is a possibility of 6,2-TNS making two different types of 1:1 complexes with β-CD prior to the formation of 2:1 complex. Failure of TMCD in forming 2:1 complex with 6,2-TNS must come from steric hindrance of the latent site on TNS for binding second host by the first-coming host.

In the course of this study, Kotake and Janzen published papers on bimodal inclusion of nitroxide radicals by β-CD studied by ESR and ENDOR.10) They found two different included species in solution, which is analogous to our results. Fluorescence spectroscopy has the advantage over ESR since it is applicable to more kinds of compounds than ESR, although poor separation of peaks of species in different environments and difficulty in quantitative determination of the ratio of the species by peak area calculation are the disadvantages. Fluorescence spectroscopy has potent power on the study of heterogeneity of the inclusion complexes in solution.

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7) Heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin is a gift from Nihon Shokuhin Kako Co., Ltd. Purity of the sample was checked by $^1H$ and $^{13}C$ NMR spectra.

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9) Addition of sodium 4-methylbenzenesulfonate or hexyltrimethylammonium bromide resulted in the decrease in the total fluorescence intensity without changing the mole ratio of the two types of complexes. This result implies that these inhibitors and 6,2-TNS compete for an identical binding site.

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