STUDY OF POLYSACCHARIDES BY THE FLUORESCENCE METHOD. V. INTERACTION OF 2-p-TOLUIDINYLNAPHTHALENE-6-SULFONATE WITH AMYLOSE AND ITS RELATED COMPOUNDS IN AQUEOUS SOLUTION

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ABSTRACT. The interaction of 2-p-toluidinylnaphthalene-6-sulfonate (TNS) with amylose and its related compounds in aqueous solution has been studied by both steady-state and transient fluorescence measurements. The fluorescence of TNS aqueous solution was enhanced by the addition of amylose, β-limit dextrin, and amylopectin. The fluorescence decay of TNS bound to these polysaccharides were well described as a sum of two-exponential functions. This suggests that there are two different microenvironments at the binding sites. The fluorescence lifetime of major component for TNS-amylose system agreed with that of major component for TNS-γ-cyclodextrin system. The mean rotational relaxation time of TNS bound to amylose is similar to that of the segmental motion of amylose chain. Based on these results, a configurational model for TNS-amylose complex has been proposed.

1. INTRODUCTION

2-p-Toluidinylnaphthalene-6-sulfonate (TNS) is virtually nonfluorescent in water, but becomes highly fluorescent when the dye is in a nonpolar microenvironment (1,2). This property has made TNS a popular structural probe for monitoring hydrophobic sites in proteins and membranes. Recently, Nakatani et al. have reported that the fluorescence of TNS is also enhanced by the presence of maltooligosaccharides, amylose, and other α-glucans (3). In this study, the interaction of TNS with amylose and its related compounds in aqueous solution investigated by measuring fluorescence spectra, excited lifetimes, and fluorescence polarizations. These measurements could offer information on the microenvironmental structure and local conformation of the site to which TNS is bound.

2. MATERIALS AND METHODS

2.1. Materials

The amylose was 'Avebe amylose', provided by Nichiden Kagaku Co. Ltd.,

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Osaka. This material was purified by recrystallization several times with butanol from aqueous solution. The crystalline residue was washed three times with ethanol, twice with ether to remove the butanol and dried for 2 days in vacuo over P_2O_5 at 70 °C. The viscosity-average molecular weight was found by viscosity measurements in dimethyl sulphoxide to be 3.9×10^5. Waxy maize starch, provided by Nichiden Kagaku Co. Ltd., Osaka, was used as amylopectin without further purifications. This material was found to have a branching structure with an average unit-chain length of 24 glucosidic residues by measuring the reducing power of the debranched products by Pseudomonas isoamylase (Hayashibara Biochemical Laboratories, Inc., Okayama). β-Limit dextrin was prepared by the digestion of the waxy maize starch with purified soybean β-amylase, hydrolyzing only the most outside branch chain. The β-amylolysis percentage was determined to be 54 % by measuring the reducing power of the digested mixture. The β-limit dextrin is considered to retain the outside branching points and the internal ramified structure of the starch molecule. α-, β-, and γ-Cyclodextrins used were those previously prepared and their purities were checked by TLC at this laboratory (4).

TNS was synthesized according to the method of McClure and Edelman (1). The purity was checked by TLC (Kiesel gel 60 F_{254}, Merck) using iso-butyl alcohol saturated with 3 % aqueous ammonia as a solvent, and was found to be a single spot.

2.2. Preparation of the Solution Measured

Aqueous amylose solutions were prepared by dissolving the amylose in 0.4 N KOH at 5 °C overnight, followed by neutralization with HCl of appropriate concentration. Appropriate amount of TNS stock solution (1 mM) was added to the amylose solution and the volume was adjusted with distilled water to the desired concentration of the solutes. Final concentrations of amylose, TNS, and KCl were 0.1 %, 5×10^{-5} M, and 0.2 M, respectively, unless otherwise specified. The measured solutions of β-limit dextrin and amylopectin were prepared by the same method as the preparation of amylose solution. In the case of cyclodextrins, they were dissolved directly in the KCl aqueous solution at pH 7.

In the pH profile experiments for the TNS-amylose system, the pH of the solution was controlled by changing the amount of HCl used to neutralize the alkaline amylose solution.

2.3. Fluorescence Measurements

Corrected emission spectra were measured using a Shimadzu spectrofluorometer (type RF-502). The excitation wavelength was 366 nm.

Time decays of fluorescence intensity were measured using a Ortec (9200) single-photon-counting nanosecond fluorometer (5). The nanosecond light pulses were provided by a free-running discharge in air. The half-width of the light pulse was about 2.5 ns. The excitation wavelength was selected to be 360-390 nm by a combination of optical filters. Fluorescence emission from a thermostatically controlled sample was

measured through cut-off filters that transmitted light above 450 nm. Excited lifetimes were calculated by curve-fitting analyses using a computer at Kyoto University (FACOM-M 200).

Fluorescence polarization measurements were made on the Shimadzu spectrofluorometer equipped with a pair of polarization filters as the polarizer and analyzer, as described previously (6). The excitation and emission wavelengths were 366 and 450 nm, respectively. The temperature of the solution in the cell was kept constant by a jacket thermostatically controlled to within an accuracy of ± 0.1 °C. The temperature was monitored by a calibrated thermistor probe immersed in the solution.

3. RESULTS AND DISCUSSION

3.1. Fluorescence Spectra

Figure 1 shows the fluorescence emission spectra of TNS aqueous solutions with amylose, β-limit dextrin, and amylopectin. The figure also includes the spectrum of TNS aqueous solution without these polysaccharides. The addition of these polysaccharides to TNS aqueous solution markedly enhances the fluorescence of TNS and sifts its maximum wavelength from 500 to 460 nm, suggesting the formation of TNS-polysaccharide complexes.

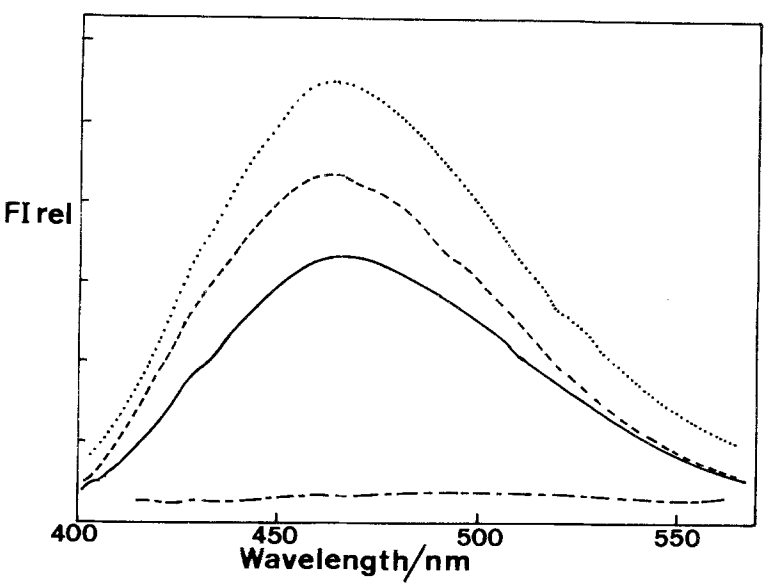

Figure 1. Corrected fluorescence spectra of TNS aqueous solutions with amylose (—), β-limit dextrin (- - -), and amylopectin (·····). (—·—); spectrum of TNS in 0.2 M KCl aqueous solution. The spectra were measured at 25 °C.

3.2. Fluorescence Lifetimes

Figure 2 shows the fluorescence decay curves of TNS aqueous solutions with amylose and amylopectin at 25 °C. The figure also shows the fitted decay curves for these experimental decays, respectively, which are given by the following equation

$$I(t) = \int_0^t P(t')F(t-t')dt' \qquad (1)$$

where $F(t)$ is the fluorescence intensity that would be observed after an infinitely short excitation, and $P(t)$ is the apparatus response function which is approximated by that of the exciting light pulse.

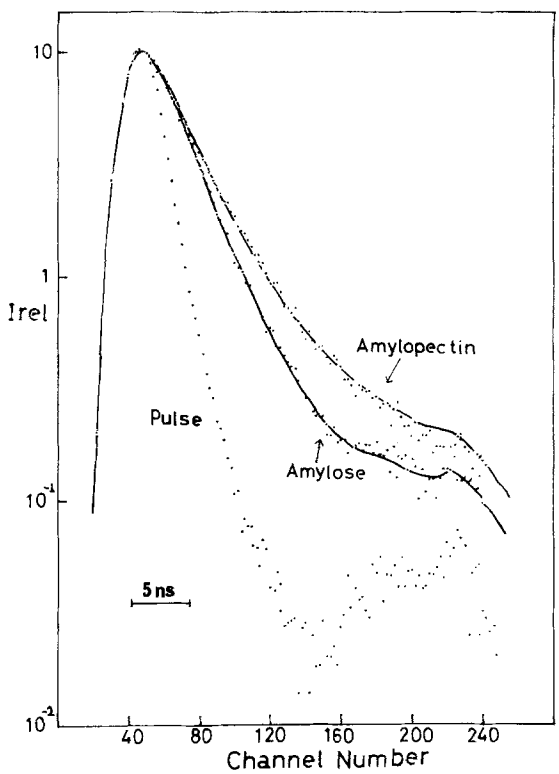

Figure 2. Fluorescence decays (····) of TNS aqueous solutions with amylose and amylopectin at 25 °C. Solid lines represent the best curves fitted on the assumption of two components of lifetime.

The calculated curves agree very well with the experimental curves. F(t) used in the study is

$$F(t) = \exp(-t/\tau_1) + A\exp(-t/\tau_2) \qquad (2)$$

where τ_1 and τ_2 are major and minor decay times, respectively, i.e., excited lifetimes of fluorescence and A is the relative amplitude of τ_2 to τ_1. The fact that the fluorescence decay of TNS in the presence of these polysaccharides is not single exponential suggests that TNS does not interact with only one kind of sites on the polysaccharide chains, but at least two kinds of sites.

Sets of decay parameters obtained for TNS aqueous solution with amylose and its related compounds, including α-, β-, and γ-cyclodextrins, are summarized in Table I. The values of lifetimes for three kinds of polysaccharides are similar , but the relative content of τ_2 increases in order of amylose, β-limit dextrin, and amylopectin. This result indicates that TNS fundamentally interacts with three kinds of polysaccharides in a similar manner, but there is a difference in the number of sites that strongly interact with TNS.

It has been shown that amylose forms inclusion complexes with iodine and many kinds of organic compounds in aqueous solution. Thus, the most probable model for the interaction of amylose with TNS is an inclusion complex. Since cyclodextrins form inclusion complexes with TNS and enhance TNS fluorescence in aqueous solution (7), it is interesting to compare fluorescence decay parameters for TNS-amylose system with those for TNS-cyclodextrin systems. The values of lifetimes for amylose are the same as those for γ-cyclodextrin. However, the relative content of the long lifetime for amylose is much smaller than that for γ-cyclodextrin. γ-Cyclodextrin is expected to form both 1:1

TABLE I. Fluorescence lifetimes of TNS aqueous solutions with various carbohydrates at 25 °C [a]

Carbohydrates	τ_1/ns	τ_2/ns	A [b]
Amylose	2.5	8.0	0.05
β-Limit dextrin	2.5	8.0	0.11
Amylopectin	2.5	8.0	0.15
α-Cyclodextrin	0.8	—	—
β-Cyclodextrin	1.7	—	—
γ-Cyclodextrin	2.5	8.0	0.40

[a] Fluorescence lifetimes were computed by the curve fitting of fluorescence decays, assuming two components of decay terms (τ_1 and τ_2).

[b] The relative amplitude of τ_2 to τ_1, bars represent an amplitude of less than 0.01.

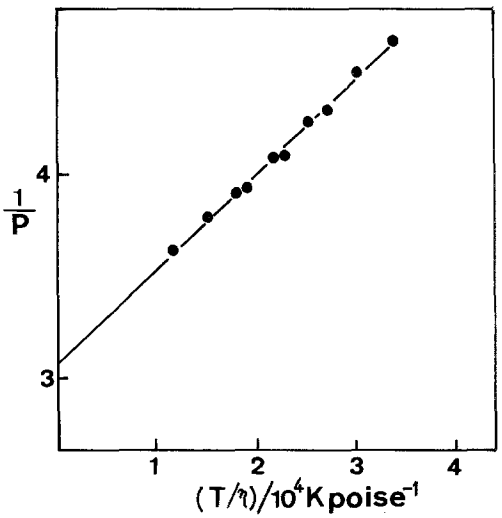

Figure 3. Plot of 1/P vs. T/η for TNS aqueous solution with amylose at 25 °C. The viscosity was varied by the addition of sucrose. T; absolute temperature, η ; the solvent viscosity.

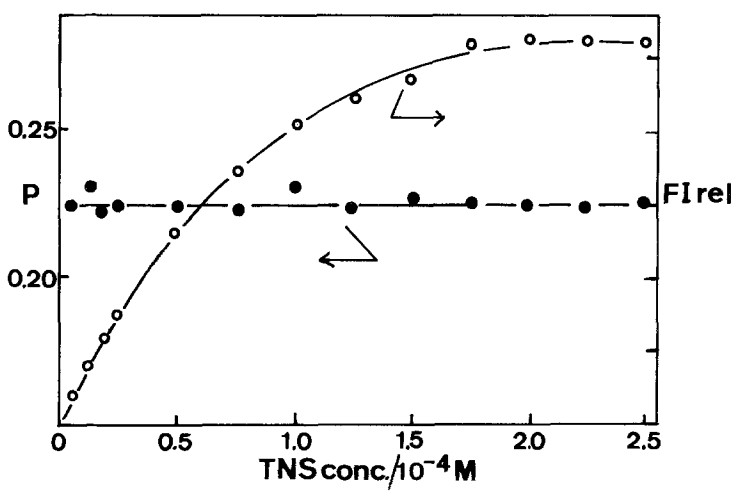

Figure 4. Fluorescence intensity (o) and polarization (●) of TNS aqueous solution with 0.1 % amylose as a function of the TNS concentration at 25 °C.

and 2:1 inclusion complexes with TNS at the experimental condition. Both of the toluidinyl group and the naphthalene ring of TNS can interact with the γ-cyclodextrin cavity. First, the toluidinyl group can be included in the γ-cyclodextrin cavity (1:1 complex), and then the naphthalene ring associated with the second cyclodextrin molecule to form the 2:1 complex (7). It can be considered that fluorescences with τ_1 and τ_2 were emitted from the 1:1 and 2:1 complexes, respectively, since the quantum yield of the 1:1 complex is smaller than that of the 2:1 complex (7). Considering the small value of A for amylose, it can be said that most of the binding site of TNS with amylose has a similar microenvironment to that of the γ-cyclodextrin-TNS 1:1 complex.

3.3. Fluorescence Polarization of the TNS-Amylose Complex

The degree of fluorescence polarization, P, of TNS aqueous solution with amylose was measured with changing solvent viscosity at constant temperature (25 °C). Figure 3 shows Perrin plot constructed from the data obtained. Since the plot is linear, the mean rotational relaxation time, $\langle\rho\rangle$, can be calculated using the following equation (6, 8)

$$\left(\frac{1}{P}-\frac{1}{3}\right) \approx \left(\frac{1}{Po'}-\frac{1}{3}\right)\left(1+\frac{3\tau}{\langle\rho\rangle}\right) \qquad (3)$$

where 1/Po' is the ordinate intercept obtained by extrapolating the linear plot, τ is the fluorescence lifetime. Using the average τ value for the TNS-amylose system at 25 °C (See Table II.), we calculated the $\langle\rho\rangle$ value to be 15 ns in 0.2 M KCl aqueous solution at 25 °C. This value is similar to that for the segmental motion of amylose chain which has been determined using the fluorescein conjugate of amylose (6, 9, 10).

Figure 4 shows the dependences of the fluorescence intensity and polarization for TNS-amylose system on the concentration of TNS. It is seen, in contrast to the fluorescence intensity, that the fluorescence polarization of the solution is constant over the range of TNS concentration examined. This fact suggests that the molecular chain of amylose does not undergo any great conformational change by the formation of complex with TNS.

3.4 Effect of Temperature

The effect of temperature on the fluorescence intensity and polarization for TNS-amylose system is shown in Figure 5. The fluorescence intensity decreases with increasing temperature. Table II shows the fluorescence lifetimes for TNS-amylose system that were determined at 25, 40, and 55 °C. The fluorescence intensity is found to decrease proportionally with decreasing the average lifetime. Thus, the decrease in fluorescence intensity can be ascribed to the change in microenvironmental properties at binding sites of TNS, but not to the decreased amount of binding TNS.

The fluorescence polarization also decreases with increasing temperature, and the decrease becomes more steep above 40 °C. The thermally induced changes in the fluorescence properties may reflect

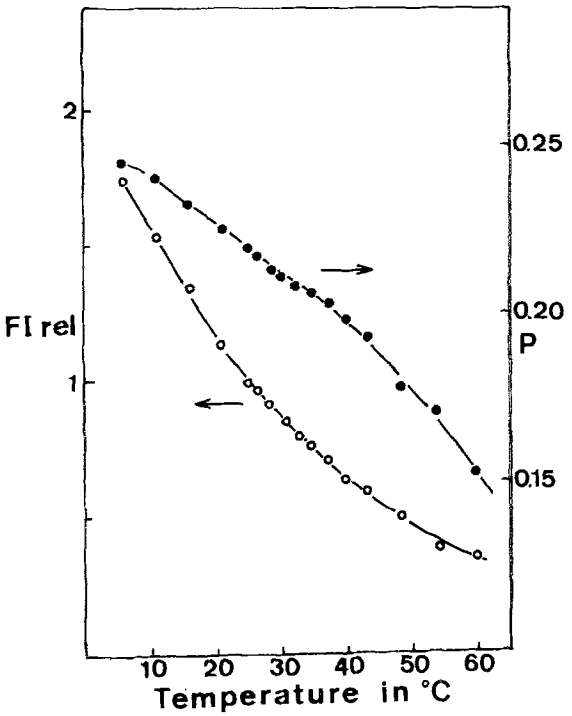

Figure 5. Effect of temperature on the fluorescence intensity (o) and polarization (●) of TNS aqueous solution with amylose.

TABLE II. Effect of temperature on the fluorescence lifetime of TNS-amylose complex

Temperature/°C	τ_1/ns	τ_2/ns	A	$\bar{\tau}$ [a]
25	2.5	8.0	0.05	2.76
40	1.5	6.5	0.03	1.65
55	1.2	5.0	0.03	1.31

[a] Average lifetime calculated by the equation;
$\bar{\tau} = (\tau_1 + A\,\tau_2)/(1+A)$.

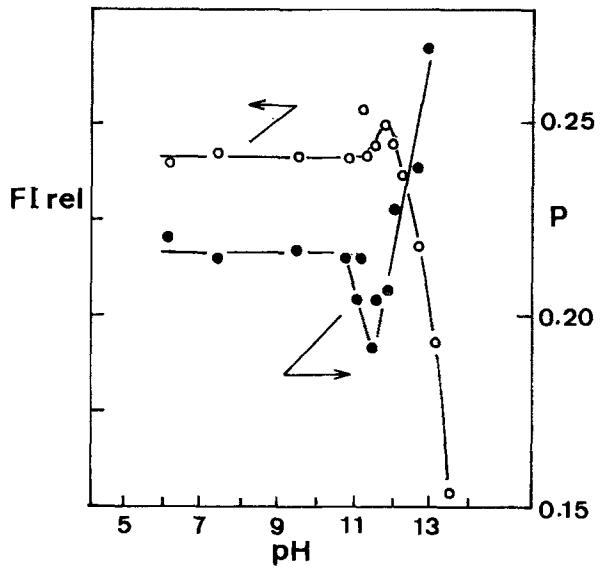

Figure 6. Effect of pH on the fluorescence intensity (o) and polarization (●) of TNS aqueous solution with amylose at 25 °C.

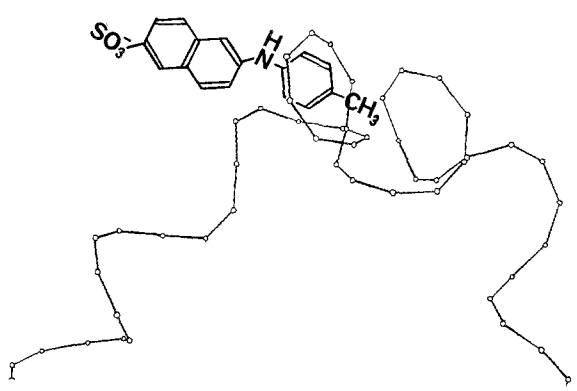

Figure 7. A model for the TNS-amylose complex. Circles represent glycosidic oxygens,and lines are virtual bonds.

the gradual change of the amylose conformation accompanying the disruption of its helical structure with increasing temperature (6, 9, 11).

3.5 Effect of pH

Figure 6 shows the pH dependences of the fluorescence intensity and polarization for TNS-amylose system. The fluorescence intensity maintains a steady level until the pH reaches 11, and then increases slightly until it reaches a peak at pH 12 before it decreases again at higher pH. The fluorescence polarization is also seen to remain constant until pH reaches 11, and then decrease steeply until pH 12. The polarization increases again at higher pH.

It has been shown that amylose in an aqueous solution exhibits considerable changes in intrinsic viscosity and optical rotation in the region of pH between 11 and 13, and this behavior has been interpreted in terms of the conformational change caused by increasing negative charges on the polymer chain (12-14). We can conclude that the changes in fluorescence intensity and polarization observed here are due to the same conformational transition of amylose.

3.6. A Model for TNS-Amylose Complex

We propose a configurational model for the TNS-amylose complex illustrated schematically in Figure 7 in order to interpret the results obtained by the fluorescence measurements. Amylose behaves as a random coil with significant sequences of pseudohelical backbone (11, 15, 16), and the toluidinyl group of TNS is included in the helical loop of the amylose chain. The microenvironment of the helical loop at binding sites is similar to that of γ-cyclodextrin cavity. This model can be also applied to TNS-β-limit dextrin and -amylopectin systems. However, the proposed model does not explain the stronger interaction of the polysaccharides with TNS which has been suggested by the presence of the long lifetime in the fluorescence decays. Interpretation of this minor component of the lifetime observed for TNS-polysaccharides systems is a continuing subject for further investigations.

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