Fluorescence Spectroscopic Study of Subtilisins as Relevant to Their Catalytic Activity in Aqueous–Organic Media

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The fluorescence spectra of subtilisin Carlsberg (STC) and subtilisin BPN′ (STB) were measured in aqueous–organic mixed solvents over a wide range of solvent composition. The tryptophan fluorophores were used as probes of the environmental polarity, and the structural changes of the enzymes were detected as changes of the difference in the emission wavelength between the enzymes and *N*-acetyl-L-tryptophan ethyl ester ($\Delta\lambda_{em}$). It was found that $\Delta\lambda_{em}$ was well correlated with the catalytic activity of the enzymes, as expressed by the hydrolysis rate of *N*-acetyl-L-tyrosine ethyl ester. For example, the characteristic dependency of the activity of STC on the solvent composition in acetonitrile–water was closely related to the change in $\Delta\lambda_{em}$. Similar correlations between $\Delta\lambda_{em}$ and the catalytic activity were observed for subtilisins in aqueous 1,4-dioxane, THF, DMF, DMA, and DMSO solutions with high correlation coefficients, except for STB in water–DMF and –DMA. The results are discussed in terms of the structural modification of the enzymes in these aqueous–organic media.

Recently, applications of enzymatic reactions to organic synthesis have attracted much attention owing to their high stereo- and regiospecificity, as compared to chemical processes. Although most enzymes are insoluble in organic solvents, it is now well recognized that enzymes can catalyze many reactions in organic solvents with restricted amounts of water.[1] For example, proteases have been utilized in organic solvents for the synthesis of esters[2—10] or peptides[11—16] and for the optical resolution of chiral compounds.[11,17—20]

Recent studies have revealed that both the activity and the specificity of enzymes are markedly affected by the nature of the organic solvent. There can be significant changes in the activity and specificity of an enzyme by switching from one solvent to another.[21—24] Enzyme catalysis is also a function of the water content in the reaction media. Not only the activity, but also the substrate specificity and enantiospecificity of enzymes, can be altered by changing the water content.[25]

It may be assumed that changes in the enzyme activity are partly due to structural modifications of the enzymes. However, since most of the reactions of interest are catalyzed by enzymes suspended in aqueous–organic media or immobilized to insoluble support materials, it is difficult to detect the possible structural changes of the enzymes by ordinary physico-chemical methods, such as NMR and CD. We previously reported that the fluorescence wavelength of α-chymotrypsin is closely related to its catalytic activity in aqueous–organic media.[26] It may be reasonably assumed that any structural modification of an enzyme due to an organic solvent would cause changes in the emission wavelength of the enzyme due to changes in the microenvironment of the tryptophan residues in the enzyme. The catalytic activity would also be altered by a structural modification of the enzymes. In this article we report on the results of a fluorescence spectroscopic study of subtilisins as relevant to their catalytic activity in aqueous–organic media. So far, although several reports have appeared concerning the fluorescence spectroscopy of enzymes in organic solvents,[27—32] the correlations between the activity and the spectral characteristics have rarely been investigated.

Experimental

Materials. Subtilisin Carlsberg (protease type 8, EC 3.4.21.14) (STC) and subtilisin BPN′ (protease type 27, EC 3.1.1.3) (STB) were purchased from Sigma Chem. Co. *N*-Acetyl-L-tyrosine ethyl ester (ATEE) and *N*-acetyl-L-tryptophan ethyl ester (ATrEE) were also purchased from Sigma. Organic solvents of guaranteed grade were obtained from Wako Pure Chem. Co. and dried on molecular sieves (3A).

Fluorescence Spectra. Steady state fluorescence spectra of ATrEE and enzymes were taken in aqueous–organic mixed solvents on a Shimadzu RF-5000 instrument with an excitation wavelength of 295 nm and a band width of 5 nm. For subtilisins, the samples contained 2.5 mg of an enzyme in 10 ml of a solvent; they were magnetically stirred in a temperature-controlled (30 °C) cell. The maximum emission wavelength of a sample was determined as an average of more than five measurements. The $\Delta\lambda_{em}$ is defined as the difference between the maximum wavelength of ATrEE and the enzyme,

$$\Delta\lambda_{em} = \lambda^{ATrEE} - \lambda^{E}. \quad (1)$$

It was found that the emission wavelengths of STC and STB are time-dependent, especially at high water contents, probably due to autolysis of the enzymes. Therefore, the fluorescence spectra were taken shortly after the preparation of enzyme solutions.

Kinetic Measurement of ATEE Hydrolysis. A solution of ATEE in a mixed solvent of water and an organic solvent was added to an aqueous solution of an enzyme (2.5 mg). The total reaction volume was 10 ml, and the final ATEE concentration was 10 mM (1 M=1 mol dm^{-3}). The mixture was incubated at 30 °C with reciprocal shaking (about 150 cycles per min). At intervals, samples were taken and filtered by poly (tetrafluoroethylene) membrane filters, and the filtrates were injected into HPLC (Shimadzu LC-6A). A Shim-pack CLC-ODS column (0.15 m×6.0 mm) was used and eluted with water–acetonitrile (50/50 by volume).

Acetanilide was used as an internal standard, and reaction components were detected with a UV detector at 270 nm. The initial reaction rate of ATEE hydrolysis was calculated from the initial increase in the amount of *N*-acetyl-L-tyrosine (AT).

Results and Discussion

In general, both tryptophan and tyrosine residues are responsible for the fluorescence of proteins; the maximum emission wavelength is a function of the excitation wavelength. STC has only one tryptophan residue (Trp-113) and 13 tyrosine residues, while STB has 3 tryptophan residues (Trp-106, 113, and 241) and 10 tyrosine residues.[33] However, it is known that the fluorescence from tryptophan residues generally predominates over that from tyrosine residues; the contribution from the latter is negligibly small, probably due to quenching or energy transfer.[34] An exception is STC, which exhibits fluorescence from tyrosine residues.[35—37] However, it has been reported that the contribution from tyrosine residues is very small when the enzyme is excited at or above 295 nm.[36—38] Therefore, in the present work, the fluorescence spectra were measured with an excitation wavelength of 295 nm in order to minimize the complexity arising from the contributions from tyrosine residues.

It is known that both the intensity and wavelength of the fluorescence of a tryptophan derivative are sensitive to the polarity of the solvent. In general, the emission shifts to a higher wavelength along with an increase in the polarity of the solvent. An example is shown in Fig. 1 for *N*-acetyl-L-tryptophan ethyl ester (ATrEE) in acetonitrile–water, where the emission wavelength increases along with an increase in the water content. Since the dielectric constant of water (80.4 at 20 °C) is larger than any of the organic solvents used in this work, similar dependencies of the emission wavelength of ATrEE on the solvent composition were observed for all of the organic solvent–water systems. Therefore, it was assumed that a shift in the emission of an enzyme to a higher wavelength is an indication of an increase in the polarity of the microenvironment of the tryptophan residues, and any structural changes of the enzyme can be detected by measuring the fluorescence wavelength.

Acetonitrile. The emission wavelengths of STC and STB were plotted against water content in acetonitrile–water mixed solvents as compared to the emission wavelength of ATrEE (Fig. 1). In this study, pure water was used as a cosolvent instead of buffer solutions in order to avoid any complexity arising from possible interactions between the enzymes and ionic species of the buffer components. Also, complexity may arise from the precipitation of buffer salts at high concentrations of the organic solvents. The maximum emission wavelengths of STC and STB in pure water were 330 and 346 nm, respectively, which were close to the emission wavelengths in buffer solutions.[37,39] This suggests that no significant modification of the enzyme structure occurs due to using pure water instead of buffer solutions.

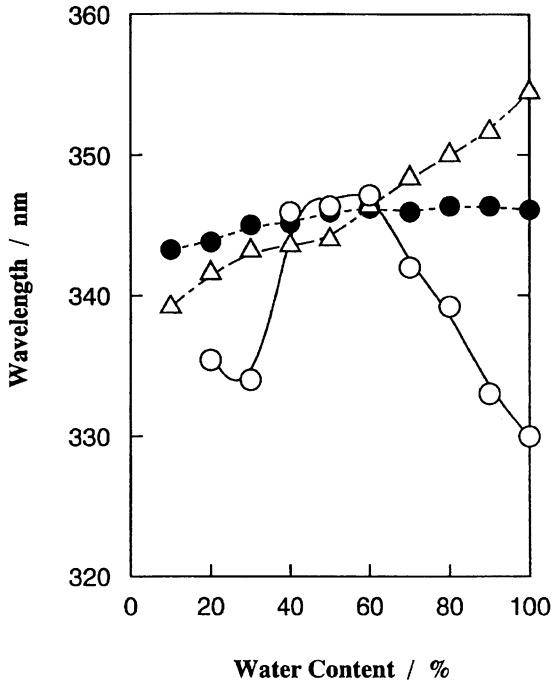

Fig. 1. Fluorescence wavelength of (○) STC, (●) STB, and (△) ATrEE in acetonitrile–water.

It can be seen from Fig. 1 that the emission wavelength of STC is very different from that of ATrEE, and is strongly dependent on the solvent composition. It may be assumed that changes in the enzyme structure, such as unfolding of the peptide chains or swelling of the enzyme by the solvent, would cause changes in the polarity of the microenvironment of the tryptophan residues, leading to shifts in the emission wavelength of the enzyme. The changes in the polarity may be associated with conformational changes of the enzyme or the solvent composition around the tryptophan residues. The latter is important when tryptophan residues are exposed to a solvent, like those in STB.[40,41] Therefore, the shifts in the emission wavelength of the enzyme and ATrEE by changing from one solvent composition to another may be expressed by

$$\Delta\lambda^{E} = \Delta\lambda^{E}(\mathrm{str}) + \Delta\lambda^{E}(\mathrm{solv}) \quad (2)$$

and

$$\Delta\lambda^{\mathrm{ATrEE}} = \Delta\lambda^{\mathrm{ATrEE}}(\mathrm{solv}), \quad (3)$$

respectively, where $\Delta\lambda(\mathrm{str})$ and $\Delta\lambda(\mathrm{solv})$ represent the contributions due to changes in the structure and solvent composition, respectively. Therefore,

$$-\Delta\Delta\lambda_{\mathrm{em}} = \Delta\lambda^{E} - \Delta\lambda^{\mathrm{ATrEE}} = \Delta\lambda^{E}(\mathrm{str}) + \left\{\Delta\lambda^{E}(\mathrm{solv}) - \Delta\lambda^{\mathrm{ATrEE}}(\mathrm{solv})\right\}. \quad (4)$$

Although a change in the solvent composition differently affects the emission wavelength, depending on the location of the tryptophan residues in the enzymes, the

second term in Eq. 4 minimizes the direct solvent effects on the emission wavelength.

Any changes in the enzyme structure around the tryptophan residue would affect the catalytic activity of the enzyme. It was therefore considered that the changes in $\Delta\lambda_{em}$ would be correlated with the enzyme activity. In Fig. 2, $\Delta\lambda_{em}$ and the rate of catalytic hydrolysis of ATEE by STC are plotted against the water content in acetonitrile–water. It can be seen that there is a good correlation between $\Delta\lambda_{em}$ and the hydrolysis rate. The result seems to indicate that the enzyme is more active when the tryptophan residue in STC is in a less-polar (more hydrophobic) environment, as compared to the bulk solvent phase. Especially, at water contents of 40—60%, both $\Delta\lambda_{em}$ and the catalytic activity of STC were very low, and by decreasing water content to 20—30%, part of the activity was recovered along with an increase in $\Delta\lambda_{em}$.

In contrast to STC, the emission of STB shifted to a lower wavelength along with a decrease in the water content (Fig. 1), though there is a parallel relation between $\Delta\lambda_{em}$ and the hydrolysis rate of ATEE by STB (Fig. 2). The change in the activity of the enzyme may be at least partly due to a structural change of the enzyme. Similarly to STC, STB exhibited higher activities at solvent compositions, which give a higher $\Delta\lambda_{em}$.

It should be noted that the low values of $\Delta\lambda_{em}$ indicate that the environmental polarities are similar for the tryptophan fluorophores in and out of the enzyme molecules. Therefore, as mentioned above, it may be assumed that, at these solvent compositions, some structural changes of the enzymes occur which increase the polarity of the microenvironment of the tryptophan residues. The tryptophan residues may be exposed to the bulk solvent phase due to conformational changes of the enzymes, or the tryptophan residues are in a specific environment of high polarity due to the polar groups in the enzymes. A modification of the enzyme structure would lead to a retardation of the enzyme activity.

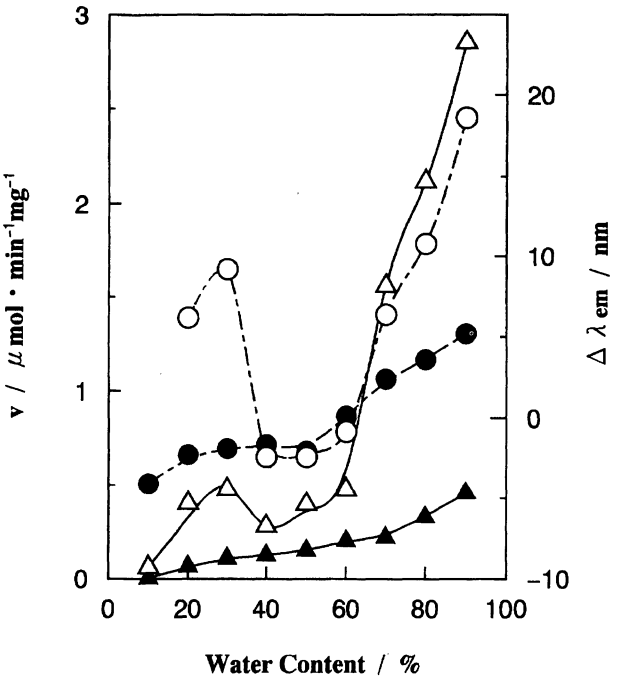

Fig. 2. Hydrolysis rate of ATEE (v) and $\Delta\lambda_{em}$ in acetonitrile–water. △: hydrolysis rate by STC; ○: $\Delta\lambda_{em}$ of STC; ▲: hydrolysis rate by STB; ●: $\Delta\lambda_{em}$ of STB.

It is known that although the enzymatic behaviors in aqueous solutions are similar for STC and STB,[42,43] the activities of the enzymes are significantly different in ethanol.[3] As shown in Figs. 1 and 2, the activity and fluorescence of the enzymes are different for STC and STB in acetonitrile–water. Both $\Delta\lambda_{em}$ and the activity of STC decrease at water contents of 40—60%. Similar profiles have been shown for α-chymotrypsin in acetonitrile–water.[26] It may be worth mentioning that these enzymes recover catalytic activity at 10—30% water, and that this has been utilized for preparative reactions of esters and peptides. In contrast, the activity of STB is very low at low water contents. This may be attributed to a difference in the surface properties of the enzymes; the amino acid residues of the STC and STB are different at 84 positions out of which 75 point outward.[44]

1,4-Dioxane and THF. The ether linkages provide 1,4-dioxane and THF with a hydrophilic nature and water-miscibility; these solvents have been utilized for some enzymatic reactions, including ester or peptide synthesis by proteases. As shown in Fig. 3, there are parallel relations between $\Delta\lambda_{em}$ and the activity of STC and STB in 1,4-dioxane–water. Similar dependencies of $\Delta\lambda_{em}$ and the activity on the solvent composition were observed for these enzymes in THF–water, as shown in Fig. 4.

DMF and DMA. DMF and DMA are useful solvents for many synthetic reactions, because they have a strong solubility power for many polar compounds, such as amino acid derivatives, which are insoluble in nonpolar organic solvents. Although DMF or DMF–water mixed solvents have been used for some enzymatic reactions, problems are often encountered due to an instability and low catalytic activity of enzymes in DMF at low water contents. The half-life of STB in anhydrous DMF was reported to be about 30 min.[11]

The catalytic activity of STC in DMF–water was strongly dependent on the solvent composition (Fig. 5), and the enzyme was deactivated at water contents below 20%. A similar tendency was observed for the activity in DMA–water (Fig. 6). Again, there seems to be a correlation between the catalytic activity and $\Delta\lambda_{em}$. The activity of STB also decreased at low water contents in both DMF– and DMA–water mixtures. However, the $\Delta\lambda_{em}$ increased inconsistently along with a decrease in the activity at water contents below 30%, leading to a relatively poor correlation between $\Delta\lambda_{em}$ and the activity at these solvent compositions.

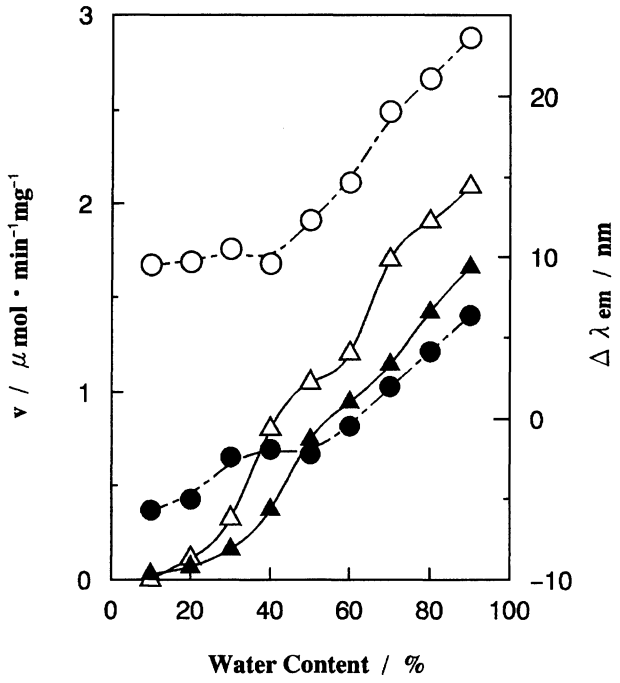

Fig. 3. Hydrolysis rate of ATEE (v) and $\Delta\lambda_{em}$ in 1,4-dioxane–water. Symbols are the same as in Fig. 2.

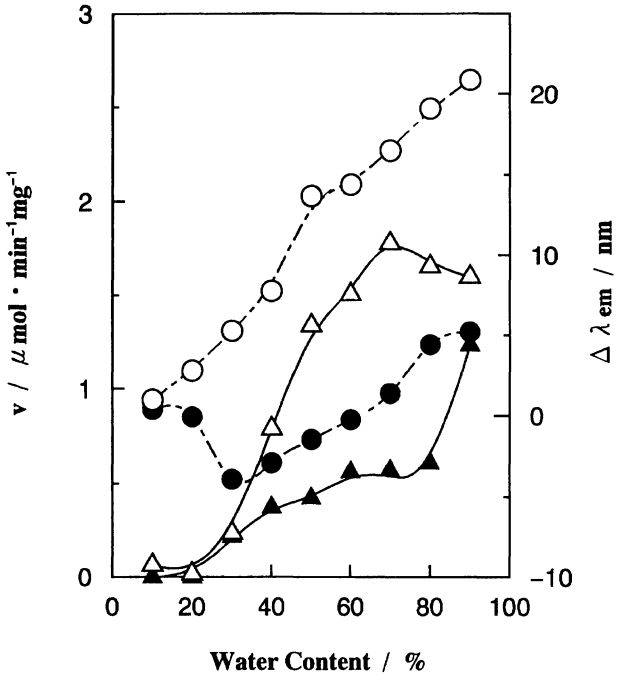

Fig. 5. Hydrolysis rate of ATEE (v) and $\Delta\lambda_{em}$ in DMF–water. Symbols are the same as in Fig. 2.

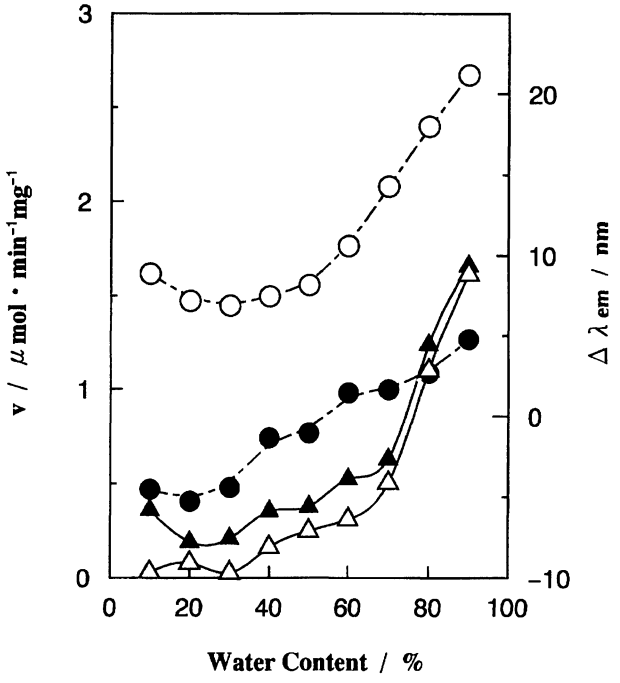

Fig. 4. Hydrolysis rate of ATEE (v) and $\Delta\lambda_{em}$ in THF–water. Symbols are the same as in Fig. 2.

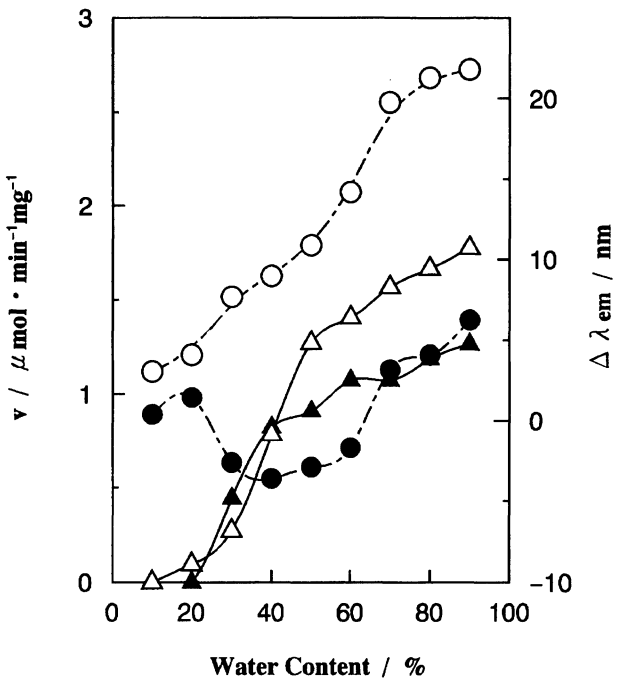

Fig. 6. Hydrolysis rate of ATEE (v) and $\Delta\lambda_{em}$ in DMA–water. Symbols are the same as in Fig. 2.

DMSO. Although DMSO is also one of the promising solvents for enzymatic reactions of polar substrates, it has been reported that it can deactivate enzymes by unfolding the peptide chains of the enzymes.[45] Figure 7 shows that the activity of both STC and STB decreased parallel to the $\Delta\lambda_{em}$ along with a decrease in the water content.

Characteristics of Fluorescence Properties of STC and STB. There are several characteristic points worth mentioning concerning the fluorescence properties of STC and STB in the aqueous–organic media studied in this work. Firstly, the dependencies of the emission wavelength, and therefore $\Delta\lambda_{em}$, on the solvent composition are much stronger for STC than for STB. For example, the emission wavelength of STC in acetonitrile–water changed by as much as 18 nm

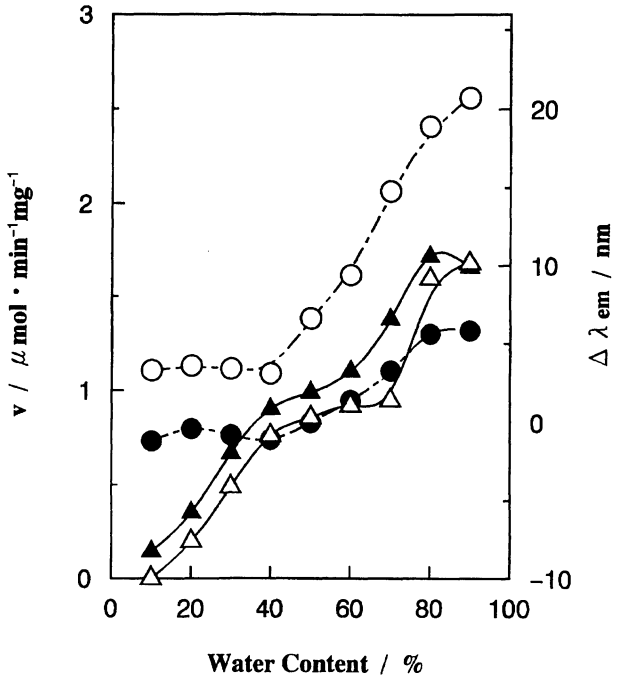

Fig. 7. Hydrolysis rate of ATEE (v) and $\Delta\lambda_{em}$ in DMSO–water. Symbols are the same as in Fig. 2.

along with a change in the solvent composition, while the change in the wavelength of STB was less than 8 nm (Fig. 1). This situation is similar for other cosolvent systems. It has been reported that the emission of STB is mainly from Trp-106 and Trp-241, and that these residues are located near to the surface of the enzyme, and are partly exposed to the solvent.[37,40,41] This may illustrate small changes and a similar solvent dependency of the emission wavelength of STB to that of ATrEE. On the contrary, the relatively large variation in the emission wavelength, as well as the low wavelength in neat water, suggests the hydrophobic environment of the Trp-113 in STC. This seems to suggest that the solvent-dependence of the emission wavelength of STC is primarily attributed to structural changes of the enzyme molecule, rather than a direct interaction with the solvent.

Secondly, the $\Delta\lambda_{em}$ of STC were almost always positive and larger than those of STB. This may also support the above consideration concerning the hydrophobic environment of the tryptophan residue in STC. Thirdly, the $\Delta\lambda_{em}$ of STB always decreased along with a decrease in the water content, and became negative below a water content of 50—65%. The negative value of $\Delta\lambda_{em}$ may be an indication of an effect of surrounding polar groups in the enzyme on the microenvironment of the tryptophan residues.

Correlation between $\Delta\lambda_{em}$ and Activity of Enzymes. The extent of linear correlations between $\Delta\lambda_{em}$ and the catalytic activity of STC and STB, as expressed by the hydrolysis rate of ATEE, was estimated by applying the data in Figs. 2, 3, 4, 5, 6, and 7 to

Table 1. Correlation between $\Delta\lambda_{em}$ and Hydrolysis Rate of ATEE[a)]

Solvent	a		b		r	
	STC	STB	STC	STB	STC	STB
Acetonitrile	0.11	0.043	0.44	0.19	0.83	0.96
1,4-Dioxane	0.13	0.15	−0.90	0.78	0.94	0.97
THF	0.072	0.12	−0.48	0.70	0.75	0.86
DMF	0.13	0.081	−0.27	0.42	0.93	0.65
DMA	0.11	0.044	−0.27	0.74	0.97	0.31
DMSO	0.071	0.17	0.17	0.75	0.90	0.90

a) Parameters in Eq. 5 calculated form data in Figs. 2, 3, 4, 5, 6, and 7.

$$v = a \cdot \Delta\lambda_{em} + b. \quad (5)$$

The constants (a and b) were determined by a least-squares method, and are listed in Table 1. The correlation coefficients (r) are also included in Table 1. It can be seen that there are fairly good correlations between $\Delta\lambda_{em}$ and the activity for both STC and STB.

The present results suggest that the fluorescence properties of STC and STB in aqueous–organic media are different for each other, but that the emission wavelengths of these enzymes are well correlated to their catalytic activity. On the basis of the assumption that the difference between the emission wavelengths of ATrEE and the enzyme is a measure of the structural change of the enzymes, the results seem to indicate that changes in the catalytic activity can be at least partly ascribed to conformational changes of the enzymes.

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References

1) P. J. Halling, *Enzyme Microb. Technol.*, **16**, 178 (1994), and references cited therein.
2) R. S. Phillips, M. S. Matthews, E. Olson, and R. L. Von Tersch, *Enzyme Microb. Technol.*, **12**, 731 (1990).
3) H. Kise, *Bioorg. Chem.*, **18**, 107 (1990).
4) E. N. Vulfson, G. Ahmed, I. Gill, P. W. Goodenough, I. A. Kozlov, and B. A. Law, *Biotechnol. Lett.*, **12**, 597 (1990).
5) H. Kise and A. Hayakawa, *Enzyme Microb. Technol.*, **13**, 584 (1991).
6) R. M. Blanco, J. M. Guisan, and P. J. Halling, *Biotechnol. Bioeng.*, **40**, 1092 (1992).
7) S. Chatterjee and A. J. Russell, *Biotechnol. Bioeng.*, **40**, 1069 (1992).
8) S. Cai, S. Hakomori, and T. Toyokuni, *J. Org. Chem.*, **57**, 3431 (1992).
9) E. Wehtje, P. Adlercreutz, and B. Mattiasson, *Biotechnol. Bioeng.*, **41**, 171 (1993).
10) P. Lozano, D. Combes, J. L. Iborra, and A. Manjon, *Biotechnol. Lett.*, **15**, 1223 (1993).
11) C. -H. Wong, S. -T. Chen, W. J. Hennen, J. A. Bibbs, Y. -F. Wang, J. L. -C. Lium, M. W. Pantoliano, M. Whitlow,

and P. N. Bryan, *J. Am. Chem. Soc.*, **112**, 945 (1990).
12) Y. Kimura, K. Nakanishi, and R. Matsuno, *Enzyme Microb. Technol.*, **12**, 272 (1990).
13) P. Clapes, P. Adlercreutz, and B. Mattiasson, *J. Biotechnol.*, **15**, 323 (1990).
14) V. Kasche, G. Michaelis, and B. Galunsky, *Biotechnol. Lett.*, **13**, 75 (1991).
15) A. O. Richards, I. S. Gill, and E. N. Vulfson, *Enzyme Microb. Technol.*, **15**, 928 (1993).
16) C. -H. Wong, M. Achuster, P. Wang, and P. Sears, *J. Am. Chem. Soc.*, **115**, 5893 (1993).
17) S. -T. Chen, S. -Y. Chen, S. -C. Hsiao, and K. -T. Wang, *Biotechnol. Lett.*, **13**, 773 (1991).
18) A. L. Gutman, E. Meyer, E. Kalerin, F. Polyak, and J. Sterling, *Biotechnol. Bioeng.*, **40**, 760 (1992).
19) Y. Tomiuchi, K. Ohshima, and H. Kise, *Bull. Chem. Soc. Jpn.*, **65**, 2599 (1992).
20) T. Kijima, K. Ohshima, and H. Kise, *J. Chem. Technol. Biotechnol.*, **59**, 61 (1994).
21) P. A. Fitzpatrick and A. M. Klibanov, *J. Am. Chem. Soc.*, **113**, 3166 (1991).
22) K. Nakamura, Y. Takebe, T. Kitayama, and A. Ohno, *Tetrahedron Lett.*, **32**, 4941 (1991).
23) T. Nagashima, A. Watanabe, and H. Kise, *Enzyme Microb. Technol.*, **14**, 842 (1992).
24) C. R. Wescott and A. M. Klibanov, *J. Am. Chem. Soc.*, **115**, 1629 (1993).
25) H. Kise and T. Nagashima, *Bull. Chem. Soc. Jpn.*, **66**, 3693 (1993).
26) Y. Tomiuchi, T. Kijima, and H. Kise, *Bull. Chem. Soc. Jpn.*, **66**, 1176 (1993).
27) S. Barbaric and P. L. Luisi, *J. Am. Chem. Soc.*, **103**, 4239 (1981).
28) P. D. Compton, R. J. Coll, and A. L. Fink, *J. Biol. Chem.*, **261**, 1248 (1986).
29) P. Pasta, S. Riva, and G. Carrea, *FEBS Lett.*, **236**, 329 (1988).
30) V. V. Mozhaev, Y. L. Khmelnitsky, M. V. Sergeeva, A. B. Belova, N. L. Klyachko, A. V. Levashov, and K. Martinek, *Eur. J. Biochem.*, **184**, 597 (1989).
31) M. M. Fernandez, D. S. Clark, and H. W. Blanch, *Biotechnol. Bioeng.*, **37**, 967 (1991).
32) R. M. Guinn, H. W. Blanch, and D. S. Clark, *Enzyme Microb. Technol.*, **13**, 320 (1991).
33) E. L. Smith, F. S. Markland, C. B. Kasper, R. J. DeLange, M. Landon, and W. H. Evans, *J. Biol. Chem.*, **241**, 5974 (1966).
34) F. W. J. Teale, *Biochem. J.*, **76**, 381 (1960).
35) K. J. Willis and A. G. Szabo, *Biochemistry*, **28**, 4902 (1989).
36) K. J. Willis, A. G. Szabo, J. Drew, M. Zuker, and M. Ridgeway, *Biophys. J.*, **57**, 183 (1990).
37) N. Genov, P. Nicolov, C. Betzel, K. Wilson, and P. Dolashka, *J. Photochem. Photobiol. B: Biol.*, **18**, 265 (1993).
38) J. M. Janot, A. Beeby, P. M. Bayley, and D. Phillips, *Biophys. Chem.*, **41**, 277 (1991).
39) P. M. Bayley, J. M. Janot, and S. R. Martin, *FEBS Lett.*, **250**, 389 (1989).
40) J. Drenth, W. G. J. Hol, J. N. Jansonius, and R. Koekoek, *Eur. J. Biochem.*, **26**, 177 (1972).
41) R. Bott, M. Ultsch, A. Kossiakoff, T. Graycar, B. Katz, and S. Power, *J. Biol. Chem.*, **263**, 7895 (1988).
42) A. O. Barel and A. N. Glazer, *J. Biol. Chem.*, **243**, 1344 (1968).
43) J. T. Johansen, M. Ottesen, and U. Svendsen, *Biochim. Biophys. Acta*, **139**, 211 (1967).
44) C. S. Wright, R. A. Alden, and J. Kraut, *Nature (London)*, **221**, 235 (1969).
45) A. A. Klyosov, N. Van Viet, and I. V. Berezin, *Eur. J. Biochem.*, **59**, 3 (1975).