Static and Kinetic Studies of the Interaction of Tryptophan with Fluorescein Mercury(II) Acetate by Fluorescence and Absorption Spectrophotometry and by Fluorescence Stopped-flow Method[1)]

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Fluorescein mercury(II) acetate (FMA) was found to combine with tryptophan (Trp) to form the FMA-Trp complex. The interaction was interpreted in terms of a reversible single-step binding mechanism. The dissociation constant of the complex estimated kinetically by means of the stopped-flow method using the forward and backward rate constants was in good agreement with that obtained by the static method. From comparison with several derivatives of tryptophan, it was found that the protonated amino group and the indole moiety of tryptophan are essential for the complex formation.

Fluorescein mercury(II) acetate (FMA), a highly fluorescent dye, is known to react with a mercapto group, accompanied by a decrease in its fluorescence intensity as well as a change in its absorption spectrum[2)]. Because it has these characteristics, FMA is used for the chemical modification of cysteine(Cys) residues of protein[3,4)]. Recently, Takeuchi and Maeda have reported that the fluorescent dye binds to some kinds of nucleic acids at the nitrogen sites of thymine and guanine residues[5)]. The fluorescent dye FMA, therefore, is useful for the discrimination of the constituent residue(s) and the conformation of proteins and nucleic acids.

Kinetic studies of the reaction of FMA with Cys by the stopped-flow method have already been made in order to discriminate[6)] the fast-reacting Cys residues in proteins.[7)] However, the authors have noticed, in the case of amylases, that even certain other residues could react with FMA.[8)] In fact, Watt and Voss found that fluorescein, which has the same aromatic rings as FMA, interacts with Trp, leading to a decrease in its fluorescence intensity.[9)] Because of these findings, the interactions between Trp and FMA have to be examined in detail before FMA can be applied to the modification of proteins including both Cys and Trp residues.

In this work, the fast complex formation between Trp and FMA was studied statically and kinetically through the decrease in the fluorescence intensity and the change in the difference absorption spectrum. The complex formation was consistent with a single-step association mechanism.

Results and Discussion

Interaction between FMA and Trp as Studied by Static Fluorescence Spectrophotometery. Figure 1 shows the fluorescence emission spectrum of FMA, which has a peak at 525 nm when excited at 495 nm (Fig. 1, 0). The fluorescence intensity at 525 nm was found to decrease with an increase in the concentration of Trp (Fig. 1, 1 and 2). The interaction of Trp with FMA also shows a difference absorption spectrum, which has a trough at 492 nm, a peak at 516 nm, and an isosbestic point at 505 nm (Fig. 2). Thus, the interaction between Trp and FMA can be followed either through the absorption or by the fluorescence change. The fluorescence measurement is preferable because of its high sensitivity.

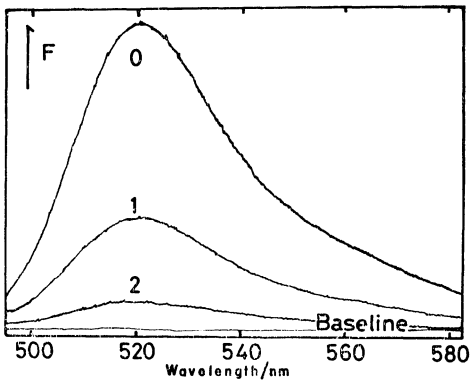

Fig. 1. Fluorescence emission spectra of FMA in the presence and absence of tryptophan.
[FMA]; 1.0 μM, [Trp]; 0; 0 M, 1; 5 μM, 2; 80 μM. Baseline means buffer solution. Excitation at 495 nm, pH 9.0, 25 °C.

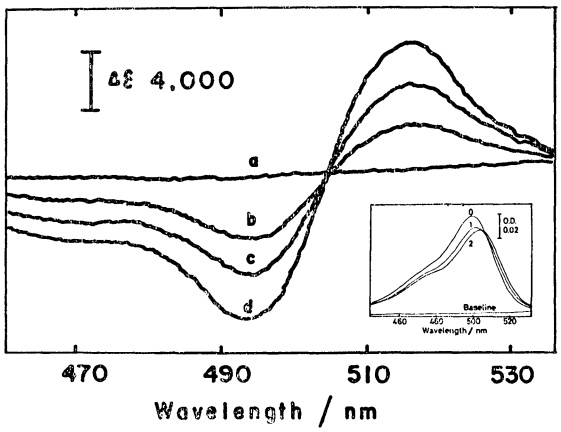

Fig. 2. Difference absorption-spectra of FMA produced by the addition of tryptophan.
[FMA]; 10.0 μM, [Trp]; a; 0 μM, b; 10 μM, c; 20 μM, d; 50 μM. Inset means the absorption spectra of FMA in the presence and absence of tryptophan. [Trp]; 0; 0 M, 1; 5 μM, 2; 80 μM, Baseline; buffer. [FMA]; 1.0 μM.

Figure 3 shows the dependence of the fluorescence intensity change (decrease), ΔF, on the initial concentration of Trp, where ΔF is expressed as the percentage of the change in the fluorescence intensity

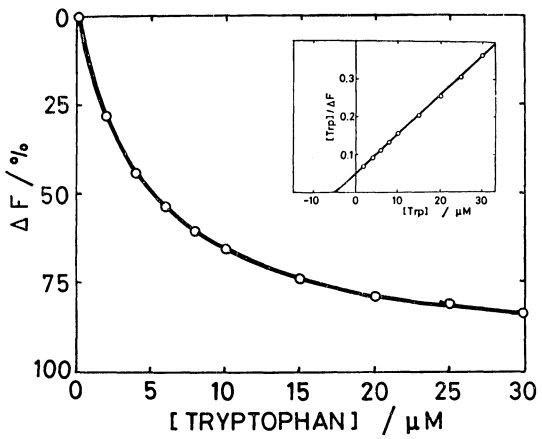

Fig. 3. Dependency of the fluorescence decrease of FMA, ΔF, upon the concentration of tryptophan. [FMA]; 1.0 μM, pH 9.0, 25 °C. Inset is the plots of [Trp]/ΔF *vs.* [Trp].

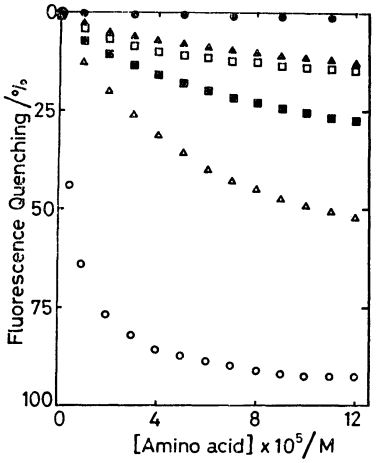

Fig. 4. Dependency of the fluorescence decrease of FMA upon the concentrations of amino acids. [FMA]; 0.2 μM, pH 9.0, 25 °C. ●; His, ▲; N^{α}-acetyl-Lys, □; Asp, ■; Phe, △; Tyr, ○; Trp.

relative to that of free FMA. The experimental points fit the characteristic hyperbolic curve, indicating a reversible complex formation between Trp and FMA:

$$\text{Trp} + \text{FMA} \rightleftharpoons \text{Trp-FMA}, \quad (1)$$

where Trp–FMA represents the Trp–FMA complex. The dissociation constant of the Trp–FMA complex, K_d, is defined as follows:

$$K_d = [\text{Trp}][\text{FMA}]/[\text{Trp-FMA}]. \quad (2)$$

A linear plot, [Trp]/ΔF against [Trp], is shown in the inset of Fig. 3, according to:

$$[\text{Trp}]/\Delta F = [\text{Trp}]/\Delta F_{max} + K_d/\Delta F_{max} \quad (3)$$

$$[\text{Trp}] \gg [\text{FMA}],$$

where ΔF_{max} is the maximum fluorescence decrease when FMA is saturated with Trp and where [Trp] is the initial concentration of Trp ([Trp]≫[FMA]). The experimental points fit a straight line, from which the K_d and ΔF_{max} values were obtained as 5.0×10^{-6} M (1 M=1 mol dm^{-3}) and 98% respectively. The solid lines in Fig. 3 (including the inset) are the theoretical ones drawn according to Eq. 3 with the obtained values of K_d and ΔF_{max}. The statically obtained experimental results are thus consistent with the mechanism represented by Eq. 1.

Complex Formation of FMA with Other Amino Acids. Tyrosine, phenylalanine, and three other amino acids besides Trp were examined for the complex formation with FMA in the manner described above. The fluorescence-intensity changes caused by the addition of each of the amino acids were measured at various concentrations. As is shown in Fig. 4, the decrease in the fluorescence intensity could be observed with all the amino acids examined, except for His. The degree of the decrease in the fluorescence intensity is strongly dependent on the nature of the amino acid. The values of K_d and ΔF_{max}, evaluated in the same way as for Trp, are summarized in Table 1. Tryptophan has the smallest K_d value and the largest ΔF_{max} value among the amino acids examined. Thus, it seems worthwhile to investigate the interaction of FMA with Trp in detail.

Table 1. The K_d and ΔF_{max} values of the FMA–amino acid complex formation

Amino acid	$K_d/10^{-6}$ M	ΔF_{max}/%
Asp	66	20
Arg	100	11
Gly	180	19
N^{α}-acetyl-Lys	95	12
Ser	25	22
Phe	87	36
Tyr	48	65
Trp	5.0	98

Excitation at 495 nm, emission at 525 nm, pH 9.0, 25 °C.

Some derivatives of Trp were also examined in order to ascertain what part of the Trp molecule contributes to the Trp–FMA complex formation. In this case, the difference absorption was used as a probe for measurement, since it is more suitable for quantitative analysis than the fluorescence measurement. The difference-absorption change measured at 492 nm, ΔA_{492}, was plotted against the concentration of the derivatives, as is shown in Fig. 5. The tryptophan methyl ester as well as tryptophan can form a complex with FMA to produce the difference absorption change, whereas neither *N*-acetyltryptophan nor the acetyltryptophan ethyl ester show any evidence of forming such a complex. These findings indicate that the amino group of the derivatives plays an essential role in the complex formation. Thus, both the indole and the amino group seem to be essential to the complex formation, whereas the carboxyl group is not essential.

Effect of pH on the Complex Formation of FMA with Trp. The dependence of ΔF on the concentration of Trp was studied at various pH's above 8, since FMA is not fluorescent at lower pH's. Figure 6 shows a part of the data showing the variation in the plots of ΔF *vs.* [Trp] at various pH's. The values of K_d and ΔF_{max} for the Trp–FMA complex at every pH were evaluated in the manner described above. The

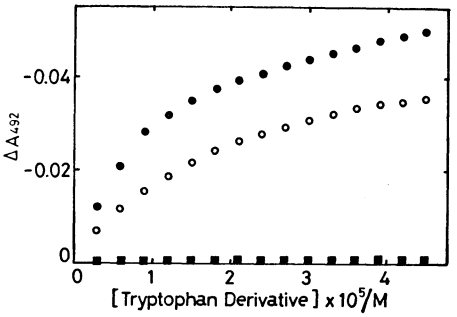

Fig. 5. Difference absorption change (ΔA) of FMA at 492 nm induced by tryptophan and its derivatives.
[FMA]; 10.0 μM, pH 9.0, 25 °C. ●; H_2N-Trp-COOH, ○; H_2N-Trp-$COOCH_3$, ■; *N*-acetyl-Trp-COOH and *N*-acetyl-Trp-$COOC_2H_5$

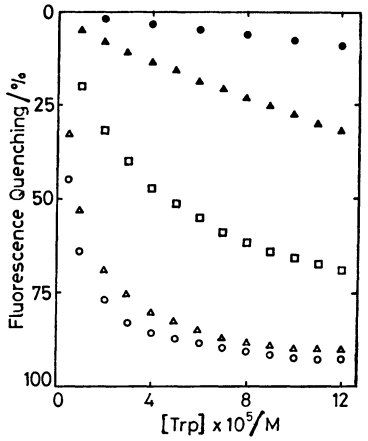

Fig. 6. Dependence of the fluorescence decrease of FMA upon the concentration of tryptophan at various pH's.
[FMA]; 0.2 μM, 25 °C. ●; pH 12.5, ▲; pH 11.7, □; pH 10.8, △; pH 9.9, ○; pH 9.0.

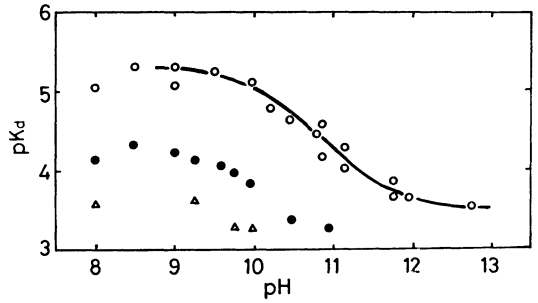

Fig. 7. pH dependency of K_d for Trp, Tyr, and Phe. Solid line was obtained by the calculation with $pK_a = 10.8$ according to the scheme, Trp-NH_2+H⇌ Trp-NH_3^+. ○; Trp, ●; Tyr, △; Phe.

pH-dependence of pK_d ($=-\log K_d$) is shown in Fig. 7, together with those obtained with Tyr and Phe. The slope of the pK_d *vs.* pH plot is nearly unity; thus, one proton, which could be Trp ($pK \simeq 10.8$), contributes to the complex formation. The pK_d value of the Trp–FMA complex is appreciably larger than those of the two other amino acids, Tyr and Phe, over the pH range examined (pH 8—11).

Kinetics of the Trp–FMA Complex Formation. The formation of the Trp–FMA complex was studied kinetically by means of the fluorescence stopped-flow method in order to evaluate the nechamism by observing the rapid decrease in the fluorescence intensity. Figure 8 shows a typical example of the reaction curves obtained in an excess of Trp over FMA. The reaction obeys first-order kinetics, as may be seen from the straight line in the Guggenheim plot (the inserted figure in Fig. 8).[10,11] The apparent first-order rate constant, k_{app}, for the interaction was evaluated at variuos Trp concentrations. The results are shown in Fig. 9. The linearity of the plot is consistent with the following single-step binding mechanism, where k_{+1} and k_{-1} are the rate constants for the forward and backward processes respectively:

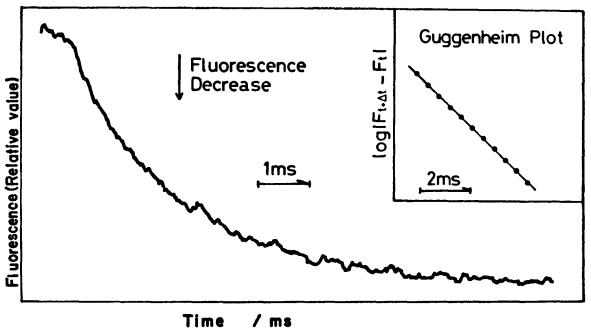

Fig. 8. A typical example of the reaction curves observed by a fluorescence stopped-flow spectrophotometer.
[FMA]; 0.2 μM, [Trp]; 12.5 μM, pH 8.5, 25 °C. Inset is the Guggenheim plot ($k_{app} = 499\ s^{-1}$).

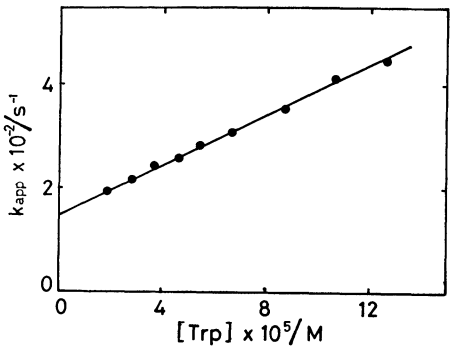

Fig. 9. Dependency of k_{app} upon the concentration of tryptophan.
The straight line was obtained by the given values, $k_{+1} = 2.37 \times 10^7\ M^{-1}\ s^{-1}$ and $k_{-1} = 149\ s^{-1}$ according to the scheme, Eq. 4 and $k_{app} = k_{+1}[Trp] + k_{-1}$, where [Trp] is the tryptophan concentration. [FMA]; 0.2 μM, pH 8.5, 25 °C.

$$Trp + FMA \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} Trp\text{-}FMA, \quad (4)$$

Based on the mechanism, k_{app} is expressed, when [FMA]≪[Trp], as follows:[12]

$$k_{app} = k_{+1}[Trp] + k_{-1}. \quad (5)$$

From the plot of k_{app} against [Trp], the rate constants, k_{+1} and k_{-1}, were evaluated by the use of the least-squares method to be $2.4 \times 10^7\ M^{-1}\ s^{-1}$ and $150\ s^{-1}$ respectively (pH 8.5). The solid line in Fig. 9 is the

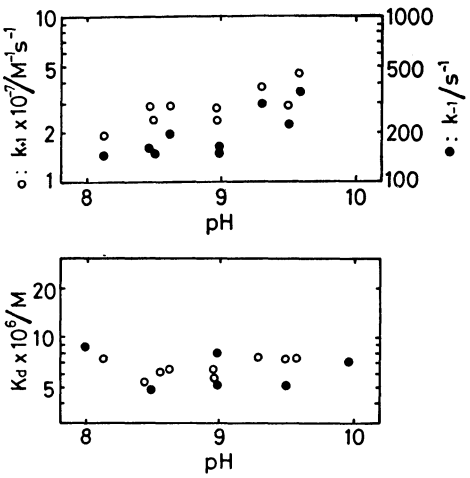

Fig. 10. pH dependencies of the rate constants, k_{+1} (open circle) and k_{-1} (closed circle) and the dissociation constant, K_d (Open circles were obtained by the kinetic observation with the stopped-flow method and closed circles were by the static observation).

theoretical one drawn according to Eq. 5, using the given rate constants. Thus, the interaction between FMA and Trp can reasonably be accounted for by a single-step binding mechanism, as has been described in Eq. 4, at least in the concentration range studied. The dissociation constant, K_d ($=k_{-1}/k_{+1}$), was evaluated from the rate constants to be 6.3×10^{-6} M (pH 8.5).

Figure 10 shows the two rate constants, k_{+1} and k_{-1} and the K_d values evaluated at various pH's in the range from 8 to 10. The K_d values obtained from the kinetic study are reasonably consistent with the values from the static measurements described above; they are nearly constant between pH 8 and 9.5, in spite of the fact that both k_{+1} and k_{-1} increase with an increase in the pH.

Experimental

Chemicals. Fluorescein mercury(II) acetate (FMA) was synthesized from uranine (fluorescein sodium salt) and mercuric acetate by the method of Karush *et al.*[2] and was purified by Sephadex G-15 column chromatography using a 20 mM aqueous ammonia solution as the eluent. The main fraction was shown by dithizone titration[5] to contain two mercury atoms per molecule; it is in agreement with the chemical structure assigned to FMA by Karush *et al.*[2] The concentration of FMA was determined spectrophotometrically using the molar absorption of 78000 at 499 nm.[2] Tryptophan (Trp) and its derivatives, tyrosine (Tyr), phenylalanine (Phe), and the other amino acids, and the chemicals used were all of a guaranteed grade, purchased from Kanto Chemical Industries Co. Ltd., Tokyo.

Measurements of Difference Absorption and Fluorescence Spectra. The difference-absorption spectrum (450—540 nm) of FMA caused by amino acid was measured in a 50 mM borate buffer, pH 9.0 at 25 °C, by means of a Union Giken SM-401 spectrophotometer. The fluorescence measurements were made with a Union Giken FS-401 fluorescence spectrophotometer. The excitation wavelength was 495 nm unless otherwise stated.

Kinetic Observation of the Interaction between FMA and Trp by the Fluorescence Stopped-flow Method. The time course of the change in the fluorescence intensity caused by the interaction of FMA with Trp was observed with a Union Giken SF-70 (piston driven type) or RA-1300 (gas-pressure driven type) stopped-flow spectrophotometer, with a 50-W tungsten lamp as the light source and a cylindrical cell with a 2 mm bore. The excitation wavelength was 495 nm, and the fluorescence emission was observed from a right angle through a cut-off filter (50% transmission at 530 nm). The reaction signals were usually accumulated 9 times to improve the signal-to-noise ratio, using a Union Giken RA-450 kinetic-data-averaging processer. All the reactions were carried out in a 50-mM borate buffer, pH 8—10, at 25 °C.

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References

1) Reported in part at the Kansai (Middle west)/Nishi Nippon (West Japan) Regional Meeting of the Agricultural Chemical Society of Japan, Okayama, Oct. 14, 1978.
2) F. Karush, N. R. Klinman, and R. Marks, *Anal. Biochem.*, **9**, 100 (1964).
3) J. R. Heitz and B. M. Anderson, *Arch. Biochem. Biophys.* **127**, 637 (1968).
4) J. R. Heitz, *J. Biol. Chem.*, **246**, 5790 (1973).
5) S. Takeuchi and A. Maeda, *Biochim. Biophys. Acta*, **454**, 309 (1976).
6) K. Hiromi, T. Kawagishi, and M. Ohnishi, *J. Biochem.* **81**, 1583 (1977).
7) S. Takeuchi and A. Maeda, *J. Biochem.*, **81**, 971 (1977).
8) Unpublished data, (M. Taniguchi, Bachelor's Thesis (1978) and Master's Thesis (1980), Kyoto University, College of Agriculture).
9) R. M. Watt and E. W. Voss, Jr., *Immunochemistry*, **14**, 533 (1977).
10) H. A. Guggenheim, *Philos. Mag.*, **3**, 538 (1926).
11) Hiromi, "Kinetics of Fast Enzyme Reactions," Kodansha-Halsted Press, Tokyo and New York (1979).
12) M. Eigen and L. DeMaeyer, "Techniques of Chemistry," ed by S. Weissberger, John Wiley & Sons, New York (1974), Vol. 6.