Interaction of Hydrophobic Cyclic Dipeptides and Acylbenzenes as Studied by Fluorescent Quenching

Toshiro Ootubo, Shunsaku Kimura, and Yukio Imanishi*
Department of Polymer Chemistry, Kyoto University, Yoshida Honmachi, Sakyo-ku, Kyoto 606
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The effect of the hydrophobic interaction on the association in aqueous media of tyrosine-containing cyclic dipeptides and 1-acyl-4-bromobenzenes having various lengths of acyl chain was investigated by quenching of the fluorescence of the former compounds by the latters. In ethanol, the change of fluorescence intensity (I_0/I) was linearly related to the quencher concentration according to the Stern-Volmer's plot, which was attributed to a dynamic quenching. In this case, structures of fluorophores and quenchers did not affect the quenching constant. However, in a buffer solution, I_0/I was linearly related to the quencher concentration according to the Perrin's plot, which was attributed to a static quenching. Perrin's quenching constants depended on the structure of fluorophores and quenchers. In some cases, a quencher molecule was bound to a fluorophore by hydrophobic interaction in a favorable arrangement for fluorescent quenching. In aqueous solution, fluorophores and quenchers formed comparatively large aggregates with nonspecific arrangement for fluorescent quenching. In this case, Perrin's quenching constants were independent of the nature of quenchers and related with the size of formed aggregates.

Recognition of substrates by enzymes and of agonists by receptors is very important as the beginning step of physiological responses. Hydrophobic and electrostatic interactions are particularly important in biological host-guest interactions. However, the contribution of the hydrophobic interaction has not quantitatively been investigated.[1] Cyclic peptides provide a useful model system for the study of the hydrophobic interaction in the enzyme-mimetic reaction, because the possible conformations of cyclic peptides are very restricted due to the cyclic structure.[2] Histidine-containing cyclic dipeptides have been investigated for the hydrolytic catalyst.[3-8] Tanihara, *et al.* showed that a substrate was bound to a cyclic peptide by hydrophobic interaction and the rate of the hydrolysis of the substrate was enhanced because of a favorable orientation of the bound substrate toward a catalytic group of the cyclic dipeptide.[9] However, since the observed reaction rate constant is a function of either the substrate binding or the intramolecular attack by a catalytic group, the hydrophobic effect on the binding step was not estimated in a quantitative way.

In the present investigation cyclic dipeptides, which consist of a tyrosine and an aliphatic amino acid, were synthesized and their interactions with 1-acyl-4-bromobenzenes having various lengths of acyl chain were investigated by the fluorescence quenching measurement. The interaction between them in aqueous media was believed to occur by hydrophobic forces. A quenching of tyrosine fluorescence by the bromobenzene moiety of the substrate is not affected by the orientation of functional groups in the associate, so that the interactions involved in the binding step can be characterized in a more quantitative way.

Experimental

Cyclic dipeptides, *cyclo*(D-Ala-CM-L-Tyr) (F(D-Ala)), *cyclo*(L-Leu-CM-L-Tyr) (F(L-Leu)), and *cyclo*(D-Leu-CM-L-Tyr) (F(D-Leu)), were synthesized according to the method reported by Kopple, *et al.*,[10] in which CM represents a carboxymethyl group introduced to the hydroxyl group of tyrosine to increase the water-solubility of cyclic dipeptides.

Quenchers, 1-acyl-4-bromobenzenes, used in the present investigation were $CH_3(CH_2)_nCOC_6H_4Br$(p) (n=0 (Q_1), 2 (Q_3), and 4 (Q_5)) and $HOOC(CH_2)_nCOC_6H_4Br$(p) (n=4 (Q_5^-) and 8 (Q_9^-)). Q_1, Q_3, and Q_5 were synthesized from bromobenzene and corresponding acyl chlorides using $AlCl_3$ as a catalyst, and Q_5^- and Q_9^- were synthesized by the hy-

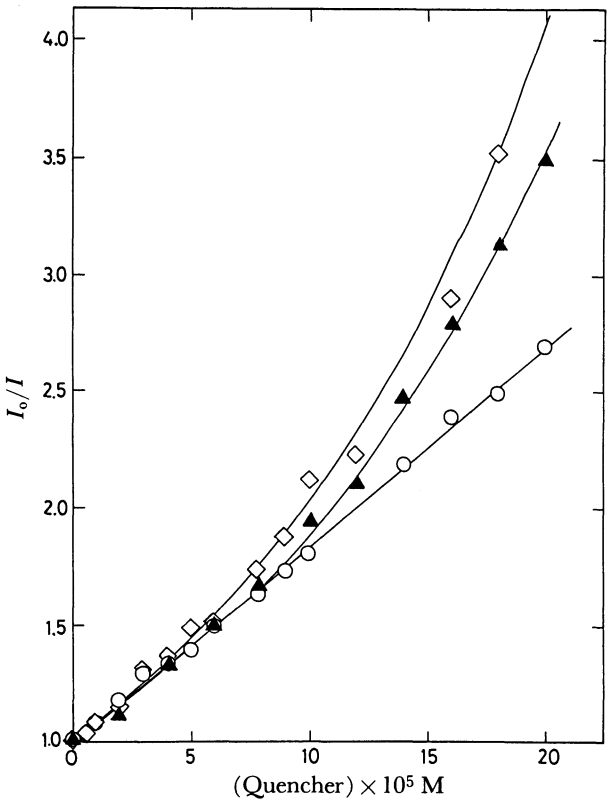


Fig. 1. Stern-Volmer's plot of the change of fluorescence intensity of F(L-Leu) with the addition of Q_5 in 20% dioxane/H_2O (◇), 20% dioxane/buffer (▲), and ethanol (○).

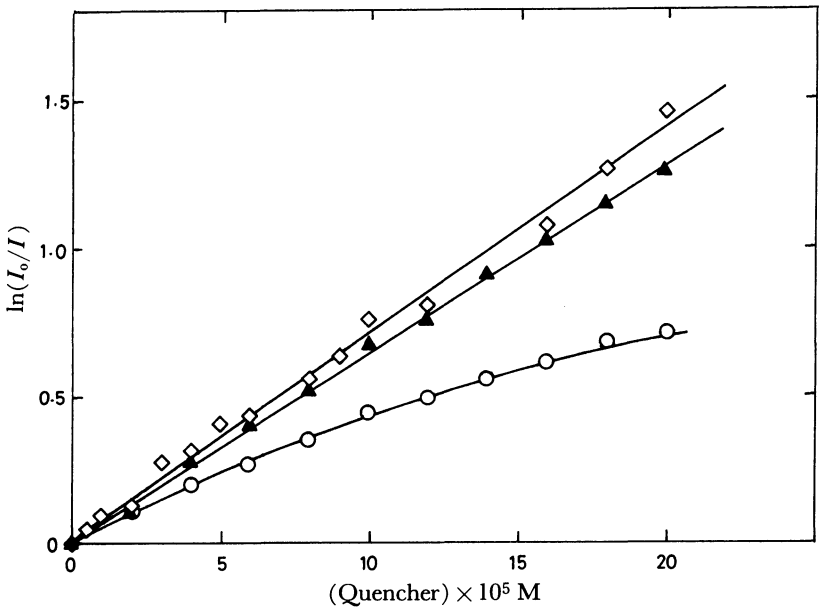


Fig. 2. Perrin's plot of the change of fluorescence intensity of F(L-Leu) with the addition of Q_5 in 20% dioxane/H_2O (◇), 20% dioxane/buffer (▲), and ethanol (○).

drolysis of the reaction product between bromobenzene and monoethyl ester chloride of the corresponding diacid catalyzed by $AlCl_3$. All of the synthesized compounds were identified by IR, ^1H NMR, and elementary analysis.

Fluorescence measurements were carried out on a Hitachi MPF-4 spectrofluorometer at 50°C. Excitation wavelengths were 277 and 278 nm in ethanol and aqueous solution, respectively. The concentration of fluorophore was 1×10^{-5} M (1 M=1 mol dm^{-3}). The quencher was added with an aliquot of ethanol solution (2×10^{-2} M). Buffer solution was prepared from tris(hydroxymethyl)aminomethane (pH 7.5, 10 mM).

Results and Discussion

Solvent Effect on the Interaction between Cyclic Dipeptides and Quenchers. The change of the fluorescence intensity of a fluorophore F(L-Leu) with the addition of a quencher Q_5 was determined and is shown in Fig. 1 in the form of the Stern-Volmer's plot. In ethanol, I_0/I (the fluorescence intensity without the quencher relative to that with the quencher) is linearly related to the quencher concentration. Therefore, the fluorophore is quenched by the quencher in diffusion-controlled collisions, which is a dynamic quenching.[11] On the other hand, in aqueous solution the plot between I_0/I and the quencher concentration gave a concave curve. However, Perrin's plot of I_0/I against the quencher concentration showed a linear relation in aqueous solution throughout this range of quencher concentration, whereas it led to a convex curve for quenching in ethanol solution (Fig. 2). Therefore, in aqueous solution, fluorophores and quenchers should form aggregates due to the hydrophobic interaction, which is a static quenching.[12]

The efficiency of quenching decreased in the order

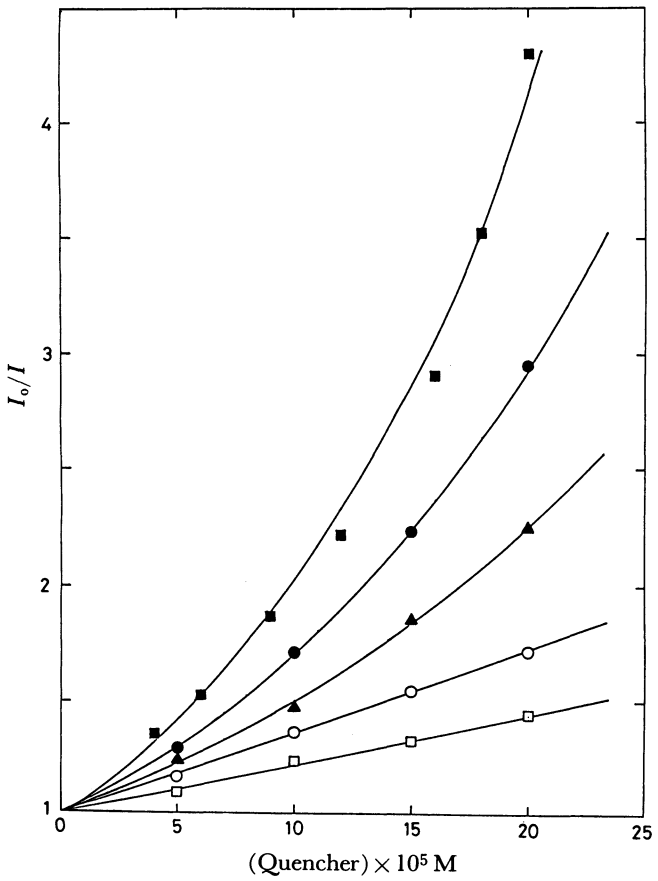


Fig. 3. Stern-Volmer's plot of the change of fluorescence intensity of F(L-Leu) with the addition of Q_5 in 20% dioxane/H_2O (■), 40% dioxane/H_2O (●), 60% dioxane/H_2O (▲), 80% dioxane/H_2O (○), and dioxane (□).

Table 1. Stern-Volmer's quenching constants ($k_Q \times 10^3\,M^{-1}$) in ethanol

Quencher	*Cyclo*(L-Leu-CM-L-Tyr)	*Cyclo*(D-Leu-CM-L-Tyr)	*Cyclo*(D-Ala-CM-L-Tyr)
$CH_3COC_6H_4Br$(p)	5.35	5.28	5.26
$CH_3(CH_2)_2COC_6H_4Br$(p)	5.31	5.19	5.28
$CH_3(CH_2)_4COC_6H_4Br$(p)	5.24	5.33	5.37

Table 2. Perrin's quenching constants ($k_Q \times 10^3\,M^{-1}$) in 20% dioxane/buffer

Quencher	*Cyclo*(L-Leu-CM-L-Tyr)	*Cyclo*(D-Leu-CM-L-Tyr)	*Cyclo*(D-Ala-CM-L-Tyr)
$CH_3COC_6H_4Br$(p)	6.14	6.00	5.77
$CH_3(CH_2)_4COC_6H_4Br$(p)	6.27	6.22	5.72
$HOOC(CH_2)_4COC_6H_4Br$(p)	5.94	5.72	5.69
$HOOC(CH_2)_8COC_6H_4Br$(p)	6.03	6.18	5.71

that 20% dioxane/H_2O>20% dioxane/buffer>ethanol. The influence of the solvent composition on the quenching of the fluorescence of F(L-Leu) by Q_5 was examined (Fig. 3). When the dioxane content was more than 80%, straight lines of the Stern-Volmer's plot was obtained. On the other hand, when the dioxane content was less than 80%, concave curves of the Stern-Volmer's plot were obtained. These results support the explanations for Figs. 1 and 2 that hydrophobic interactions are involved in the quenching process in aqueous solution.

Quenching in Ethanol. Stern-Volmer's quenching constants in ethanol were determined from the slope of the Stern-Volmer's plot and are summarized in Table 1. In all combinations of fluorophores and quenchers, a linear relation was obtained between I_0/I and the quencher concentration, and the quenching constants were almost the same for all combinations. Therefore, it is concluded that the fluorescence is quenched by the diffusion-controlled collision with a quencher, and that the structure of fluorophores and quenchers does not affect the quenching constant.

Quenching in 20% Dioxane/Buffer.

Perrin's quenching constants in 20% dioxane/buffer were determined from the slope of the Perrin's plot and are summarized in Table 2. In the case of F(D-Ala) the quenching constants by Q_1 and Q_5 or $Q_{\bar{5}}$ and $Q_{\bar{9}}$ were almost the same without the dependence on the length of acyl chain of the quencher. On the other hand, in the cases of F(L-Leu) and F(D-Leu) the quenching constants with Q_5 were larger than those with Q_1, and the quenching constants with $Q_{\bar{9}}$ were larger than those with $Q_{\bar{5}}$. These results can be explained by the difference of hydrophobic interactions between the cyclic dipeptides and the quenchers. The isobutyl substituent of F(L-Leu) or F(D-Leu) is hydrophobic enough to represent the difference of interaction due to different lengths of acyl chain. However, the methyl substituent of F(D-Ala) is not hydrophobic enough to represent different degrees of interaction.

When Q_1 and Q_5 were used as quenchers, the quenching constants decreased in the order that F(L-Leu)>F(D-Leu)>F(D-Ala). The same order was also found in the case of $Q_{\bar{5}}$ as a quencher. However, the quenching constant of F(D-Leu) with $Q_{\bar{9}}$ was larger than that of F(L-Leu). This is also shown in Fig. 4, which shows the change of Perrin's quenching constant as a function of the composition of F(D-Leu)/F(L-Leu) mixture. These results suggest that the orientation of F(D-Leu) with respect to $Q_{\bar{9}}$ might be specific as a result of the hydrophobic interaction between the long acyl chain of $Q_{\bar{9}}$ and the isobutyl substituent of F(D-Leu). In this case the bromobenzene moiety of the quencher could come close to the tyrosine residue. This consideration agrees with the result that *cyclo*(D-Leu-L-His) was a more efficient catalyst than *cyclo*(L-Leu-L-His) in the hydrolysis of *p*-nitrophenyl laurate and decanoate.[6,8] The efficient hydrolysis by *cyclo*(D-Leu-L-His) was explained in terms of the hydrophobic substrate bound by the hydrophobic interaction with isobutyl substituent of the cyclic dipeptide to a sterically favorable position for the intramolecular nucleophilic attack by the imidazolyl substituent.

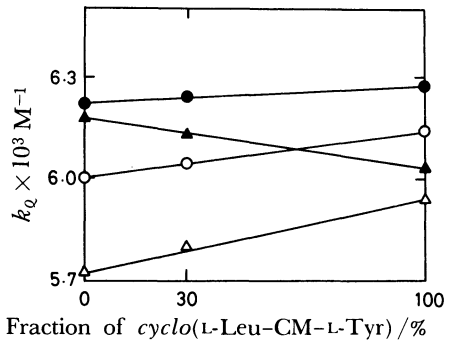


Fig. 4. Perrin's quenching constants of F(L-Leu), F(D-Leu), and their 3/7 mixture with the addition of Q_1 (○), Q_5 (●), $Q_{\bar{5}}$ (△), and $Q_{\bar{9}}$ (▲) in 20% dioxane/buffer.

Perrin's quenching constant of F(D-Leu) or F(L-Leu) with Q_5 was larger than that with $Q_{\bar{5}}$. The association of the fluorophore with $Q_{\bar{5}}$ might be weaker than that with Q_5 due to the electrostatic repulsion between the negative charges of the fluorophore and $Q_{\bar{5}}$.

Table 3. Perrin's quenching constants ($k_Q \times 10^3$ M^{-1}) in 20% dioxane/H_2O

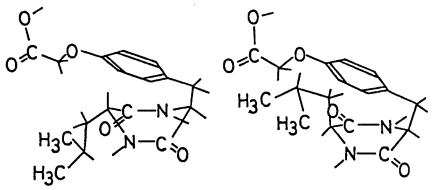
Quencher	*Cyclo*(L-Leu-CM-L-Tyr)	*Cyclo*(D-Leu-CM-L-Tyr)	*Cyclo*(D-Ala-CM-L-Tyr)
$CH_3COC_6H_4Br$(p)	7.00	6.32	6.04
$CH_3(CH_2)_2COC_6H_4Br$(p)	6.99	6.34	6.06
$CH_3(CH_2)_4COC_6H_4Br$(p)	7.01	6.34	6.07

Table 4. Coupling constants (Hz) of $C^{\alpha}H$ and $C^{\beta}H$ protons, the fraction of side chain conformers (%), and chemical shifts (ppm) of $C^{\alpha}H$ of cyclic dipeptides

	Tyr residue				The other residue		
	$J_{\alpha-\beta}$		U/%	F/%	$C^{\alpha}H$	$C^{\alpha}H$	Solvent
Cyclo(L-Leu-CM-L-Tyr)	4.15	5.34	39.3	60.7	4.22	3.51	C_2D_5OD
	3.92	4.88	31.8	68.2	4.49	3.85	D_2O (pH=7.5)
Cyclo(D-Leu-CM-L-Tyr)	4.19	4.63	32.6	67.4	4.28	2.94	C_2D_5OD
	3.96	4.64	30.9	69.1	4.63	3.33	D_2O (pH=7.5)
Cyclo(D-Ala-CM-L-Tyr)	3.67	4.88	30.6	69.4	4.17	2.85	C_2D_5OD
	3.86	4.68	30.3	69.7	4.49	3.22	D_2O (pH=7.5)

Quenching in 20% Dioxane/H_2O. Perrin's quenching constants in 20% dioxane/H_2O were determined by the same method as employed in the preceding section, and are shown in Table 3. The quenching constants decreased in the order that F(L-Leu)> F(D-Leu)>F(D-Ala), and this order was obtained with any kinds of quenchers examined. Furthermore, the quenching constants are independent of the length of acyl chain of quenchers, indicating the absence of any specific interactions. Carboxyl groups do not completely dissociate under experimental conditions, and a large aggregate of cyclic dipeptides and quenchers might be formed by hydrophobic interaction. The aggregate is not expected to take any defined structure, which might be the reason for the absence of specific interaction in quenching process.

Different quenching constants between F(L-Leu) and F(D-Leu) should be attributable to different orientations of the side chains in the cyclic dipeptides, which were investigated by the H-C^{α}-C^{β}-H coupling constants ($J_{\alpha-\beta}$) of NMR spectra. The aromatic group can take either a folded form (F) or two unfolded forms (U). In the folded form, the aromatic group protrudes over the plane of the cyclic dipeptide. The fractions were calculated according to Pachler's equation[13] and are shown in Table 4. The predominant conformation was found to be the folded form for all cyclic dipeptides, and the solvent effect on the conformation was not remarkable. Another evidence for the aromatic group stacking over the 2,5-piperazinedione ring is obtained from the chemical shifts of $C^{\alpha}H$. The chemical shifts of $C^{\alpha}H$ of the Tyr residue and the other residue are shown in Table 4. The chemical shifts of Ala-$C^{\alpha}H$ of F(D-Ala) and Leu-$C^{\alpha}H$ of F(D-Leu) were found to shift to a higher magnetic field compared with 3.96 ppm for Leu-$C^{\alpha}H$ of *cyclo*(D-Leu-L-Leu) and 3.92 ppm for Leu-$C^{\alpha}H$ of *cyclo*(L-Leu-L-Leu), which do not contain an aromatic amino acid, in CD_3OD, and 3.51 ppm for Leu-$C^{\alpha}H$ of F(L-Leu) in C_2D_5OD. This observation may be explained in terms that in the cases of F(D-Ala) and F(D-Leu), $C^{\alpha}H$ of the Ala or Leu residue and the aromatic group of Tyr residue stick out on the same side of the cyclic skeleton, and that the aromatic group protrudes over the $C^{\alpha}H$, that is, indicating a folded structure. The folded conformations are depicted in Fig. 5, which may explain a larger quenching constant of F(L-Leu) than F(D-Leu). Since the molecular size of F(L-Leu) is more compact than that of F(D-Leu), the former is expected to form a larger hydrophobic aggregate than the latter, which, in turn, makes Perrin's active sphere overlap with each other and increases the ability of quencher apparently.



cyclo(D-Leu-CM-L-Tyr) *cyclo*(L-Leu-CM-L-Tyr)

Fig. 5 The folded conformations of F(D-Leu) and F(L-Leu).

In the case of the cyclic peptide, the specific interaction could be realized under the suitable combination of the hydrophobic and electrostatic interaction with the substrate.

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