Steric-Control for the Enantioselective Hydrolysis of Amino Acid Esters in Hybrid Membrane Systems

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The enantioselective hydrolysis of the amino acid esters, *p*-nitrophenyl-*N*-dodecanoyl-D(L)-phenylalaninates (C_{12}-D(L)-Phe-PNP) catalyzed by active tripeptide, *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-histidyl-L-leucine (Z-PheHisLeu) in the presence of coaggregates (hybrid membranes) composed of native phospholipid, L-α-dimyristoylphosphatidylcholine (DMPC) and nonionic surfactant, polyoxyethylene (8) lauryl ether ($C_{12}(EO)_8$) was easily controlled by regulating the reaction temperature and changing the composition of coaggregates. Furthermore, excellent correlations were observed between the enantioselectivity in the hydrolysis of C_{12}-D(L)-Phe-PNP catalyzed by Z-PheHisLeu in the presence of coaggregates and physical properties of hybrid membranes. It is assumed that catalytic activities of tripeptide catalyst in hybrid membranes should be regulated by changing the microenvironments of reaction fields.

Key words enantioselective hydrolysis; hybrid membrane; stereochemical control

Stereoselective reactions have attracted considerable attention in connection with understanding the origins of the stereoselectivity observed with proteolytic enzymes.[1—5] In the course of our study on the stereoselective hydrolysis (deacylation) of amino acid and dipeptide esters with the functional molecular assemblies composed of synthetic surfactants and reactive species, we emphasized that the stereochemical (enantiomeric and diastereomeric substrates) control could be attained by changing the composition of the coaggregates[6—8] and regulating ionic strength[9] and temperature.[10,11] Especially, an extremely high enantioselectivity was attained at the optimum composition of coaggregate.[6]

However, the values of k_t and k_s (the first-order rate constants with and without the peptide catalyst, respectively) are fairly close for the enantioselective hydrolysis in the aggregate systems of synthetic surfactants. Then, it is impossible to disregard the errors of k_t and k_s values when we discuss catalytic activities of the artificial membranes bound enzyme on the basis of $k_{a,obsd}$ values.

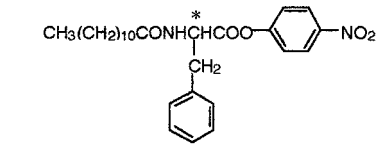
In this study, we employed native phospholipids instead of synthetic surfactants for the hydrolysis of amino acid esters (C_{12}-D(L)-Phe-PNP)[12] with tripeptide (Z-PheHisLeu)[12] in the coaggregate systems. The advantage of this experiment should enable to evaluate of the catalytic activities directly on the basis of $k_{a,obsd}$ values because of the large difference of the k_t and k_s values, that is, the k_t/k_s ratios were above 30 and 4 folds for the L- and D-substrates, respectively.

Recently, in the course of our study on artificial membranes composed of native phospholipids, very useful knowledges for medical engineering applications *in vitro* and *in vivo* have been obtained. For example, artificial lipid membranes were used as a drug carrier in drug delivery systems, such as 1,3-bis(2-chloroethyl)-1-nitrosourea encapsulated in hybrid liposomes (artificial membranes) which should be effective against malignant glioma *in vitro* and *in vivo*.[13] Moreover, the artificial membranes themselves were found to be useful as a new type of anticancer drug without side effects.[14,15]

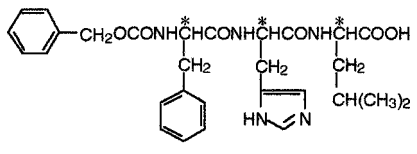
In this study, we report on the remarkable effects (composition of hybrid membranes and reaction temperature) on the enantioselective hydrolysis of C_{12}-D(L)-Phe-PNP catalyzed by Z-PheHisLeu in the hybrid membranes composed of phospholipid (DMPC) and nonionic surfactant ($C_{12}(EO)_8$) at pH 7.4. The relations between the enantioselectivity and the microenvironment of the reaction fields are discussed.

Results and Discussion

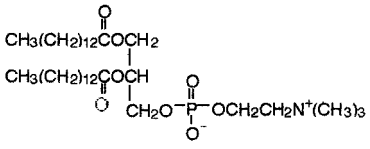
The composition dependence of coaggregates on the enantioselective hydrolysis of C_{12}-D(L)-Phe-PNP catalyzed by Z-PheHisLeu was investigated in hybrid membranes of DMPC/$C_{12}(EO)_8$, as shown in Fig. 1. The concentration of $C_{12}(EO)_8$ is expressed as mol% of total lipids. Both the second-order rate constants ($k_{a,obsd}$) and enantioselectivity ($k^L_{a,obsd}/k^D_{a,obsd}$) values increased as the $C_{12}(EO)_8$ concentration was raised in the range of 10—30 mol%. No clear solution was obtained in the $C_{12}(EO)_8$ concentration range over 30 mol% (phase separation occurred in this region), but high second-order rate constants and enantioselectivity were ob-



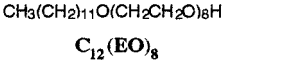
C_{12}-D(L)-Phe-PNP



Z-PheHisLeu



DMPC



$C_{12}(EO)_8$

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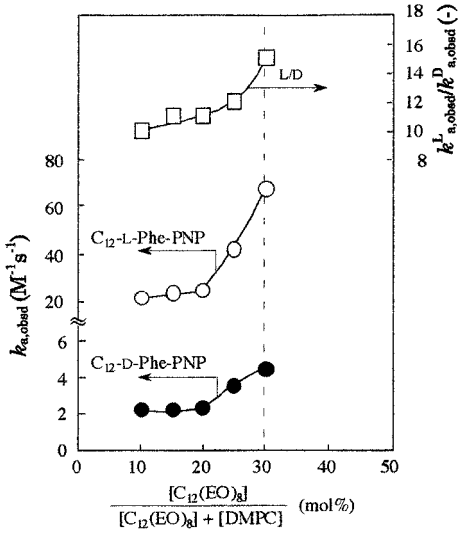

Fig. 1. Composition Dependence of Rate Constants ($k_{a,obsd}$) and Enantioselectivity ($k^{L}_{a,obsd}/k^{D}_{a,obsd}$) for the Hydrolysis of C_{12}-D(L)-Phe-PNP Catalyzed by Z-PheHisLeu in DMPC/$C_{12}(EO)_8$ Hybrid Membranes at 25 °C and μ=0.01

[DMPC]=1.0×10^{-3} M, [Z-PheHisLeu]=2.0×10^{-4} M, [C_{12}-D(L)-Phe-PNP]=1.0×10^{-5} M.

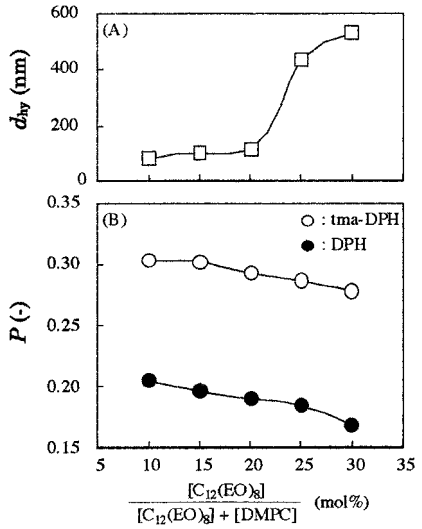

Fig. 2. Composition Dependence of Apparent Hydrodynamic Diameter (A) and Fluorescence Polarizations of DPH and tma-DPH (B) in DMPC/$C_{12}(EO)_8$ Hybrid Membranes at 25 °C and μ=0.01

[DMPC]=1.0×10^{-3} M.

tained on the verge of phase separation.

The hydrodynamic diameter (d_{hy}) of the hybrid membranes was evaluated by dynamic light scattering (dls). The d_{hy} value was sharply increased in the $C_{12}(EO)_8$ concentration range of 20—30 mol%, and interestingly, the enantioselectivity for the hydrolysis of C_{12}-D(L)-Phe-PNP was also extremely enhanced in the same region of coaggregate composition (Fig. 2A). On the other hand, the fluorescence polarizations (P) observed from 1-[(4-trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene iodide (tma-DPH) placed in the pseudo-hydrophobic domain near the membrane surface and DPH placed in the inner hydrophobic domain gently decreased as the $C_{12}(EO)_8$ concentration was raised. These results indicate that the enhancement of fluidity of hybrid membranes should be important to increase the enantioselectivity for the hydrolysis of C_{12}-D(L)-Phe-PNP (Fig. 2B). These observations suggest that the size and the fluidity of hybrid membranes should change upon the addition of $C_{12}(EO)_8$ and result in a large enhancement of enantioselectivity at the $C_{12}(EO)_8$ concentration range of 20—30 mol%. From these results, it is clear that the catalytic activity could be controlled by changing the composition ratio of DMPC and $C_{12}(EO)_8$ in hybrid membranes systems and the high enantioselectivity in the artificial membranes was attained on the verge of phase separation region as well as the enhancement of catalytic activity in the native enzymes.

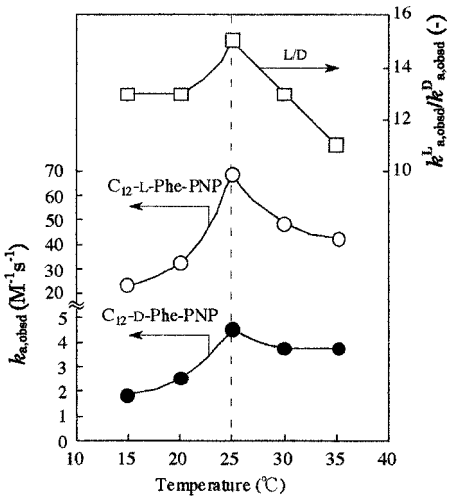

Fig. 3. Temperature Dependence of Rate Constants ($k_{a,obsd}$) and Enantioselectivity ($k^{L}_{a,obsd}/k^{D}_{a,obsd}$) for the Hydrolysis of C_{12}-D(L)-Phe-PNP Catalyzed by Z-PheHisLeu in 70 mol% DMPC/30 mol% $C_{12}(EO)_8$ Hybrid Membranes at pH 7.4 and μ=0.01

[DMPC]=1.0×10^{-3} M, [Z-PheHisLeu]=2.0×10^{-4} M, [C_{12}-D(L)-Phe-PNP]=1.0×10^{-5} M.

The temperature effect on the enantioselective hydrolysis of C_{12}-D(L)-Phe-PNP catalyzed by Z-PheHisLeu was investigated in the hybrid membranes of 70 mol% DMPC/30 mol% $C_{12}(EO)_8$, as shown in Fig. 3. It is noteworthy that the temperature dependence of both the second-order rate constants and enantioselectivity for the hydrolysis of C_{12}-D(L)-Phe-PNP were bell-shaped with a maximum ($k^{L}_{a,obsd}/k^{D}_{a,obsd}$=15) at 25 °C. From this result, Tc of the hybrid membranes of 70 mol% DMPC/30 mol% $C_{12}(EO)_8$ could be estimated kinetically to be *ca.* 25 °C,[16] though it was impossible to estimate Tc of these hybrid membranes on the basis of differential scanning calorimetry (DSC) method.

Interestingly, the temperature dependence of d_{hy} value was also bell-shaped with a maximum at 25 °C (Fig. 4A). On the other hand, the P values observed from tma-DPH and DPH decreased along with the elevation of temperature, as shown in Fig. 4B. However, the inflection in the correlation of P against temperature was observed around 25 °C. This result indicates that the fluidity of the hybrid membranes of 70 mol% DMPC/30 mol% $C_{12}(EO)_8$ is delicately changed around 25 °C, that is somewhat higher than the phase transition temperature (Tc; 23 °C) of pure DMPC membrane estimated on the basis of DSC method. These results suggest that the enantioselective hydrolysis of C_{12}-D(L)-Phe-PNP could be controlled by regulating the temperature of hybrid membranes systems, and the highest enantioselectivity was

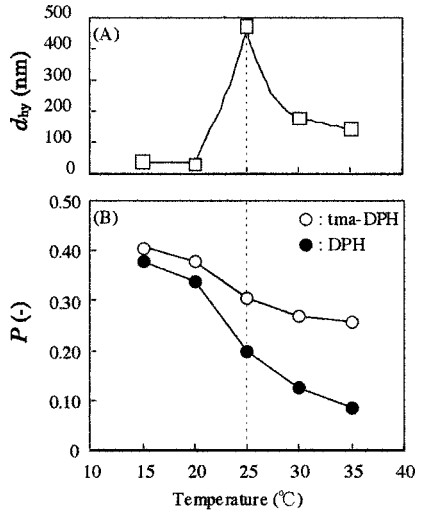

Fig. 4. Temperature Dependence of Apparent Hydrodynamic Diameter (A) and Fluorescence Polarizations of DPH and tma-DPH (B) in 70 mol% DMPC/30 mol% $C_{12}(EO)_8$ Hybrid Membranes at pH 7.4 and $\mu=0.01$ [DMPC]$=1.0\times10^{-3}$ M.

observed around the phase transition temperature.

Conclusion

The stereochemical control for the enantioselective hydrolysis of amino acid esters could be established by changing the composition of coaggregates and by regulating the reaction temperature. In particular, a very interesting feature of this study was that the remarkably large enhancement of the enantioselectivity could be attained between stable and unstable regions (around the phase separation and/or the phase transition temperature) of the artificial membranes.

Experimental

Materials The enantiomeric substrates were prepared from *N*-(benzyloxycarbonyl)-D(L)-phenylalanine by the esterification of the COOH group with *p*-nitrophenol as described in ref. 1. Satisfactory analytical data were obtained for C_{12}-D-Phe-PNP: mp 108.0—108.2 °C; $[\alpha]_D^{23}$ +10.8° ($c=2$, $CHCl_3$). *Anal.* Calcd for $C_{27}H_{36}N_2O_5$: C, 69.21; H, 7.74; N, 5.98. *Anal.* Found: C, 69.07; H, 7.67; N, 5.96. C_{12}-L-Phe-PNP: mp 107.0—107.5 °C; $[\alpha]_D^{23}$ −10.8° ($c=2$, $CHCl_3$). *Anal.* Found: C, 68.98; H, 7.77; N, 5.96.

The sample solutions were prepared by dissolving both DMPC and $C_{12}(EO)_8$ in tris(hydroxymethyl)aminomethane (Tris)-KCl buffer with the sonication (BRANSONIC Model B2200 aparatus, 80W) at 45 °C for 60 min and filtered through a 0.45 μm filter.

Rates of *p*-nitrophenol liberation from *p*-nitrophenyl esters were measured at 400 nm with a Hitachi 150-20 UV spectrophotometer. The reaction obeyed the usual pseudo-first order rate law, and the apparent second-order rate constant ($k_{a,obsd}$) for the hydrolysis of an ester substrate was evaluated by Eq. 1,

$$k_{a,obsd}=(k_t-k_s)/[\text{nucleophile}]_0 \quad (1)$$

where k_t and k_s refer, respectively, to the observed first-order rate constants for the hydrolytic cleavage (hydrolysis) of C_{12}-D(L)-Phe-PNP with and without a nucleophile and $[\text{nucleophile}]_0$ indicates the initial nucleophile concentration.

The dynamic light-scattering measurements were performed with BROOKHAVEN BI-90 particle sizer and a He–Ne laser light source (Spectra-Physics Model 127-35). The hydrodynamic diameter (d_{hy}) was calculated by Stokes–Einstein relation, Eq. 2,

$$d_{hy}=kT/3\pi\eta D \quad (2)$$

where k is Boltzmann's constant, T is absolute temperature, η is the solvent viscosity, and D is the diffusion coefficient.

The fluorescence spectra were measured on a Hitachi F-2000 spectrophotometer. The emissions at 434 nm originating from 1-[(4-trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene iodide tma-DPH and at 430 nm originating from 1,6-diphenyl-1,3,5-hexatriene (DPH) were monitored upon excitations at 360 and 357 nm, respectively. The fluorescence polarizations (P) of tma-DPH and DPH were measured after the sonication of the hybrid membrane solutions and calculated by Eq. 3,

$$P=(I_{vv}-C_fI_{vv})/(I_{vv}+C_fI_{vh}) \quad (3)$$

where I is the fluorescence intensity and the subscripts v and h refer to the orientations, vertical and horizontal, respectively, for the excitation and analyzer polarizers in this sequence: *e.g.*, I_{vh} indicates the fluorescence intensity measured with a vertical excitation polarizer and a horizontal analyzer polarizer. C_f is the grating correction factor, given by I_{hv}/I_{hh}.

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References and Notes

1) Moss R. A., Talkowski C. J., Reger D. W., Pewell C. E., *J. Am. Chem. Soc.*, **95**, 5215—5224 (1973).
2) Breslow R., Czarnik A., *J. Am. Chem. Soc.*, **125**, 1390—1391 (1983).
3) Ueoka R., Matsumoto Y., Harada K., Akahoshi H., Ihara Y., Kato Y., *J. Am. Chem. Soc.*, **114**, 8339—8340 (1992).
4) Scrimin P., Tecilla P., Tonellato U., *J. Org. Chem.*, **59**, 4194—4201 (1994).
5) Ueoka R., Goto K., Tanoue O., Miki A., Yoshimitsu S., Imamura C., Ihara Y., Murakami Y., *Chem. Lett.*, **1999**, 73—74 (1999).
6) Ueoka R., Matsumoto Y., Moss R. A., Swarup S., Sugii A., Harada K., Kikuchi J., Murakami Y., *J. Am. Chem. Soc.*, **110**, 1588—1595 (1988).
7) Goto K., Matsumoto Y., Ueoka R., *J. Org. Chem.*, **60**, 3342—3346 (1995).
8) Ueoka R., Mori S., Moss R. A., *Langmuir*, **10**, 2892—2898 (1994).
9) Ueoka R., Matsumoto Y., Yoshino Y., Hirose T., Kikuchi J., Murakami Y., *Chem. Lett.*, **1986**, 127—130 (1986).
10) Matsumoto Y., Ueoka R., *J. Org. Chem.*, **55**, 5797—5799 (1990).
11) Tanoue O., Baba M., Tokunaga Y., Goto K., Matsumoto Y., Ueoka R., *Tetrahedron Lett.*, **40**, 2129—2132 (1999).
12) The enantioselectivity for the hydrolysis of C_{12}-D(L)-Phe-PNP by Z-PheHisLeu was highest among the enantiomeric substrates and the peptide catalysts employed in our study, as mentioned in ref. 6.
13) Kitamura I., Kochi M., Matsumoto Y., Ueoka R., Kuratsu J., Ushio Y., *Cancer Res.*, **56**, 3986—3992 (1996).
14) Matsumoto Y., Imamura C., Ito T., Taniguchi C., Ueoka R., *Biol. Pharm. Bull.*, **18**, 1456—1458 (1995).
15) Imamura C., Kemura Y., Matsumoto Y., Ueoka R., *Biol. Pharm. Bull.*, **20**, 1119—1121 (1997).
16) Kunitake T., Okahata Y., *J. Am. Chem. Soc.*, **99**, 3860—3861 (1977).