Hybrid Liposomes Coupled to Steric Control with High Enantioselectivity

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With respect to the hydrolysis of enantiomeric substrates (*p*-nitrophenyl *N*-dodecanoyl-D(L)-phenylalaninate; C_{12}-D(L)-Phe-PNP) by the tripeptide catalyst (*N*-(benzyloxycarbonyl)-L-phenylalanyl-L-histidyl-L-leucine; Z-PheHisLeu), a remarkably high enantioselectivity ($k^{L}_{a/obsd}/k^{D}_{a/obsd} = 28$) along with marked rate-enhancement of the hydrolytic cleavage of C_{12}-D(L)-Phe-PNP was obtained with specific coaggregates of 32 mol % L-α-dipalmitoylphosphatidylcholine (DPPC) and 68 mol % α-[4-(1,1,3,3-tetramethylbutyl)phenyl]-ω-hydroxypoly(oxy-1,2-ethanediyl) (TritonX-100). The enantioselectivity was maximized at the phase transition temperature (T_e) in the 65 mol % DPPC/35 mol % TrixonX-100 and 32 mol % DPPC/68 mol % TritonX-100 coaggregate systems. The hydrophobicity and fluidity of the coaggregates can apparently be changed around T_e on the basis of isokinetic temperature and fluorescence parameter studies.

The study of native lipid membranes (liposomes) has greatly contributed to the growth of knowledge in such interdisciplinary areas as biophysics, biochemistry, medicinal chemistry, cell biology, and immunology. In addition, enzyme model studies in the micellar[1–4] and vesicular[5] systems continue to be of interest with respect to development of stereoselective reaction sites for the hydrolysis of enantiomeric esters and a better understanding of the origins of the catalytic specificity in proteolytic enzymes.

Recently, success has been achieved in obtaining almost complete L-enantiomer-selective catalysis, which was attained by controlling the reaction microenvironment in specific membrane systems composed of vesicular and micellar surfactants.[6–8] For example, in our study of enantioselective catalysis in coaggregate systems, the following results were obtained: (1) Excellent correlations were obtained between the stereoselectivity for the hydrolysis of the amino acid esters and the apparent mean hydrodynamic diameters of membranes;[9–11] (2) The enantioselectivity was markedly enhanced by employing peptide catalysts having specific amino acid residues including L-histidine;[12] (3) The origin of the high stereoselective hydrolysis can be simulated by computer modeling studies;[13,14] with respect to the isokinetic discrimination for the enantioselective hydrolysis, noteworthy aspects are as follows: (4) The effect of the addition of micelles[15] or cholesterol[16] to membrane systems was related to the change of membrane fluidity on the basis of isokinetic temperature (β). (5) The location of the reaction sites of catalysts in coaggregates was discriminated by β.[17]

In this study, we extend the hydrolytic cleavage of long-chained substrates catalyzed by Z-PheHisLeu to include hybrid liposomes composed of native lipid (L-α-dipalmitoylphosphatidylcholine (DPPC)) and nonionic micellar surfactant (α-[4-(1,1,3,3-tetramethylbutyl)phenyl]-ω-hydroxypoly(oxy-1,2-ethanediyl) (TritonX-100)). The effect of the hybrid liposomes on the enantioselective hydrolysis is discussed. The temperature-sensitive enantioselective hydrolyses are observed in the hybrid liposomes composed of DPPC and TritonX-100.

Experimental Section

Materials. *p*-Nitrophenyl *N*-Dodecanoyl-D(L)-phenylalaninate (C_{12}-D(L)-Phe-PNP) and *o*-Nitrophenyl *N*-Dodecanoyl-D(L)-phenylalaninate (C_{12}-D(L)-Phe-ONP). The enantiomeric substrates (C_{12}-D(L)-Phe-PNP and C_{12}-D(L)-Phe-ONP) were prepared from *N*-(benzyloxycarbonyl)-D(L)-phenylalaninate by the esterification of the COOH group with nitrophenol and dicyclohexylcarbodiimide,[18] followed by hydrobromination of the NH_2 group,[19] and then acylation of the NH_2·HBr group with dodecanoic anhydride.[18] Satisfactory elemental analyses and specific rotations were obtained for C_{12}-D(L)-Phe-PNP and C_{12}-D(L)-Phe-ONP. C_{12}-D-Phe-PNP: mp 107.0–108.5 °C, $[\alpha]^{23}_D$ + 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. Found: C, 69.07; H, 7.67; N, 5.96. C_{12}-L-Phe-PNP: mp 106.5–108.0 °C, $[\alpha]^{23}_D$ −10.8° (*c* 2, $CHCl_3$). Anal. Found: C, 68.98; H, 7.77; N, 5.96. C_{12}-D-Phe-ONP: mp 83.0–84.2 °C, $[\alpha]^{23}_D$ +29.12° (*c* 2, $CHCl_3$). Anal. Found: C, 68.97; H, 7.59; N, 6.14. C_{12}-L-Phe-ONP: mp 81.3–82.0 °C, $[\alpha]^{23}_D$ −29.90° (*c* 2, $CHCl_3$). Anal. Found: C, 68.70; H, 7.56; N, 6.11.

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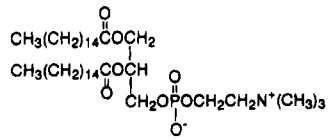
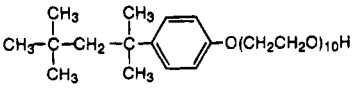
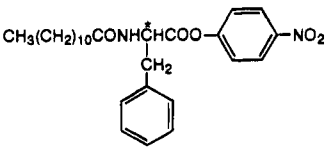


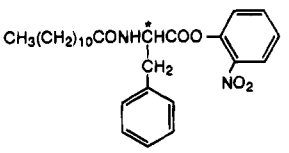
DPPC



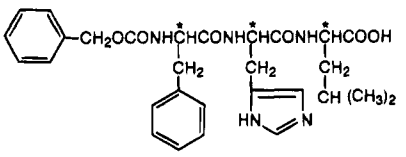
TritonX-100



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



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



Z-PheHisLeu

Commercially available *N*-(benzyloxycarbonyl)-L-phenylalanyl-L-histidyl-L-leucine (Z-PheHisLeu, Bachem), L-α-dipalmitoylphosphatidylcholine (DPPC, Nippon Oil and Fats Co.) and α-[4-(1,1,3,3-tetramethylbutyl)phenyl]-ω-hydroxypoly(oxy-1,2-ethanediyl) (TritonX-100, Wako Chemicals) were used without further purification.

Kinetic Measurement. Rates of nitrophenyl liberation from nitrophenyl esters were monitored at 400 nm (for C_{12}-D-(L)-Phe-PNP) or 410 nm (for C_{12}-D(L)-Phe-ONP) with Hitachi 150-20 UV and Shimadzu UV-150-20 spectrophotometers. Each run was initiated by adding an acetonitrile solution (0.01 mL) of a substrate ester to the reaction medium of phosphate buffer (3.40 mL) containing peptide catalyst, phosphatidylcholine, and surfactant. The reactions 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,

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

where k_t and k_s refer, respectively, to the observed first-order rate constants for the hydrolytic cleavage (hydrolysis) of enantiomeric substrates with and without a catalyst, and $[Cat]_0$ represents the initial catalyst concentration.

The clear stock solutions were prepared by dissolving Z-PheHisLeu catalyst into the lipid membranes composed of DPPC and TritonX-100 in 0.01 M phosphate buffer (0.01 M KCl) with sonication (BRANSONIC Model B2200 apparatus, 80 W) at 50 °C for 60 min. The stock solutions were employed for kinetic measurements after standing for 1 d.

Fluorescence Measurements. Polarization. The fluorescence spectra were measured on a Hitachi F-2000 spectrophotometer; the emission at 431 nm originating from 1-[(4-trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene iodide (tma-DPH) was monitored upon excitation at 361 nm. The fluorescence polarization (P) of tma-DPH was measured after the sonication of the lipid-membrane solutions and calculated by eq 2,

$$P = (I_{vv} - C_f I_{vv})/(I_{vv} + C_f I_{vh}) \quad (2)$$

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.[20,21] C_f is the grating correction factor, given by I_{hv}/I_{hh}.

Dynamic Light-Scattering Measurements. 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 particle hydrodynamic diameter (d_{hy}) is given by the Stokes–Einstein relation, eq 3,

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

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

The sample solutions were prepared by the ultrasonic treatment (with BRANSONIC Model B2200 apparatus, 80 W) of DPPC and TritonX-100 in 0.01 M phosphate buffer (0.01 M KCl) at 50 °C for 60 min and were filtered through a 0.45 μm filter before being measured.

Differential Scanning Calorimetry. The sample solutions for differential scanning calorimetry (DSC) were prepeared in the same manner as used in the dls measurements. After standing for 1 d at room temperature, the sample solution was sealed in a Ag sample pan and DSC thermograms were obtained with a heating rate of 1 °C/min with a SEIKO SSC5200 DSC120. The details of the DSC measurement are given elsewhere.[22]

Results and Discussion

Composition Effect of Hybrid Membranes on Enantioselective Hydrolysis. It is well known that natural and synthetic lipid-surfactants form various aggregates, vesicles, micelles, etc. in aqueous solution, and these aggregates are useful model systems for the investigation of biological phenomena on cell membranes and globular proteins. In the course of our studies on the enantioselective hydrolysis of amino acid esters catalyzed by peptide catalyst in synthetic surfactant aggregates, we found that stereochemical control was established by changing the composition of the coaggregates composed of single- and double-chained surfactants.[9–11] In this study, we tried to clarify the correlation between the composition of coaggregate systems and stereoselective catalysis in the native lipid membranes composed of phosphatidylcholine (DPPC) and nonionic surfactant (TritonX-100). With respect to the coaggregate structure composed of phosphatidylcholine and polyoxyethylene alkyl ether including Triton X-100, globular vesicles were observed by electron microscopy as described eleswhere.[23]

The composition effect of the hybrid liposomes composed of DPPC and TritonX-100 on the enantioselective hydrolysis of C_{12}-D(L)-Phe-PNP catalyzed by Z-PheHisLeu at 25 °C was examined and the results are summarized

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Table 1. Composition Dependence on 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 Hybrid Liposomes Composed of DPPC and TritonX-100[a]

[TritonX-100]	$k_{a/obsd}$ (M^{-1} s^{-1})		
[DPPC] + [TritonX-100] (mol %)	L-isomer	D-isomer	L/D
35	49	5.5	8.9
45	57	3.3	17
57	60	2.6	23
68	69	2.5	28
80	56	2.5	25
100[b]	50	2.1	24

[a] Conditions: 25 °C, pH 7.4, 0.01 M phosphate buffer (0.01 M KCl), 3% (v/v) CH_3CN-H_2O, [Z-PheHisLue] = 1×10^{-4} M, [C_{12}-D(L)-Phe-PNP] = 1×10^{-5} M, [DPPC] = 1×10^{-3} M. [b] [TritonX-100] = 4×10^{-3} M.

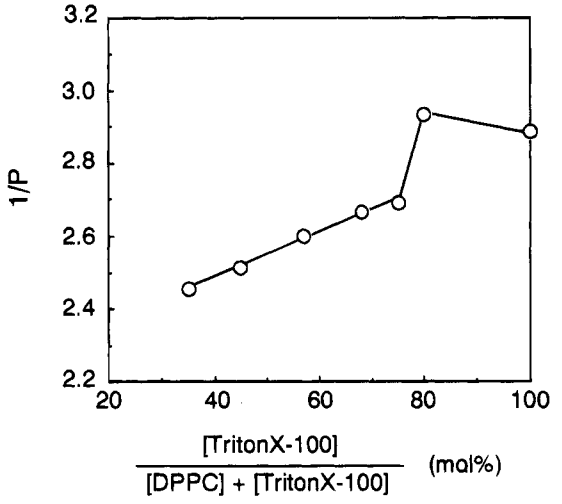

Figure 1. Fluidity (reflected in 1/*P*) versus composition of the hybrid liposomes at 25 °C and pH 7.4 (0.01 M phosphate-KCl buffer). [DPPC] = 1×10^{-3} M.

in Table 1. The concetration of TritonX-100 is expressed as mol % of total lipids. The second-order rate constants ($k_{a/obsd}$) for the hydrolysis of C_{12}-L-Phe-PNP were bell-shaped with a maximum at the TritonX-100 concentration of 68 mol %, though the rates for the hydrolysis of C_{12}-D-Phe-PNP were almost constant over the entire composition range examined. No clear solution was obtained in the TritonX-100 concentration range from 0 to 34 mol %. As a result, the enantioselectivity was maximized ($k^L_{a/obsd}/k^D_{a/obsd} = 28$) at 68 mol % TritonX-100. The fluorescence polarization (*P*) observed from 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,3,5-hexatriene iodide (tma-DPH) when placed in the pseudohydrophobic domain near the membrane surface[24] sharply decreased at a composition of 68 mol % TritonX-100/32 mol % DPPC hybrid liposomes (Figure 1). This result suggests that the fluidity of the hydrophobic region might change at the TritonX-100 concentration of 68 mol %.

However, the composition effect of the hybrid liposomes composed of DPPC and TritonX-100 on the enantioselective hydrolysis of C_{12}-D(L)-Phe-PNP catalyzed by Z-PheHisLeu at 50 °C is quite different from that at 25 °C as shown in Figure 2. The enantioselectivity increased as the TritonX-100 concentration was raised and was maximized ($k^L_{a/obsd}/k^D_{a/obsd} = 16$) in the system of pure TritonX-100 micelles, though the rate constant for the hydrolysis of C_{12}-L-Phe-PNP was bell-shaped with a

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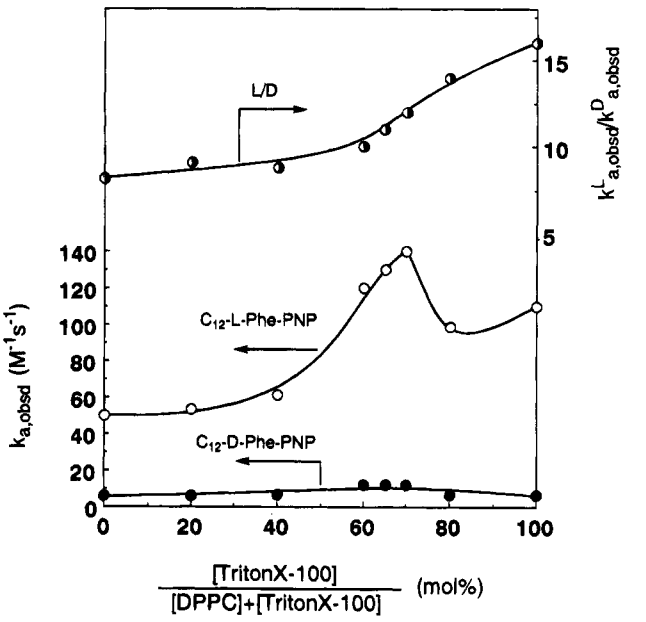

Figure 2. Rate constants ($k_{a/obsd}$ M^{-1} s^{-1}) (left-hand ordinate) and enantioselectivity ($k^L_{a/obsd}/k^D_{a/obsd}$) (right-hand ordinate) versus composition of the hybrid liposomes for the hydrolysis of C_{12}-D(L)-Phe-PNP catalyzed by Z-PheHisLeu at 50 °C and pH 7.4 (0.01 M phosphate-KCl buffer) in 3% (v/v) CH_3CN-H_2O. [Z-PheHisLeu] = 1×10^{-4} M, [Substrate] = 1×10^{-5} M, [DPPC] = 1×10^{-3} M.

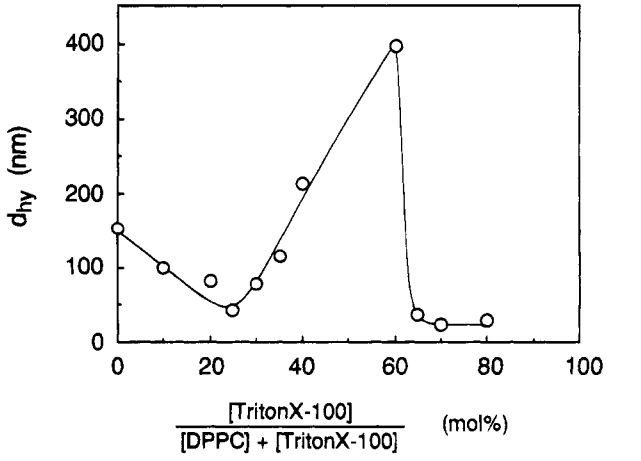

Figure 3. Apparent hydrodynamic diameter (d_{hy}) versus composition of the hybrid liposomes at 50 °C and pH 7.4 (0.01 M phosphate-KCl buffer). [DPPC] = 1×10^{-3} M.

maximum at a TritonX-100 concentration of 70 mol %. The hydrodynamic diameter (d_{hy}) of the DPPC/TritonX-100 hybrid liposomes were estimated by dls. Interestingly, the d_{hy} value was also bell-shaped with a maximum around 60 mol % TritonX-100 concentration (Figure 3). The above-mentioned results suggest that stereochemical control of the enantioselective hydrolysis of amino acid ester can be modulated by changing the composition of coaggregates in native lipid membrane systems or synthetic surfactants.

Temperature Effect in the Hybrid Membrane Systems. The composition effect of the hybrid membranes on the enantioselective hydrolysis of C_{12}-D(L)-Phe-PNP catalyzed by Z-PheHisLeu was also obtained in synthetic surfactants.[9-11] Thus, we examined the temperature effect on the enantioselective hydrolysis of C_{12}-D(L)-Phe-PNP catalyzed by Z-PheHisLeu in the hybrid membranes composed of DPPC and TritonX-100.

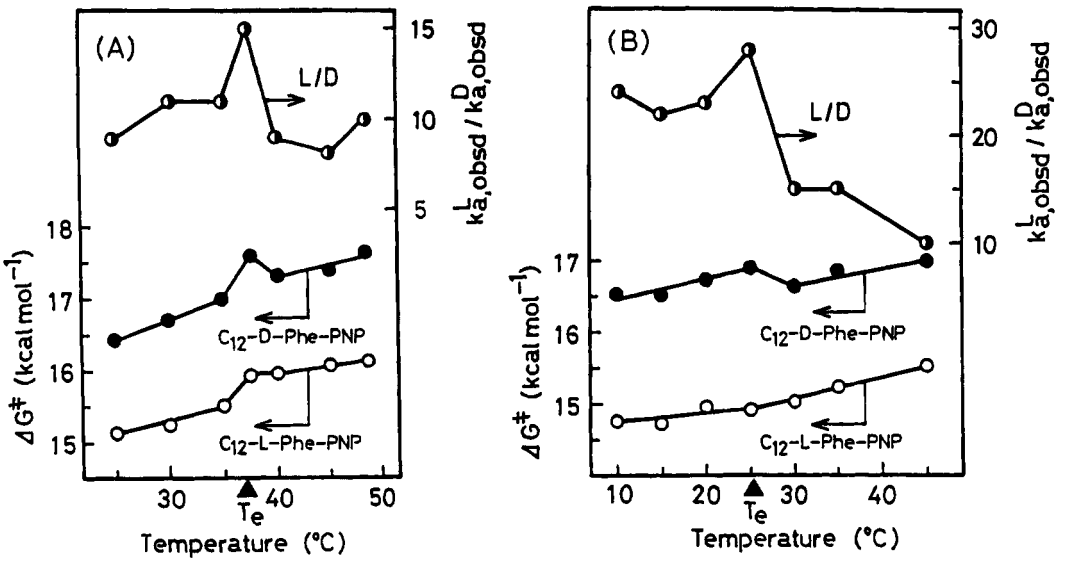

Figure 4. Temperature dependence of enantioselectivity (L/D) and free energy of activation ($\Delta G^{\ddagger}$) for the hydrolysis of C_{12}-D(L)-Phe-PNP catalyzed by Z-PheHisLeu in the hybrid liposomes composed of 65 mol % DPPC and 35 mol % TritonX-100 (A) and 32 mol % DPPC and 68 mol % TritonX-100 (B).

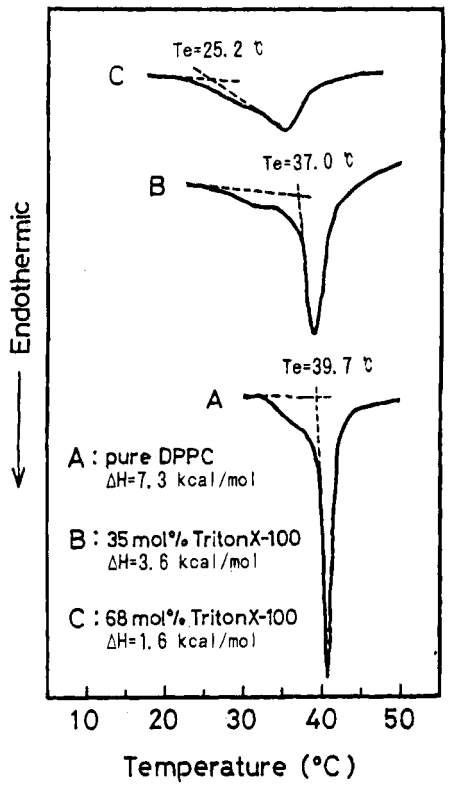

Figure 5. DSC curves for pure DPPC (A) and the hybrid liposomes composed of 65 mol % DPPC and 35 mol % TritonX-100 (B) and 32 mol % DPPC and 68 mol % TritonX-100 (C).

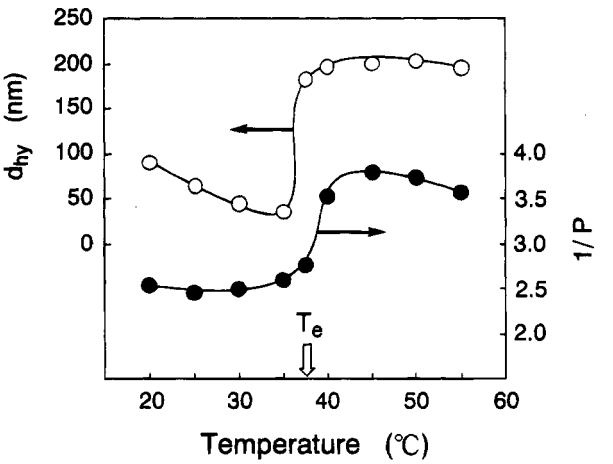

Figure 6. Temperature dependence of d_{hy} (left-hand ordinate) and $1/P$ value (right-hand ordinate) for the hybrid liposomes composed of 65 mol % DPPC and 35 mol % TritonX-100.

As shown in Figure 4, the temperature dependence of enantioselectivity was bell-shaped with a maximum at 37.5 °C and 25 °C in the 65 mol % DPPC/35 mol % TritonX-100 (A) and 32 mol % DPPC/68 mol % TritonX-100 (B) coaggregates, respectively. The phase transition temperatures (T_e), the so-called thermodynamic equilibrium temperatures, were determined to be 37.0 °C and 25.2 °C in the 65 mol % DPPC/35 mol % TritonX-100 and 32 mol % DPPC/68 mol % TritonX-100 hybrid membranes, respectively, on the basis of DSC spectra (Figure 5). Figure 4 also showed the correlation between the free energy of activation ($\Delta G^{\ddagger}$) and temperature in the hydrolysis of C_{12}-D(L)-Phe-PNP. Interestingly, the inflection temperature ranges of 35–40 °C (T_e: 37.0 °C) in the 65 mol % DPPC/35 mol % TritonX-100 coaggregate system and 20–30 °C (T_e: 25.2 °C) in the 32 mol % DPPC/68 mol % TritonX-100 coaggregate system were in the neighborhood of 37.5 °C and 25 °C with a maximum of enantioselectivity in the respective coaggregate systems.

The fluorescence polarization (P) in the hybrid membranes composed of 65 mol % DPPC and 35 mol % TritonX-100 is shown in Figure 6. It is noteworthy that the P value abruptly changed around T_e. This result suggests that the fluidity of the hydrophobic region is altered around the phase transition.

From these results, it is seen that stereochemical control of enantioselective hydrolysis of amino acid esters can be achieved by changing the temperature of lipid membrane systems in addition to that of synthetic vesicular systems.[25]

Thermodynamic Discrimination. The discrimination of the reaction field of micelles, vesicles, and coaggregates has been demonstrated for the enantioselective

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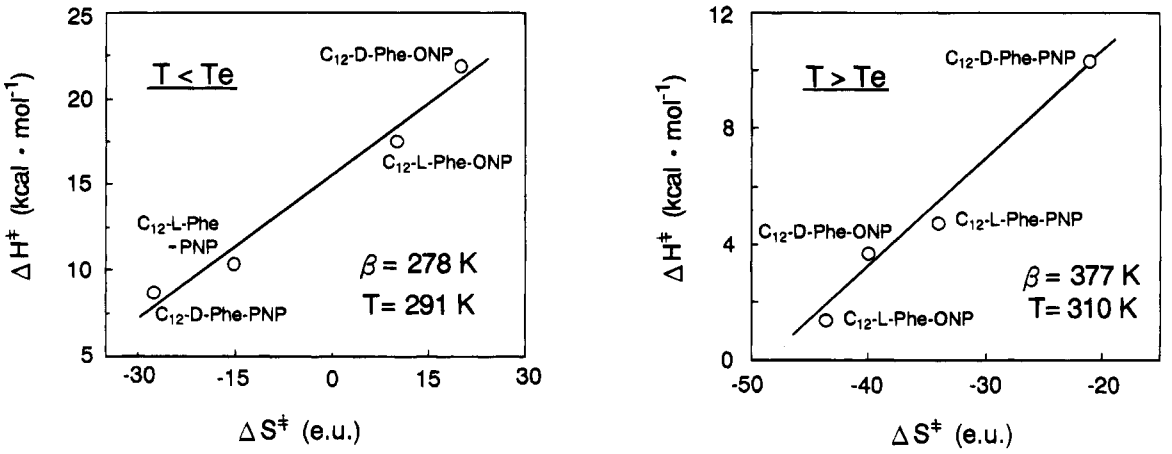

Figure 7. Isokinetic relationships for the hydrolysis of C_{12}-D(L)-Phe-PNP and C_{12}-D(L)-Phe-ONP catalyzed by Z-PheHisLeu in the 32 mol % DPPC/68 mol % TritonX-100 hybrid liposomes.

Table 2. Isokinetic Temperature[a]

hybrid liposomes		β (K)	$\bar{T}$ (K)
65 mol % DPPC	$T < T_e$	274	303
35 mol % TritonX-100	$T > T_e$	318	318
32 mol % DPPC	$T < T_e$	278	291
68 mol % TritonX-100	$T > T_e$	377	310

[a] Conditions: pH 7.4, 0.01 M phosphate buffer (0.01 M KCl), 3% (v/v) CH_3CN-H_2O.

hydrolysis of *p*-nitrophenyl *N*-acyl-D(L)-phenylalaninates on the basis of isokinetic temperature (β).[15-17] Furthermore, the location of catalysts in the coaggregates could be estimated from the correlation between β and the average value of experimented temperature ($\bar{T}$).[17] In the present study, the thermodynamic discrimination of the reaction field is expanded to include native lipid membrane systems composed of DPPC and TritonX-100. The activation parameters ($\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$) were calculated according to eq 4,

$$\Delta G^{\ddagger} = 2.303RT \log(kT/hk_{a/obsd}) = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (4)$$

where k and h stand for the Boltzmann and Planck constants, respectively.

Isokinetic relationships appear to hold for the hydrolysis of C_{12}-D(L)-Phe-PNP and C_{12}-D(L)-Phe-ONP in the coaggregates composed of 32 mol % DPPC/68 mol % TritonX-100 as shown in Figure 7. The correlation coefficients in the relationship between $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were estimated to be more than 0.99 for the hydrolysis in all the systems employed. The isokinetic temperature (β) was evaluated by eq 5,[26]

$$\Delta H^{\ddagger} = \Delta H^{\ddagger}_0 + \beta\Delta S^{\ddagger} \quad (5)$$

where $\Delta H^{\ddagger}_0$ is simply the intercept of $\Delta H^{\ddagger}$ corresponding to $\Delta S^{\ddagger} = 0$. The β values obtained are summarized in Table 2. It is known that hydrophobic interactions are mainly entropy driven while lyophobic ones are mainly enthalpy driven.[27,28] On the basis of the β value in connection with $\bar{T}$, it can be presumed that the hydrolysis in the crystalline gel phase ($\bar{T} < T_e$) may be governed by the entropy of activation, that is, $\bar{T}$ (303 K) exceeded β (274 K), and $\bar{T}$ (291 K) exceeded β (278 K) in the coaggregates composed of 65 mol % DPPC/35 mol % TritonX-100 and 32 mol % DPPC/68 mol % TritonX-100, respectively. However, the hydrolysis in the liquid crystalline phase ($\bar{T} > T_e$) for the coaggregates composed of 32 mol % DPPC/68 mol % TritonX-100 may be governed by the enthalpy of activation, that is, β (377 K) exceeded $\bar{T}$ (310 K), though the correlations between β and $\bar{T}$ for the hydrolysis in the coaggregates composed of 65 mol % DPPC/35 mol % TritonX-100 was $\beta = \bar{T}$.

These results suggest that the hydrophobicity of the coaggregates composed of DPPC and TritonX-100 are altered around T_e.

Conclusion

With respect to the hydrolysis of enantiomeric substrates (C_{12}-D(L)-Phe-PNP) by the tripeptide catalyst (Z-PheHisLeu), (a) a remarkably high enantioselectivity ($k^{L}_{a/obsd}/k^{D}_{a/obsd}$ = 28) along with marked rate-enhancement of the hydrolytic cleavage of C_{12}-L-Phe-PNP was obtained with specific coaggregates of 32 mol % DPPC and 68 mol % TritonX-100. (b) The enantioselectivity was maximized at T_e in the 65 mol % DPPC/35 mol % TritonX-100 and 32 mol % DPPC/68 mol % TritonX-100 coaggregate systems. (c) The hydrophobicity and fluidity of the coaggregates composed of DPPC and TritonX-100 could be changed around T_e on the basis of β and P value. These results suggest that the enantioselectivity is enhanced around fluctuation between unstable and stable regions,[29] because T_e along with the highest enantioselectivity should be related to the inflection point of d_{hy} and P value.

It is significant that stereochemical control of the enantioselective hydrolysis of amino acid esters could be established by temperature regulation and by altering the composition of the coaggregate in the native lipid membrane systems.

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