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Reactivity of Soybean Lipoxygenase-1 to Linoleic Acid Entrapped in Phosphatidylcholine Vesicles

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The linoleic acids embedded in the SUVs of soy-PC, DMPC, and DPPC served as substrate for soybean lipoxygenase-1 (L-1). The initial velocity of the catalytic reaction and the concentration of the substrate showed a hyperbolic relation. The K_m values of L-1 for the linoleic acids in soy-PC, DMPC, and DPPC vesicles were 0.07, 0.09, and 0.11 mM, respectively, being comparable with that for Tween-20 micellar linoleic acid. Soy-PC and DMPC competitively inhibited the enzyme with K_i values of 0.20 and 0.13 mM, respectively, whereas DPPC had no effect. DSC analysis revealed the phase separation of linoleic acid and DPPC in vesicles in the temperature range in which the enzyme reaction was carried out. This may account for the lack of inhibitory effect of DPPC on the enzyme. From the temperature dependence of the specific activity of the enzyme, the E_a values of the catalytic reaction were estimated to be 26.7 and 35.3 kJ·mol^{-1} for soy-PC and DPPC vesicles, respectively. For linoleic acid-DMPC vesicles, a two-phase temperature dependence of the activity across the transition temperature of the mixed vesicles was suggested.

Key words: linoleic acid, lipoxygenase-1, oxygenation, phosphatidylcholine, vesicles.

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Lipoxygenases [linoleate:oxygen oxidoreductase, EC 1.13.11.12] are widely distributed in plants and animals and catalyze the incorporation of molecular oxygen into the 1*Z*,4*Z*-pentadiene moiety of unsaturated fatty acid, yielding conjugated hydroperoxydiene fatty acid (*1-3*). Kinetics of lipoxygenases have been quite well investigated (*1, 4, 5*), but most studies have been carried out in micelle systems using detergent, which is different from the *in vivo* medium.

Attempts have been made to estimate the catalytic activities of water-soluble enzymes toward water-insoluble and highly lipophilic substrates in hydrophobic and biomimetic environments. The main approaches involve the use of reversed micelles (*6, 7*) or vesicles (*8, 9*). Catalytic properties of soybean lipoxygenase-1 (L-1) have been studied in hydrated reversed micelles (*10, 11*). According to Luisi *et al.* (*10*), L-1 exhibits typical Michaelis-Menten type behavior to the overall concentrations of linoleic acid in the reversed micellar solution consisting of aerosol-OT/isooctane. On the other hand, Kurganov *et al.* (*11*) has demonstrated that the substrate is distributed in both the reversed micelles and the organic solvent and that the reaction velocity of L-1 depends on the Hill equation.

Enzymology for vesicular substrates has been extensively studied using different phospholipases (*8, 9, 12*). Both PLA_2 and PLC hydrolyze phospholipids in SUVs at a significantly low catalytic rate compared to that for the micellar substrates (*13*). The activity is influenced by the temperature relative to the thermotropic phase transitions of the phospholipids (*14, 15*).

The substrate fatty acids of lipoxygenases, both esterified and free, are derived from the membrane lipids. Thus far, no attempt has been made to use membrane system for lipoxygenases. The aim of this study is to establish a new approach for investigating the reaction of lipoxygenase with substrates embedded in sonicated vesicles as a model for biological membranes. L-1 used here has been classified as a 15-lipoxygenase, which introduces dioxygen into the ω-6 position of the acyl chain of the polyunsaturated fatty acid. Since L-1 acts on free fatty acids but not on esterified substrates, the free fatty acid on which L-1 acts must be liberated from the membrane phospholipid in advance and remain in the lipid bilayer during the enzymatic peroxidation *in vivo*.

MATERIALS AND METHODS

Materials—Soybean lipoxygenase-1, a product of Sigma Chemical, Type V (630,000 units/mg protein, 2.13 mg/ml) was stored at 4°C and used without purification. Before use, it was diluted 50 times with 50 mM sodium phosphate buffer, pH 7.0, kept in an ice-cold bath, and a 4-μl aliquot was used for each experiment. Purified soy-PC was isolated from the ethanol extract of a crude preparation, Lecion-P (Riken Vitamin), by silica gel column chromatography (*16*). It was located as a single spot on the thin-layer

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Abbreviations: PLA_2, phospholipase A_2; PLC, phospholipase C; soy-PC, soybean phosphatidylcholine; DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; SUV, small unilamellar vesicle; K_m, Michaelis constant; DSC, differential scanning calorimetry; E_a, activation energy.

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chromatography. Synthetic L-α-phosphatidylcholines, DPPC and DMPC (99.0% pure), were purchased from Sigma Chemical and used without purification. Linoleic acid, biochemical grade (99.0% pure) from Wako Pure Chemical, was stored at -20°C under argon and used without purification. Other reagents were of analytical grade.

Preparation of Vesicular and Micellar Dispersions of Linoleic Acid—SUVs of phosphatidylcholines containing linoleic acid were freshly prepared before use as follows. Phosphatidylcholine (12.8 μmol of soy-PC, DPPC, or DMPC) and linoleic acid were placed with a small amount of chloroform in a test tube so that the molar ratio of linoleic acid to phospholipid would be 0.15, 0.30, or 0.60. Chloroform was evaporated by first gassing with nitrogen and then *in vacuo* for 30 min. The film of phospholipid-linoleic acid mixture formed on the inner wall of the test tube was hydrated with 1 ml of 0.1 M sodium borate buffer, pH 9.0, by vortexing under a stream of nitrogen for 1 min, then subjected to ultrasonic irradiation using a Branson Sonifier Model 450 (Branson Ultrasonic) equipped with a microprobe tip. The last procedure was carried out under a stream of argon for 10 min at 55, 0, or 35°C, above the transition temperature for DPPC, soy-PC, or DMPC, respectively. The vesicle dispersion was diluted up to 80 times with 0.1 M sodium borate buffer, pH 9.0, then subjected to the ultrasonic irradiation for 3 min before use. In the experiment for the temperature dependence of the enzyme activity, a more concentrated vesicle dispersion (10 mM linoleic acid/33.3 mM phospholipid) was used. The stock solution of micellar linoleic acid (2.5 mM) was prepared by dispersing linoleic acid in 0.1 M sodium borate buffer, pH 9.0, in the presence of 0.25% Tween 20 in the usual way and stored at -20°C under argon. It was freshly diluted with 0.1 M sodium borate buffer, pH 9.0, before use.

Enzyme Reaction and Assay—The vesicular or micellar dispersion containing various concentrations of linoleic acid, 0.5 ml, was incubated with 4 μl of the diluted enzyme (0.17 μg of protein) for 4 min at 25°C. For determination of temperature dependence of the enzyme activity, 0.3 ml of 10 mM linoleic acid/33.3 mM phospholipid mixed vesicle dispersions or 2.5 mM linoleic acid-0.25% Tween 20 micelle dispersion was incubated with 4 μl of the diluted enzyme (0.17 μg protein) for 4 min in the range of 5 to 30°C. The initial velocity of the reaction was determined by spectrophotometrically monitoring the product separated by HPLC (Jasco): 1 μl of the reaction mixture was applied to a Chemcosorb 5-ODS-H column (4×15 mm) (Chemco) and eluted with methanol-water-acetonitrile (73.5 : 14 : 12.5, v/v/v) containing 0.25 mM sodium perchloric acid at a flow rate of 0.8 ml/min. The linoleic acid hydroperoxide in the effluent was estimated from the peak area in the chromatogram using ε_{max} at 234 nm = 25,000 (*17*).

Measurement of Size Distribution of Vesicle Preparations—Gel permeation chromatography was carried out with Sepharose-4B (*18*). A column (22×160 mm) was first saturated with the vesicle dispersion of linoleic acid and soy-PC (0.77 mM linoleic acid/1.28 mM soy-PC) to eliminate the adsorptive effects of the gel, then equilibrated with 10 mM sodium borate buffer, pH 9.0. One milliliter of the dispersion was applied to the column and eluted with the same buffer as above at a flow rate of 10 ml/h. Fractions of 1.4 ml of effluent were monitored at 300 nm (*18*). Dry materials were determined by a colorimetric method using sodium dichromate (*19*). Electron-microscopic observation was done in the usual way. In brief, a Formvar film carbon-coated 150-meshed copper grid was pretreated with an ion coarter (Eiko 1B-3) for hydration. The dispersion of the vesicles was diluted with 1% ammonium molybdate adjusted to pH 9.0. A drop of the mixture was placed on the film and excess solution was drained off. After drying, the grid was immediately examined with a Hitachi H-700H electron microscope operated at 100 kV and 15,000 magnification. The size distribution of the vesicles was estimated from the micrograph. It was also determined by quasi-elastic light scattering using a Nicomp Model 370 submicron particle sizer (Particle Sizing Systems) with a single vesicle mode.

DSC Measurement—Calorimetric data were obtained by using a DSC 120 differential scanning calorimeter (Seiko Electric) equipped with a thermal-analysis data station and calibrated with indium. One milligram of DPPC, various amount of linoleic acid affording a molar ratio to DPPC of 0, 0.05, 0.15, 0.30, or 0.60, and 50 μl of chloroform were put into an aluminum sample pan. The mixture was spread over the inner wall of the pan and allowed to stand for 10 min at 70°C to remove chloroform. The dried film was hydrated with 50 μl of 0.1 M sodium phosphate buffer, pH 9.0, in a sealed pan, then sonicated in a bath-type sonifier for 30 min and allowed to stand overnight at room temperature. The final concentration of DPPC was adjusted to 2%. Samples were heated and cooled between 0 and 60°C at a fixed rate of 1 deg·K·min^{-1}. Before each heating and cooling run, the samples were held at the relevant starting temperature for at least 3 min to attain thermal equilibrium.

RESULTS

Properties of Vesicles—Figure 1 shows the gel-chromatographic profile of the ultrasonic dispersion consisting of soy-PC and linoleic acid. Three A_{300} peaks are observed (arrows A, B, and C). This profile resembles that for the egg yolk phosphatidylcholine and oleic acid mixture (*20*). The dry materials appear in peaks A and B, mostly in the latter (Fig. 1, closed circles). The peak A coincides with the void volume of the column. Materials in peak A appeared under the electron microscope to consist of vesicles of various sizes around 140 nm in diameter (data not shown). The vesicles in peak B are more uniform in size with a mean diameter of 32 nm under the electron microscope. This is consistent with the light scattering profile (inset in Fig. 1), which shows that the mean diameter of the vesicles is 31 nm, and more than 90% of vesicles range from 24 to 38 nm in diameter. Materials seen in peak C correspond to free fatty acid (*20*), being negligible in the dry material assay. Similar results were found for the vesicle preparations made of linoleic acid and DPPC or DMPC (data not shown). These observations prove that the phospholipids can entrap linoleic acid in a molar ratio of up to 0.6.

Reactivity of Soybean Lipoxygenase-1 toward Vesicular Linoleic Acid—The linoleic acid entrapped in SUVs of various phospholipids at different molar ratios was subjected to the enzymatic peroxidation. Figure 2 shows the initial velocity of the L-1 reaction with linoleic acid in soy-PC and DPPC vesicles as a function of increasing concentration of

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linoleic acid. The molar ratio of linoleic acid to the phospholipid was fixed for each run. The relationship of the velocity and the substrate concentration follows Michaelis-Menten's equation for both lipids. The v vs. [S] curves for soy-PC vesicles varied with the molar ratio of linoleic acid to phospholipid (Fig. 2A), whereas the curves for DPPC vesicles did not depend on the molar ratio (Fig. 2B). These results indicate a difference between soy-PC and DPPC in their effects on the catalytic activity of L-1:soy-PC is inhibitory and DPPC is not. The double reciprocal plots for linoleic acid-soy-PC move upward parallel to the $1/v$ axis as the ratio of [soy-PC] to [linoleic acid] increases (inset in Fig. 2A). Similar behavior was observed for linoleic acid entrapped in DMPC vesicles (data not shown).

As soy-PC and DMPC were shown to have an inhibitory effect on the enzyme, the manner of the inhibition was examined. Figure 3 summarizes the Lineweaver-Burk plots for L-1 activity toward linoleic acid in soy-PC and DMPC vesicles at fixed concentrations of phospholipids. It is clear that both soy-PC (Fig. 3A) and DMPC (Fig. 3B) are competitive inhibitors. The kinetic parameters for linoleic acid incorporated into soy-PC and for the inhibition of soy-PC were estimated from the plots of v^{-1} vs. [soy-PC] at the various fixed concentrations of linoleic acid (Dixon plots) (Fig. 4) using the relationship shown in Eq. 1: V_{max} = 101 μmol·min^{-1}·mg^{-1}, K_m = 0.07 mM and K_i = 0.20 mM.

$$\frac{1}{v}=\frac{K_m}{V_{max}K_i[S]}[I]+\frac{1}{V_{max}}\left(1+\frac{K_m}{[S]}\right) \tag{1}$$

In the same way, the parameters for L-1 activity toward linoleic acid in DMPC vesicles and the inhibition of DMPC were obtained: V_{max} = 115 μmol·min^{-1}·mg^{-1}, K_m = 0.09 mM, and K_i = 0.13 mM. When the molar ratio of the inhibitor to the substrate is fixed the double reciprocal form of the velocity equation for competitive inhibition is shown as Eq. 2.

$$\frac{1}{v}=\frac{K_m}{V_{max}}\frac{1}{[S]}+\frac{\left(1+\frac{\alpha K_m}{K_i}\right)}{V_{max}} \tag{2}$$

where α = molar ratio of inhibitor to substrate by mole.

Since the slope expression does not contain an α term and the $1/v$-axis intercept increases with an increase in the value of α, the plots at various fixed molar ratios are expected to be parallel. The result shown in Fig. 2A inset is consistent with this prediction.

As Fig. 2B shows, the catalytic activity of L-1 is independent of the concentration of DPPC. The plots of [S]/v vs. [S] derived from the values shown in Fig. 3B at all the concentrations of DPPC lie on a single line, yielding K_m of 0.11 mM and V_{max} of 97 μmol·min^{-1}·mg^{-1}.

Table I summarizes the kinetic parameters obtained here. The specific activities (V_{max}) of L-1 toward the vesicular substrates are similar for three phospholipid systems tested and comparable to that toward linoleic acid in Tween 20 mixed micelles. One can postulate that the competitive inhibition with soy-PC is due to the esterified linoleoyl residues that comprise 47.8% of the acyl residues

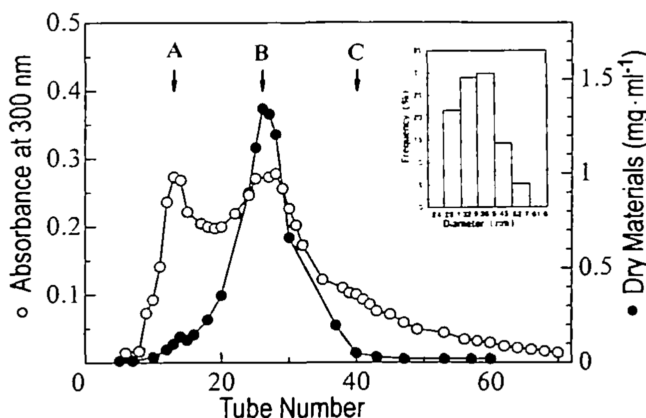

Fig. 1. **Sepharose-4B gel permeation chromatography of ultrasonic dispersion consisting of soy-PC and linoleic acid.** One milliliter of the dispersion (1.28 mM soy-PC/0.77 mM linoleic acid/10 mM sodium borate buffer, pH 9.0) was applied to the column (22 × 160 mm) and eluted with the same buffer as above. Fractions of 1.4 ml effluent were monitored at 300 nm, and dry materials were estimated by a colorimetric method using sodium dichromate. Symbols: ○, absorbance at 300 nm; ●, dry materials. Inset: Size distribution profile of the dispersion determined by quasi-elastic light scattering with a single vesicle mode.

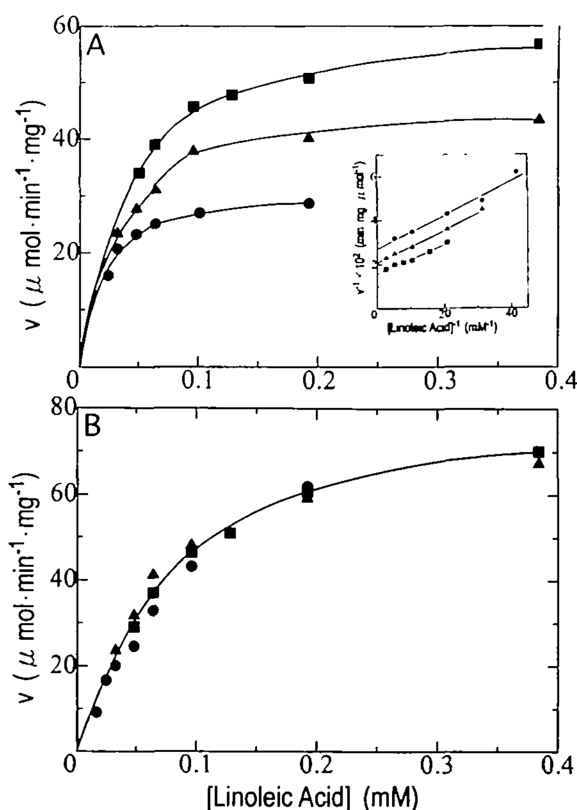

Fig. 2. **Dependence of initial velocity of L-1 on concentration of linoleic acid entrapped in soy-PC (A) or DPPC (B) at fixed molar ratio of linoleic acid to phospholipid for each run.** The vesicle dispersion, 0.5 ml, containing various concentrations of linoleic acid was incubated with 4 μl of diluted enzyme (0.17 μg of protein) for 4 min at 25°C. Enzyme activity was determined by spectrophotometrically analyzing linoleic acid hydroperoxide in 1 μl of the reaction mixture after its separation by HPLC. Symbols: molar ratio of linoleic acid to phospholipid: 0.15 (●), 0.30 (▲), and 0.60 (■). Inset in Fig. 2A: Double reciprocal plots derived from values presented in Fig. 2A.

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in soy-PC (*21*). It seems more likely, however, that the liquid-crystalline physical state of the vesicles is a dominant factor in the inhibition, since DMPC inhibits the enzyme in a similar manner. Both soy-PC and DMPC are melted at the reaction temperature of 25°C. In soy-PC and DMPC vesicles the substrate linoleoyl and the phospholipid acyl chains are molecular-dispersed and miscible in the liquid crystalline matrix of the vesicles, resulting in inhibition by the interaction among the acyl chains. On the other hand, the dipalmitoyl moiety of DPPC in the ordered gel matrix seems not to interfere with the catalytic reaction of L-1, because of its lack of interaction with the linoleoyl chain of the substrate. This leads to the following experiment.

Differential Scanning Calorimetry—The phase transition behavior of DPPC mixed with linoleic acid was investigated by DSC. Representative DSC scans are given in Fig. 5. The curve for pure DPPC (curve a) shows a sharp endothermic peak centering at 42°C. As the ratio of linoleic acid is increased, the main peak broadens and shifts to lower temperature. The minor peak has the midpoint at 37°C for pure DPPC. It also becomes broader and shallower when linoleic acid is mixed at a molar ratio to DPPC of 0.05 (curve b), and it disappears completely when the ratio is raised to 0.15 or above (curves c, d, and e). Table II summarizes the transition parameters for the DPPC vesicles with and without linoleic acid. The decrease in T_c of DPPC with the increase in the ratio of linoleic acid is in agreement with that reported by Fukui *et al.* (*22*). The enthalpies of transition (ΔH) for the DPPC-linoleic acid mixtures are similar to that for the pure DPPC. This behavior and the broadening of the endothermic peak by addition of linoleic acid strongly suggest that phase separation of DPPC takes place. Phase separation is known to occur in the intermolecular mixing of acyl chains with widely different phase transition temperatures in a lipid system (*23*). It is probable that, at temperatures below T_c, the solvent water easily penetrates into the phase-separated linoleic acid-DPPC matrix, after which the solute

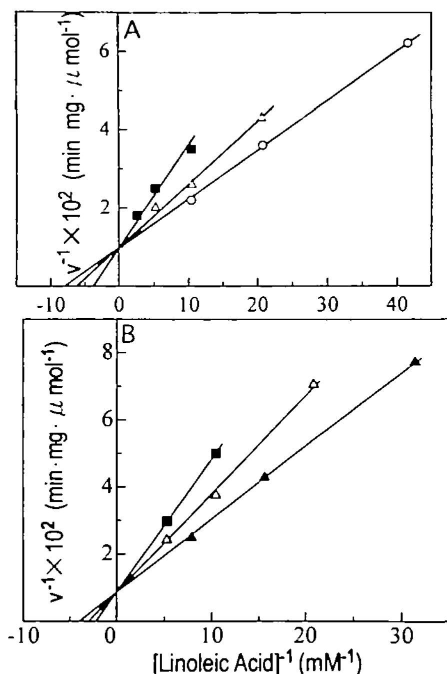

Fig. 3. **Lineweaver-Burk plots of L-1 activity toward linoleic acid entrapped in soy-PC (A) or DMPC (B) vesicles at various molar ratios of linoleic acid to phospholipid.** The reaction conditions and determination of enzyme activity were the same as in Fig. 2. Symbols: concentrations of phospholipids: 0.64 mM (■), 0.32 mM (△), 0.21 mM (▲), and 0.16 mM (○).

TABLE I. **Kinetic parameters of lipoxygenase-1 for linoleic acid embedded in phospholipid vesicles and dispersed in Tween-20 mixed micelles.**

	V_{max} (μmol·min^{-1}·mg^{-1})	K_m (mM)	K_i (phospholipid) (mM)
Soy-PC	101	0.07	0.20
DMPC	115	0.09	0.13
DPPC	97	0.11	–
Micelle	40.5	0.05[a]	–

[a]From Ref. *26*.

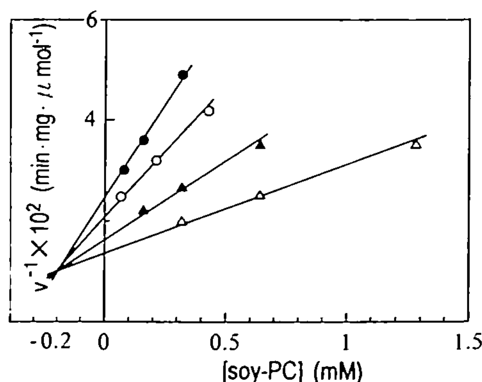

Fig. 4. **Dixon plots for linoleic acid-soy-PC vesicles at fixed concentrations of linoleic acid.** Symbols: concentration of linoleic acid: 0.048 mM (●), 0.064 mM (○), 0.096 mM (▲), and 0.192 mM (△).

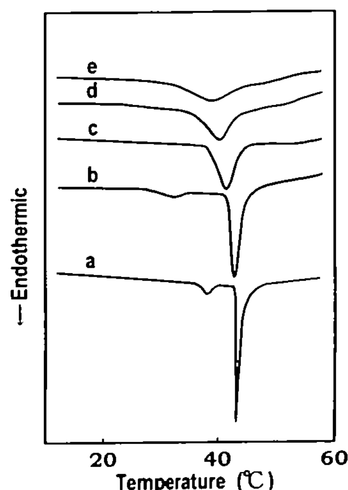

Fig. 5. **Calorimetric scans of ultrasonic dispersions of DPPC in the presence of linoleic acid.** Curves (a), (b), (c), (d), and (e) correspond to molar ratios of linoleic acid to DPPC of 0, 0.05, 0.15, 0.30, and 0.60, respectively. Final concentration of DPPC was adjusted to 2%. Heating and cooling rates were fixed at 1°C·min^{-1}.

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TABLE II. **Phase transition data obtained by DSC for linoleic acid-DPPC mixtures.**[a]

Linoleic acid (molar ratio to DPPC)	0	0.05	0.15	0.30	0.60
T_c (°C)	41.9	41.2	39.9	38.9	37.3
ΔH (kJ·mol^{-1})	25.1	25.4	25.2	24.7	24.6

[a]Final concentration of DPPC: 2%.

enzyme binds to linoleic acid without any interference by the clustered palmitoyl chains of DPPC. In contrast, at temperature above T_c, the catalytic activity of the enzyme toward linoleic acid in DMPC and soy PC may be inhibited by the acyl moieties of the phospholipids, which monomerically disperse and interact with the linoleoyl chains of the substrate in the layered matrices of the vesicles.

Effects of Temperature on Catalytic Activity of L-1 toward Vesicular Substrate—Figure 6 shows Arrhenius plots for the catalytic activity of L-1 toward linoleic acid entrapped in the vesicles and dispersed in the Tween-20 mixed micelles. The E_as were estimated by approximation to the straight line for the points in the range of 278 to 298 K. The point at 303 K was neglected since the enzyme was considered to be denatured. They are 27 kJ·mol^{-1} for the soy-PC and 35 kJ·mol^{-1} for DPPC. The latter value is comparable with that for the micelles, 37 kJ·mol^{-1} (Fig. 6A). For DMPC (Fig. 6B), the E_a value is evaluated to be 27 kJ·mol^{-1} if an approximately linear relationship holds throughout the range of the temperatures tested, similar to that for soy-PC. The plot for DMPC, however, shows a convex upward deviation from the straight line. Assuming that a break point exists around 288 K in the temperature dependence of the L-1 activity, the two values of E_a for DMPC are estimated to be 13.9 and 37.1 kJ·mol^{-1} for the lower and upper regions of temperature, respectively. This break might be associated with the phase transition of DMPC-linoleic acid mixture. It has been reported that the main transition of DMPC shifts to 17°C when it is mixed with linoleic acid (molar ratio to DMPC = 0.6) from that of pure DMPC (24°C) (*24*). It seems likely that the phase separation takes place below the T_c, by analogy with the behavior of linoleic acid-DPPC as seen in the last section (Fig. 5).

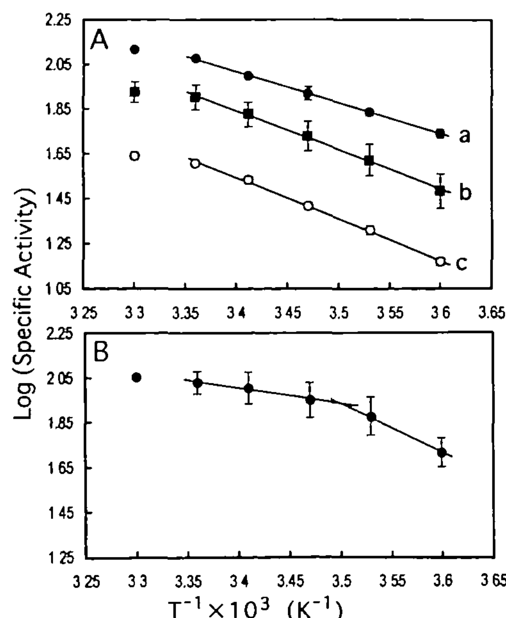

Fig. 6. **Arrhenius plots for L-1 activity toward linoleic acid entrapped in phospholipid vesicles or dispersed with Tween 20.** (A) Soy-PC vesicles (a), DPPC vesicles (b), and Tween-20 micelles (c). (B) DMPC vesicles. Mixtures of 4 μl of diluted enzyme (0.17 μg of protein) and 0.3 ml of 10 mM linoleic acid/33.3 mM phospholipid vesicle dispersions or 2.5 mM linoleic acid/0.25% Tween-20 mixed micelle dispersion were incubated for 4 min at 5, 10, 15, 20, 25, and 30°C.

DISCUSSION

It has been reported that the catalytic reaction of L-1 toward linoleic acid incorporated in the hydrated reversed micelles obeys the empiric Hill's equation for the solvent-bound substrate concentration (*11*). We present here evidence that the linoleic acids fixed in SUV matrices of phosphatidylcholines serve as a substrate for L-1 in a similar manner to the micellar substrate. A hyperbolic dependence of the initial velocity of the reaction on the concentration of linoleic acid was found for soy-PC, DMPC, and DPPC, regardless of their physical state.

A remarkable observation is the kinetically different effects of soy-PC, DMPC, and DPPC on L-1:soy-PC and DMPC competitively inhibit L-1, whereas DPPC has no effect. The expanded head group (*25*) of soy-PC or DMPC in the melted state may prevent the access of L-1 to the substrate acyl chain embedded in the lipid matrix. Another plausible explanation for the inhibition is interaction between the acyl chains of the liquid-crystalline substrate and soy-PC or DMPC. The fact that alcohols (*26*) and alkyl compounds (*26-28*) with medium chain lengths competitively inhibit L-1 may support the latter possibility.

It has been reported that the inner monolayer of SUV remains intact during the reaction of PLA_2 without trans-bilayer movement (flip-flop) of phospholipid molecules between the inner and outer monolayer (*29*). If the same situation occurs in the present experiment, *i.e.*, if only linoleic acid in the outer monolayer is the effective substrate, corrections should be made to the substrate concentrations and the K_m values. The numbers of molecules occupying the outer and inner monolayers for a small vesicle of phospholipid are calculated to be 3,820 and 2,490, respectively, by using 15 nm for the radius, 2 nm for the bilayer thickness, and 0.74 and 0.61 nm^2 for the effective surface areas per lipid head group for the outer and inner monolayers, respectively (*30*). Hence, the effective concentrations of linoleic acid are considered to be 0.6 of the overall concentration, and the values of K_m are reestimated to be 4 to 7×10^{-5} M. These values are comparable with those previously reported for the micellar systems (*26, 31, 32*). Thus, it was proved that the linoleic acids dispersed in the micelles and fixed in the lipid membrane of the SUV have the same affinity with L-1. The specific activities for the vesicles and the micelles also lie within the comparable range.

It is noteworthy that two different E_as are inferred for the reaction of L-1 with linoleic acid-DMPC vesicles above and below the T_c of the vesicles. The value of E_a for linoleic acid in the gel-state DPPC vesicles was shown to be higher than that in the liquid-crystalline soy-PC vesicles. It is unclear whether the two-phase behavior of E_a across the T_c

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of the vesicles is a common phenomenon in the reaction of L-1 and vesicular linoleic acid.

Further work is needed to clarify the interfacial reaction of L-1 on the biological membranes, using various natural phospholipids mixtures and liposomes including cholesterol.

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