**Comparison of Partitioning Behaviors of L-/D-Trp
in Solvent-Water System and Liposome Membrane System**

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The partitioning behaviors of L-/D-Trp in a solvent-water system modified with a phospholipid and a liposome membrane system were compared. Trp was partitioned to the interface of chloroform-water system supplemented with phospholipid. As the methanol ratio in the chloroform phase increased, the Trp was gradually partitioned to the organic phase while the chiral selectivity (the partitioning ratio of L-/D-Trp) was not increased. In contrast, the liposome membrane system was found to induce a significant chiral selectivity of L-Trp against D-Trp during their partitioning from the aqueous phase to the lipid membrane. This result clearly indicates the significance of the liposome membrane, a self-assembly of higher molecular order, as an effective platform for selective separation (or recognition).

1. Introduction

Amino acids are fundamental building blocks of proteins (enzymes) in biological systems. Many amino acids contribute to the regulation of some biological functions. All amino acids, except for glycine, have a chiral center at their α -carbon atom. Almost all amino acids exist in the L-enantiomer form while the building blocks (nucleic acid) of DNA exist in the D-enantiomer form. The above homo-chirality in biological systems (i.e. amino acids, nucleic acids) has already been investigated from a viewpoint of the origin of life [1-4].

Phospholipid molecules are the main components of biomembranes and common amphiphilic molecules derived from biological systems. There have been several studies on the up- or down-regulation of the function of the gene expression machinery [5], RNA [6], peptides [7] and enzymes [8] on

phospholipid membranes. These substances are recognized via electrostatic interactions, hydrogen bonds and hydrophobic interactions [9-10]. However, each contribution requires investigation in a simpler target molecule. In practice, the distinct orientation of each chiral oligopeptide in phospholipid membranes has been reported by using a liposome, a self-assembly of phospholipid molecules [11]. For the effective recognition of small stereo-chemical differences, it is important to understand the contribution of various bindings around the recognition site and the role of assembly formation on the lipid membrane in more detail.

The purpose of this study was to investigate the intermolecular interaction between phospholipids and tryptophan (Trp), which is an amino acid and is advantageous for quantitative and qualitative analyses because of the aromatic indole ring as a side chain. The solvent-water system, supplemented with a phospholipid, and the liposome membrane solution system were used as the extraction system targeting Trp as a model molecule. Chiral selectivity of Trp was also investigated by analyzing the partitioning behaviors of each Trp enantiomer (L-form and D-form).

Figure 1. Schematic Illustration of the partitioning behavior of L,D-Tryptophan (Trp) by phospholipids (PL) in different systems. (a) Phospholipids were dissolved in chloroform and formed a thin PL aggregation phase after mixing the phases. (b) Methanol was dissolved in chloroform/water solution with PL and the aggregation phase became thicker. (c) Liposome was prepared by hydrating the PL thin layer in the aqueous phase.

2. Experimental

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). L-Trp and D-Trp was purchased from Peptide Institute (Suita, Osaka, Japan). Chloroform and methanol were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan) and used without further purification.

With regard to the solvent-water system, DPPC was dissolved in chloroform or chloroform/methanol (27 mM). The ratio of methanol was varied from 0.05 up to 0.25. L-/D-Trp were dissolved in water as 3 mM solution. The Trp concentration in the aqueous phase was measured by UV absorbance at 280 nm, based on their calibration curves. Then the distribution ratio (*D*) of Trp at each enantiomer (*D_L* for L-Trp and *D_D* for D-Trp) was calculated by the following equation.

$$D = \frac{C_0 - C_1}{C_1}$$

where C_0 is the initial Trp concentration in the aqueous phase and C_1 is the equilibrium concentration of Trp in the aqueous phase after extraction.

In relation to the liposome preparation, a solution of DPPC in chloroform was dried in a round-bottom flask by rotary evaporation under vacuum. The lipid thin film was kept under high vacuum for at least three hours, and then hydrated at room temperature with water. The vesicle suspension was frozen at $-80\text{ }^{\circ}\text{C}$ and thawed at $50\text{ }^{\circ}\text{C}$ to enhance the transformation of small vesicles into large multi-lamellar vesicles (MLVs). This freeze-thaw cycle was performed five times. MLVs were used to prepare the small unilamellar vesicles (SUVs) by extruding the MLV suspension 11 times through two layers of polycarbonate membranes with mean pore diameters of 100 nm using an extruding device (Liposofast; Avestin Inc., Ottawa, Canada). After incubating the liposome solution (4.5 mM) and the amino acid solution (0.5 mM) at $25\text{ }^{\circ}\text{C}$ for 48 hours, the solution was filtered by using an ultrafiltration membrane (molecular cut: 50 kDa). The amino acid concentration of the filtered solution was determined by using the UV absorbance and the distribution ratio (D) of Trp for each enantiomer (D_L for L-Trp and D_D for D-Trp) using the following equation.

$$D = \frac{C_0 - C_A}{C_A}$$

where C_0 is the initial Trp concentration and C_A is the equilibrium concentration of Trp in the filtered solution.

3. Results and Discussion

Trp is known as a hydrophobic amino acid although its distribution ratio has a relatively low value in the organic-water system ($\text{Log } P = -1.1$) (except for partitioning at the isoelectric point). The effect of additives, such as the amphiphilic phospholipid (DPPC) and methanol, in the organic phase on the distribution ratio of Trp consisting of both enantiomers ($D_{L/D\text{-Trp}}$) was investigated in three types of systems, which are described in Figure 1. When chloroform (dielectric constant $\epsilon=5$) was used as the organic phase, a thin cloudy layer was formed on mixing the two phases (Figure 1(a)). This was probably caused by the formation of phospholipid aggregates at the interface on the solvent side. When methanol ($\epsilon=33$) was added to the chloroform, the formation of the cloudy layer was enhanced with an increase in the ratio of methanol (Figure 1(b)). The liposome membrane system has a hydrophobic layer with nano-order (approximately 4nm) on its surface (Figure 1(c)).

The distribution ratio of Trp (D_{Trp}) in these systems was determined from the mass balance by measuring the Trp concentration in the aqueous phase (Figure 2). At the lower methanol ratio ($X_{\text{met}} = 0 \sim 0.1$), the D_{Trp} value was found to be small (approximately 0.2). When the methanol ratio was increased, the D_{Trp} value became larger, with some expansion of the aggregation layer, and the value reached a plateau ($D_{\text{Trp}} = 0.6 \sim 0.7$) at a methanol ratio above 0.2. It is thought that the enhancement in the formation of the aggregation layer is promoted by the efficient contact between DPPC and Trp molecules, thus implying that

Figure 2. Distribution ratio of tryptophan (D_{Trp}) and its chiral selectivity (D_L/D_D) in different solvents of chloroform mixed with methanol. As the methanol ratio (X_{met}) increased, the dielectric constant (ϵ) is increased.

Figure 3. Chiral selectivity (D_L/D_D) and the yield of tryptophan (ratio of concentration between in water and in lipid phase) at the solvent of chloroform (a), chloroform mixed methanol (b) and liposome membrane (c) corresponding to Fig.1.

the binding between the lipid and Trp molecules became stronger and the partitioning behavior of Trp was enhanced. At methanol ratios over 0.25, the cloudy layer disappeared on mixing due to the reduction in the difference in the dielectric constant between the two phases. The chiral selectivity of Trp partitioning was also investigated by determining the ratio of the D_{Trp} value of L-Trp to that of D-Trp. The partition coefficient of D-Trp became slightly larger ($D_L/D_D < 1.0$) at lower methanol ratio. As the ratio was increased, the D_L/D_D value increased and approached to 1.0 ($X_{\text{met}} = 0.25$). It was thus found that the specific partitioning of the chiral Trp was not observed in the DPPC-modified organic-water system and was slightly improved with an increase in the ratio of methanol to chloroform in spite of the low chiral selectivity for L-/D-Trp.

Although DPPC, as a key molecule, is the same as the above-mentioned solvent-water system, a liposome is stable in water and forms a nano-scale vesicle structure by self-assembly of the phospholipid. The liposome bilayer membrane has an ordered structure through molecular alignment and can also be characterized as gradient polarity layers (Figure 1(c)). Trp dissolved in the aqueous phase was partitioned to the liposome membrane by mixing them for 48 hours. In this liposome membrane system, the yield of Trp recovery, calculated by its partitioning behavior from the aqueous phase to the phospholipid phase, was found to be high especially in the case of L-Trp, while that of D-Trp was extremely low (Figure 3). As for the chiral selectivity, the liposome

solution indicated an extremely high value ($\sim 10^4$) as compared with pure chloroform and chloroform mixed with methanol. It is thought that the liposome membrane could provide a suitable environment for the partitioning of the hydrophobic Trp and the electrostatic and hydrophobic interactions neighboring to the chiral C carbon of the lipid molecule could be related to its dramatic chiral recognition. Although further investigation is needed, the use of the liposome membrane system was thus shown to achieve a specific partitioning of L-Trp, different from the solvent-water system, probably, because of the “order” of the lipid molecules at the interface.

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