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Thermodynamics of the binding of Substance P to lipid membranes

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Abstract: The thermodynamic functions for the binding of the peptide Substance P (SP) on the surface of lipid vesicles made of various types of lipids were obtained by using isothermal titration calorimetry. The reaction enthalpies measured from the experiments were -0.11 to -4.5 kcal mol^{-1}. The sizes of the lipid vesicles were measured with dynamic light scattering instrument in order to get the correlation between the reaction enthalpies and the vesicle sizes. The bindings of SP on the lipid vesicles with diameter of 37 to 108 nm were classified into the enthalpy-driven reaction or the entropy-driven reaction according to the size of the lipid vesicles. For the enthalpy-driven binding reaction, the significance of the electrostatic interactions between SP and lipid molecules was affirmed from the experimental results of the DMPC/DMPG/DMPH and DMPC/DMPS/DMPH vesicles as well as the importance of the hydrophobic interactions between hydrophobic groups of SP and lipid molecules.

Key words: thermodynamics, partitioning, Substance P, lipid vesicle

1. Introduction

Physicochemical parameters involved in the reaction of the biomembrane peptide, which latches on to the biomembrane and shows a variety of biological reactions, are extremely useful in understanding not only the biological reaction process of the biomembrane peptide but also the biological characteristics of the biomembrane.[1,2] Ongoing research attempts are aiming to understand how differences in the peptide structure and the number of electric charges could impact the reaction between the biomembrane and the peptide, upon attachment of the peptide to the biomembrane.[3-6] In addition, the reactivity of the biomembrane peptide, which depends on the characteristics of the biomembrane, has been determined using various biomembranes and model biomembranes. Specifically, research findings are being reported using various model biomembranes to identify differences in reaction, depending on the type of lipid molecule, ion density, and existence of some other substance in the biomembrane.[7-9] Among these types of research, thermodynamic research on the process of reaction between the biomembrane and the biomembrane peptide plays an important role in identifying the reaction mechanism and the major factors involved. For instance, the thermodynamic function is measured and interpreted to identify the level of impact of the

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peptide's structural change during the reaction (random coil → helical structure) on the partition coefficient from the aqueous solution to the model biomembrane.[10]

In general, it is not appropriate to physicochemically study the biomembrane peptide reaction by using the actual biomembrane. This is because it is extremely difficult to independently identify the underlying physicochemical elements of the biomembrane peptide bonding owing to the complexity of the biomembrane. Usually, research is conducted using model lipid membranes made up of phospholipids constituting the biomembrane—for instance, micelles, vesicles, and bicelles.[11] The aforementioned model biomembranes facilitate thermodynamic research on reaction characteristics depending on the type of lipid molecule and the level of reaction depending on the size or curvature of the model lipid membrane. Among others, unilamellar vesicles are relatively more useful because they can be easily prepared in different sizes. The lipid membrane curvature changes with a change in the vesicle size. If the lipid membrane curvature changes, the degree to which the biomembrane peptide penetrates into the model lipid membrane changes and then the characteristics of interaction between the peptide and the lipid molecule change. Seelig *et al.* interpreted these characteristics using the chemical terms of classical hydrophobic interactions and nonclassical hydrophobic interactions.[1] Such interpretation allows researchers to identify the effects of reaction enthalpy and entropy on the bonding of the biomembrane peptide onto a model lipid membrane. It is also possible to identify that the effects of enthalpy and entropy vary depending on the type of lipid molecule constituting the lipid model membrane (differences in the size and ion distribution of the lipid's hydrophilic region or the shape and size of the hydrophobic region). To facilitate such physicochemical understanding, molecular dynamics simulation has been used in some researches.[12]

This study examined the thermodynamic characteristics of the reaction between Substance P (SP), one of the biomembrane peptides, and vesicles made up of various phospholipids. This study measured the dynamic light scattering (DLS) spectrum to identify the size of the vesicle, the circular dichroism (CD) spectrum to identify the structural changes in the peptide during the SP bonding process, and the isothermal titration calorimetry (ITC) spectrum to identify changes in enthalpy and entropy during the reaction.

SP is a neuropeptide made up of 11 amino acids and is involved in various physiological processes such as muscle contraction, salivation, blood flow, inflammation, and pain transmission.[13,14] SP is generally known to react with the receptor in the biomembrane and plays a role in transmitting signals through the biomembrane. Several studies confirmed that biomembrane lipid molecules affected the function of SP.[15, 16] When SP bonded with the lipid membrane surface, vast changes in the structure of SP and reaction enthalpy were observed, depending on the physicochemical characteristics of the model lipid membrane. By measuring the thermodynamic function of the reaction between SP and the vesicle comprising various lipid molecules, this study intended to enhance the basic understanding of the type of interactions involved in the bonding between the biomembrane peptide and the lipid membrane.

2. Experiment

2.1. Reagents and samples

Substance P was purchased from Sigma-Aldrich and used without further purification. 1,2-dimyristoyl-sn-glycerol-3-phosphatidylcholine (DMPC), 1,2-dimyristoyl-sn-glycerol-3-phosphoglycerol (DMPG), 1,2-dimyristoyl-sn-glycerol-3-phosphatidylserine (DMPS), and 1,2-dihexanol-sn-glycerol-3-phosphatidylcholine (DHPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL).

The SP sample was prepared by adding SP to phosphate buffered saline (PBS) (10 mM phosphate, 100 mM NaCl, pH 7.0). The final SP concentration was 100 μM or 400 μM. Large unilamellar vesicles (LUVs) up to 100 nm in diameter were prepared through the following process: After lipids dissolved in chloroform were mixed at the set ratio, chloroform was evaporated under nitrogen gas and additionally

removed under vacuum conditions. After the organic solvent was removed, PBS was added to the lipid compound, vortexed for 30 minutes at 38 °C and frozen under liquid nitrogen. The compound was thawed and then vortexed again. This process was repeated several times. The resulting solution was extruded at least 9 times using the Avanti Mini-Extruder. The pore size of the polycarbonated filter used in the study was 100 nm. The final concentrations of the lipids obtained were 10 mM and 20 mM.

2.2. Experimental instruments and measurements

The size of the vesicle prepared as described above was measured using the DynaPro NanoStar instrument (Wyatt Technology, GmbH, Europe). The CD spectrum was acquired using the Jasco J-715 spectrometer. The wavelength of the spectrum ranged from 200 nm to 260 nm. The SP concentration of the solution used to measure the CD spectrum was 50 μM whereas its lipid concentration was 5 mM. Three measurements were taken and the average was used for analysis. The ITC spectrum was acquired using the Auto ITC 200 calorimeter (General Electric). For the ITC measurement, 200 μL of the 100 μM SP solution was added at the bottom of the cell and the vesicle with a concentration of 10–20 mM was filled in the syringe at the top. Next, 2 μL of the vesicle solution was added at the bottom of the cell every 5 minutes to measure the changes in calories. The cell at the bottom was stirred continuously at 400 rpm. All the experiments above were conducted at 25 °C.

2.3. Binding model

To obtain the thermodynamic function values from the ITC isothermal data, the binding model described below was applied.[17, 18] This model assumes that the peptide reacts with the surface of the vesicle consisting of lipids as in the following equation: nL+P↔PL$_n$. In other words, it assumes that a peptide bonds with n lipid molecules on the vesicle surface. In this equation, L represents the lipid molecule, P represents the peptide, and PL$_n$ represents the bonding of a peptide and n lipid molecules. In this case, the following Langmuir binding isotherm is derived:

$$\frac{c_{P,b}}{(c_{L,total}/n)} = \frac{Kc_{P,f}}{1+Kc_{P,f}} \tag{1}$$

In the equation above, $c_{L,total}/n$ indicates the maximum number of bondable peptides. $c_{P,b}$ indicates the concentration of bonded peptides, $c_{L,total}$ indicates the total concentration of lipid molecules, K indicates the equilibrium constant, and $c_{P,f}$ indicates the concentration of the non-bonded peptides adjacent to the surface of the lipid membrane, which is assumed to be the same as that of peptides in the bulk solution. Since n lipid molecules bond with a peptide, $c_{P,b} = \frac{c_{L,b}}{n}$. $c_{L,b}$ indicates the concentration of the lipid molecule bonded with the peptide. Eq. (1) could be written as:

$$K = \frac{c_{P,b}}{(c_{L,total} - c_{L,b})c_{P,f}} = \frac{c_{P,b}}{(c_{L,total} - c_{L,b})(c_{P,total} - c_{P,b})} \tag{2}$$

In this equation, $c_{P,total}$ refers to the total peptide concentration. The concentration of the bonded lipid molecule from Eq. (2) could be described using the mole ratio of total lipid molecules to peptide molecules in the solution $x = \frac{c_{L,total}}{c_{P,total}}$ as:

$$c_{L,b} = \frac{1}{2}\left(\frac{1}{K} + nc_{P,b} + c_{P,total}x\right) - \frac{1}{2}\left\{\left(\frac{1}{K} + nc_{P,b} + c_{P,total}x\right)^2 - 4nc_{P,total}^2 x\right\}^{1/2} \tag{3}$$

The heat of reaction after the ith lipid drop is added is derived by multiplying the number of moles participating in the reaction by reaction enthalpy as:

$$\Delta q(i) = \{c_{L,b}(i) \times V_{cell}(i) - c_{L,b}(i-1) \times V_{cell}(i-1)\} \times \Delta H_{lipid} \tag{4}$$

In the equation above, $\Delta q(i)$ refers to the calories arising from the addition of the ith lipid, $c_{L,b}(i)$ and $c_{L,b}(i-1)$ refer to the concentrations of the lipid bonded with the peptide after ith and i-1th lipids are added, respectively, $V_{cell}(i)$ and $V_{cell}(i-1)$ refer to the volumes of the solution in each case, and ΔH_{lipid} refers to the reaction enthalpy per lipid mole. The concentration of the lipid bonded with the peptide at each stage is obtained by Eq. (3). By interpreting ITC data using Eq. (4), the equilibrium constant (K),

number of lipid molecules bonded with a peptide (n), and reaction enthalpy (ΔH_{lipid}) could be calculated.

3. Results and Discussion

This study identified the vesicles and measured their size using DLS. *Fig.* 1 shows the size distribution of vesicles made up of various lipids. It was found that vesicles were even in size except for vesicles consisting of DMPC/DMPS/DHPC. Vesicles consisting of DMPC, DMPG, or DMPS had an average diameter of approximately 100 nm. While the average diameter of the DMPC vesicles was 126 nm, that of the DMPC/DMPG vesicles was 108 nm. The size of DMPC/DMPG vesicles, which included DMPG with a negative electric charge in the head group, was found to be decreased, possibly because of the electrostatic interaction between the lipid molecules having a negative electric charge. This indicates that the vesicle membrane had become more solid, imparting more stability to the lipid membrane.

The lipids containing DHPC, however, were much smaller than 100 nm in size although they were prepared by passing through the 100-nm pore size polycarbonated filter. The average diameter of the DMPC/DMPG/DHPC vesicles was 50.1 nm whereas that of the DMPC/DMPS/DHPC vesicles was 36.9 nm. It is known that a bicelle in the size of several nm is formed when DHPC and DMPC are mixed to prepare a model lipid membrane. However, in this study, given the size of the lipid aggregate, it could be said that the vesicles were formed rather than bicelles. It has been reported that the physicochemical state of the solution requires extremely fine adjustment to prepare a bicelle when two lipid molecules of different sizes are mixed.[19] As DHPC, with a smaller tail group, intercalated into the space between DMPC and DMPG lipid molecules, the curvature of the vesicle was shown to increase. An increase in the vesicle's curvature implied a decrease in the size of the vesicle. Consequently, the DMPC/DMPG vesicle whose diameter was 108 nm was reduced into the DMPC/DMPG/DHPC vesicle whose dimeter was 50.1 nm. The vesicle showing a decreased size owing to the mixture of DHPC was considered to have a more stable and solid lipid membrane.

As illustrated in *Fig.* 1, vesicles containing DMPS were smaller than those containing DMPG because of structural differences in the head group of the two lipid molecules. Both DMPG and DMPS possess a

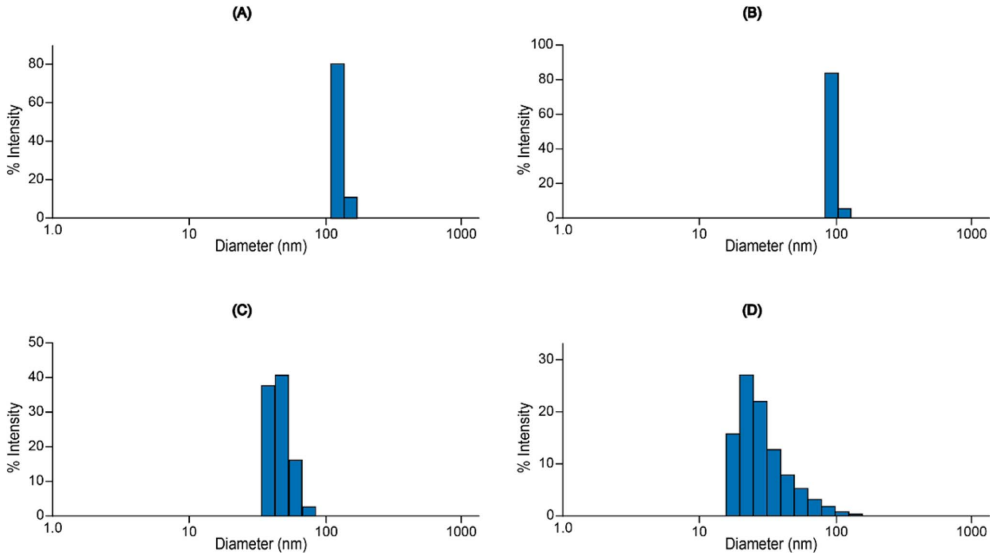

Fig. 1. Dynamic light scattering measurements: hydrodynamic diameters of lipid vesicles made of DMPC (A), DMPC/DMPG (B), DMPC/DMPG/DHPC (C), and DMPC/DMPS/DHPC (D).

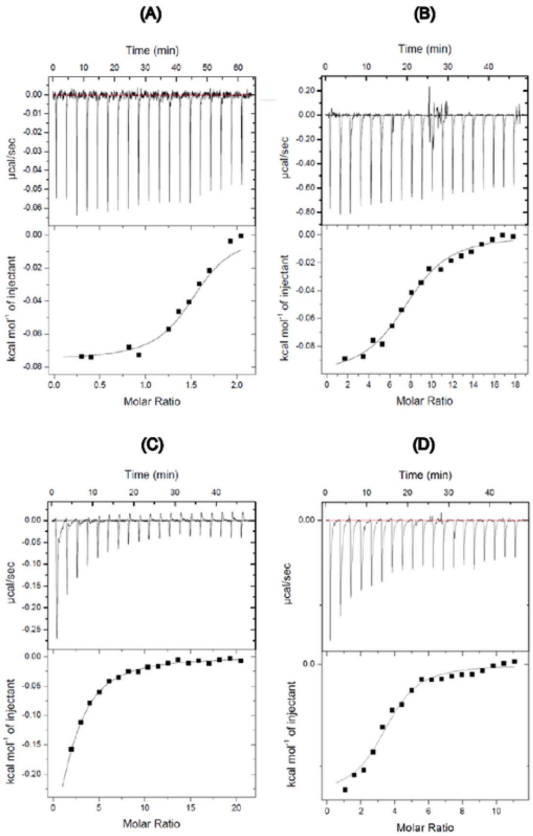

Fig. 2. Isothermal titration calorimetry of SP solutions containing the lipid vesicles made of DMPC/DHPC (A), DMPC/DMPG (B), DMPC/DMPG/DHPC (37.5:12.5:50) (C), and DMPC/DMPS/DHPC (56.3:18.7:25) (D), Each peak corresponds to the injection of 2 μL of lipid suspension into the reaction cell (V_{cell}=0.2 mL) containing the SP solution. Solid lines are the theoretical binding isotherm.

head group showing a negative electric charge; however, DMPG has one anion only whereas DMPS has one cation and two anions. Hence, in comparison with DMPG, DMPS is expected to show stronger electrostatic interaction with zwitterionic DMPC and other neighboring DMPS molecules, which appears to have affected the vesicle size.

Fig. 2 shows the ITC experimental data obtained from the reaction of SP with various vesicles and the theoretical binding isotherm. Results demonstrate that the theoretical reaction model mentioned above fits quite well within the margin of error for the experimental data. Thermodynamic function values derived from this theoretical binding isotherm are summarized in *Table* 1. Reaction enthalpies acquired directly from the experiment are values in the 5th column and indicate the reaction enthalpies per lipid mole (ΔH_{lipid}). Reaction enthalpies in the 6th column indicate the reaction enthalpies per SP molecule mole (ΔH_{pept}) and are calculated by multiplying values in the 3rd column (n) by values in the 5th column (ΔH_{lipid}). The Gibbs energy in the 8th column (ΔG_{pept}) is calculated using the equilibrium constant in the 3rd column (K). Reaction entropy in the 7th column (ΔS_{pept}) is calculated by reaction enthalpy and the Gibbs energy.

As demonstrated by the ITC data, all the reactions where SP bonds with the vesicle surface were exothermic reactions with negative reaction enthalpy. Except for the DMPC/DMPS/DHPC vesicle, all the other types of vesicle showed low reaction enthalpy. This suggests that reaction entropy leads the bonding of SP, instead of reaction enthalpy. By contrast, reaction enthalpy leads the reaction in the case of DMPC/DMPS/DHPC vesicle.

Generally, when the vesicle size is small, reaction

Table 1. DLS and ITC results at 25 °C

Vesicles [a]	Diameter (nm)	n	K (M^{-1})	ΔH_{lipid} (cal mol^{-1})	ΔH_{pept} (kcal mol^{-1})	ΔS_{pept} (cal mol^{-1} K^{-1})	ΔG_{pept} (kcal mol^{-1})
DMPC:DHPC	nd[b]	1.5	3.5×10^5	-75	-0.11	25	-7.6
DMPC:DMPG	108	7.8	7.5×10^3	-101	-0.79	15	-5.3
DMPC:DMPG:DHPC	59	1.7	4.7×10^3	-580	-1.0	13	-5.0
DMPC:DMPG:DHPC	50	8.4	3.8×10^4	-145	-1.2	17	-6.2
DMPC:DMPS:DHPC	37	3.5	9.4×10^4	-1270	-4.5	8	-6.8

[a]The molar ratios of lipids. DMPC:DHPC=50:50, DMPC:DMPG=75:25, DMPC:DMPG:DHPC=37.5:12.5:50 (diameter=59 nm), DMPC:DMPG:DHPC=56.3:18.7:25 (diameter = 50 nm), DMPC:DMPS:DHPC=56.3:18.7:25
[b]Non-detected

enthalpy has been known to lead the reaction.[6] According to Seelig *et al.*, nonclassical hydrophobic interactions are usually important in a reaction led by reaction enthalpy.[20] Nonclassical hydrophobic interactions are represented by van der Waals interactions between the peptide's hydrophobic residue and the lipid molecule's hydrophobic tail group. When the vesicle size is small, the large curvature of the vesicle would allow the hydrophobic tail group embedded inside the lipid bilayer to be exposed to the surface, thus facilitating the hydrophobic interaction between the peptide and lipid. On the contrary, when the vesicle is big, the membrane peptide's bonding is known to be led by reaction entropy.[6] During the reaction between the membrane peptide and lipid membrane, reaction entropy increases owing to an increase in the entropy of water, as water molecules orderly bonded with the lipid membrane surface and the membrane peptide surface fall off the interface between membrane surface and peptide owing to the bonding of the two molecules.[1] In the case of a large vesicle, it is suggested that the membrane peptide bonds with the large area of the vesicle surface owing to its small curvature.

When DMPC/DMPG/DHPC and DMPC/DMPS/DHPC vesicles were compared, there were huge differences in reaction enthalpy, possibly owing to the decrease in vesicle size. However, not only van der Waals interaction but also electrostatic interactions between DMPS and SP appear to have contributed to this variation. In a molecular dynamics simulation research on the interaction between the fusion peptide gp41-FP and the aerosol-OT micelle, Barz *et al.* confirmed that the size of the lipid molecule surface area affected the peptide structure.[12] In the lipid bilayer, the lipid molecule surface area is related to the curvature on the surface of the lipid bilayer. Usually, the lipid molecule surface area increases with an increase in the lipid bilayer curvature. As such, electrostatic interactions between the peptide and lipid molecule could also increase. According to the molecular dynamics simulation research of Broemstrup *et al.*, DMPS molecules are known to bond more strongly with Na^+ than DMPG molecules do.[21] It

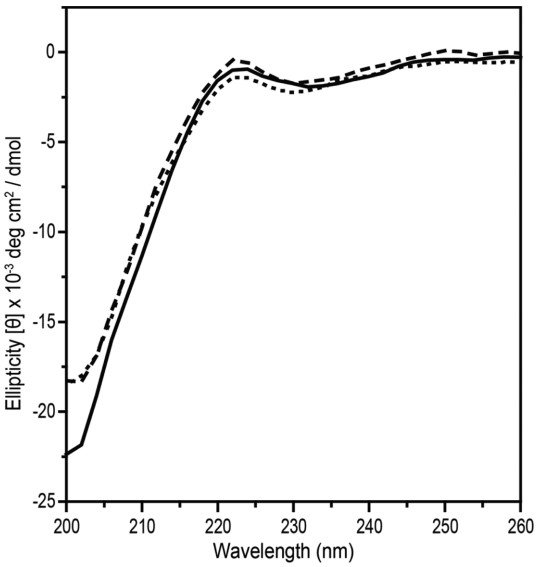

Fig. 3. CD spectra of SP in PBS solution (solid), in DMPC/DMPG/DHPC solution (dot), and in DMPC/DMPG/DHPC solution (dash).

means that the anion in the carboxyl group of the DMPS molecule strongly bonds with the Na^+ cation. Therefore, it is expected that a similar phenomenon would occur with the SP peptide having several cations, which is consistent with the results of the current study. Wymore *et al.* also reported that electrostatic interaction and hydrogen bonding between the peptide and the head group of the sodium dodecylsulfate micelle had a key impact on the structure and fluidity of SP.[22] Owing to changes in the vesicle size, the reaction enthalpy showed considerable changes. It is therefore difficult to interpret all the changes in reaction enthalpy by using only van der Waals interaction arising from changes in the vesicle size. Gibbs energy and entropy values in this study seem to have been acquired appropriately, as they were found to be similar to the values reported for the membrane peptide.[23]

Fig. 3 shows the CD spectrum of the mixture of the lipid vesicles and SP. This spectrum demonstrates that SP mostly depicts a random coil structure, regardless of the existence of the vesicle, suggesting that SP does not penetrate deep into the lipid

membrane when it bonds with the vesicle. Electrostatic interaction between the SP cation and the negative electric charge in the head group of the lipid molecule is thought to contribute more to the bonding of SP with the vesicle than the hydrophobic interaction between the hydrophobic residue of SP and the tail group of the lipid molecule. In summary, DMPS molecules possibly exhibit greater electrostatic interactions with SP than DMPG molecules.

4. Conclusions

In the bonding between the DMPC-based vesicle and SP, it was confirmed that reaction enthalpy was greatly determined by the size of the lipid vesicle. In vesicles of 50 nm or bigger, reaction entropy contributed to the peptide bonding reaction on the model lipid membranes much more than reaction enthalpy did. By contrast, in vesicles of less than 50 nm, reaction enthalpy led the reaction. These findings are consistent with the established fact that hydrophobic interaction is important in SP bonding as the vesicle gets smaller. Moreover, this study confirmed, based on differences in contribution between two lipid molecules DMPG and DMPS, that the electrostatic interaction between SP and lipid molecule is also important for the SP bonding on the model lipid membranes.

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