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Thermodynamics of Partitioning of Substance P in Isotropic Acidic Bicelles

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The temperature dependence of the partition coefficients of a neuropeptide, substance P (SP), in isotropic acidic bicelles was investigated by using a pulsed field gradient nuclear magnetic resonance diffusion technique. The addition of negatively charged dimyristoylphosphatidylserine to the neutral bicelle changed the SP partitioning a little, which implies that the hydrophobic interaction between the hydrophobic residues of SP and the acyl chains of lipid molecules is the major interaction while the electrostatic interaction is minor in SP binding in a lipid membrane. From the temperature dependence of the partition coefficients, thermodynamic functions were calculated. The partitioning of SP into the acidic bicelles is enthalpy-driven, as it is for small unilamellar vesicles and dodecylphosphocholine micelles, while peptide partitioning into a large unilamellar vesicle is entropy-driven. This may mean that the size of lipid membranes is a more important factor for peptide binding than the surface curvature and surface charge density.

Key Words : Substance P, Bicelle, Partitioning, Diffusion, NMR

Introduction

Understanding the interaction of peptides with biological membranes is one of the foundations for understanding a number of biological processes, such as insertion and folding of peptides and proteins in biomembranes, membrane fusion through the membrane fusion peptides, destruction of biomembranes, and membrane-mediated peptide-receptor formation. A large number of biochemical studies have been conducted in recent years, using a variety of techniques, on the interaction of peptides with model membranes.[1,2] Nuclear magnetic resonance (NMR) has been a major technique in such studies.[3-5]

For high resolution NMR studies the model membranes have to reorient isotropically and randomly with a correlation time shorter than the nanosecond time scale in a solution. Due to these limits, micelles or small unilamellar vesicles (SUVs) have been used as model membranes in the NMR studies.[6-8] Among them, a dodecylphosphocholine (DPC) micelle, having a zwitterionic headgroup, was the best membrane mimic for structural or dynamic studies of membrane peptides. Even though micelles and SUVs are very efficient mimic systems, there is a problem in that they all have curved surfaces. As many other studies have reported, the curvature of the membrane surface may affect the action of the peptide on the biomembranes. In order to separate the effect of membrane curvature from other factors influencing the binding of peptides on the membrane surface, a new membrane mimic is required which has a similar size to a micelle (because of its isotropic rapid rotational motion in solution) but a flat surface. Recently, a new membrane mimic named bicelle, which has a disc form, was introduced and has been widely used, particularly in studies on the partitioning (or binding) of peptides to this bicelle.[9-14]

A large number of investigations on peptide binding have been performed in order to understand the effects of the net charge of hydrophilic residues, the hydrophobicity of hydrophobic residues, the binding conformation of peptides, and the surface charge density and aggregation type of the lipid membrane.[5,9,15,16] However, it is difficult to separate the effects of each factor due to their cooperative nature. In our previous study we compared the experimental results of the bicelle system with those of the micelle system in order to investigate the effect of surface curvature on the peptide binding onto biomembranes.[17] To understand the results from the microscopic perspective, the thermodynamic functions of peptide partitioning, such as Gibbs free energy, enthalpy, and entropy, were obtained.

In the partitioning of amphiphatic peptides onto lipid bilayers, the Gibbs free energy change of the process is affected by two factors: the changes in enthalpy and entropy. In large-sized membranes such as a unilamellar vesicle (LUV), small negative or nearly zero changes in enthalpy and large positive changes in entropy have been observed in peptide partitioning.[16] This is referred to as the classical hydrophobic effect, in which the driving force for the spontaneous reaction is the large increase of entropy that results from the disordering of the bulk water deleted from the peptide. In contrast to large-sized vesicles, large negative changes of the enthalpy and the entropy were observed in small vesicles.[16,18] This is referred to as the nonclassical hydrophobic effect, in which the driving force is the van der Waals interactions between the nonpolar residues of the peptide and the hydrophobic core of the lipid bilayer. However, the molecular origin of the phenomena is unclear.[19]

Substance P (SP) is involved in many important physiological processes including pain transmission, inflammation, blood flow, salivation, and various muscle contractions.[20,21]

SP is also known to activate three membrane-embedded receptor subunits with different levels of potency.[21] The interaction of an amphiphatic peptide, SP, containing eleven residues, with lipid monolayers or bilayers has been investigated extensively in the past.[4,5,22,23] The lipid membrane has been known to play an important role in the biological activity of SP either by increasing the concentration of SP at the surface of the membrane or by inducing and stabilizing the physiologically active conformation of SP.

In this work, we report the temperature dependence of the partition coefficient of SP on bicelles made of 1,2-dimyristoyl-*sn*-gylcerol-3-phosphatidylcholine, 1,2-dimyristoyl-*sn*-gylcerol-3-phosphatidylserine and 1,2-dihexanol-*sn*-gylcerol-3-phosphatidylcholine lipids using the pulsed-field gradient (PFG) NMR diffusion method. From these temperature-dependent partition coefficients, the thermodynamic functions of the partitioning of SP in the neutral and acidic bicelles were determined and compared to our previous results[17] in order to understand the effects of the surface charge, the surface curvature, and the size of lipid model membranes on the SP binding.

Materials and Methods

Sample Preparation. Substance P was obtained from Sigma chemical company. 1,2-Dimyristoyl-*sn*-gylcerol-3-phosphatidylcholine (DMPC), 1,2-dimyristoyl-*sn*-gylcerol-3-phosphatidylserine (DMPS), and 1,2-dihexanol-*sn*-gylcerol-3-phosphatidylcholine (DHPC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). Samples were made by adding an aqueous SP solution to lipid bicelle solutions which were prepared by mixing the DMPC, DMPS, and DHPC in a chloroform solution in order to make solutions with a ratio of q = [DMPC+DMPS]/[DHPC] = 0.5 and ratios of r = [DMPS]/[DMPC] = 0/100, 15/85, and 30/70. The total amount of lipids was 15% (w/v) and the molar ratio of the peptide to the lipids was 1:40. The final concentration of SP was 2.8 mM in the bicelle solutions with 0.1 M of NaCl. The pH was adjusted to 6.0 with sodium acetate buffer solution and 10% of D_2O was added for field locking in NMR experiments. The mixed solutions were heated for 30 min at 38 °C, incubated for 15 min at 4 °C and vortexed. These processes were carefully performed several times until the lipid bicelle solutions were cleared.[24] For the diffusion rate measurement, a minute amount of hexamethyldisilane (HMDS) was added to the bicellar solutions as a probe molecule in order to measure the diffusion rate of a whole bicelle.[25]

NMR Spectroscopy. All the NMR experiments were carried out using a Bruker DRX 500 NMR spectrometer equipped with a broad-band inverse probe and pulsed field *z*-gradient capability. The gradient strength used was determined by comparing the known diffusion coefficient of water, 1.9×10^{-9} m^2/s, at 25 °C with the experimentally measured one. For diffusion experiments, a modified version of a double-stimulated-echo (DSTE) pulse sequence with bipolar gradient pulses was used to effectively suppress the convection artifacts.[26] Diffusion times of 400-1000 ms were used for the peptide and the lipid bicelle and diffusion times of 40-80 ms were used for water. The gradient duration was 2 ms and the settling time was set to 30 ms. A sine-shaped spoiler gradient with 6.6 G/cm was applied for 4 ms in each z-storage period. The longitudinal eddy-current delay was set to 30 ms. Scans of 32 and 256 with phase cycling were collected for the diffusion signals of lipid and peptide, respectively. The gradient strength in a series of experiments was incremented from 0.5 to 25 G/cm in 10 steps. Temperature-dependent experiments were performed in the range of 296-326 K in increments of 5 K. The temperature was maintained to within ± 0.1 K during the experiments.

Determination of Peptide Partition from Diffusion Measurements. The partition coefficients of SP in the bicelle solutions were calculated from the diffusion coefficients determined by measuring the decay of the PFG echo signal in the diffusion experiment using a DSTE pulse sequence and fitting the gradient-dependent signal attenuation, f_G, with the following equation through a nonlinear least-squares fitting method[26]:

$$f_G = \exp\left[-Dq^2\left(T+\frac{4\delta}{3}+\frac{5\tau_1}{4}+\frac{\tau_2}{4}\right)\right], \quad (1)$$

where D is the diffusion coefficient, T is the diffusion time, δ is the gradient duration, and τ_1 and τ_2 are the settling times. In this equation, q is defined as $q = \gamma\delta g$, where γ is the magnetogyric ratio of the nucleus and g is the gradient strength. To avoid relaxation effects in the diffusion measurement, the gradient strength was varied with the diffusion time being constant in the DSTE pulse sequence. The experimental and theoretical decays of PFG echo signals of bicelle and SP are shown in Figure 1.

The analysis of the peptide diffusion data in the bicelle system is based on the two-site model.[27] The decay of the PFG echo signal is basically two-exponential in the two-site model but when the exchange between two sites is fast compared to the chemical shift differences and to the diffusion time, like in our case, the decay of the PFG echo signal can be safely approximated as single exponential decay. Thus

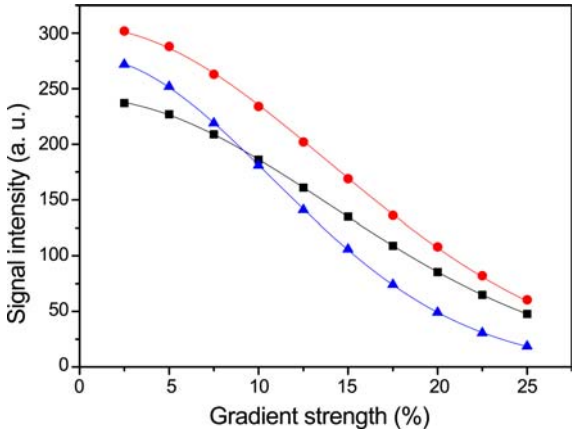

Figure 1. The experimental and theoretical (solid curves) decays of PFG echo signals of DMPC (■), HMDS (●), and SP (▲) in 0% neutral bicelles at 311 K.

the measured diffusion coefficient, D_{obs}, obtained from the decay of SP can be given by[27]

$$D_{obs} = f_b D_b + (1-f_b)D_f, \quad (2)$$

where D_f and D_b denote the diffusion coefficients of the peptide in the free and bound forms, respectively. Here f_b is the fraction of the bound peptide. D_b can be taken as being equal to the diffusion coefficient of the bicelle, $D_{bicelle}$, which can be directly measured from the signal decays of DMPC lipid, or more accurately from the signals of a small amount of a hydrophobic molecule which is known to be completely solubilized within the hydrophobic core of the membrane. HMDS has been widely used as a probe molecule for this purpose in diffusion studies of membrane mimic systems.[25] Two diffusion coefficients of the bicelle measured using the two methods were in agreement with each other within the experimental error. D_f was obtained from diffusion measurements of free peptide and water in the buffer solutions using the following equation[28]:

$$D_f = D_f^0 \frac{D_{water}^b}{D_{water}^0}, \quad (3)$$

where D_f^0 and D_{water}^0 are the diffusion coefficients of free peptide and water in the buffer solution, respectively, and D_{water}^b is the diffusion coefficient of water in the bicelle solution. The partition coefficient of peptide in bicelles, p, is defined as[6]

$$p = [P]_{bicelle}/[P]_{aqueous}. \quad (4)$$

The peptide concentrations [P] in the respective phases can be determined through their relations to f_b which is calculated from the measured diffusion coefficients with Eq. (2),

$$[P]_{bicelle} = n_p \times f_b / V_{bicelle}, \quad (5a)$$

$$[P]_{aqueous} = n_p \times (1-f_b)/V_{aqueous}, \quad (5b)$$

where n_p is the total number of moles of peptide in the sample, $V_{bicelle}$ and $V_{aqueous}$ are the phase volumes of the bicelles and the aqueous bulk, respectively, and their ratio is approximated by their weight fraction.[6] For the determination of the free energy of partitioning, the error introduced by this approximation is about 1%, which has been proved to be within the level of experimental error in the PFG diffusion measurements.

Thermodynamic Functions from Temperature-dependent Measurements. The thermodynamic functions for the partitioning can be calculated from the temperature-dependent partition data. The free energy change of partition, ΔG, is related to the partition coefficient by the following equation:

$$\Delta G = -RT \ln(p), \quad (6)$$

Then the thermodynamic functions are determined from a nonlinear least-squares fit of the following equation[29]:

$$\Delta G = \Delta H - T\Delta S + \Delta C_p(T-T_{ref}) - T\Delta C_p \ln(T/T_{ref}), \quad (7)$$

where T and T_{ref} are the observed and standard state reference temperatures, respectively. The standard state reference temperature was chosen to be 298 K for comparison with the previous data.[17]

Results

From the ^{31}P spectra of the lipids and the diffusion coefficients of the bicelle and water, we could calculate the composition and the size of the bicelle. The contents of DMPS among the long chain lipids in the three acidic bicelle samples we made were determined to be 0%, 15%, and 24%, respectively, from the analysis of ^{31}P spectra. At first, the molar ratio between DHPC and [DMPC+DMPS] was determined by measuring the intensities of two peaks at around 1 ppm. The peak at 0.9 ppm in ^{1}H NMR spectrum was from the methyl protons of the [DMPC+DMPS] acyl chains and the peak at 1.0 ppm was from DHPC acyl chains. Second, ^{31}P spectrum gave three peaks from DMPC (0.68 ppm), DHPC (0.76 ppm), and DHPS (1.28 ppm). (S1) By integrating the areas of three peaks the molar ratio among the DMPC, DHPC, and DHPS could be determined. The effective hydrodynamic radius was nearly constant at about 37 Å over the experimental temperature range, which is consistent with the fact that a disc-like aggregate of lipids was stably retained over the temperature range.[30,31] From this effective radius of the bicelle we determined the molar ratio, q, of long chain lipids to short chain lipids by considering the headgroup areas of DMPC lipid in the flat surface and DHPC lipid in the rim surface.[32] The molar ratio was 0.45. This means that about 10% of long chain lipids were lost in the centrifugation process. The error from the loss is within the current level of experimental error. The overall tumbling of the bicelle was probed to be isotropic from the sharp Lorentzian lineshape of the ^{31}P spectrum (not shown here) within the experimental temperature range. Binding of SP was found to occur only on the flat surface composed of DMPC and DMPS lipids from the intermolecular ^{1}H-^{1}H NOESY cross-peaks between only DMPC molecules in the bicelle and SP molecules.[33]

The diffusion coefficients, the binding fractions, and the partition coefficients of SP in the [DMPC+DMPS]/DHPC bicelles over the experimental temperature range are given in Table 1. The decay of PFG echo signals of DMPC was fitted with a single-exponential function as shown in Figure 1 and the diffusion coefficient of the bicelles was obtained. The diffusion coefficient of a bicelle obtained using the DMPC signals was nearly same to that obtained using HMDS signals within the experimental errors (Figure 1) and thus its value was confirmed as the diffusion coefficient of a lipid bicelle. The decay of PFG echo signals of SP in the bicelle system was well fitted by a single exponential with only a very small deviation (Figure 1). This indicates that a fast exchange model between two states of SP, the free state and the bound state, is appropriate for describing the experimental phenomenon. The exchange rate between the free form and the bound form was found to be larger than 150 s^{-1} by ana-

Table 1. The diffusion coefficients (in 10^{-11} m^2/s), the binding fractions, and the partition coefficients of substance P in isotropic [DMPC+DMPS]/DHPC bicelles

Bicelle type	Quantity	Temperature (K)						
		296	301	306	311	316	321	326
0%[a]	D_b	3.94	4.68	5.41	6.23	7.11	8.00	9.05
	D_f	22.0	27.0	29.5	31.9	37.9	40.9	44.0
	D_{obs}	6.34	7.56	8.96	10.4	12.4	14.3	16.9
	f_b	0.882	0.871	0.853	0.840	0.824	0.810	0.779
	p	109	98.5	84.7	76.6	68.2	62.3	51.5
15%	D_b	3.91	4.62	5.41	6.30	7.08	8.02	9.26
	D_f	22.3	27.1	30.0	32.8	38.3	41.6	43.7
	D_{obs}	5.76	7.13	8.44	9.99	12.0	13.9	16.1
	f_b	0.899	0.888	0.877	0.860	0.842	0.825	0.802
	p	130	116	104	90.0	78.0	68.7	59.0
24%	D_b	4.08	4.85	5.57	6.56	7.29	8.53	9.46
	D_f	20.9	27.2	29.9	32.4	38.6	42.3	44.5
	D_{obs}	5.59	7.19	8.33	9.90	11.8	13.9	15.9
	f_b	0.910	0.895	0.886	0.871	0.857	0.841	0.815
	p	148	125	114	98.4	87.5	77.1	64.5

[a]The percentages of DMPS in [DMPC+DMPS] lipids.

lyzing the chemical shifts of the amide protons and the decay of the PFG echo signal of the aromatic protons of phenylalanine in SP (not shown). The temperature dependence of the diffusion coefficients measured at different temperatures follows the Arrhenius relationship, which means that the convection effect was effectively removed in our diffusion measurements by using the DSTE pulse sequence.[26] From the comparison of the diffusion coefficients of free SP and bicelle, the effective size of the bicelle was at least 5 times larger than that of the free SP, which means that the assumption that the diffusion rate of bicelle is kept same regardless of the binding of SP is valid. The partition coefficient of SP into the bicelle is of the order of 10 to 100, which is lower than those in the DPC and SDS micelles. The temperature dependences of the partition coefficients are given in Figure 2.

The thermodynamic functions of the peptide partitioning determined by using the Eqs. (6) and (7) at 298 K are presented in Table 2. The values of ΔG in the acidic bicelle solutions are about −2.8 kcal/mol. According to Seelig *et al.*,[23] each hydrophobic residue of the peptide taking part in the hydrophobic interaction with the hydrophobic core of the lipid membrane makes a contribution to the Gibbs free

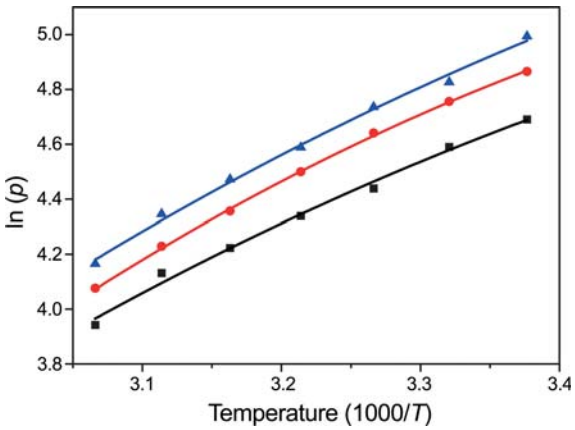

Figure 2. The temperature dependence of the partition coefficients of SP in 0% neutral bicelles (■), in 15% acidic bicelles (●), and in 24% acidic bicelles (▲). Solid curves are theoretical ones best fitted with eqs. (6) and (7).

energy change of partitioning of approximately −0.6 kcal/mol. Our results are consistent with the SP binding model in which the main interactions are the hydrophobic interactions between the side chains of four residues (Phe7, Phe8, Leu10, and Met11) and the membrane lipids and there is no deep insertion of an overall backbone of SP into the hydrophobic core.

Discussion

The binding of peptides has been observed to depend on the surface charge density of the membrane. As the acidic lipid fraction increases, the Gibbs free energy of peptide partitioning decreases, which means that the peptide favors the acidic lipid membranes more than neutral membranes. The electrostatic interaction between the positively charged residues of SP and the negatively charged headgroup of DMPS is expected to stabilize the binding state of SP. This fact is confirmed by the decrease in the enthalpy of partition that accompanies an increase in DMPS contents. However, the additional electrostatic interaction between SP and DMPS lipids is a small contribution to the partitioning of SP into our bicelle systems. The addition of 24% DMPS caused an increase of only 6% of the Gibbs free energy of partition because the decrease of the enthalpy of partition was compensated by the decrease of the entropy of partition.

However, the study on the structures of SP bound to the membrane in a neutral bicelle and an acidic bicelle revealed

Table 2. The thermodynamic functions for the partitioning of substance P from water to isotropic [DMPC+DMPS]/DHPC bicelles at 298 K

Bicelle type	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})	ΔC_p (cal K^{-1} mol^{-1})
0%[a]	-2.73 ± 0.05	-3.9 ± 0.6	-3.7 ± 1.9	-60 ± 43
15%	-2.84 ± 0.05	-4.0 ± 0.2	-3.9 ± 0.6	-84 ± 13
24%	-2.89 ± 0.25	-4.3 ± 0.5	-4.6 ± 1.7	-63 ± 38
DPC micelles[b]	-3.75 ± 0.08	-2.58 ± 0.10	4.0 ± 0.1	-5 ± 4

[a]The percentages of DMPS in [DMPC+DMPS] lipids. [b]Data for DPC micelles at 298 K were taken from Ref. 6.

a somewhat different feature. In our previous study we could get a well-structured conformation of SP by modeling the molecular structure using the NOESY spectra observed in the state bound to the acidic bicelle membrane with 24% DMPS lipids.[33] However, we could not get a well-structured conformation of SP in the neutral bicelle membrane.

The two results obtained from the diffusion experiments and the structure determination experiments appear to be somewhat contradictory to each other. However, the apparent contradiction could be explained in the sense that two experimental data give us different information. The diffusion measurement only tells us the binding fraction of the peptide and does not provide precise information about the character of peptide binding, such as the binding depth into the membrane or the conformation of the peptide bound to the model membrane. Even though the same fraction of peptide binds to the membrane, the conformation and/or the stability of the conformation of the peptide can be different according to their binding patterns. Therefore, our results may be interpreted as implying that the electrostatic interaction between peptides and the negatively-charged lipids mainly contributes to the stability of the conformation of peptides bound to the membrane and gives well-defined structure. The present findings combined with our previous results suggest that the electrostatic interaction between SP and lipids in the bicelle is expected to enhance the SP binding but the effect on the SP partitioning is not large because the entropy of partition decreases due to enhanced ordering of the lipid molecules and the creation of an ordered structure of SP by the strong electrostatic interaction between the peptide and the lipids.

The large contribution of the enthalpy of partition to the Gibbs free energy of partition and the negative values of the entropy of partition in our bicelle systems may indicate that the partitioning of SP to the bicelle is enthalpy-driven, as it is in the micelle or POPC/POPG (3:1) SUV.[16] This means that the partitioning of SP onto the small acidic bicelle is mainly due to the nonclassical hydrophobic interactions of the van der Waals interactions between the nonpolar residues of the peptide and the hydrophobic core of the lipid bilayer.[16]

In past works, the binding of amphiphatic peptides on the membrane mimics was shown to be enthalpy-driven in a small vesicle with a diameter of 30 nm, but entropy-driven in a large vesicle with a diameter of 400 nm.[16] Seelig and coworkers explained this difference as due to the difference in the degrees of packing of lipids, which directly related to the curvature of the membrane surface and thus the internal bilayer pressure.[16] As the size of a vesicle increases and thus the curvature of the membrane surface decreases, the packing constraints of the lipids are relaxed and the internal tension increases.[19] Hence, considerably more energy is required to insert a foreign molecule between the lipids of a planar membrane, compensating in part or totally the gain in van der Waals energy.[16] Because of this reason, entropy contribtuion to the Gibbs free energy in a large size of vesicles should be large to compensate the enthalpy increment but in a small size of vesicles enthalpy contribution is dorminant. They, however, concluded that even though there was an enthalpy-entropy compensation mechanism the molecular origin of this effect was not clear.[19]

Generally, the entropy change of the peptide binding onto a LUV with a small surface curvature is positive due to the disordering of bulk water and membrane lipids,[6,16] which leads to the classical hydrophobic mechanism. Even though the binding surface of SP in the bicelle system is flat, the entropy change of our system was negative, similar to that of an SUV with a diameter of 30 nm which has a highly curved surface. This means that the nonclassical interaction and entropy contribution proposed by Seelig and coworkers[1] cannot be interpreted as depending only on the curvature of the membrane surface in small sized model membranes such as bicelles and micelles. Our results suggest that it is likely that the size of the model membrane plays a more important role than the curvature of the membrane mimic system in the partitioning of peptides in small membrane mimics.

Negative entropy of partition in small membranes such as isotropic bicelles and SUVs may mean that the ordering of lipid molecules by the lipid-peptide interaction dominates over the disordering of water released from the peptide surface. In small model membranes the degree of the ordering of lipid molecules is thought to be much more sensitive to the membrane curvature than it is in the large membranes and thus the increase in the degree of the ordering of the lipid molecules by peptide binding will be much larger than in the case of large membranes. A small positive entropy of partition in DPC micelles is likely since the increase in order caused by peptide binding is small because DPC molecules in a micelle aggregation are already well-ordered due to its molecular shape, as DMPC molecules are well-ordered in an LUV.

The heat capacity changes, ΔC_p, in our bicelle systems are similar to the values obtained in the POPC vesicle.[1] The negative values of ΔC_p imply that some of the bonding or nonbonding interactions between lipid molelcules are disrupted on the peptide binding to lipid bilayers, which could be caused by the burial of hydrophobic residues of SP into the bilayers and the release of water molecules from the hydrophobic core or the surface of the bicelle.[23]

Conclusions

Small acidic bicelles made out of DMPC, DHPC, and DMPS exist over the experimental temperature range of 23 to 53 °C. The Gibbs free energy, enthalpy, and entropy of peptide partitioning show that the SP binding on the small acidic lipid bicelles with a flat surface is enthalpy-driven and that the nonclassical interaction between the SP and the lipid molecules is a major driving force as in the small unilamellar vesicles with a curved surface. Negative surface charge of the bicelle membrane has a small effect on the partitioning of SP into our bicelle systems. The results of the present study suggest that the size of a model membrane is likely to be a more important factor than the curvature of the membrane surface in the partitioning of SP into small membrane

mimics. The negative entropy change may indicate that a high degree of the ordering of the lipid molecules occurs by the binding of SP into the less ordered bicelle.

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