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The determination of binding constants of micellar-packaged gramicidin A by ^{13}C-and ^{23}Na-NMR

Naijie Jing, Kari U. Prasad [1], Dan W. Urry *

Laboratory of Molecular Biophysics, The University of Alabama at Birmingham, VH 300, Birmingham, AL 35294-0019, USA

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Abstract

Based on the malonyl gramicidin A structure of a single-stranded head-to-head hydrogen bonded right-handed, $\beta^{6.3}$-helix in dodecyl phosphocholine (DPC) lipid micelles (Jing et al. (1994) Biophys. J. 66, A353), the determination of cation binding sites for gramicidin A (GA) in DPC micelles becomes a significant step in the study of ion transport through the model channel. First, the investigation of cation binding sites in DPC micellar packaged gramicidin A was achieved by ^{13}C-NMR experiments at 30° C using four C-13 labeled GA samples. Then, the analyses based on two different equations, one for single and one for double occupancy, were employed to evaluate the correct occupancy model for GA in DPC micelles. The results clearly indicate double occupancy to be correct for Na^+ ion as well as for K^+, Rb^+, Cs^+ and Tl^+ ions. Finally, the binding constants for Na^+ ion were also estimated by the measurement of the longitudinal relaxation time (T_1) using ^{23}Na-NMR of the same sample at the same temperature as used for the ^{13}C-NMR study. The binding constants obtained from ^{23}Na-NMR are essentially equivalent to those determined from the ^{13}C-chemical shifts.

Keywords: Gramicidin A, C-13 labeled; Dodecyl phosphocholine micelle; Ionic titration; Circular dichroism

1. Introduction

Gramicidin A (GA) is a linear, hydrophobic pentadecapeptide, HCO-L-Val[1]-Gly[2]-L-Ala[3]-D-Leu[4]-L-Ala[5]-D-Val[6]-L-Val[7]-D-Val[8]-L-Trp[9]-D-Leu[10]-L-Trp[11]-D-Leu[12]-L-Trp[13]-D-Leu[14]-L-Trp[15]-$NHCH_2CH_2OH$, which forms an ion-selective transmembrane channel in lipid membranes [1,2]. The mechanics of ion transport through the GA channel have been studied intensively. Several theoretical treatments have been proposed for years [3–8]. Even though the two-site three-barrier model is more generally accepted, locating the position of the binding site in the GA channels and detecting the interaction of the two occupied ions become critical issues in interpreting the mechanism of ion transport in the single-file channel. The location of cations bound in the gramicidin dimer was observed by X-ray diffraction studies using the crystal of the K^+ and Cs^+ ion-gramicidin complexes grown in methanol; this showed that the length of the GA channel was 26 Å and the distance from the ion to the end of the GA channel was 2.5 Å [9]. The conformation of the ion-gramicidin complexes in methanol (or in methanol/chloroform) was later confirmed to be an antiparallel double helix [10,11]. The ion binding sites in gramicidin A in LPC (L-α-lysophosphatidylcholine) lipid vesicles were also observed by ^{13}C-NMR using C-13 labeled gramicidin samples [12,13]. The center of the binding sites was first located between the carbonyl groups of Trp[11] and Trp[13] residues. The distance between the two binding sites was estimated to be 23 Å, for the structure of a head-to-head, left-handed, single-stranded $\beta^{6.3}$-helical dimer and to be 16 Å for the right-handed structure. Recently, the analysis of X-ray diffraction data on gramicidin A in dilauroylphosphatidylcholine at a liquid crystalline phase shows the distance between the two bound Tl^+ ions to be about 19 Å [14]. The conformation of gramicidin A in SDS detergent micelles using two dimensional NMR was demonstrated to be a right-handed, head-to-head dimer, single-stranded β-helix with 6.3 residues per turn [15]. The structure of malonyl gramicidin A incorporated in dodecyl phosphocholine (DPC) lipid micelles was also determined to be a right-handed, single-stranded β-helix, head-to-head dimer by two-dimensional proton NOE spatial connectivities [16]. Based on the deduced structure of gramicidin A in DPC

* Corresponding author. Fax: +1 (205) 9344256.

[1] Present address: Magainin Sciences, Inc., 5110 Campus Drive, Plymouth Meeting, PA 19462, USA.

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micelles, the monovalent-cation binding sites were located, the interaction between the two occupied ions was detected, and the distance between the two symmetrically related binding sites was estimated in this study by means of the measurements of ion-induced chemical shifts using the four ^{13}C-labeled GA samples. Also, the binding constants were determined by the data analyses.

2. Materials and methods

The four syntheses of gramicidin A, each of which contains a 90% ^{13}C enriched carbonyl carbon at the position of Trp^{9}, Trp^{11}, Trp^{13}, or Trp^{15} amino acid residue, were obtained and characterized in this laboratory, as described previously [12,13]. All chemicals used were optical grade and were dried in an oven before using. The dodecyl phosphocholine (DPC), $(CH_2)_{11}$-$PO_4(CH_2)_2N(CH_3)_3$ was purchased from Avanti Polar-lipid (Birmingham, Alabama) and used without further purification.

2.1. Sample preparation

The desired amounts of dodecyl phosphocholine lipid (0.2 M) and ^{13}C-enriched gramicidin A (0.006 M) were dissolved in 1 ml of 2,2,2-trifluoroethyl alcohol (TFE) and were incubated for 3 h at 25° C. Then, TFE was removed under high vacuum overnight, and 0.7 ml of water (D_2O 99.83%) was added to the samples. Next, the samples were sonicated for five times 3 min at power level 5 with a macrotip by the sonicator cell disrupter (W-225R), then incubated in a water bath at 70° C about 70 h. After being centrifuged at 5000 rpm for 30 min at 27° C, the supernatant was extracted for an incorporation check using circular dichroism (CD). The CD spectra of the mixtures of the supernatant and water (20/150 ml) were recorded by a Cary 60 spectropolarimeter at room temperature using a 0.1-mm optical path length cell. The concentration of gramicidin channels (dimers) in the supernatant was measured by absorbance after a 15-fold dilution of the sample in methanol, using a molar extinction coefficient of 45 000 cm^{-1} M^{-1} at 280 nm.

2.2. Nuclear magnetic resonance

The ^{13}C-NMR were carried out at 25 MHz on a JEOL FX100 instrument using a 10-mm multinuclear probe. The experiments were maintained at 28–30° C by a JEOL UT-3B temperature controller. The spectra were obtained under the following conditions: using a memory of 8 K, a 5000 Hz spectral width, an 18 μs pulse width for 90°, and an interval time of 2.82 s. An input power of 15 W was applied continuously for decoupling broad-band protons. The reference ^{13}C-NMR spectra for each C-13 labeled gramicidin and different ion titrations were obtained in the presence of 0.5 mM NaCl. The titrations of sodium, potassium, rubidium, and cesium ions were carried out by adding dry NaCl, KCl, RbCl, and CsCl. The concentration was increased from 1 mM to 2 M with 22 to 25 data points; however, Tl^+ ion concentration was titrated by thallium acetate in D_2O and increased from 0.15 mM to 150 mM. The graphs of the chemical shifts, $\Delta\delta$, induced in ^{13}C-enriched carbonyl C-1 resonance versus ion concentrations, were plotted by a Tektronix computer system.

The longitudinal relaxation time (T_1) of Na^+ ion was performed using ^{23}Na-NMR at 26.3 MHz on a JEOL FX-100, using ^{13}C-Trp^{11} gramicidin A incorporated into DPC micelles in D_2O. The relaxation time T_1 was measured by the inversion recovery method (180°-τ-90° pulse sequence) with 28 μs pulse width for 90°, and the pulse interval for complete relaxation was taken as greater than $5 \times T_1$ at each NaCl concentration. The reference relaxation time T_{1f} for free ^{23}Na was 50 μs, as obtained from 10 mM NaCl in the presence of DPC micelles in D_2O.

2.3. Data analysis

Because of the single-stranded 6.3 residue per turn helix the monovalent cations must pass through the GA channel in single-file with direct interaction of carbonyl oxygen with cations. Accordingly, ^{13}C chemical shifts of the car-

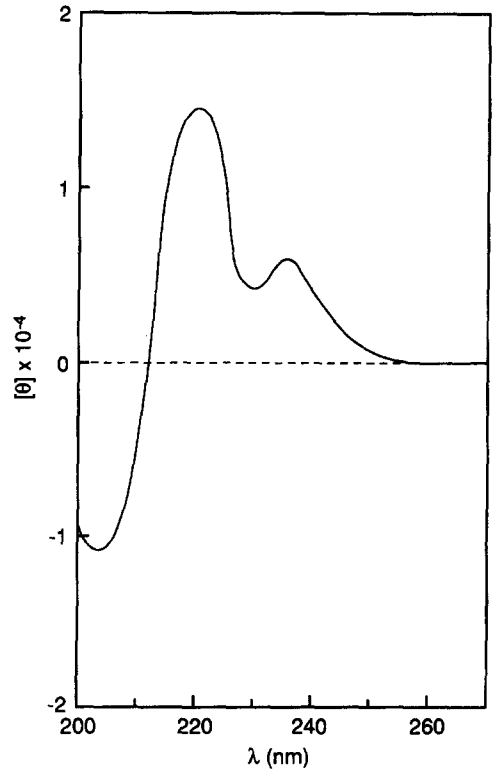


Fig. 1. CD spectrum of sonicated dispersions of [1-^{13}C]Trp labeled gramicidin A incorporated into DPC lipid micelles (1:35) in D_2O, recorded at room temperature by Cary 60 spectropolarimeter.

bonyl carbons, induced by the interaction between ions and the peptide channel, can be used to identify the binding sites. The magnitude of the chemical shift depends on the site occupancy and the binding constants. The data analysis employed to evaluate the correct occupancy model for GA channel is based on the two different equations, one for single and one for double occupancy, as described previously [17]. The equation for double occupancy utilizing the ^{13}C chemical shifts of gramicidin A in DPC micelles is given by

$$\Delta\delta = \frac{C_1}{C_T} \cdot \Delta\delta_t + \frac{C_2}{C_T} \cdot \Delta\delta_w$$

where C_1 and C_2 respectively, are the concentrations of singly and doubly occupied channel molecules; C_T is the

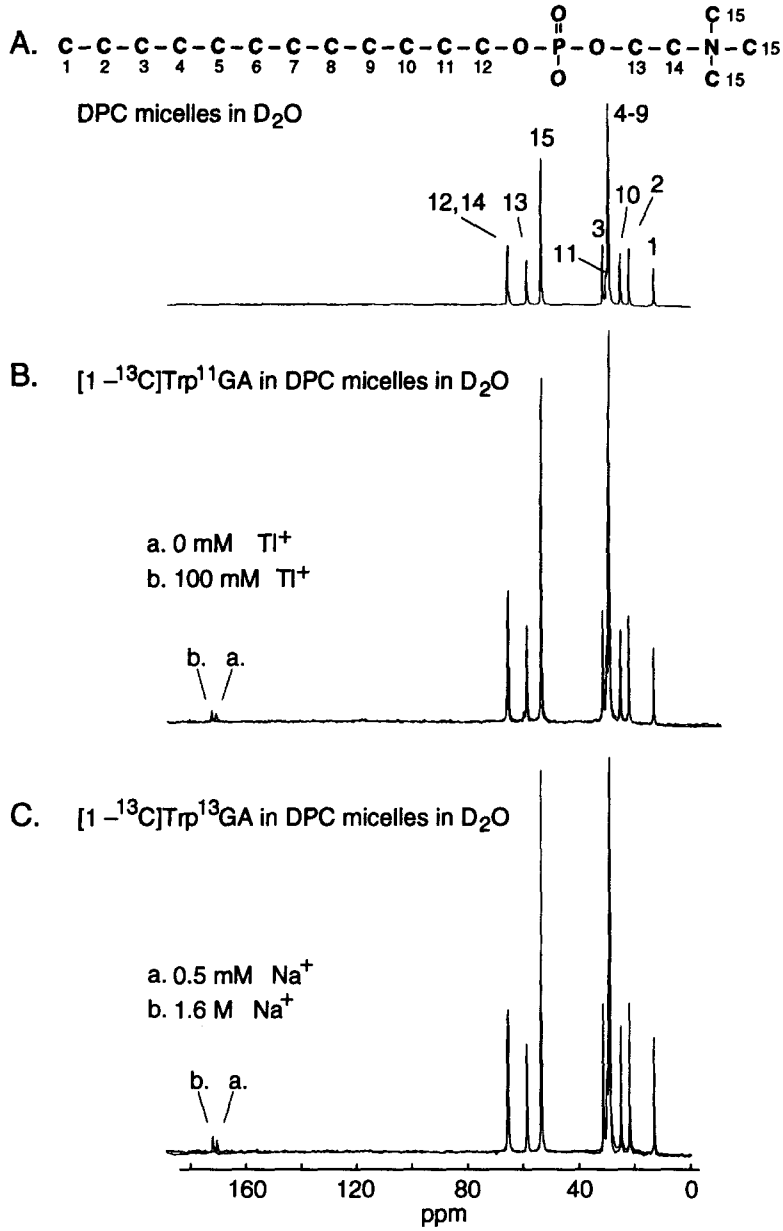


Fig. 2. ^{13}C-NMR spectra at 25 MHz recorded by JEOL FX-100 spectrometer at 30° C. (A) The spectrum of the sonicated dodecyl phosphocholine (DPC) micelles in D_2O without gramicidin A, in which no signal shows in low field. (B) The two overlapped spectra of [1-^{13}C]Trp^{11} GA incorporated into DPC micelles in the present of 0 mM (a) and 100 mM (b) Tl^+ concentrations. The small signal of C-13 enriched carbonyl carbon (C = O) has a chemical shift ($\Delta\delta$) of 1.51 ppm in the two different concentrations. (C) The two overlapped spectra of [1-^{13}C]Trp^{13} GA in DPC micelles at 0.5 mM (a) and at 1.6 M (b) of Na^+ concentration. The chemical shift ($\Delta\delta$) of (C = O) between them is 1.54 ppm. The C-13 resonances of fatty acid chains of dodecyl phosphocholine are not found in any shift in all the spectra, and only the C-13 enriched carbonyl carbon is observed in a downfield shift as the ion concentration is increased.

concentration of the total channel molecules; $\Delta\delta_t$ is the chemical shift for the singly occupied channels, and $\Delta\delta_w$ is the chemical shift in the doubly occupied channels [17]. When only one site is occupied, the two carbonyl carbons (one in each monomer of the dimer) have the same chemical shift due to the rapid exchange (i.e., xo ⇔ ox), so the tight binding constant, K_b^t, for the singly occupied state is $\frac{C_1}{2\cdot[X]\cdot C_0}$, in which C_0 is the empty channel concentration and $[X]$ is the concentration of free ions. At high concentration, the channels come to the doubly occupied state, the weak binding constant, K_b^w, is equal to $\frac{2\cdot C_2}{[X]\cdot C_1}$ which is approximately $\frac{2\cdot C_2}{[X]_T\cdot C_1}$ where $([X]_T$ is the total ion concentration) because the fraction of bound ions is very small relative to the high concentration of free ions [18]. The equation of single occupancy employed to analyze the ^{13}C chemical shift data is given by

$$\Delta\delta = \frac{C_1}{C_T}\cdot\Delta\delta_b,$$

where δ_b is the maximal ^{13}C chemical shift for each sample and the binding constant, K_b, for the single occupancy is $\frac{C_1}{[X]\cdot C_0}$ in which $C_0 = C_T - C_1$; $[X] = [X]_T - C_1$.

The fitting procedure is to input the binding constants and the chemical shifts estimated from the experimental data $-K_b^t$, K_b^w, $\Delta\delta_t$, and $\Delta\delta_w$ for double occupancy; and K_b and δ_b for single occupancy –then to use an optimizing program to search for the best fit. The best fitting curves are plotted by a Tektronix computer system, and the

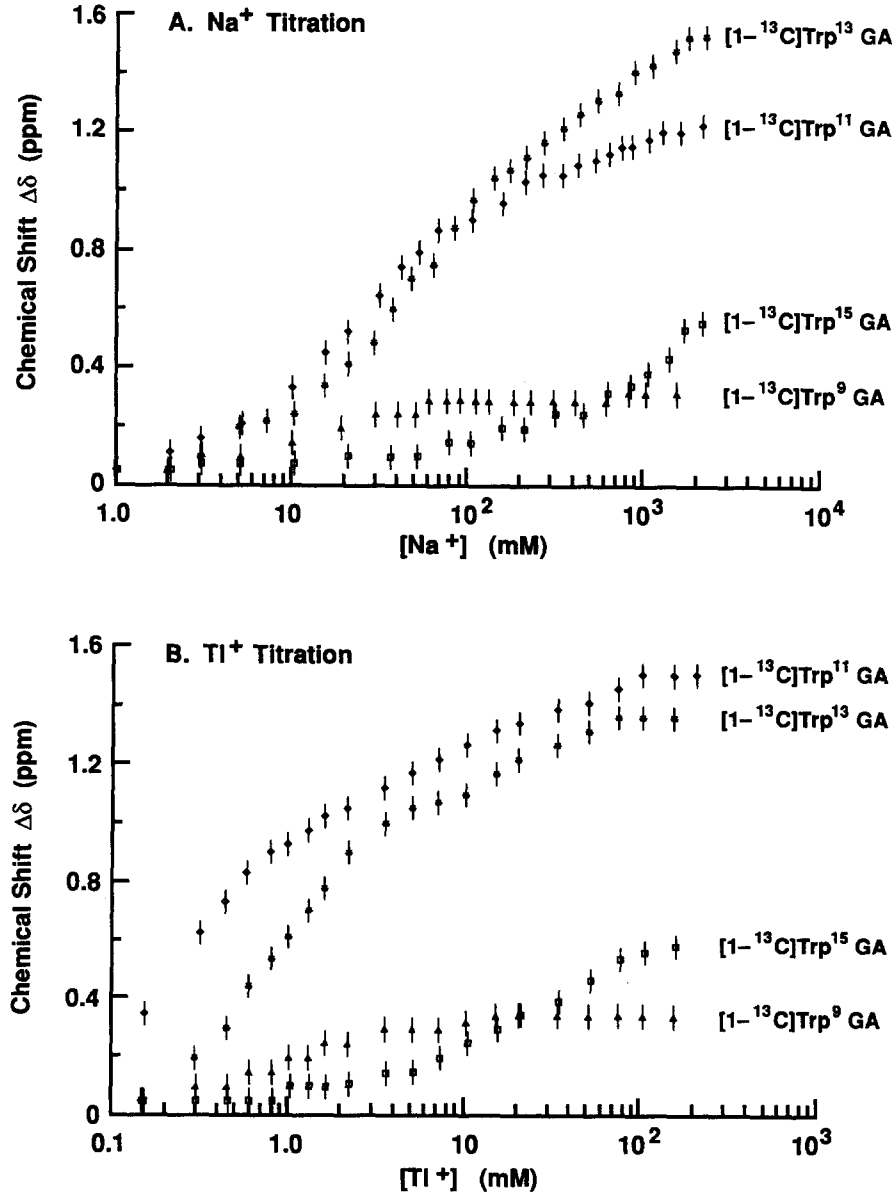


Fig. 3. The chemical shift $\Delta\delta$ (ppm) of carbonyl carbon (C = O) of [1-^{13}C]Trp^9 GA, [1-^{13}C]Trp^{11} GA, [1-^{13}C]Trp^{13} GA, and [1-^{13}C]Trp^{15} GA induced as a function of Na^+ ion concentration (A) and of Tl^+ ion concentration (B). The range of titration of Na^+ ion is from 1 mM to 2 M and that of Tl^+ ion is from 0.15 mM to 150 mM (see text for details).

average errors calculated from the difference between the analytical values and the experimental data show the fitting quality for the two occupancy models.

3. Results

3.1. The conformation of gramicidin A in DPC micelles

Circular dichroism (CD) is a convenient method for detecting the conformation of gramicidin molecules in lipid micelles. The CD spectrum of gramicidin A in DPC micelles displays positive extreme at 218 and 235 nm with a weak negative inflection between them (230 nm), and negative elipticity below 208 nm (Fig. 1); this is the same as the CD spectrum of malonyl gramicidin A in DPC micelles (Jing and Urry, unpublished data). As a result, the conformation of gramicidin A incorporated in DPC micelles is the same as that of malonyl gramicidin A in DPC micelles, which was confirmed to be a right-handed, head-to-head dimerized, single-stranded β-helix with 6.3 residues per turn by two-dimensional proton NMR [16].

3.2. The dependence of C-13 chemical shifts on Na^+ and Tl^+ concentrations

The ^{13}C-NMR spectra show cation-induced chemical shifts of ^{13}C-enriched carbonyl carbons of gramicidin A in DPC micelles (Fig. 2). Fig. 2A is the ^{13}C-NMR spectrum of free DPC micelles in D_2O without gramicidin molecules (A). In Fig. 2B and C, the two spectra of the same ^{13}C-labeled gramicidin A in DPC micelles with different ion concentrations were recorded over one another. The small signals in the low field are from the 90% ^{13}C-enriched carbonyl carbon of gramicidin A. The lower intensity of the small signals is due to the ratio of GA/lipid (1:35). From these spectra, it has been found that only the signal of ^{13}C-labeled carbonyl carbon of gramicidin A has a chemical shift (δ_i) to a lower field as a function of ion concentration, and all the resonance signals of the DPC micelles remain unshifted when the ion concentration is increased so that the signal of the fatty acid chain, C^4-C^9, was set at 29.10 ppm as a reference. In the presence of 0.5 mM NaCl, the chemical shift (δ_0) of carbonyl carbon of [1-^{13}C]Trp^{11} GA is 170.82 ppm referring to the signal of C^4-C^9 as 29.10 ppm, and it shifts 1.51 ppm to a lower field as Tl^+ ion concentration is increased from 0 to 100 mM. The chemical shift (δ_0) of carbonyl carbon of [1-^{13}C]Trp^{13} GA in 0.5 mM NaCl is 170.33 ppm, and it shifts 1.54 ppm to a lower field when the concentration of NaCl is raised to 1.6 M.

Since the diameter of the hole of gramicidin channels is approx. 4 Å, only the bare cation can enter into the channels, and water molecules can pass through the channels in front or behind a cation as a single-file mechanism [1,2]. Therefore, the interaction between cations and the peptide occurs directly within the gramicidin channels. Based on the libration theory of the GA channel [2], the carbonyl oxygen of amino acid residues rotate into the channel to form the ion-peptide complexes at the binding sites when ion entering; this hypothesis was recently supported by the experimental observations on solid-state ^{15}N-NMR [19,20] and by the molecular dynamic computation [21]. The interaction of the type N-C = O $\cdots$ Na^+ (or Tl^+) will change the shielding constants ($\Sigma\sigma$) and decrease the electron density surrounding the carbonyl carbon; so the signal of the carbonyl carbon shifts to lower field in the ^{13}C-NMR spectra. The position of carbonyl carbon exhibiting the largest chemical shift, $\Delta\delta(=\delta_i-\delta_0)$, is considered to be nearest the center of the ion binding site.

The results of the ^{13}C-NMR titration experiments were

Table 1
Estimates of ion-binding constants for gramicidin A channel interaction with Na^+ and Tl^+ ions in different lipid environments

Ions per channel	Na^+ (M^{-1})		Tl^+ (M^{-1})		Lipid membrane or micelles	Experimental method	Reference
	K_b^t	K_b^w	K_b^t	K_b^w			
2	63	10			[a] Lyso PC	^{23}Na-NMR	[6]
2			~ 1000	70	[a] Lyso PC	^{13}C-NMR	[2]
2	30	2.6			[a] Lyso PC	^{23}Na-NMR	[22]
2	≤ 30		500–1000	≤ 50	[b] DPhC	dialysis	[23]
2	120	0.20	9000	25	[c] GMO	BLM	[24]
1 and 2	10		550		[c] GMO	BLM	[25]
3			2.8		[c] GMO	BLM	[25]
1			500		[c] GMO	BLM	[26]
1			900		[a] Lyso PC	^{205}Tl-NMR	[27]
1	31.6		506		[a] Lyso PC	^{205}Tl-NMR	[28]
1	5				[b] DPhC	BLM	[29]
2	25	2.6			[d] DPC	^{23}Na-NMR	in this study
2	40	4	1000	30	[d] DPC	^{13}C-NMR	in this study

[a] Lyso PC, L-α-lysophosphatidylcholine vesicles. [b] DPhC, diphytanoyl phosphatidylcholine membranes. [c] GMO, glycerolmonooleate membranes. [d] DPC, dodecyl phosphacholine micelles.

plotted as the chemical shifts ($\Delta\delta$) of ^{13}C-enriched carbonyl carbons versus Na^+ and Tl^+ ion concentrations (Fig. 3), using the samples of C-13 labeled gramicidin A in DPC micelles. Similar observations were found in the plots: (1) The ^{13}C chemical shift versus Na^+ concentration is similar to that versus Tl^+ concentration for each of the GA samples. But the titration of Na^+ ion runs from 1 mM to 2 M while that of Tl^+ ion increases from 0.15 mM to 150 mM. Hence, the affinity for Tl^+ binding in the GA channel is at least 10 times larger than for Na^+ ion, in agreement with early studies (see Table 1). (2) The larger chemical shifts occur in the titration studies of [1-^{13}C]Trp^{11} and [1-^{13}C]Trp^{13} GA for both Na^+ and Tl^+ ions, as observed in LPC vesicles [13,30]. The center of the binding site is between the carbonyl groups (C = O) of the Trp^{11} and Trp^{13} residues. (3) In the Na^+ titration study (Fig. 3A), the chemical shift $\Delta\delta$ of [1-^{13}C]Trp^9 GA increases from 0.05 ppm at 0.5 mM to 0.29 ppm at 60 mM; then remains essentially constant for the rest of the titration. The ion concentration corresponding to the mid-point of the sigmoidal shift is 15 mM; the mid-point is the point at half of the difference between the maximum and

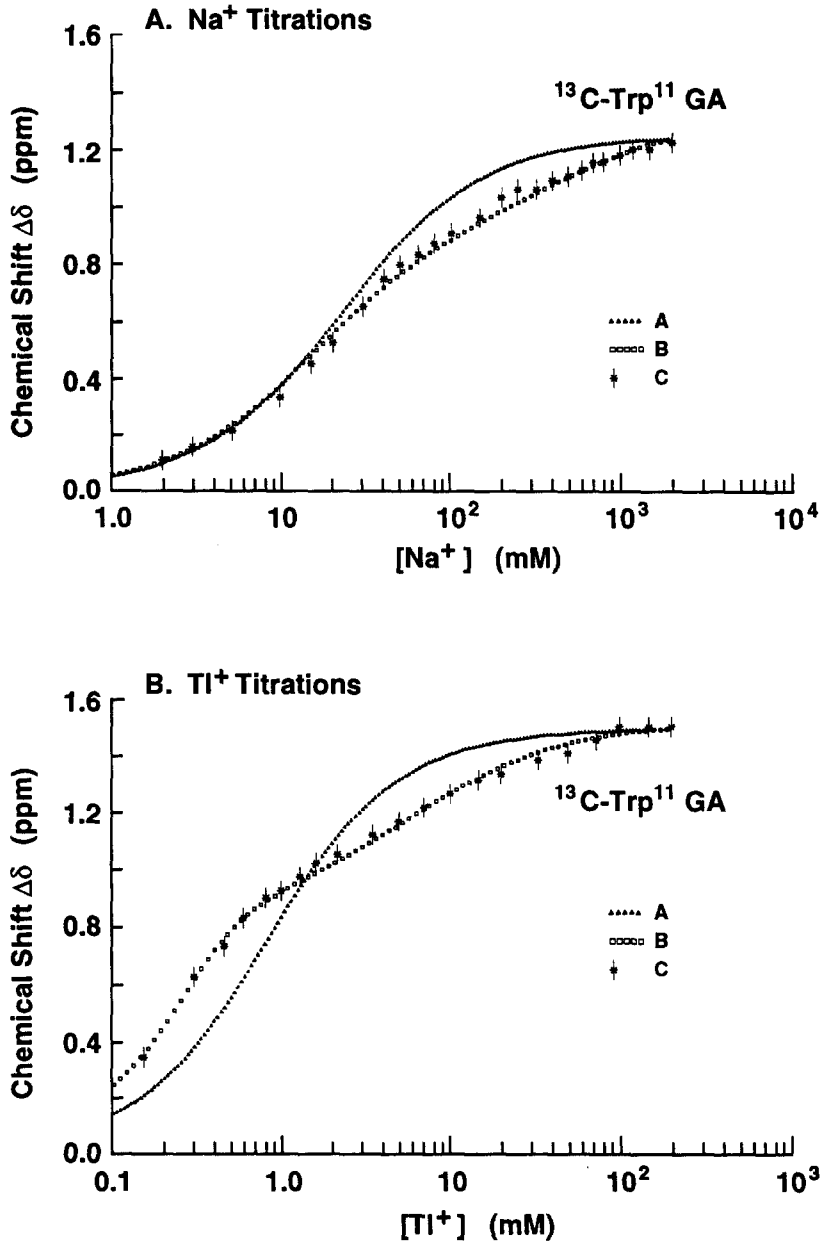


Fig. 4. The data analyses of ^{13}C chemical shift, $\Delta\delta$, of the carbonyl carbon of [1-^{13}C]Trp^{11} GA in DPC micelles as a function of ion concentration. The two theoretical equations correspond to a single occupancy model (dotted line, A) and a double occupancy model (squared line, B). A star with a standard deviation bar (C) stands for experimental points. In the Na^+ titration (A), the mean deviation of the single and double occupancy fittings are 8.4% and 2.1%. In the Tl^+ titration (B), the average deviation of the single and double occupancy fittings are 12.2% and 1.7%. The binding constants, K_b^1 and K_b^w, obtained from the double occupancy are 40/M and 4/M for Na^+ and 1000/M and 30/M for Tl^+.

minimum chemical shifts, $1/2(\Delta\delta_{mix} - \Delta\delta_{min})$ However, the chemical shift, $\Delta\delta$, of [1-^{13}C]Trp^{15} GA starts increasing from 0.15 ppm at 76 mM to 0.56 ppm at 2 M with the mid-point at 600 mM. In the Tl^+ titration study (Fig. 3B), the $\Delta\delta$ of [1-^{13}C]Trp^9 GA increases from 0.15 mM to 10 mM; then, it remains unchanged. That of [1-^{13}C]Trp^{15} GA starts increasing at 7 mM. The mid-points are 1 mM for Trp^9 GA and 33 mM for Trp^{15} GA. A reasonable interpretation is that at low ion concentrations, the most probable states of gramicidin channels are from being empty to being singly occupied as the first ion goes into the channel. An ion at the singly occupied state is bound in the sequence of Trp^{9,11,13} residues; as a result, $\Delta\delta$ of carbonyl carbon of [1-^{13}C]Trp^9 GA increases while that of [1-^{13}C]Trp^{15} GA remains unshifted. As the ion concentration is raised, the second ion enters the channel, and the channel states shift from being singly occupied to being doubly occupied. The interaction between the two ions pushes each other slightly out to include the Trp^{15} residues. In high concentration, the chemical shifts of carbonyl carbon of [1-^{13}C]Trp^{15} GA increase and that of [1-^{13}C]Trp^9 GA remain unchanged.

3.3. Determination of the occupancy model and the binding constants

Since the center of the binding sites was located between the carbonyl groups of Trp^{11} and Trp^{13}, both titration data of ^{13}C-Trp^{11} and ^{13}C-Trp^{13} GA in DPC micelles are suitable for the data analysis. According to the plots of Na^+ and Tl^+ ion-induced chemical shifts of carbonyl carbon vs. ion concentration (Fig. 3), the chemical shifts of the carbonyl carbon of [1-^{13}C]Trp^{11} GA were chosen for the analysis because the carbonyl carbon of Trp^{11} has larger chemical shifts for both Na^+ and Tl^+ ions.

The reciprocals of the ion concentrations corresponding to the mid-points were employed as the estimated binding constants. The mid-point of [1-^{13}C]Trp^9 GA was the best one for the binding constant, K_b^t, of the singly occupied state, and that of [1-^{13}C]Trp^{15} GA was the best one for the binding constant, K_b^w of the doubly occupied state. In this way, the estimated binding constants, 67/M and 1.7/M for Na^+; 1000/M and 30/M for Tl^+, were input into the fitting programs; then, the best fitting curves were searched and plotted. The curves fitting to the chemical shifts of the carbonyl carbon of [1-^{13}C]Trp^{11} GA as a function of the concentrations of NaCl and TlAc are shown in Fig. 4. In Na^+ titration, the optimum fitting constants for double occupancy are $K_b^t = 40/M$, $K_b^w = 4/M$, $\Delta\delta_t = 0.9$ ppm and $\Delta\delta_w = 1.32$ ppm, and for the single occupancy are $K_b = 47.2/M$ and $\Delta\delta_b = 1.25$ ppm. According to the deviation analysis, the average errors for the double and the single occupancy fittings are 2.1% and 8.4%; the large deviation in the single occupancy fitting appeared from 20 mM to 1000 mM. For Tl^+ ion, the best fitting constants for double occupancy are $K_b^t = 1000/M$, $K_b^w = 30/M$, $\Delta\delta_t = 0.9$ ppm and $\Delta\delta_w = 1.52$ ppm; those for single occupancy are $K_b = 150/M$ and $\Delta\delta_b = 1.5$ ppm. The average error for the double occupancy fitting is 1.7% and for the single occupancy fitting is 12.2%.

In the absence of ion occupancy, the two binding sites of the GA channel are identical due to the structural 2-fold symmetry. Therefore, the phenomenological tight and weak sites really represent the difference between single and double ion occupancy of the channel. On double occupancy, the binding site carbonyl C-13 chemical shifts increase for the carbonyls at greater distance from the 2-fold symmetry axis of the structure, presumably due to repulsion between the ions on double occupancy. This repulsion causes the binding constant to be weaker for double occupancy and also causes the location of the sites for double occupancy to be several Å further apart than for single occupancy. Accordingly, the carbonyl carbon chemical shift for the weak site, $\Delta\delta_w$, can be greater than that of the tight site, $\Delta\delta_t$, when monitoring a carbonyl at greater distance from the 2-fold symmetry axis

The information obtained from these analyses are that (1) double occupancy is the correct model for the GA channel. Single occupancy is not relevant. Also, no more than two Tl^+ ions were observed in the GA channel (see Table 1). (2) Compared with the binding constants estimated by different methods in different lipid membranes, the binding constants obtained by the analyses, being 40/M and 4/M for Na^+; 1000/M and 30/M for Tl^+, are quite reasonable (see Table 1). (3) The binding constants estimated from the middle points of [1-^{13}C]Trp^9 and [1-^{13}C]Trp^{15} GA are essentially equivalent to the binding constants from the data analyses. Therefore, the ^{13}C chemical shift of carbonyl carbon of Trp^9 was induced by an ion at the singly occupied state and that of Trp^{15} was shifted as the second ion entered to form the doubly occupied state.

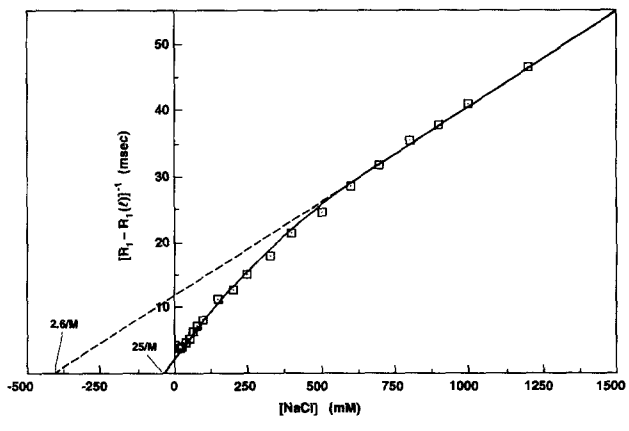
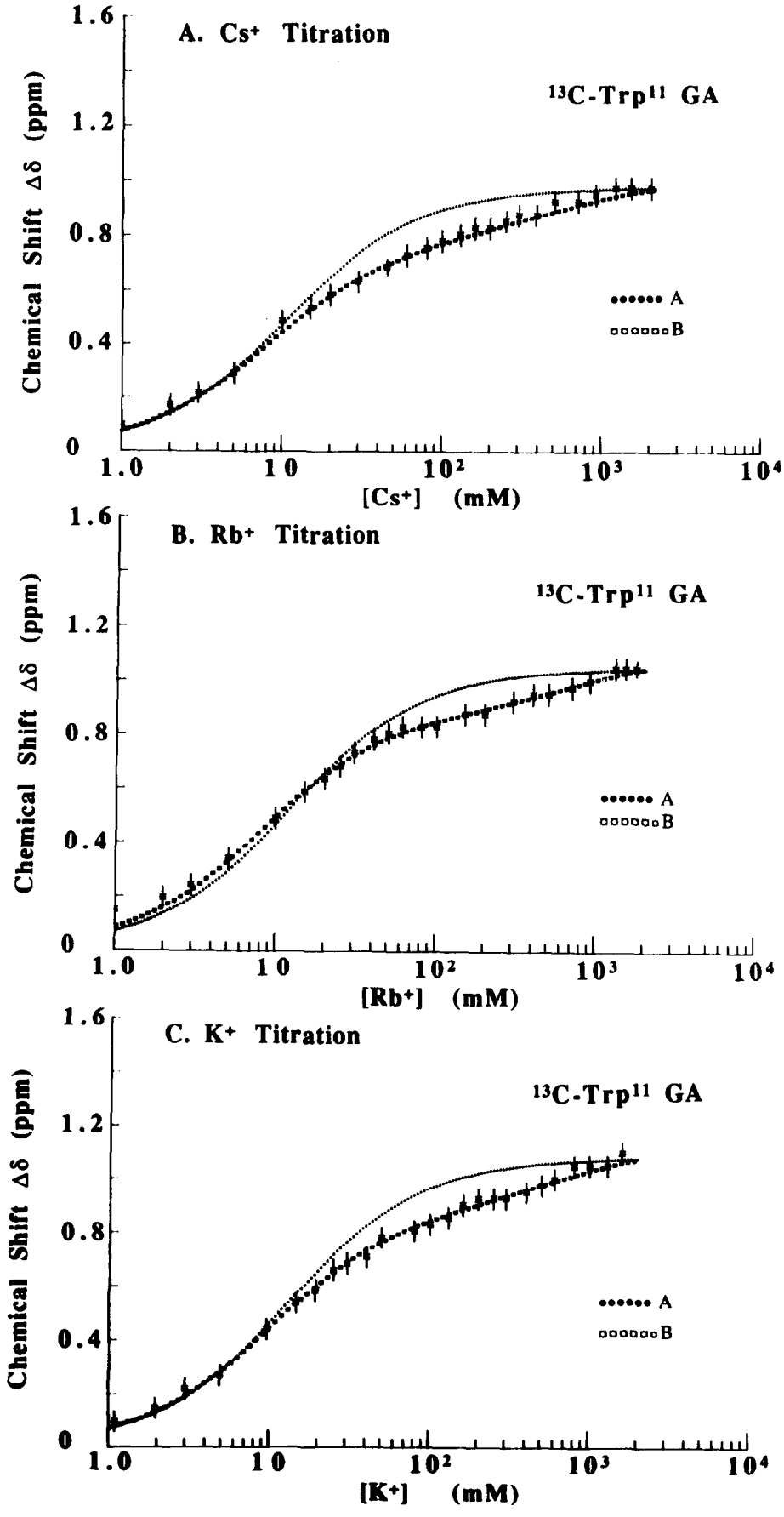


Fig. 5. The plot of excess longitudinal relaxation rates of sodium ion interaction with [1-^{13}C]Trp^{11} GA in DPC micelles at 30° C, measured by ^{23}Na-NMR. The binding constants, K_b^t and K_b^w are given as 25/M and 2.6/M. $R(1)$ ($= 1/T_{1f}$, 20/s) is the reciprocal of the longitudinal relaxation time, T_1, in presence of DPC micelles in the sample with 10 mM NaCl in D_2O but without gramicidin molecules (see text for details).


A. Cs+ Titration
13C-Trp11 GA
Chemical Shift Δδ (ppm)
1.6
1.2
0.8
0.4
0
1.0
10
10²
10³
10⁴
[Cs+] (mM)
•••••• A
oooooo B
B. Rb+ Titration
13C-Trp11 GA
Chemical Shift Δδ (ppm)
[Rb+] (mM)
•••••• A
oooooo B
C. K+ Titration
13C-Trp11 GA
Chemical Shift Δδ (ppm)
[K+] (mM)
•••••• A
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3.4. The binding constants determined by ^{23}Na relaxation study

The measurement of the longitudinal relaxation time (T_1) to estimate the binding constants of the GA channel has been described before [5,6,22]. In this study, the longitudinal relaxation times (T_1) of Na^+ ion were measured by ^{23}Na-NMR, using the [1-^{13}C]Trp^{11} GA in DPC micelles at the same temperature as used for ^{13}C-NMR experiments. The plot of the excess longitudinal relaxation rate (ELR) was expressed as

$$(R_1 - R_{1f})^{-1} = \frac{1}{C_T(R_{1b} - R_{1f})}\left(Na_T + K_b^{-1}\right)$$

where C_T is the total channel concentration, Na_T is the sodium concentration; $R_1 = 1/T_1$, and R_{1f} and R_{1b} are ^{23}Na longitudinal relaxation rates for the free and bound states [31]. The binding constants, K_b^t and K_b^w, were obtained from the reciprocal of the extrapolated negative x-axis intercepts, as 25/M and 2.6/M (Fig. 5). These are very close to the binding constants determined from the ^{13}C chemical shift analysis (Table 1). Since the gramicidin transmembrane channel is a 2-fold symmetric system and has a single-file transport mechanism [2], the value of the relaxation time, T_1, of cations with a spin number $I > 1/2$ can provide the information of an ion-channel interaction at binding site (see Discussion).

3.5. C-13 chemical shifts induced by K^+, Rb^+ and Cs^+ ions

The potassium, rubidium, and cesium ion titration studies were carried out by ^{13}C-NMR at 30° C, using C-13 enriched carbonyl carbon of Trp^{11} GA in DPC micelles. In these studies (Fig. 6), it was found that the chemical shifts ($\Delta\delta$) of carbonyl carbon induced by increasing K^+, Rb^+, and Cs^+ ion concentrations are the same with one another, and also the same as that induced by changing Na^+ ion concentration; this suggests that all the monovalent cations have common binding sites in the GA channel, which are independent on ion species. It was also found that all of the titration data are indicative of double occupancy, rather than single occupancy at high concentrations. The average deviations between the analytical curves and the experimental data from 20 mM to 1000 mM are less than 1.5% for the double occupancy fittings, and larger than 8% for the single occupancy fittings (8.1% for K^+, 8.3% for Rb^+, and 10.6% for Cs^+). As a consequence, the double occupancy is taken as the correct model for all the monovalent cations including for Na^+. The binding constants of the gramicidin A in DPC micelles are 60/M and 3/M for K^+; 80/M and 2.5/M for Rb^+; 80/M and 4/M for Cs^+.

4. Discussion

The measurements of ^{13}C chemical shifts of carbonyl carbons to detect the interaction between K^+ ions and valinomycin in CF_3CD_2OD and to locate the ion binding site in the GA channel in LPC vesicles have been demonstrated before [12,13,30,32]. The CD spectrum of Fig. 1 shows that the gramicidin A packaged by DPC micelles forms the same structure as malonyl gramicidin A in DPC micelles, which has been identified as a head-to-head, single-stranded, right-handed, $\beta^{6.3}$-helical dimer [16]. Accordingly, the carbonyl groups of Trp^{9,11,13,15} residues are largely buried in the lipid micelles. Comparing the ^{13}C spectrum of free DPC micelles with those of GA incorporated into DPC micelles at low and high ion concentrations (Fig. 2), only the chemical shift of ^{13}C-label carbonyl carbon of gramicidin A was induced by changing ion concentration; the other ^{13}C signals exhibited no detectable shift. Therefore, the observed ion-induced chemical shifts occur as ions enter the channels in single file and interact with the peptide at the binding sites, as described in the Results. Moreover, the binding constants of gramicidin A in DPC micelles for Na^+ ion were estimated by two independent methods: (1) the measurement of longitudinal relaxation time (T_1) of Na^+ ion as a function of concentration; and (2) the dependence of ^{13}C chemical shift on ion concentration. It is known that the relaxation rate, $1/T_1$, of quadrupolar ions is strongly influenced by the magnitude of the quadrupolar coupling constant ($\frac{e^2qQ}{}$) and the value of the correlation time (τ_c) for fluctuations in the electric field gradient experienced by the quadrupolar ion. The value of τ_c increases when the small quadrupolar ion is bound by a large slow-moving peptide. The value of the electric quadrupolar moment, eQ, is fixed for Na^+ ion, but the electric field gradient, eq, at the nucleus will have a larger value since the interaction between the ions and the peptide carbonyls of the channel may cause the distortion of the closed shells of electrons around the nucleus or the change of the charge distributions. The increases in τ_c and eq are caused by the ion-channel interaction at binding sites, so the T_1 of the bound ions is much shorter than that of the free ions. The nuclear relaxation rate for the ion, $R_1 = 1/T_1$, is composed of two parts, the bound ion fraction and the free ion fraction. According to James-Noggle's function, the constant, K_b, provides information of the ion-peptide binding interaction at a binding site. As

Fig. 6. The data analyses of ^{13}C chemical shift, $\Delta\delta$ (C), of the carbonyl carbon of [1-^{13}C]Trp^{11} GA in DPC micelles as a function of Cs^+, Rb^+, and K^+ ion concentrations. The mean deviations of the single (dotted line, A) and double (squared line, B) occupancy fittings between the analytical curves and the ^{13}C chemical shifts are 10.6% and 1.2% for Cs^+ (A); 8.3% and 1.4% for Rb^+ (B); 8.1% and 1.5% for K^+ (C). The binding constants, K_b^t and K_b^w obtained from the double occupancy are 80/M and 4/M for Cs^+, 80/M and 2.5/M for Rb^+, and 60/M and 3/M for K^+.

a 2-fold symmetric system and a single-file interaction for the GA channel, the values of the constants, K_b^t and K_b^w were considered as the binding constants in low and high concentrations. Since the two methods were carried out with the same sample at the same temperature, and the data are comparable (Table 1), it is obvious that ^{13}C chemical shifts were induced by the ion–peptide interaction at the binding sites within the GA channel. Also the measurement of the relaxation time (T_1) on ionic NMR is a convenient method for obtaining the binding constants for ion transport channels.

Based on the conformation of gramicidin A in DPC micelles, the distance between the two binding sites is about 16 Å, matching the position between the Trp^{11} and Trp^{13} residues for the right-handed helix, and the distance of the binding site to the entrance is about 5 Å. An important observation in this study is that the repulsive electrostatic interaction between the two ions within micellar-package gramicidin A pushes the pair of ions slightly outward from the sequence of $Trp^{9,11,13}$ for one ion to the $Trp^{11,13,15}$ sequence in the presence of two ions (Fig. 3). This observation shows clearly that at high ion concentration, two cations can be bound in the GA channel simultaneously and that electrostatic repulsion will occur between the two occupied ions. According to the theoretical analyses, the tight binding constants, K_b^t, for Na^+, K^+, Rb^+ and Cs^+ are 40/M, 60/M, 80/M and 80/M in a sequence as $Na^+ < K^+ < Rb^+ = Cs^+$, which are similar to the ^{205}Tl NMR study [33]. The weak binding constants, K_b^w for these ions are 4/M, 3/M, 2.5/M, and 4/M. At the singly occupied state, the ion-channel interaction is a main interaction in the peptide channel and a larger cation has a tighter coordination with the peptide channel, as predicted by the peptide libration mechanism [2]. As the second ion enters, the ion–ion interaction has a strong influence on the ion-channel interaction and binding for the second ion is less favorable with the free energy change ($-RT\ln K_b^w$). Using the binding constants from the data analyses, the differences between the free energy change for the singly occupied state and for the doubly occupied state ($-RT\ln K_b^w/K_b^t$) are about 1.4, 1.8, 1.9, and 1.8 kcal/mol for Na^+, K^+, Rb^+, and Cs^+ ions due to the effect of the ion-ion repulsion at a 16 Å distance. However, further study of the ion–ion interaction influencing the rate of ion permeation will be reported using GA analogs with different channel lengths.

Whether the channel is right-or left-handed has no material effect on the interpretation of these or previously published data, except to know whether the observed interaction between the ions on double occupancy occurs at a distance of 16 or 23 Å.

It is also important to derive the correct binding model of gramicidin A in DPC micelles, which was achieved by the analyses of ^{13}C chemical shift vs. ion concentration. Two different equations were employed to fit the ^{13}C chemical shifts. The analytical calculation of single occupancy depends on the ratio of singly occupied channels to the total channels as a function of ion concentration; however, that of the double occupancy is based on a sum of the chemical shifts of a singly occupied fraction and a doubly occupied fraction. The ratio of the two fractions varies with ion concentration. Compared with the fitting curves for the ion titrations, the fittings of the single occupancy have a larger deviation to ^{13}C chemical shifts in high ion concentration due to ignoring the influence of the second ion within GA channels. The deviations for double occupancy fittings are less than 2%. Clearly, the double occupancy is an acceptable model for the GA channel system, which supports the earlier proposed kinetic models [3,5,6]. To match the righthanded structure of gramicidin A in DPC micelles, the distance between the two binding sites is about 16 Å and the distance of the binding site to the entrance is about 5 Å. The possibility of another two sites located outside of the entrances, as proposed by Sandblom et al. [4,34], still remains, particularly if the ions at these sites retain their hydration shells.

Finally, for the thallium ion, the binding constants of gramicidin A in DPC micelles were also determined by the data analyses. The tight binding constant, K_b^t, is 1000/M, in agreement with the earlier studies (Table 1). The dissociation constant for the first ion bound in GA channels is about 1 mM; that is the same as the mid-point of ^{13}C-Trp^9 GA in DPC micelles in Tl^+ titration (Fig. 3B). When the second ion enters, the two ions push one another slightly outward and both are bound in the two symmetric positions of the GA channel so that the binding constant of the doubly occupied state, K_b^w decreases about 33 times to 30/M As a consequence, the repulsive interaction of ion-ion over 16 Å within the GA channel causes some adjustment of binding site for double occupancy and decreases the binding affinity for the second ion, which agrees with the prediction of Veatch and Durkin [23].

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