Drickamer, K., Mamon, J. R., Binns, G., & Leung, J. O. (1984) *J. Biol. Chem. 259*, 770–778.
Furth, A. J. (1980) *Anal. Biochem. 109*, 207–215.
Geuze, H. J., Slot, J. W., Strous, G. J. A. M., Peppard, J., von Figura, K. Hasilik, A., & Schwartz, A. L. (1984) *Cell (Cambridge, Mass.) 37*, 195–204.
Hardy, M. R., Townsend, R. R., Parkhurst, S. M., & Lee, Y. C. (1985) *Biochemistry 24*, 22–28.
Harford, J., & Ashwell, G. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1557–1561.
Harford, J., Lowe, M., Tsunoo, H., & Ashwell, G. (1982) *J. Biol. Chem. 257*, 12685–12690.
Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29–79.
Hildreth, J. E. K. (1982) *Biochem. J. 207*, 363–366.
Holland, E. C., Leung, J. O., & Drickamer, K. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7338–7342.
Hudgin, R. L., Pricer, W. E., Jr., & Ashwell, G. (1974) *J. Biol. Chem. 249*, 5536–5543.
Hwang, D. L., Tay, Y.-C., Barseghian, G., Roitman, A., & Lev-Ran, A. (1985) *J. Recept. Res. 5*, 367–380.
Kawasaki, T., & Ashwell, G. (1976) *J. Biol. Chem. 251*, 1296–1302.
Kolb-Bachofen, V. (1981) *Biochim. Biophys. Acta 645*, 293–299.
Leung, J. O., Holland, E. C., & Drickamer, K. (1985) *J. Biol. Chem. 260*, 12523–12527.
Lichtenberg, D., Robson, R. J., & Dennis, E. A. (1983) *Biochim. Biophys. Acta 737*, 285–304.
Matsuura, S., Nakada, H., Sawamura, T., & Tashiro, Y. (1982) *J. Cell Biol. 95*, 864–875.
McIntosh, D. B., & Davidson, G. A. (1984) *Biochemistry 23*, 1959–1965.
Oka, J. A., & Weigel, P. H. (1983) *J. Biol. Chem. 258*, 10253–10262.
Pang, D. T., & Shafer, J. A. (1984) *J. Biol. Chem. 259*, 8589–8596.
Ray, D. A., & Weigel, P. H. (1985) *Anal. Biochem. 145*, 37–46.
Ray, D. A., Oka, J. A., & Weigel, P. H. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 2043.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Schwartz, A. L., Fridovich, S. E., & Lodish, H. F. (1982) *J. Biol. Chem. 257*, 4230–4237.
Seglen, P. O. (1973) *Exp. Cell Res. 82*, 391–398.
Spiess, M., & Lodish, H. F. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6465–6469.
Steer, C. J., Kempner, E. S., & Ashwell, G. (1981) *J. Biol. Chem. 256*, 5851–5856.
Wall, D. A., Wilson, G., & Hubbard, A. L. (1980) *Cell (Cambridge, Mass.) 21*, 79–93.
Weigel, P. H. (1980) *J. Biol. Chem. 255*, 6111–6120.
Weigel, P. H. (1986) in *Vertebrate Lectins* (Olden, K., & Parent, B., Eds.) Van Nostrand/Reinhold, New York (in press).
Weigel, P. H., & Oka, J. A. (1982) *J. Biol. Chem. 257*, 1201–1207.
Weigel, P. H., & Oka, J. A. (1983a) *J. Biol. Chem. 258*, 5089–5094.
Weigel, P. H., & Oka, J. A. (1983b) *J. Biol. Chem. 258*, 5095–5102.
Weigel, P. H., Ray, D. A., & Oka, J. A. (1983) *Anal. Biochem. 133*, 437–449.
Weigel, P. H., Clarke, B. L., & Oka, J. A. (1986) *Biochem. Biophys. Res. Commun.* (submitted for publication).

Investigation of the Interaction between Thallous Ions and Gramicidin A in Dimyristoylphosphatidylcholine Vesicles: A Thallium-205 NMR Equilibrium Study†

Dikoma C. Shungu, James F. Hinton,* Roger E. Koeppe II, and Francis S. Millett
Department of Chemistry and Biochemistry, University of Arkansas, Fayetteville, Arkansas 72701
Received March 18, 1986; Revised Manuscript Received June 6, 1986

ABSTRACT: This study reports the first *direct observation* of multiple occupancy of the gramicidin A channel by Tl^+ ions. ^{205}Tl NMR has been used to study the equilibrium binding of Tl^+ by gramicidin A incorporated in sonicated dimyristoylphosphatidylcholine vesicles. It is shown that only multiple-channel occupancy can account for the ^{205}Tl chemical shifts measured. The data are analyzed to yield the equilibrium association constants of 450–600 and 5–20 M^{-1} for the binding of the first and the second ions at 34 °C, respectively.

Gramicidin A, a linear pentadecapeptide antibiotic isolated from *Bacillus brevis*, has a well-known amino acid sequence (Sarges & Witkop, 1965). In natural and artificial lipid membranes, it dimerizes to form ion-transporting transmembrane channels (Hladky & Haydon, 1970, 1972; Bamberg & Lauger, 1973; Urry, 1971; Veatch & Stryer, 1977; Krasne et al., 1971). The channels exhibit a number of properties that make them an ideal model for studying cation transport across biological membranes. They are selective to monovalent cations, impermeable to anions and blocked by divalent cations (Myers & Haydon, 1972; Bamberg & Lauger, 1977; Eisenman et al., 1977; Sandblom et al. 1977); they show high ionic flux rates, ca. 10^7 ions/s^{-1}, concentration dependence of permeability ratios, and single file transport (Finkelstein & Andersen, 1981; Neher et al., 1978; Urban et al., 1980; Andersen, 1984 and references therein). The structure of the membrane-bound, ion-permeable channel has now been established to consist of two left-hand helices, joined head-to-

† We gratefully acknowledge the support of the National Science Foundation through Grant PCM-8300065 (J.F.H.) and the National Institutes of Health through Grant GM-34968 (R.E.K.).

0006-2960/86/0425-6103$01.50/0

head, with 6.3 residues/turn (Urry, 1971; Szabo & Urry, 1979; Bamberg et al., 1977; Weinstein et al., 1979, 1980, 1985), although a recent 2-D ^1H NMR report disputes the accepted left handedness of the helices (Arseniev et al., 1985).

Equilibrium and kinetic properties of the ion-binding channels have been extensively studied (Hinton et al., 1982; Venkatachalam & Urry, 1980; Urry et al., 1985; Veatch & Durkin, 1980; Procopio & Andersen, 1979; Eisenmann et al., 1978; Hladky et al., 1979; Sandblom et al., 1977; Levitt, 1978). Depending on the model adopted to fit the observed data, various channel occupancy numbers have been predicted.

Recently, we have used ^{205}Tl NMR to determine the equilibrium association constants for the binding of Tl^+ ions by micellar-packaged gramicidins A–C (Hinton et al., 1986a). All of the observed shifts could be accounted for by assuming single occupancy of the channels. Also, using a combination of this technique and the competitive displacement of Tl^+, we have been able to derive the equilibrium binding constants for the group 1 metal cations to gramicidin A in aqueous dispersions of lyso-PC[1] (Hinton et al., 1986b). The choice of the latter environment for gramicidin packaging was dictated by factors previously discussed (Hinton et al., 1986a; Spisni et al., 1979; Masotti et al., 1980).

Here, we report the first NMR spectroscopic study of the binding of Tl^+ by gramicidin A incorporated in DMPC vesicles. This system presents several attractive features: (i) The conformational state of gramicidin in DMPC has been well characterized (Weinstein et al., 1979, 1980, 1985; Wallace et al., 1981; Nabedryk et al., 1982). The dimers exist in the channel state in this phospholipid. (ii) The small size of the vesicles (250–500 Å) allows them to remain suspended in aqueous solutions, and their short reorientation correlation times permit NMR measurements using standard solution techniques. (iii) The bilayers are almost as thick as the channels are long. This observation is quite significant since the mean channel lifetimes decrease with increasing bilayer thickness (Elliot et al., 1983). It has been estimated that on going from a 26- to a 64-Å lipid layer thickness the gramicidin A channel mean lifetime decreases from 60 s to 30 ms (Hladky & Haydon, 1972). (iv) A gel–liquid crystalline phase transition at 23 °C for DMPC (Janiak et al., 1976) makes possible handling and measurements at physiological temperatures.

Veatch and Durkin (1980) used the DMPC system in equilibrium dialysis experiments to directly measure the Tl^+ association constant to gramicidin A. The results were interpreted in terms of the existence of only one set of tight-binding sites per dimer in the range of Tl^+ concentrations investigated (up to 20 mM), with an association constant of 500–1000 M^{-1}. Values for Na^+ and Rb^+ were considered too low for accurate determination by this method. The equilibrium dialysis study appears to be the only *direct* measurement of binding constants previously reported for the DMPC system.

The present work uses ^{205}Tl NMR to measure chemical shifts as a function of Tl^+ ion activities, at constant gramicidin A concentration. The results of these "titrations" are used to answer two of the classical questions about binding sites on a macromolecule: "How many?" and "How tightly?". A model is developed that accounts for the magnitudes and signs of the observed shifts. Comparisons are made with previous equilibrium (Veatch & Durkin, 1980; Monoi, 1985) and kinetic (Eisenman et al., 1978; Levitt, 1978) treatments. Similarities and differences between the present model and that developed for micellar-packaged gramicidins (Hinton et al., 1986a) are established.

Experimental Procedures

Materials. Gramicidin was purchased either as gramicidin D from Sigma or as gramicidin from U.S. Biochemical Corp. These commercial preparations contain mixtures of gramicidins A–C. Separation and purification of the individual analogues were achieved as previously described (Koeppe & Weiss, 1981; Turner et al., 1983). The samples used in this NMR study consisted of 92% gramicidin A and 8% gramicidin C as assessed by analytical HPLC (Koeppe & Weiss, 1981). Dimyristoylphosphatidylcholine (DMPC) was obtained form Sigma and was used without further purification. $TlNO_3$ (Alfa Ultrapure) was twice recrystallized from water and then dried to constant weight at 90 °C (40 mmHg). Water was deionized and twice distilled in an all glass apparatus.

Incorporation of Gramicidin A in DMPC Vesicles. Gramicidin A (117 mg) and 950 mg of DMPC were dissolved in 2 mL of 1:1 $CHCl_3$–CH_3OH; this yielded a molar ratio of about 20 DMPC/polypeptide monomer. The solvent was removed by rotary evaporation at 50 °C. Any residual solvent was evaporated by vacuum pumping at room temperature for 24 h. The dry film that resulted was resuspended in 5 mL of N_2-flushed H_2O and incubated at 37 °C for 120 min to hydrate the lipid. Gramicidin A was incorporated into DMPC vesicles by a combination of two methods: (1) the sonication method of Barrow and Lentz (1980) used to produce small (unilamellar) vesicles using a cup horn; (2) a variation of the heat incubation technique of Masotti and co-workers (1980).

The DMPC–gramicidin A mixture was briefly sonicated and then quantitatively transferred to a polycarbonate centrifuge tube. Next, with a Branson W-185 cell disruptor equipped with a cup horn, the mixture was sonicated for 3.5 h at 37 °C. This was about 3 times longer than necessary to achieve sample clarity. The resulting yellowish blue preparation was incubated at 60 °C for 12 h and then sonicated again at 37 °C for 2 h. The 60 °C incubation step was necessary to ensure completion of the incorporation process (Masotti et al., 1980). Finally, the gramicidin A–vesicle mixture was centrifuged at 35000*g* for 1 h at 30 °C to remove any remaining multilamellar structures and unincorporated polypeptide. The supernate was withdrawn for NMR sample preparation. The final concentration of gramicidin A in the sample was determined by UV (ϵ_{max} = 20 840) on a Cary 210 spectrophotometer. Generally, it was found that 80–90% (w/w) of the gramicidin A originally present was incorporated in the liposomes. The conformational state of the dimers was checked by CD on a Jasco J-500 spectropolarimeter interfaced to a model DP-500N data processor. A second sample, which was gramicidin free, was prepared by sonicating an aqueous dispersion of 950 mg of DMPC for 5.5 h at 37 °C. This sample was also subjected to centrifugation. The gramicidin A containing vesicle preparations were stable both above and below the gel–liquid crystalline-phase transition temperature (23 °C) of DMPC. The gramicidin A free samples, however, needed to be kept above the phase transition temperature of the lipid. Failure to do so resulted in a rapid formation of large lipid aggregates as evidenced by sample clouding.

NMR Samples and Measurements. The preparation of samples for ^{205}Tl NMR has been recently described (Hinton et al., 1986a). Because of the relatively low solubility of $TlNO_3$ in water (250 mM at 25 °C), samples with Tl^+ concentrations above 50 mM were prepared by adding 1 mL of 1:4 (H_2O–DMPC)–gramicidin A to preweighed solid thallous nitrate. The Tl^+ content of this sample was 150 mM.

[1] Abbreviations: lyso-PC, L-α-lysophosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; NMR, nuclear magnetic resonance; CD, circular dichroism.

This concentrated mixture was diluted with aliquots of appropriate ratios of H_2O, Tl^+, and gramicidin A containing vesicles to yield the desired Tl^+ and gramicidin A concentrations.

To correct for the effect of the lipid on the observed ^{205}Tl shifts, identical samples were prepared of the gramicidin A free vesicle suspension. These are the "lipid correction" samples. All of the samples in the NMR tubes were then sonicated in an ultrasonic bath for 10 min, at 37 °C, to ensure that there were ions both inside and outside the vesicles. The samples were stored in a Dewar at 50 °C for the duration of the NMR measurements.

^{205}Tl spectra were recorded as a function of Tl^+ activities at 34 °C. The amount of gramicidin A was identical and invariant. ^{205}Tl shifts induced by the interaction of Tl^+ ions with the channels were obtained by subtracting, for each pair, the shifts of the lipid correction samples from those of gramicidin A containing samples. In order to assess the efficacy of this correction for lipid effects, ^{31}P NMR spectra of the phospholipid head groups were obtained for a number of pairs of gramicidin-free/gramicidin-containing samples, in the presence of varying amounts of Tl^+. These measurements were made on A JEOL FX-90Q spectrometer. All of the ^{205}Tl chemical shifts were referenced to the resonance frequency of Tl^+ ion in water at infinite dilution. Downfield shifts were assigned positive values. The spectrometer used for ^{205}Tl NMR is a Bruker HFX-90 modified for pulsed FT operation. The probe was temperature controlled by a Bruker temperature control unit. That ^{205}Tl NMR is an extremely sensitive probe for studying biological systems has been demonstrated in this laboratory (Hinton & Briggs, 1979; Hinton & Metz, 1980; Briggs et al., 1980).

Binding Models and Computational Methods. The chemical equilibria describing the interaction between Tl^+ ions and the gramicidin A dimers (GA) in DMPC can be written as

$$Tl^+ + GA \overset{K_1}{\rightleftharpoons} TlGA^+ \quad (1)$$

$$TlGA^+ + Tl^+ \overset{K_2}{\rightleftharpoons} Tl_2GA^{2+} \quad (2)$$

where K_1 and K_2, the stepwise association constants, are given by

$$K_1 = [TlGA^+]/[Tl^+][GA] \quad (3)$$

and

$$K_2 = [Tl_2GA^{2+}]/[TlGA^+][Tl^+] \quad (4)$$

Equations 1–4 are suggested by two types of binding models. The *one-site* model or model I, described by eq 1 and 3, assumes that only *one* ion at a time can bind to the single set of tight sites in the gramicidin channel. Model II or the *two-site* model–eq 1–4—is based on the assumption that both single and double occupancies of the channel can be observed.

For both of these models, equations relating the observed ^{205}Tl chemical shifts (δ_0) to the binding parameters, K_1, K_2, δ_1, and δ_2, have been fully derived elsewhere (Hinton et al., 1986a); only their useful forms will be given here.

For model I

$$\delta_0 = [TlGA^+]\delta_1/[Tl^+]_0 \quad (5)$$

and for model II

$$\delta_0 = ([TlGA^+]\delta_1 + 2[Tl_2GA^{2+}]\delta_2)/[Tl^+]_0 \quad (6)$$

δ_1 and δ_2 are the intrinsic chemical shifts of Tl^+ in the complexes $TlGA^+$ and Tl_2GA^{2+}, respectively. $[Tl^+]_0$ is the total Tl^+ concentration in solution. Prior to using these equations, ionic concentrations were converted to activities by the extended Debye-Hückel law (Bockris & Reddy, 1970).

The curve-fitting procedure for model I is straightforward (Hinton et al., 1982, 1986a; Johnston et al., 1975) and thus will not be given here. Data fitting for model II, on the other hand, involved (a) assuming values for K_1 and K_2; (b) simultaneously solving eq 3 and 4 for $[TlGA^+]$ and $[Tl_2GA^{2+}]$; (c) substituting the values of $[TlGA^+]$ and $[Tl_2GA^{2+}]$ into eq 6 to obtain δ_1 and δ_2 by nonlinear least squares; and (d) calculating δ_0 of the model and the standard deviations of the fit. Steps (a)–(d) were repeated for a wide range of K_1 and K_2 until those values of the fitting parameters that best reproduced the observed shifts were found [see Johnson et al. (1975) for complete details]. The criteria for reliability of the parameters obtained were those of Deranleav (1969). We have used these criteria previously (Hinton et al., 1986a) in studies of the binding of Tl^+ to gramicidin analogues.

All of the computations were carried out interactively, under Conversational Monitor System (CMS), on an IBM system/370 computer. Since a multidimensional parameter space was to be searched for eventual "true" minima, a Lee Data Model 1216 terminal with full-screen editing capability was used for easy data and parameter manipulation; at the display console, parameters could be altered either individually or collectively to observe their immediate effect on the fits. Data-fitting progress could thus be closely followed.

Simultaneous solution of equations and nonlinear least squares were performed using subroutines ZSPOW and ZXSSQ, respectively; both are part of the International Mathematical and Statistical Library (IMSL, Houston, TX).

Results

Two methods are known to date that are capable of achieving a high enough concentration of gramicidin A channels in phospholipids for equilibrium binding studies: heat incubation for lysolecithins (Spisni et al., 1979; Hinton et al., 1982, 1985a) and ultrasonic irradiation of the sample for lecithins (Veatch & Durkin, 1980). Initially we used the latter method alone to incorporate gramicidin A in DMPC. But, a procedural variation was introduced as it became apparent that extended incubation of NMR samples at 50 °C further promoted channel formation. It was observed that the ^{205}Tl chemical shift of a sample prepared by sonication alone was less than that of the same sample after it had been stored at 50 °C. At low Tl^+ the magnitude of this shift difference was up to 2.5 ppm (over 100 Hz) and seemed to be dependent on the length of the incubation period. This is strong evidence that heating further promotes channel formation. The present observation and that of others (Masotti et al., 1980) suggested the introduction of the 60 °C incubation step in our incorporation method (vide supra). Comparison of the CD spectra of the samples prepared as described here with the CD spectra reported by Wallace et al. (1981) suggest that the gramicidin dimers were essentially in the conducting channel conformation. However, the difficulties in the interpreting the CD spectra in terms of the details of conformation are realized. Addition of Tl^+ to the samples had no apparent effect on these spectra. This observation, which is also in agreement with the findings of Wallace et al. (1981), suggests that cation binding to the gramicidin A channels is not accomplished by any sort of conformational changes.

The results of the measurements of the ^{205}Tl chemical shifts as a function of the Tl^+ ion concentrations are shown in Figure 1. The most striking feature about these data is the presence, at high Tl^+ concentrations, of negative shifts. The sign of these shifts suggests that, at some point along the binding curve, there occur new types of interactions that induce upfield

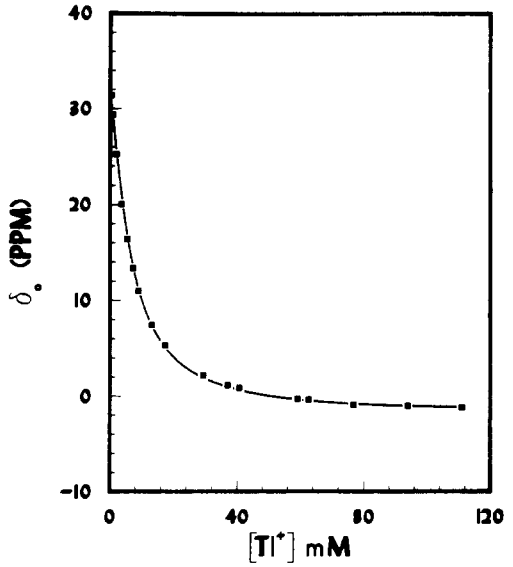

FIGURE 1: Concentration dependence of ^{205}Tl chemical shifts at 35 °C. The filled squares are the observed data points. Their size does not represent the experimental uncertainty. The solid (theoretical) line was calculated as described in the text.

displacements in the resonance frequency of the Tl^+ ions. In the present system, such a reversal in the direction of the observed chemical shifts can be rationalized only if one of the following two assumptions is made: (1) The lipid correction samples do not fully correct for the effects of the Tl^+–phospholipid head group interactions. In other words, the chemical shifts of the free Tl^+ ions in the lipid correction samples and in the gramicidin A containing samples are not identical, in which case the observed upfield shifts would simply be a manifestation of this difference. At low Tl^+, higher gramicidin A to Tl^+ ratios and stronger ion channel interactions effectively mask such lipid correction inaccuracies so that only downfield shifts are observed. (2) There exists a type of binding site in the gramicidin A channels that is accessible to Tl^+ ions only at high concentrations. The resonance frequencies of Tl^+ bound to these sites are, however, shifted upfield relatve to that of the Tl^+ ions in water at infinite dilution.

Experimental evidence points toward the latter possibility as that which applies in the present study. Figure 2 shows the ^{31}P NMR spectra of the phospholipid head groups obtained both from the lipid correction samples and from the gramicidin A containing samples. For each pair, it can be seen that the ^{31}P NMR spectra are identical. This indicates that the Tl^+ interactions with the lipid head groups are identical in both samples of each pair. It may thus be concluded that the observed upfield (negative) shifts are not an artifact of inaccuracies in the correction for lipid effects. The similarities in the NMR characteristics of the ^{31}P signals suggest that all of the Tl^+ interactions with the lipid vesicles are taken into account by the lipid correction samples; however, this evidence is not unequivocal. The presence of both positive and negative ^{205}Tl shifts clearly eliminates the possibility of model I being correct in fitting these data. Equation 5, which describes this model, predicts that the observed shifts (δ_0) can only be either all positive ($\delta_1 < 0$) or all negative ($\delta_1 < 0$). Experimentally, however, this is not the case. The results of the present study were, therefore, analyzed solely on the basis of model II.

Data Analysis: Two-Site Model. In nonlinear least-squares methods, the best estimates of the fitting parameters are those that minimize the sum of the squares of the deviations between the experimentally observed data and the model. In the present

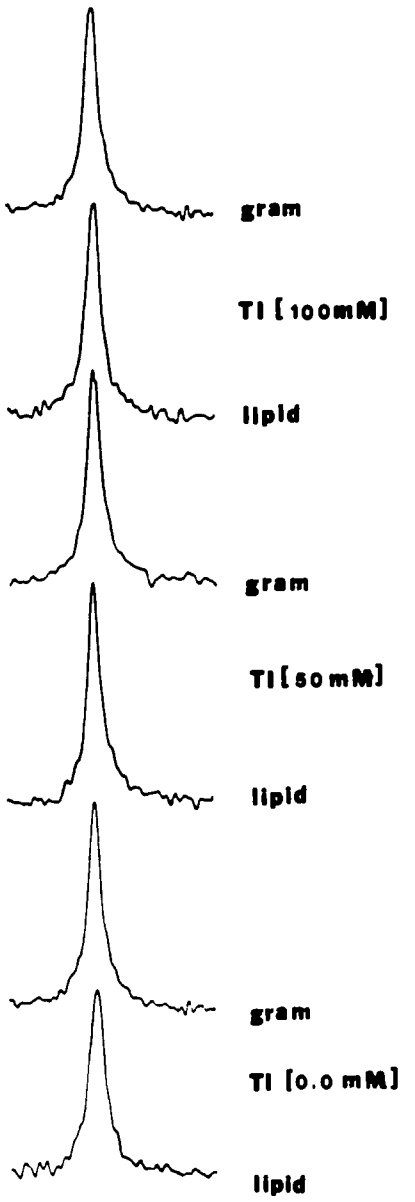

FIGURE 2: ^{31}P NMR spectra of the DMPC polar head groups, recorded in the presence and absence of gramicidin A.

analysis this criterion alone proved to be insufficient in yielding *meaningful* values of the parameters. For instance, a minimized sum of the squares was obtained with values contrary to the model. K_1, the association constant for the strong-binding site, was much less than K_2; δ_1 was unrealistically large (over 300 ppm). Sometimes a minimum was found with only one end of the calculated curve fitting the data. These examples show that a multiparametric analysis such as this one must be approached with great caution if meaningful parameters are to be obtained. We, therefore, found it necessary to establish two criteria that a fit would have to meet before it was considered *meaningful*: (1) As the binding of a second ion was thought to weaken the ion site interactions, $K_2 \ll K_1$ was expected. (2) The fits should be best at high Tl^+ since these data points are measured with higher accuracy (good signal/noise ratio and narrow NMR lines).

Figure 1 shows a plot of the fit that best met these criteria. The theoretical line was calculated with $K_1 = 500\ M^{-1}$, $K_2 = 7\ M^{-1}$, $\delta_1 = 53$ ppm, and $V_2 = -95$ ppm. As can be seen the fit is quite good throughout the binding curve. The fit shown in Figure 2 is not unique. Other values of K_1 and K_2 in the ranes of 450–600 and 5–20 M^{-1}, respectively, also produced

acceptable fits. The magnitude of δ_1 did not vary much. δ_2, however, assumed values ranging from −22 ppm (K_2 = 20 M^{-1}) to −118 ppm (K_2 = 5 M^{-1}). These parameters were obtained with many repetitions of the experiment.

Discussion

The enriched-^{13}C NMR experiments of Urry and co-workers (Urry et al., 1982) elegantly and conclusively determined the location of the cation binding sites in the gramicidin A channel. The question of how many ions can simultaneously occupy these sites is however still under controversy. Equilibrium dialysis studies in DMPC (Veatch & Durkin, 1980) and ^{205}Tl NMR investigations (Hinton et al., 1982, 1986a) in lyso-PC micelles argue that, in the ranges of Tl^+ concentrations covered (up to 20 and 50 mM, respectively), only one occupied site per channel can be observed; diionic, reversal potential, and ^{13}C NMR measurements (Hladky et al., 1979; Eisenman et al., 1978; Urry et al., 1985), on the other hand, predict that at least two Tl^+ ions can simultaneously be found in the channel. For Na^+ ions, likewise, single (Procopio & Andersen, 1979) and double (Venkatachalam & Urry, 1980; Urry et al., 1985; Levitt, 1978; Hladky et al., 1979; Monoi, 1985) occupancies have been predicted. Regretably, most of these predictions have seemed to be model dependent.

The results of the present NMR investigation, for the first time, provide evidence for the *direct* observation of multiple occupancy of the gramicidin A channel. The key to ruling out several possible binding models has been the observation of both positive and negative ^{205}Tl chemical shifts. ^{31}P NMR experimental evidence has ruled out the lipid effects as the cause for the measured negative shifts. On the basis that under no circumstances can it ever account for the observed combination of upfield and downfield chemical shifts, model I has also been effectively ruled out. It is only by assuming double-channel occupancy that the experimental data could be calculated. Model II is therefore the correct model, and the observed upfield shifts are a direct indication of the formation of higher order complexes.

The value range of 450–600 M^{-1} for K_1 at 34 °C is in excellent agreement with estimates of 550 at 25 °C (Eisenmann et al., 1978), 400–500 (Levitt, 1978) and 500–1000 M^{-1} at 23 °C (Veatch & Durkin, 1980), and 570 M^{-1} (Monoi, 1985). It is also interesting to note that there is agreement with our recent determination (582 M^{-1} at 34 °C) in lyso-PC (Hinton et al., 1986a) although two different models were used to fit the two sets of data. However, values for K_1 and K_2 at 70 °C of 5000 and 70 M^{-1}, respectively, have been reported by Urry et al., (1985) from an analysis of [^{13}C]carbonyl chemical shifts of gramicidin A packaged in lyso-PC as a function of Tl^+ concentration. These values at 70 °C seem rather high compared to those obtained at lower temperatures. A K_2 of 5–20 M^{-1} does not agree with the prediction of Eisenman et al. (1978) of 550 M^{-1} for the binding of a second ion to the opposite end of the channel. An equilibrium binding constant of 550 M^{-1} represents a dissociation constant (K_{2_d}) of 1.8 mM, and therefore, experiments using low Tl^+ concentrations, ca. up to 10 mM, should be able to easily determine it. Although Veatch and Durkin (1980) used concentrations higher than that, they could not detect the binding of a second ion. This indicates that the value of 1.8 mM (Eisenman et al., 1978) for K_{2d} may be an overestimation. The upper limit of 20 M^{-1} (K_{2d} = 50 mM) determined in the present study is more plausible in that it can partially explain why multiple occupancy was not observed at 20 mM Tl^+ (Veatch & Durkin, 1980), the ion site interaction for the doubly bound complex being too small to be measured at low ionic concentrations. One would have to go up to at least 50 mM Tl^+ to detect it. The relatively high dissociation constant obtained here is also consistent with a case of negative allosteric interactions between ions. Through electrostatic repulsion, the ion that is already bound to the channel inhibits additional Tl^+ from binding. It is only when all the strong-binding sites have been saturated ($[Tl^+]_0 > K_{2d}$) that the second ion can get into the channel. The flattening of the binding isotherm at high Tl^+ (Figure 1) corresponds to this saturation threshold. Beyond it, doubly bound complexes start forming. It is also beyond it that upfield shifts are observed.

The factors that determine the magnitudes and signs of the so-called bound shifts are not well understood. However, the number, the geometric arrangement, and the basicity of the electron-donating groups at the cation binding site have been established to play an important role in the determination of the $^{205}Tl^+$ chemical shift (Dechter and Zink, 1976). Nevertheless, it can be said that they provide information about the immediate neighborhood of Tl^+ ions in the singly and doubly bound complexes. A study of the ^{205}Tl chemical shift as a function of the ligand basicity has shown that carbonyls induce downfield shifts (Briggs, 1978). A downfield shift of +51 ppm for δ_1 is consistent with this observation since there is evidence (Urry et al., 1982a,b) that carbonyls are responsible for cation binding in the gramicidin channel. Its magnitude also seems reasonable since the binding curve can conceivably extrapolate to this value at the infinite dilution limit. As for δ_2, −22 ppm (or −118 ppm for that matter) is so uncharacteristic of the $^{205}Tl^+$ shifts in aqueous solutions that it would certainly have to mean that the binding of a second ion to the channel *drastically* alters the immediate environment of Tl^+. Recently, it has been suggested that no significant ion–ion interactions take place upon double occupancy of the gramicidin channel and that the binding of a second ion is more likely to induce a conformational change of the channels (Monoi, 1985). Such a suggestion is, however, inconsistent with CD spectral data that show no change upon addition of cations to a gramicidin–vesicle suspension (Wallace et al., 1981; this work). Veatch and Durkin (1980), on the other hand, attributed their inability to observe multiple occupancy to repulsive Coulombic effects arising from ion–ion interactions within the channel. Such effects (Levitt, 1978; Pullman & Etchebest, 1983; Etchebest, 1983; Jordan, 1984) could alter the energy profile in the channel and affect the magnetic character of the sites in such a way as to influence the extremely environment-sensitive ^{205}Tl chemical shift. The magnitude and the sign of δ_2 can also be mathematically explained by eq 6. Since the mole fractions of the doubly bound species are low at high Tl^+ concentrations, δ_2 would have to be relatively large and negative if upfield shifts are to be observed. The uncertainties in K_2 and δ_2 are a good illustration of the degree of difficulty associated with the measurement of relatively weak ion site interactions. It is only the inherently high sensitivity of the $^{205}Tl^+$ chemical shift to its environment that made this determination possible.

The results of the experiments presented show that ^{205}Tl NMR can be used to *directly* observe multiple occupancy of the gramicidin channel; that in DMPC at least two ions simultaneously bind; and that the binding of the second ion *may* drastically alter the energy profile of the channel and the magnetic character of the binding sites.

Acknowledgments

We thank Dr. Soon Han and Dr. Neil Purdie at the Chemistry Department of Oklahoma State University for recording the CD spectra. We thank Ruth Corder for puri-

fying the gramicidin A used in these experiments. We also thank Dr. Milton Johnston for making available to us his probram, LISA2, which was used in the data analysis.

Registry No. DMPC, 13699-48-4; Tl, 7440-28-0; gramicidin A, 11029-61-1.

REFERENCES

Andersen, O. S. (1984) *Annu. Rev. Physiol. 46*, 531.

Arseniev, A. S., Barsukov, I. L., Bystrov, V. F., Lomize, A. L., & Ovchinnikov, Y. A. (1985) *FEBS Lett.* 186, 168.

Bamberg, E., & Lauger, P. (1973) *J. Membr. Biol. 11*, 177.

Bamberg, E., & Lauger, P. (1977) *J. Membr. Biol. 35*, 351.

Bamberg, E., Apell, H.-J., & Alpes, H. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2402.

Barrow, D. A., & Lentz, B. R. (1980) *Biochim. Biophys. Acta 597*, 92.

Bockris, J. O., & Reddy, A. K. (1979) *Mod. Electrochem.*, 1.

Briggs, R. W. (1978) Ph.D. Thesis, University of Arkansas.

Briggs, R. W., Etzkorn, F. A., & Hinton, J. F. (1980) *J. Magn. Reson. 37*, 523.

Dechter, J. D., & Zink, I. J. (1976) *Inorg. Chem. 15*, 1690.

Deranleav, D. A. (1969) *J. Am. Chem. Soc. 91*, 4044.

Eisenman, G., Sandblom, J., & Neher, E. (1977) in *Metal–Ligand Interactions in Organic and Biochemistry* (Pullman, B., & Goldblum, N., Eds.) Part II, pp 1–36, Dordrecht, Holland.

Eisenman, G., Sandblom, J., & Neher, E. (1978) *Biophys. J. 22*, 307.

Elliot, J. R., Needham, D., Dilger, J. P., & Haydon, D. A. (1983) *Biochim. Biophys. Acta 735*, 95.

Finkelstein, A., & Andersen, O. S. (1981) *J. Membr. Biol. 59*, 155.

Hinton, J. F., & Briggs, R. W. (1979) *J. Solution Chem. 8*, 479.

Hinton, J. F., & Metz, K. R. (1980) *J. Solution Chem. 9*, 197.

Hinton, J. F., Young, G., & Millett, F. S. (1982) *Biochemistry 21*, 651.

Hinton, J. F., Koeppe, R. E. II, Shungu, D., Whaley, W. L., Paczkowski, J. A., & Millett, F. S. (1986a) *Biophys. J. 49*, 571.

Hinton, J. F., Whaley, W. L., Shungu, D., Koeppe, R. E., & Millett, F. S. (1986b) *Biophys. J.* (in press).

Hladky, S. B., & Haydon, D. A. (1970) *Nature (London) 225*, 451.

Hladky, S. B., & Haydon, D. A. (1972) *Biochim. Biophys. Acta 274*, 412.

Hladky, S. B., Urban, B. W., & Haydon, D. A. (1979) in *Ion Permeation through Membrane Channels* (Stevens, C. F., & Tsien, R. W., Eds.) pp 89–104, Raven, New York.

Janiak, M., Small, D., & shipley, G. (1976) *Biochemistry 15*, 4575.

Johnston, M. D., Shapiro, B. L., Shapiro, M. J., Proulx, T. W., Godwin, A. D., & Pearce, H. L. (1975) *J. Am. Chem. Soc. 97*, 542.

Jordan, P. C. (1984) *J. Membr. Biol. 78*, 91.

Koeppe, R. E. II, & Weiss, L. S. (1981) *J. Chromatogr. 208*, 414.

Krasne, S., Eisenman, G., & Szabo, G. (1971) *Science (Washington, D.C.) 174*, 412.

Levitt, D. G. (1978) *Biophys. J. 22*, 220.

Masotti, L., Spisni, A., & Urry, D. W. (1980) *Cell. Biophys. 2*, 241.

Monoi, H. (1985) *Biophys. J. 48*, 643.

Myers, V. B., & Haydon, D. A. (1972) *Biochim. Biophys. Acta 274*, 313.

Nabedryk, E., Gingold, P. M., & Breton, J. (1982) *Biophys. J. 38*, 243.

Neher, E., Sandblom, J., & Eisenman, G. (1978) *J. Membr. Biol. 40*, 97.

Procopio, J., & Andersen, O. S. (1979) *Biophys. J. 25*, 8a (Abstract).

Pullman, A., & Etchebest, C. (1983) *FEBS Lett. 163*, 199.

Sandblom, J., Eisenman, G., & Neher, E. (1977) *J. Membr. Biol. 31*, 383.

Sarges, R., & Witkop, B. (1965) *J. Am. Chem. Soc. 87*, 2011.

Spisni, A., Khaled, M. A., & Urry, D. W. (1979) *FEBS Lett. 102*, 321.

Szabo, G., & Urry, D. W. (1979) *Science (Washington, D.C.) 203*, 55.

Turner, G. L., Hinton, J. F., Koeppe, R. E. II, Parli, J. A., & Millett, F. S. (1983) *Biochim. Biophys. Acta 756*, 133.

Urban, B. W., Hladky, S. B., & Haydon, D. A. (1980) *Biochim. Biophys. Acta 602*, 331.

Urry, D. W. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 672.

Urry, D. W., Prasad, K. 1., & Trapane, T. L. (1982a) *Proc. Natl. Acad. Sci. U.S.A. 79*, 390.

Urry, D. W., Walker, J. T., & Trapane, T. L. (1982b) *J. Membr. Biol. 69*, 225.

Urry, D. W., Trapane, T. L., Venkatachalam, C. M., & Prasad, K. U. (1985) *Can. J. Chem. 63*, 1976.

Veatch, W. R., & Stryer, L. (1977) *J. Mol. Biol. 113*, 89.

Veatch, W. R., & Durkin, J. T. (1980) *J. Mol. Biol. 143*, 411.

Venkatachalam, C. M., & Urry, W. D. (1980) *J. Magn. Reson. 41*, 313.

Wallace, B. A., Veatch, W. R., & Blout, E. R. (1981) *Biochemistry 20*, 5754.

Weinstein, S., Wallace, B. A., Blout, E. R., Morrow, J. S., & Veatch, W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4230.

Weinstein, S., Wallace, B. A., Morrow, J. S., & Veatch, W. R. (1980) *J. Mol. Biol. 143*, 1.

Weinstein, S., Durkin, J. T., Veatch, W. R., & Blout, E. R. (1985) *Biochemistry 24*, 4374.