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Sodium Binding Sites of Gramicidin A: Sodium-23 Nuclear Magnetic Resonance Study†

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ABSTRACT: In ethanol–water mixtures (90:10), the gramicidin dimer binds Na^+ cations at well-defined sites, with a binding constant $K = 4\ M^{-1}$. Partial desolvation of Na^+ occurs upon binding, as judged from the magnitude of the quadrupolar coupling constant (1.7 MHz) for bound sodium. The binding sites are identified with the outer sites flanking the channel entrances. The rate constants for binding and release are $k_+ \leq 2.2 \times 10^9\ M^{-1}\ s^{-1}$ and $k_- \leq 5.5 \times 10^8\ s^{-1}$, respectively.

Gramicidin, one of the very first antibiotics to be isolated (Dubos, 1939), greatly enhances the permeability of natural (Chappell & Crofts, 1965; Harold & Baarda, 1967; Harris & Pressman, 1967) and artificial (Mueller & Rudin, 1967) membranes toward monovalent cations. In fact, gramicidin is a mixture of six pentadecapeptides constituted by regularly alternating D and L residues, the main constituent being valine-gramicidin A (Gross & Witkop, 1965; Sarges & Witkop, 1964a,b, 1965a–d): formyl-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-(L-Trp-D-Leu)$_3$-L-Trp-ethanolamide.

Ionic permeability conferred on membranes by gramicidin doping (Tregold, 1977) occurs through association of gramicidin into dimers which form channels (Bamberg & Lauger, 1973; Tosteson et al., 1968) responsible for the transport process. The two existing proposals for the channel-forming dimer, either head-to-head (Urry, 1971; Bamberg et al., 1977) or a double helical antiparallel β structure (Veatch & Blout, 1974), agree as to the length of the cylindrical channel, approximately 30 Å.

A recent model for the gramicidin A channel (Sandblom et al., 1977) includes a specific cationic binding site at each entrance to the channel. We adduce here direct evidence, from sodium-23 NMR measurements (Laszlo, 1978), for the existence of sodium-binding sites compatible with this proposal. Exchange of the sodium cations between these outer sites and the bulk solution is rapid compared with the transport process. We provide also a measurement of the rate constants for binding and release of Na^+ by these sites.

Experimental Procedure

Gramicidin, a gift from Société Rapidase (Seclin, France), has the composition: 70% gramicidin A, 10% gramicidin B, and 20% gramicidin C. We study its sodium-binding properties in ethanol–water binary mixtures (90:10) chosen both for solubility purposes and for the aqueous environment. Samples are prepared 24 h in advance from stock solutions containing either 100 or 50 mg of gramicidin/mL together with 10^{-2} M $NaClO_4$, diluted with 10^{-2} M $NaClO_4$ solutions. Ethanol (Baker Analyzed, reagent grade) has a water content of 0.05%. Sodium perchlorate (Merck, reagent grade) is dried under vacuum for 16 h immediately before use. Solvent stock mixtures are made by adding ethanol up to a final volume of 50.0 mL to 5.0 mL of deionized water. Samples are kept in the dark, at room temperature. After introduction in the NMR tubes, equilibration to the probe temperature (36 ± 1 °C) is ensured by waiting 15 min before the first spectrum is recorded. NMR spectra are obtained on Fourier transform instruments, at 23.81 MHz with a Bruker HFX-90 spectrometer field-locked on the deuterium signal of D_2O contained in a coaxial cell and at 62.86 MHz with a Cameca 250 spectrometer, without lock; 3500 to 95 000 transients are recorded, depending upon the conditions. All the NMR absorptions are Lorentzian and obey the criterion $w_{1/8} < 1.10 \times w_{1/2}\sqrt{7}$, where $w_{1/8}$ and $w_{1/2}$ are the line widths at $^1/_8$ and $^1/_2$ the total height (Delville et al., 1979).

Viscosities and densities are measured on these samples immediately after the NMR spectra have been recorded. Absolute viscosities are obtained from joint use of a Desreux-Bischoff viscosimeter (Desreux & Bischoff, 1950) and a pycnometer, both calibrated with water and thermostated at 36.0 ± 0.1 °C.

Results and Data Analysis

The sodium-23 line width in these experiments obeys the following set of equations applicable to transverse relaxation (Delville et al., 1979)

$$\frac{1}{T_2} = \frac{1 - p_B}{T_{2F}} + p_B\left[\frac{0.6}{T'_{2B} + \tau_B} + \frac{0.4}{T''_{2B} + \tau_B}\right] \quad (1)$$

$$\frac{1}{T'_{2B}} = \frac{\pi^2}{5}\chi^2\left[\tau_c + \frac{\tau_c}{1 + \omega^2\tau_c^2}\right] \quad (2)$$

$$\frac{1}{T''_{2B}} = \frac{\pi^2}{5}\chi^2\left[\frac{\tau_c}{1 + 4\omega^2\tau_c^2} + \frac{\tau_c}{1 + \omega^2\tau_c^2}\right] \quad (3)$$

where χ is the quadrupolar coupling constant (e^2qQ/h) for

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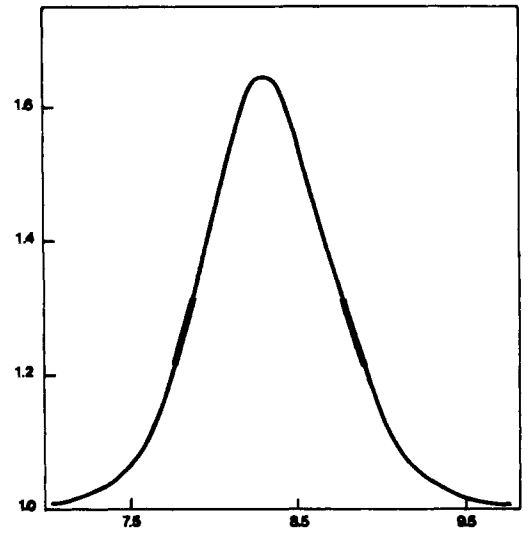

FIGURE 1: Plot of the ratio of the relaxation rate enhancements R_e at 62.86 and 23.81 MHz against minus the decimal logarithm of the correlation time τ_c. The segments indicated on the curve by a thickened line are the intersection with the experimental values, indicated together with error bars; they correspond to non-Lorentzian (left portion) and quasi-Lorentzian (right portion) absorptions.

bound sodium, T_{2F} is the transverse relaxation time of the free ion, T'_{2B} and T''_{2B} are the rapid and slow components, respectively, of the transverse relaxation for the bound sodium, p_B is the mole fraction of bound sodium, τ_B is its residence time on the slowly reorienting species, τ_c is the apparent correlation time for the bound sodium, and ω is the Larmor frequency. The observed Lorentzian absorptions have line widths at half-height $w_{1/2}$ related to T_2 by

$$\frac{1}{\pi T_2} = w_{1/2} \tag{4}$$

The relaxation rates for the free sodium ion $1/T_{2F}$ are corrected for the measured viscosities of the solution assuming Debye–Stokes–Einstein proportionality. The residence time τ_B can be neglected in eq 1 with respect to T'_{2B} and T''_{2B}; from the data below it will be seen that $\tau_B \sim 10^{-9}$ s, when T'_{2B} and $T''_{2B} \sim 10^{-5}$ s. At low gramicidin concentrations (<56 mg/mL) when $p_B \ll 1$, the enhancement of the relaxation rate can then be expressed by

$$\left(\frac{1}{T_2}\right)_e = \frac{1}{T_2} - \frac{1}{T_{2F}} = p_B\left[\frac{0.6}{T'_{2B}} + \frac{0.4}{T''_{2B}}\right] \tag{5}$$

Combining eq 2, 3, and 5, we obtain τ_c from the ratio R_e of the relaxation rate enhancements at two widely differing frequencies, 23.81 and 62.86 MHz (Delville et al., 1979; Grandjean et al., 1977) (Figure 1).

A first check on the quality of the procedure used is the excellent linear correlation between $(1/T_2)_e^{23.81}$ and $(1/T_2)_e^{62.86}$ ($\rho > 0.995$ for six points corresponding to variable gramicidin/$NaClO_4$ ratios), going through the origin, with a slope $R_e = 1.28 \pm 0.05$. Fortunately, the choice between the two values of the correlation time τ_c indicated by Figure 1, i.e., 14.4 ± 2.5 or 1.6 ± 0.3 ns, is unambiguous in this case: with the former value, a non-Lorentzian line shape would occur, contrary to observation; only with the latter and smaller value, an absorption band close to a true Lorentzian is expected (Delville et al., 1979) in accordance with our observations.

Another approach yields the same value of R_e, and hence of the correlation time τ_c, together with values for the mole fraction of bound sodium p_B and the quadrupolar coupling

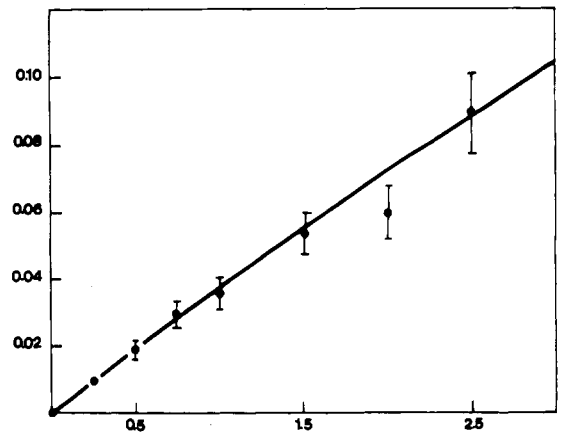

FIGURE 2: Plot of the reduced line width w_R against the molar ratio $(G_2)_t/(Na^+)_t$ at 62.86 MHz [$(Na^+)_t = 10^{-2}$ M]. Discrete points relate to w_R calculated from $w_{1/2}$ (experimental), $(w_{1/2})_F$, and $(w_{1/2})_B$ (Simplex-optimized values). The uncertainties combine the 5% error on the experimental line-width measurement and the standard deviation on the reproducibility of the Simplex adjustment. The solid line represents w_R calculated from the Simplex-adjusted value of K.

Table I: Limiting Line Widths and Binding Constant

parameter	23.81 MHz	62.86 MHz	ratio
$(w_{1/2})_F$ (Hz)	33.2 ± 1.2	31.5 ± 1.3	unity
$(w_{1/2})_B$ (Hz)	5310 ± 139	3956 ± 134	1.34 ± 0.08
K (M^{-1})	4.00 ± 0.25	4.02 ± 0.43	unity

constant χ. Let us assume that gramicidin is present as the dimer G_2, to which sodium ions bind according to

$$G_2 + Na^+ \overset{K}{\rightleftharpoons} G_2, Na^+ \tag{6}$$

then the reduced line width is given by (Live & Chan, 1976)

$$w_R = \frac{(w_{1/2})_F - (w_{1/2})}{(w_{1/2})_F - (w_1/_2)_B} = \frac{1}{2}\left[A - \left(A^2 - \frac{4(G_2)_t}{(Na^+)_t}\right)^{1/2}\right] \tag{7}$$

where

$$A = 1 + \frac{1}{K(Na^+)_t} + \frac{(G_2)_t}{(Na^+)_t} \tag{8}$$

$(Na^+)_t$ and $(G_2)_t$ are the analytical concentrations in sodium cation and in gramicidin dimer, respectively, and $(w_{1/2})_B$ is the line width extrapolated to unit mole fraction of bound sodium. Multiparametric Simplex adjustment (Deming & Morgan, 1973; Cornélis & Laszlo, 1978) of $(w_{1/2})_F$, $(w_{1/2})_B$, and K reproduces satisfactorily the observed dependence of the reduced line width on the molar ratio $(G_2)_t/(Na^+)_t$, at both frequencies used (Figure 2). The corresponding parameters are given in Table I. While they incorporate the actual experimental uncertainties, the range of errors given in Table I results from the reproducibility of the fitting procedure, when varying the input parameters. Close agreement between the values of $(w_{1/2})_F$ and K obtained at both frequencies shows that the model is at least internally consistent, whereas the $(w_{1/2})_B$ values are in a ratio $R_e = 1.34 \pm 0.08$, equal within the combined uncertainties with the above value of 1.28 ± 0.05. Hence, $\tau_c = 1.9 \pm 0.5$ ns. Taken together with the values of $(w_{1/2})_B$, it yields a quadrupolar coupling constant $\chi = 1.7 \pm 0.2$ MHz.

Discussion

How does the apparent correlation time for bound sodium $\tau_c = 1.9 \pm 0.5$ ns compare with the reorientational correlation time for its host, the gramicidin dimer? Indeed, gramicidin exists as the dimer G_2 in alcoholic (methanol, ethanol) solutions

(Veatch & Blout, 1974) such as we use. A water-normalized reorientational correlation time $\tau_R \simeq 30$ ns describes the isotropic motion as a rigid body of G_2, at 20 °C (Fossel et al., 1974).[1] Under our experimental conditions, the value of τ_R should be little different, viz., about 31 ns. Clearly, τ_c for the Na^+ cation is much smaller than τ_R, whose exact value is therefore not crucial to the argument, for the antibiotic dimer. For the equilibrium of eq 6, these parameters are related through (Forsén, 1978; Forsén & Lindman, 1978)

$$\tau_c^{-1} = \tau_B^{-1} + \tau_R^{-1} + \tau_i^{-1} \quad (9)$$

where τ_i characterizes the internal motion of the sodium binding site relative to the overall motion of the biomolecular ionophore. In the presence of fast internal motion, τ_i is expected to be a fraction of a nanosecond. Since the observed correlation time τ_c is in the nanosecond range, we must conclude that the sodium binding polar groups on the polypeptide reorient at the same slow rate as the overall motion. Hence, neglecting the third term in eq 9, τ_c appears to be determined predominantly by a residence time $\tau_B = 1.8 \pm 0.2$ ns for sodium cations on gramicidin dimers. Thus the rate constant for desorption of sodium $k_- = (\tau_B)^{-1} = (5.5 \pm 0.5) \times 10^8$ s^{-1} and, from the value of the equilibrium constant K (Table I), the binding rate constant $k_+ = (2.2 \pm 0.2) \times 10^9$ M^{-1} s^{-1}. Such a value of k_+, close to the diffusion-controlled limit (Chock et al., 1977), is reasonable.

The quadrupolar coupling constant χ reflects the asymmetry of the electric field gradient at the location of the quadrupolar nucleus. In the absence of any good calculation of this term and of the Sternheimer antishielding factor entering it (Laszlo, 1978), only qualitative conclusions are to be inferred from values of χ. Here, this parameter has a rather high value of 1.7 MHz as compared with the full range of 0.2–2 MHz (Laszlo, 1978): it is uniquely consistent with partial dehydration of Na^+ upon binding to a presumably polar site on the ionophore. If the sodium cation were not to shed one or several solvation molecules, one would expect a value closer to the lower limit of this range, smaller than ~0.5 MHz. The value of 1.7 MHz reported here is close to those reported for ^{23}Na with a number of neutral ionophores (Haynes et al., 1971).

Thus, we come up with a model for the interaction between the univalent sodium cation and gramicidin dimer, in which true binding occurs between the Na^+ ion and a site on gramicidin A, at a rate close to the diffusional limit. Our experiments monitor fast sodium exchange between the free state in the protic medium and a bound state on the channel-forming antibiotic. We do not believe that we are seeing any substantial contribution from ion transport itself; even though the electrostatic energy barriers that a univalent cation has to jump over in passage through such a channel embedded in a lipid membrane have been significantly lowered in recent calculations, to about 7 kT (Levitt, 1978a), the vast majority of sodium ions will go through the least-energy pathways available to them. In the present case of the gramicidin A dimer, interpretation of conductance data hinges upon the postulate of a specific cationic binding site at each entrance to the channel (Sandblom et al., 1977; Neher et al., 1978; Levitt, 1978b) in addition to binding sites inside the channel (Hladky, 1972). The simplest explanation of our results would be that the sodium cations are sampling the more easily accessible outer sites, at a rate more than sufficient for these outer sites to serve as reservoirs with respect to slower diffusion from outer sites into inner sites (Levitt, 1978b) and thus through the channel. Support for this identification of the sodium-binding sites in this study with the outer sites earlier posited (Sandblom et al., 1977; Levitt, 1978b) is afforded by the nice order of magnitude agreement between our measured value of K, 4 M^{-1}, and the sodium binding constant, 2.9 M^{-1}, used for fitting conductance data in symmetrical single salt solutions (Neher et al., 1978). Inclusion of the gramicidin A dimer in a lipid membrane should not greatly modify the physical properties of these outer sites, and our qualitative conclusions should thus also apply to the ion channels in nerve membranes.

[1] Considering the estimated dimensions of the gramicidin dimer, the calculated reorientational correlation time is smaller by an order of magnitude; the abnormal slowness of molecular rotation is ascribed to extensive solvation (Fossel et al., 1974). This discrepancy between the predicted and observed τ_R values, taken together with the well-established adaptation in size of DL alternant dimeric polypeptides toward solvent molecules (Lotz et al., 1976), renders difficult comparisons of G_2 hydrodynamics in widely differing solvents.

Added in Proof

Sodium binds to gramicidin dimers in an emulsified medium, according to ^{23}Na NMR results (Monoi & Vedaira, 1979) which agree with our findings of fast exchange of sodium ions between the bulk solution and a high electric field gradient site on the antibiotic.

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Investigation of Solvent Accessibility of the Fluorotyrosyl Residues of M13 Coat Protein in Deoxycholate Micelles and Phospholipid Vesicles†

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ABSTRACT: We have utilized a nonperturbing nuclear magnetic resonance technique, specifically measuring sensitivity of the chemical shift of fluorotyrosyl residues to change in solvent from H_2O to D_2O, to demonstrate that the tyrosyl residues of fluorotyrosyl M13 coat protein in phospholipid vesicles are not accessible to solvent i.e., are buried in the hydrophobic portion of the bilayer. The two fluorotyrosyl residues of the protein *did* show partial exposure to solvent (42% and 65% with respect to aqueous *m*-fluorotyrosine) when the protein was incorporated into deoxycholate micelles, pointing to differences in conformation of micellar protein with respect to vesicle-associated protein. M13 coat protein in phospholipid vesicles was not sensitive to lactoperoxidase-catalyzed iodination, supporting the NMR results. Coat protein in deoxycholate micelles showed release of fluorotyrosyl residues upon Pronase digestion, but only after an observed change in environment. The observed changes suggest that proteolytic digestion studies of membrane proteins should be interpreted with the possibility of artifacts related to conformational changes in mind. M13 coat protein in phospholipid vesicles did *not* demonstrate release of fluorotyrosine by Pronase, again pointing to differences between protein in micelles and in vesicles and corroborating the NMR result.

The nonlytic, filamentous coliphage M13 offers an excellent model system for the study of membrane–protein interactions. The process of phage assembly takes place entirely within the inner membrane of the *Escherichia coli* host, and the major coat protein (gene 8) is stored during infection as a cytoplasmic membrane spanning protein (Smilowitz et al., 1972; Smilowitz, 1974; Wickner, 1976). The coat protein is a 50 amino acid polypeptide of known sequence (Figure 1) and its physical properties have been extensively characterized (Knippers & Hoffmann-Berling, 1966). In addition, it is easily prepared in large quantities from intact phage, which are recovered from the growth medium.

In a previous publication (Hagen et al., 1978), we described the in vivo preparation of a fluorotyrosyl derivative of M13 coat protein and also a method for incorporation of high levels of this protein into small, uniformly sized synthetic phospholipid vesicles. Using high-resolution fluorine-19 nuclear magnetic resonance spectroscopy, we showed that the fluorotyrosyl probe was sensitive to the lipid phase state and proposed a physical model for the motional properties of the fluorotyrosine side chains in the phospholipid bilayer. The model which we proposed was characterized by correlation times for rotation about the $\alpha\beta$ and $\beta\gamma$ bonds of the fluorotyrosyl residues of 50×10^{-9} and 2×10^{-9} s, respectively, indicating increasing mobility away from the polypeptide backbone. Further, to account for all the features of the observed relaxation of the ^{19}F probe, we had to invoke rapid intermolecular fluorine–proton dipolar interactions. Based on the primary sequence (Figure 1) and on studies by other workers involving proteolytic digestion of M13 coat protein in deoxycholate micelles (Woolford & Webster, 1975), we hypothesized that the fluorotyrosyl residues were interacting with lipid methylene protons rather than solvent and estimated that these lipid protons had a minimum lateral diffusion coefficient of 3×10^{-9} cm^2 s^{-1}.

In this manuscript, we demonstrate that fluorotyrosyl residues of M13 coat protein *are* buried in the hydrophobic portion of the bilayer and present the application of a nonperturbing NMR[1] approach to study the exposure of individual

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