TEMPERATURE-INDUCED INCORPORATION OF GRAMICIDIN A INTO LYSOLECITHIN MICELLES DEMONSTRATED BY ^{13}C NMR

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Received 3 April 1979

1. Introduction

Lipid–protein interactions play a fundamental role in the organization and function of biological membranes. Gramicidin A, a pentadecapeptide, forms a transmembrane channel by adopting a single-stranded β-helical structure whose helix axis coincides with the channel axis [1–3]. It was proposed [4] that gramicidin A first forms large aggregates at the interface of the lipid bilayer then releases the aggregates that necessarily penetrate the lipid layer in order to form active channels. This aggregation could be viewed as the hydrophobic association of gramicidin A sidechains in an aqueous phase. Being familiar with elevated temperature inducing hydrophobic association in elastin peptides [5,6], it was our purpose to investigate whether or not elevated temperatures could drive the gramicidin A molecules to incorporate into micelles and result in transmembrane channels.

Simple ^{13}C spectra of lipid molecules could be utilized to monitor changes in chemical shifts and/or signal intensities of carbon resonances, if such changes were concurrent with the incorporation of gramicidin A molecules in the membrane systems. Therefore, we undertook to study the incorporation of gramicidin A in a micellar system of L-α-lysolecithin in deuterated water as a function of temperature. The results are reported here.

2. Materials and methods

L-α-lysolecithin from egg yolk was purchased from Sigma Chemical Company, St Louis, and used without further purification. D_2O (99.7%) was purchased from Merck Canada/Isotopes, Quebec. Gramicidin A was obtained from ICN Pharmaceutical, Cleveland.

2.1. *Preparation of micelles and gramicidin A complex*

L-α-lysolecithin was dispersed in D_2O and sonicated for 5 min in a bath-type sonifier (Ladd Research Foundation, Burlington, VT). The average concentration of lysolecithin was kept at 100 mM which was determined by measuring the total phosphorus content according to a modified Fiske-Subbarow method [7]. Weighted amounts of gramicidin A, in the dried form, were added to a fixed volume of D_2O containing micelles in order to have different molar ratios of phospholipid to gramicidin A. The suspension was shaken in a vortex mixer for several minutes, sonicated in a bath-type sonifier for 5 min, then used immediately for the ^{13}C NMR. The sample was then incubated for 8 h at 68°C in a water bath and the ^{13}C spectrum was obtained. In separate experiments KCl salt was added to the suspension in fixed volumes of a D_2O stock solution, to give 10 mM final conc. in the sample.

2.2. *^{13}C NMR measurements*

Since ^{13}C NMR line width and intensity are mainly dependent on dipolar relaxation and this relaxation mechanism is modulated by the relative mobility of the nuclei, namely the correlation time (τ) [8,9], it is then possible to interpret the broadening and the associated decrease of intensity of the resonances, as being due to the decrease in the mobility experienced by the nuclei under investigation. In the membrane

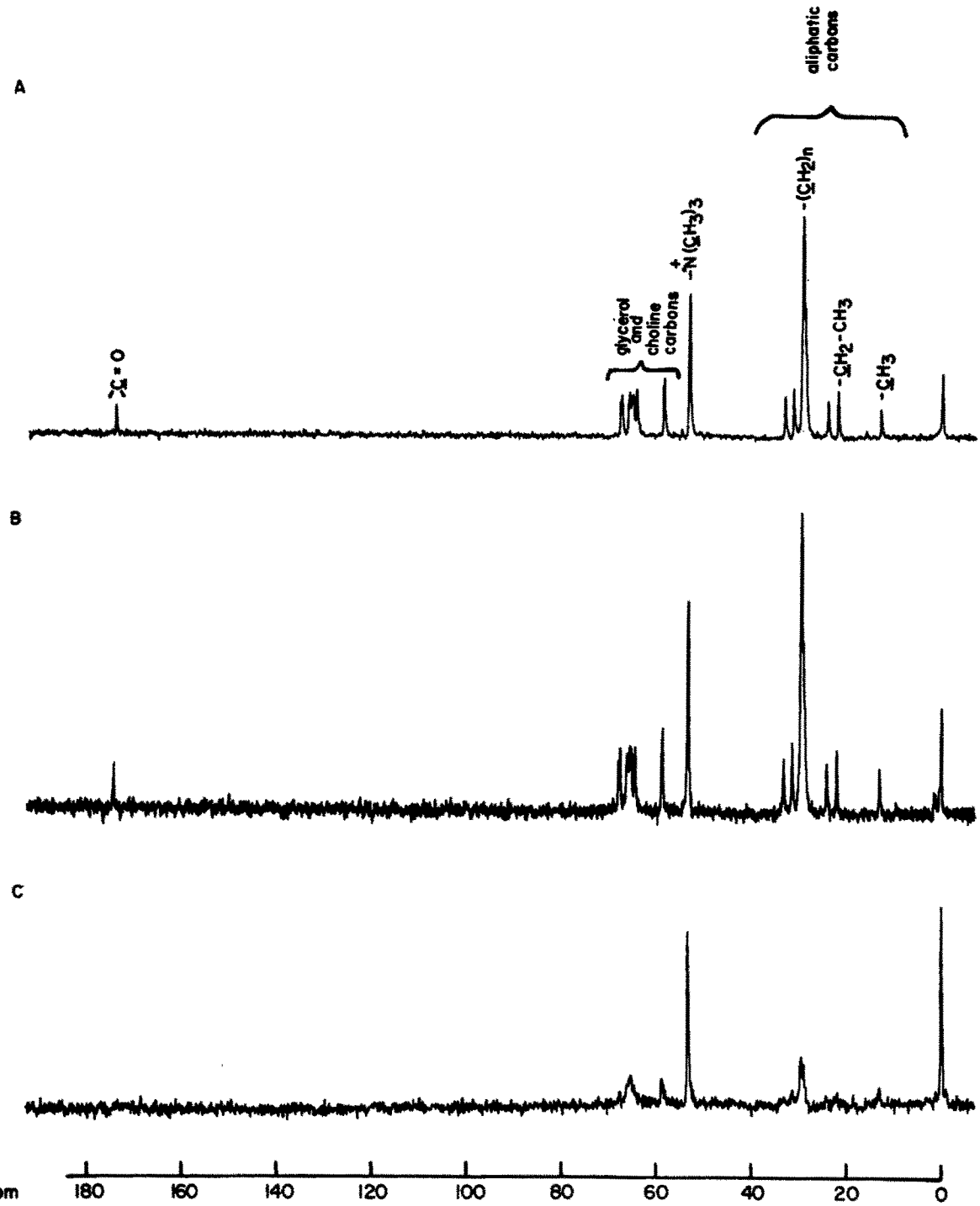

Fig.1. 25.15 MHz ^{13}C NMR spectra of: (A) L-α-lysolecithin micelles in D_2O at 29°C, 50 mg lysolecithin/ml; (B) with gramicidin A at 29°C, 50 mg lysolecithin/ml, 1.8 mg gramicidin A/ml; (C) with gramicidin A, 18 mg lysolecithin/ml, 6.2 mg gramicidin A/ml, at 28°C after incubation at 68°C for 8 h.

system the resonance assigned to the $-\overset{+}{N}(\underline{C}H_3)_3$ carbons of the choline moiety, being on the surface of the membrane, appears to be relatively less sensitive to temperature changes or to incorporation of molecules into the bilayer. It is therefore, reasonable to consider the $-\overset{+}{N}(\underline{C}H_3)_3$ carbon resonance as a standard with which to compare the behavior of the other carbon atoms of the lipid phase. The ratios of the signal heights of $-(\underline{C}H_2)_n-$, $-\underline{C}H_2-CH_3$ and $-\underline{C}H_3$ carbon resonances of the aliphatic chains to the height of the $-\overset{+}{N}(\underline{C}H_3)_3$ resonance of the choline were thus used to monitor the effect of the interaction of the polypeptide with the phospholipids.

^{13}C NMR spectra were recorded on a JEOL PFT-100 spectrometer, operating at 25.15 MHz and equipped with a JEOL VT-3B variable temperature unit. All the spectra were obtained by broad band noise decoupling of protons using 8000 K data points and a pulse width of 20 μs for a 90° tilt.

3. Results

The ^{13}C NMR spectra of the lysolecithin micelles in D_2O, with and without gramicidin A prior to incubation at a higher temperature and obtained at a probe temperature of 29°C, are presented in fig.1A,B. Assignments of the signals of interest, as indicated in fig.1A have been made by comparison with the peak positions of reported [10] sonicated egg phosphatidylcholine liposomes in D_2O. Figure 1C shows the ^{13}C spectrum, obtained at a probe temperature of 28°C, of lysolecithin micelles in D_2O with gramicidin A preincubated at 68°C for 8 h.

Changes in the siginal intensities are given in table 1 as the ratios of peak heights of $-(\underline{C}H_2)_n-$, $-\underline{C}H_2-CH_3$ and $-\underline{C}H_3$ carbon resonances of lipid moiety to the $-N(\underline{C}H_3)_3$ carbon resonance of the choline moiety. Similar experiments were also performed in the presence of KCl at 29°C and 58°C; the results are summarized in table1.

4. Discussion

A dramatic change in the linewidths and intensities of aliphatic carbon resonances can be observed in fig.1C as a result of incubation of the micelles containing gramicidin A at 68°C for 8 h. Although there occurs a drastic decrease of signal height of all aliphatic carbon resonances as listed in table 1, only the intensity ratio of the $-(\underline{C}H_2)_n-$ carbon resonance to the $-N(\underline{C}H_3)_3$ is considered here for the sake of brevity of discussion. A decrease in the magnitude of the ratios together with line broadening is an indication of a decreased mobility of the aliphatic carbons. This decreased mobility can be viewed as the results of deeper penetration and/or the stronger interaction of the gramicidin A molecules with the hydrophobic core of the phospholipid micelles.

A slightly higher value of the ratios for the micellar system alone and with KCl at 58°C (see table 1) indicates the expected higher mobility of the aliphatic chains at an elevated temperature. However,

Table 1
Intensity ratios of ^{13}C NMR resonances of L-α-lysolecithin micelles[a]

Samples	Temp. (°C)	$-(\underline{C}H_2)_n-$ / $-N(\underline{C}H_3)_3$	$-\underline{C}H_2-CH_3$ / $-N(\underline{C}H_3)_3$	$-\underline{C}H_3$ / $-N(\underline{C}H_3)_3$
L-α-lysolecithin micelles	29	1.56	0.31	0.19
	58	2.13	0.34	0.19
L-α-lysolecithin micelles	29	1.39	0.33	0.23
+ KCl	58	2.78	0.33	0.19
L-α-lysolecithin micelles	29	1.43	0.29	0.20
+ gramicidin A	28[b]	0.30	0.08	0.11
L-α-lysolecithin micelles	29	1.41	0.25	0.17
+ KCl + gramicidin A	58	0.63	0.11	0.12

[a] Lysolecithin:gramicidin A molar ratio was 110:1
[b] After incubation for 8 h at 68°C with a lysolecithin:gramicidin A molar ratio of 10:1

on addition of gramicidin A with KCl present, the intensity ratio of $-(\underline{C}H_2)_n-$ to $-N(\underline{C}H_3)_3$ drops down from 1.39 at 29°C to 0.63 only when the temperature is raised to 58°C, otherwise the value at 29°C remains virtually the same within the experimental error (see table 1).

To ascertain further that a higher temperature, not ions, is responsible for bringing about such changes, experimental results without KCl show the reduction of intensity ratio of $-(\underline{C}H_2)_n$ to $-N(\underline{C}H_3)_3$ from 1.43 at 29°C to 0.30 at 28°C after preincubated at 68°C for 8 h (see fig.1, table 1). It is, therefore, apparent that the incorporation of gramicidin A molecules into the lipid is induced by elevated temperature. The gramicidin A incorporation into the lipid core is irreversible in nature which is demonstrated by the similarity of results obtained at a higher and a lower temperature. This phenomenon could also be time dependent, if it is assumed that the aggregates of gramicidin A molecules, at the interface, disperse slowly into the lipid core as a result of the hydrophobic association at elevated temperature. In fact experiments performed at different periods of incubation show that the perturbation of the hydrophobic core increases with time.

In particular, this report demonstrates the incorporation of gramicidin A molecules and, expectedly, functional channels into phospholipid structures such that they may be characterized in solution by means of different physico-chemical techniques. In general, the report demonstrates the importance of temperature and time in building up the hydrophobic interactions involved in the lipid–protein association and as such should be a consideration in problems of membrane reconstitution.

Acknowledgement

This work was supported in part by the National Institutes of Health grant no. HL-11310.

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