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Raman-spectroscopic evidence for the incorporation of alamethicin into dimyristoylphosphatidylcholine bilayers

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The interaction of alamethicin with dimyristoylphosphatidylcholine dispersions was investigated by Raman spectroscopy. The temperature dependence of the C–H stretching spectra demonstrates the incorporation of the polypeptide into the hydrophobic core even in the absence of a transmembrane potential gradient.

The question of how the pore-forming antibiotic alamethicin partitions into a lipid bilayer in the absence of an electrical potential gradient across the membrane has been subject to much controversy in the past. Evidence has been given for a mere surface activity without insertion into the hydrophobic core of the bilayer [1–3] as well as for a full incorporation of at least a portion of the polypeptide into the membrane [4,5]. In this note I present Raman spectroscopic results that suggest an interpretation in terms of insertion of alamethicin into a dimyristoylphosphatidylcholine (DMPC) bilayer leaflet, as is also required by recent models for the molecular details of ion translocation by alamethicin across black lipid membranes [6].

The alamethicin used in these experiments was a generous gift of Dr. G.B. Whitfield, Jr. and Dr. J.E. Grady of the Upjohn Co., and was used as obtained. DMPC obtained from Fluka, Neu-Ulm, was checked for purity on a TLC plate and used without further treatment. Paucilamellar liposomes of DMPC were prepared as follows: 2 mg of the lipid (or the desired lipid-alamethicin mixture) were dissolved in chloroform. The solvent was evaporated under a stream of nitrogen and the resulting thin lipid film was first kept in vacuo for 1 h and then in a humid atmosphere at 50°C for 2 h. The hydrated film was then dispersed in about 3 ml H_2O (millipore quality, pH 5.5) corresponding to a lipid concentration of less than 1% (w/v). After a final overnight equilibration at 50°C the milky dispersion was put into a cavity of a Teflon adapter, linked to a quartz cappilary, fitted into a centrifugation tube. Upon centrifugation at about $10\,000 \times g$ in a swing-out rotor the lipid was collected at the bottom of a 1 mm inner diameter quartz capillary as an approx. 4 mm long pellet. This procedure was chosen to ensure (i) a good equilibration between alamethicin and lipid under excess water conditions [4,19] and (ii) a sufficient concentration of DMPC liposomes in the laser beam for the Raman scattering experiment.

Raman spectra of the samples in the sealed, thermostated (±0.5 C deg) capillaries were recorded by photon-counting in a Canberra Multichannel Analyzer 8100 with a Jobin Yvon

Abbreviation: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine.

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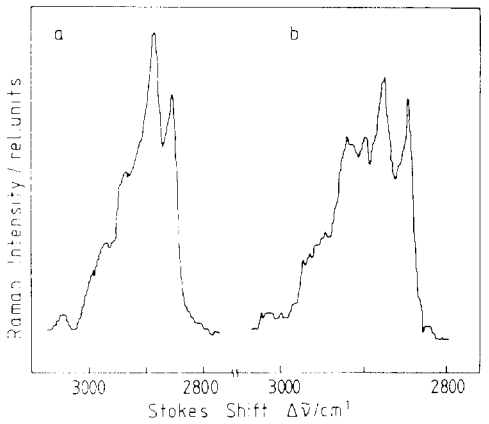

Fig. 1. (a) Raman spectrum of DMPC in the C–H stretching region at $T = 22°C$, i.e. in the ordered phase. (b) As in (a), but the lipid was prepared with 4 mol% alamethicin.

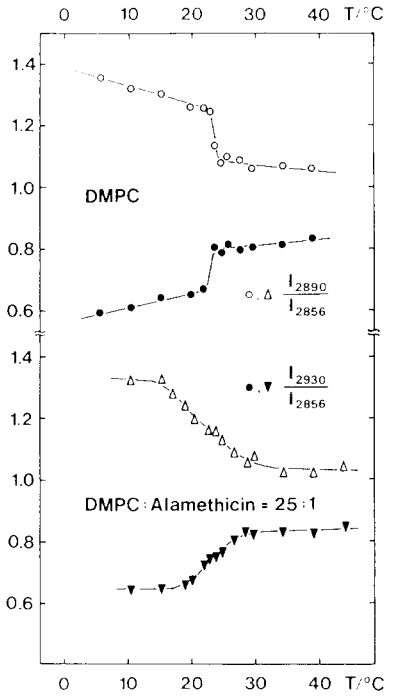

Fig. 2. Temperature dependence of the peak height intensity ratios T_{2890}/I_{2856} and I_{2930}/I_{2856} as obtained from spectra like those presented in Fig. 1 for pure DMPC (○———○ and ●———●) and a 25:1 DMPC alamethicin mixture (△———△ and ▼———▼), respectively.

Ramanor HG 2 S spectrometer equipped with a Spectra Physics Model 164 argon-ion laser operated usually with 200 mW at 488 nm [7]. Stokes-shifted Raman spectra (resolution 3–5 cm^{-1}) were taken as a function of temperature in the range 2780–3030 cm^{-1} where the C–H stretching vibrations can be observed. Intensity peak height ratios given are the average of at least three measurements.

Temperature-dependent Raman spectra for various phospholipids in the region of the C–H stretching modes are given in the literature (see, for example, Refs. 7–9). Fig. 1a shows one example of pure DMPC at 22°C which is just below the main phase transition temperature of 23.8°C [10]. By plotting the intensity ratios I_{2890}/I_{2856} and I_{2930}/I_{2856} as a function of the sample temperature one obtains the well-known break of these empirical order parameters [9] at the phase transition temperature. As expected for a cooperatively melting lipid the temperature width of the transition region is rather narrow, compared to the intrinsic width of this very pure lipid (0.2 C deg, ref. 11), however, slight temperature gradients within the sample due to laser heating broaden this range to approx. 1.5 C deg.

The addition of 4 mol% alamethicin changes the Raman spectrum of the DMPC hydrocarbon tails considerably. For a direct comparison with the pure lipid Fig. 1b shows the obtained spectrum also at $T = 22°C$. Clearly, some features are changed in a characteristic way. If one plots again the two peak height ratios as a function of temperature as it is done in Fig. 2 one can see that (i) the lipid bilayers still undergo a transition from an ordered to a fluid phase, (ii) the width of the transition range is tremendously increased starting already at about 15°C and extending over 32°C, (iii) the transition midpoint, however, is virtually unchanged.

The above presented results unambigously indicate that contrary to recently published work [3] Raman spectroscopic data do indicate an interaction of alamethicin with the hydrocarbon tails of DMPC bilayers. However, the question wether we see an insertion of the polypeptide into the membrane needs to be discussed further.

A trivial explanation for the broadened phase transition of DMPC in the presence of 4 mol% alamethicin would be the convertion of all liposomal material into small or mid-sized unilamellar

vesicles. This would give rise also to observed wide transition range (though with changed values for the peak height ratios) [12]. This, however, can be excluded because an electron microscopic control of the dispersions revealed no change of the size distribution or lamellarity of the liposomes in the presence of alamethicin.

More serious is the objection that a mere adsorption of the polypeptide to the membrane water interface or a partial penetration into the headgroup region would effect also the hydrocarbon chain ordering and hence would modify the C–H Raman spectra in the observed way. By comparison with other Raman data obtained with model-proteins whose location in the membrane is well characterized by additional experimental techniques one is led, however, to the conclusion that alamethicin penetrates into the hydrophobic core even in the absence of an electric field.

One example of a pure electrostatic interaction between a positively charged polypeptide and the negative charges of the lipid headgroup is the system polylysine/phosphatidic acid at pH 9 [13]. What is found also by Raman spectroscopy is a stabilization of the ordered phase of the bilayer which is manifested in an increase of the transition temperature by approx. 10 C deg with an almost unchanged transition width [7]. This is not what we observe in the case of alamethicin.

Another clear situation is given for the interaction of gramicidin A with phosphatidylcholine bilayers. It is generally accepted that this antibiotic penetrates deep into the hydrocarbon part of the membrane and even spans the bilayer after a dimerization reaction [14]. Raman spectroscopic investigations with gramicidin in DMPC [15] as well as in DPPC [16] have shown basically the same effect on the hydrocarbon chains that I have found upon the incorporation of alamethicin: a broadening of the transition range with only a minor change of the midpoint temperature.

Finally, another ionophore the cyclic depsipeptide valinomycin should be considered. Nuclear magnetic resonance studies [17] have shown that valinomycin interacts with dipalmitoylphosphatidylcholine bilayers predominantly in the region of the polar head groups whereas in the case of DMPC a penetration into the hydrophobic region of the membrane is observed. Now, Raman spectroscopic studies with both lipids [18] have shown a clear difference in the influence of valinomycin on the C–H stretching modes of the two systems: In the first case (interfacial adsorption) almost no change in the temperature dependence of the I_{2883}/I_{2847} and I_{2936}/I_{2847} intensity ratios could be found [18]. Only for DMPC a substantial broadening of the transition curve was observed upon the incorporation of valinomycin. In conclusion, the presented Raman spectroscopic data of the DMPC C–H stretching vibrations region demonstrate that alamethicin penetrates into the bilayer even in the absence of an electric field across the membrane.

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