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EVIDENCE FOR A NUCLEATION STEP IN A LIPID-PROTEIN INTERACTION- KINETICS OF THE INCORPORATION OF POLYMYXIN INTO PHOSPHATIDIC ACID BILAYER VESICLES

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SUMMARY

The kinetics of the incorporation of polymyxin into negatively charged phosphatidic acid bilayer vesicles has been investigated by a fluorescence polarization technique. Polymyxin is taken up within a second by the outer lipid layer of a vesicle. The incorporation time determined by the measurement of fluidity changes is independent of polymyxin concentration and of temperature in the range between 35° and 60° C. A diffusion controlled process has to be considered. The fluidity change after mixing the vesicles and polymyxin in solution exhibits a lag time period. This gives evidence for a cooperative binding process. The inner lipid layer of a vesicle is only affected at temperatures where the protein bound lipids are melted. A very slow transfer of polymyxin within 20 hours is observed.

INTRODUCTION

The assembly of a biological membrane has received considerable attention but the molecular basis of the interaction between lipids and proteins is still under investigation. Recently we reported a response curve of a phosphatidic acid membrane to increasing amounts of polymyxin, an antibiotic peptide, which deviates from a Langmuir adsorption isotherm. (1,2) The latter was established for the binding of poly-Lysine, a model substance for extrinsic proteins. The binding curve of polymyxin, however, is found to be S-shaped, demonstrating a cooperative binding process. The behavior of the system upon binding of polymyxin can be considered as a phase transition from a rigid to a fluid lipid matrix. The binding function resembles the case of a graded response as described by Changeaux et al. (4) for the cooperativity in membranes. The experimental response curves of phosphatidic acid membranes to

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polymyxin are in excellent agreement with the theoretical curves obtained by the application of the Bragg-Williams theory of cooperative binding processes. (1,5)

This paper reports evidence for the cooperativity of this special lipid-protein interaction obtained from kinetic experiments. An induction phase is observed upon binding of polymyxin. The lag time between addition of the antibiotic and the observable change in lipid fluidity is again an indication for the cooperativity of the binding process. The appearance of such an induction phase or lag time is a very well established phenomenon for a nucleation step in crystallography as well as in the self-association process of proteins in electrolyte solutions. (6) These processes are considered to be cooperative.

MATERIALS AND METHODS

Dipalmitoylphosphatidic acid was obtained from Fluka, Germany. The lipid was solved in chloroform/methanol and diphenylhexatrien (Aldrich) was added to come to one mole% of the lipid. The solution was evaporated by a nitrogen stream and dried in a descicator under reduced pressure. Buffer was added and the sample was dispersed with a Branson sonifier at T = 65° C for two minutes with a microtip at 30W. The lipid concentration was 1 mg/ml. A borate/boric acid buffer pH 9.0 and an ionic strength of 0.03 M was used. The kinetic experiments were performed with a stopped flow apparatus attached to a Schoeffel fluorescence spectrometer RRS 1000. This instrument was equipped with two photomultiplier/monochromator systems arranged perpendicular to the excitation light beam. Therefore, we were able to measure, simultaneously, the fluorescence intensity parallel and perpendicular to the polarization of the excitation light. The polarization degree $(I_{//} - I_{\perp})/(I_{//} + I_{\perp})$ was calculated by an analog computer and followed with time or with temperature, respectively.

RESULTS AND DISCUSSION

The phase transition curve of pure dipalmitoylphosphatidic acid vesicles at pH 9.0 and an ionic strength of I = 0.03 M is shown in Figure 1. The polarization degree of the probe diphenylhexatrien is plotted as a function of temperature. A phase transition temperature of $T_t = 63^{\circ}$ C is obtained. Addition of polymyxin in a one-to-one molar ratio relative to the lipid causes a decrease of the phase transition temperature to 37° C. The phase transition becomes much broader, whereas the melting of the peptide bound lipids occurs between 30 and 42° C. The amount of polymyxin added exceeds the amount of polymyxin that can incorporate into the lipid matrix. A complex of three

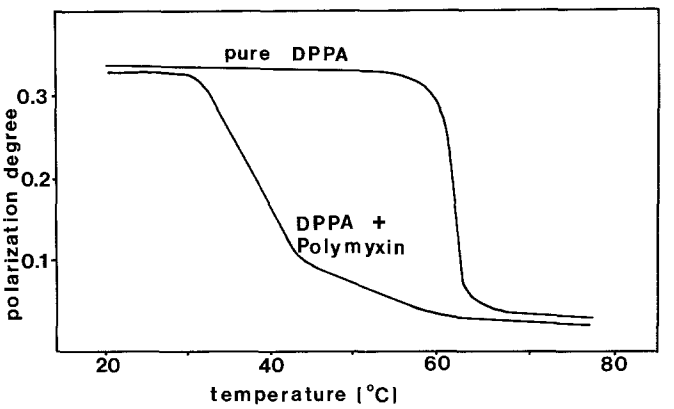

Figure 1 - Phase transition curves of pure dipalmitoylphosphatidic acid vesicles and of dipalmitoylphosphatidic acid vesicles after addition of polymyxin in a one-to-one molar ratio relative to the lipid. The polarization degree is measured using diphenylhexatriene as probe.

lipids bound to one polymyxin molecule was found. (1) We then used the higher amount to permit a complete incorporation in the mixing experiments. The sample shown in Figure 1 was sonified after polymyxin addition. Both the inner and outer lipid layers of the vesicles were affected. No melting of free phosphatidic acid was observed at this polymyxin concentration. A significant increase in membrane fluidity in the temperature range between 35° and 60° C was caused by an addition of polymyxin. At 60° C, for example, the polarization degree can well be taken as a measure for the incorporation of polymyxin into the lipid bilayer membrane.

Two examples for such kinetic experiments are given in Figure 2 at two temperatures. According to Figure 1, at 36° C we have to expect a change in relative polarization from P = 0.34 to P = 0.23 if polymyxin incorporates into both the inner and outer layers. Experimentally, we find a decrease which is approximately one-half of that expected if polymyxin is incorporated into both of these layers. The same is valid for 60° C. In the sonified dispersion, P decreases from 0.3 to 0.05, whereas the mixing experiment yields a decrease only to P = 0.18. These experiments clearly demonstrate that polymyxin incorporates only into the outer lipid layer of the vesicles. The process takes place within a second.

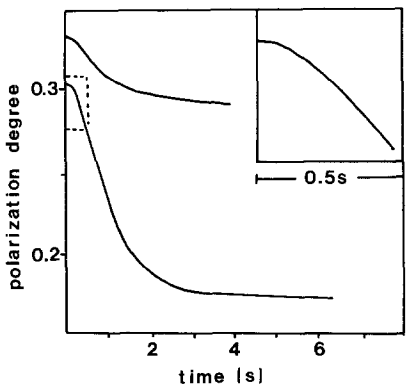

Figure 2 - Change in polarization degree after addition of polymyxin at T = 36° C and T = 60° C. The decrease in polarization degree demonstrates an increase in the membrane fluidity. The insert shows the initial part of the 60° C measurement. Note the lag time of these measurements, giving evidence for a cooperative binding process initiated by the formation of the nucleus for the lipid-peptide-complex.

In the given temperature range we observed no change in the time of incorporation either with polymyxin concentration or with ionic strength. This means that at least at polymyxin concentrations exceeding the maximum number of available binding places in the lipid matrix (a one-to-three protein to lipid complex), the process of incorporation may be diffusion controlled.

We followed the polarization degree over a period of 30 hours. No further change was observed at temperatures $T < 50^{\circ}$ C, demonstrating that the polymyxin could not penetrate into the inner layer. At $T = 50^{\circ}$ C, we observed a further small decrease of the polarization degree by approximately 30% within 20 hours. At this temperature, where the lipid matrix containing the antibiotic peptide nearly reaches its maximum fluidity, a very slow transfer may occur. Nevertheless, the lipid matrix was not totally melted as can be demonstrated by a temperature increase to $T > 63^{\circ}$ C. A further decrease in the polarization degree of the probe to the final value expected from Figure 1 for sonified, and therefore completely equilibrated membranes, was observed.

The highlight of our kinetic experiments was the discovery of a lag time in the early phase of the polymyxin incorporation (e.g., insert in Figure 2). Such a lag time is characteristic for many cooperative processes,

for example the crystallization or polymerization of actin. (6) The early phase of such a process is characterized by the formation of crystallization or condensation nuclei. Once a stable nucleus is formed, it facilitates the growth of a polymer by addition of more subunits.

In our model presented in references 1 and 2 the incorporation of a polymyxin molecule into a phosphatidic acid layer causes a change in membrane symmetry leading to an attractive force between two different polymyxin-lipid-complexes. The formation of the first complex may be considered as a nucleus for the formation of the lipid-peptide cluster within the lipid matrix. Further incorporation of polymyxin decreases the elastic stress in the lipid layer and therefore facilitates the incorporatoin of further peptide molecules.

Our results (shown in Figure 2) are a direct evidence for the existence of a nucleation step in a lipid-peptide-interaction. The incorporation of a polymyxin added to the aqueous phase of a phosphatidic acid vesicle preparation is delayed by approximately 0.5 sec. The incorporation into the outer layer of the vesicle is completed within the next second. The existence of such a lag time is related to the cooperativity of the polymyxin binding process.

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