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EFFECTS OF PRESSURE AND POTASSIUM CHLORIDE ON THE AGGREGATION OF POLY(γ-BENZYL-L-GLUTAMATE) IN LIPOSOMAL BILAYERS

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Fluorescence depolarization studies were made on dimyristoylphosphatidylcholine liposomes containing four kinds of dansylated poly(γ-benzyl-L-glutamate) with different degrees of polymerization or hydrocarbon chain lengths under high pressure at up to 981 bar (1 bar = 10 MPa). Potassium chloride promoted the aggregation of the synthetic peptides in liposomal bilayers at both atmospheric and high pressure. The chain lengths of the hydrocarbons of the peptides had more influence than their degrees of polymerization on aggregation.

1. Introduction

Previously, we reported the phase-transition temperatures and mobilities of the hydrocarbons of liposomes of dimyristoylphosphatidylcholine (DMPC) containing vitamin E and cholesterol at high pressures [1,2]. Here we describe the behavior of peptides in model membranes at high pressure, which were examined by fluorescence techniques in the presence of KCl. Since measurement of fluorescence depolarization provides information about the mobility of the microenvironment around fluorescent probes, this method has often been used to determine the phase-transition temperatures of liposomal bilayers and the mobility of molecules in membranes. Protein is one of the main components of biological membranes, and its physiological activities are strongly dependent on its modes of existence in the membranes and binding to lipids. To obtain information on the behavior of peptides in model membranes, we examined the influence of pressure, temperature and added salt on four kinds of synthetic peptides (dansylated poly(γ-benzyl-L-glutamate), DNS-PBLG) in liposomal bilayers of DMPC by a fluorescence technique. The results provided information about the locations and mobilities of the peptides, their phase separation and their microenvironment.

2. Materials and methods

DMPC was obtained from Sigma Chemical Co. and was used without further purification. Four kinds of PBLG were prepared by Leuch's method: i.e., polymerization of the *N*-carbonic anhydride of benzyl glutamate in dioxane with primary alkyl amine initiators (see table 1). These PBLGs were dansylated in chloroform or *N*,*N*-dimethylformamide by adding dansyl chloride (DNS-Cl: 1-dimethylaminonaphthalene-5-sulfonyl chloride) acetone solution dropwise under cool conditions. The solutions of DNS-PBLGs obtained were evaporated and the residues were dissolved in *N*,*N*-dimethylformamide and precipitated from water.

Chloroform solutions (2 cm^3) of DNS-PBLG

Table 1

Synthetic peptides used

Poly(γ-benzyl-L-glutamate)
H(NHCHCO)$_n$-NH-R′ (with R on CH)
R = $-CH_2-CH_2-COO-CH_2-Ph$
R′ = $-C_{12}H_{25}$, $-C_6H_{13}$
(a) PBLG (n = 5, C_{12})
(b) PBLG (n = 7, C_6)
(c) PBLG (n = 20, C_{12})
(d) PBLG (n = 21, C_6)

and DMPC (total 7.4×10^{-6} mol) were evaporated and residual traces of chloroform were removed by evaporation in vacuo (24 h). The dried mixtures of lipids and synthetic peptides were dispersed in a series of concentrations of aqueous KCl solution (4 cm^3) in a bath-type sonifier for a few minutes, and then subjected to ultrasonication in a probe-type sonifier.

A high-pressure vessel with three optical windows (fig. 1) was set in a fluorescence spectrophotometer to determine fluorescence polarization [3]. The apparatus was accurately aligned with the aid of an He-Ne laser. The body and parts of the high-pressure vessel were all made of 17-4PH stainless-steel, and the vessel had three window plugs (A, B, C). The orifice D with an inside diameter of 2 mm was set in the light path of the plug A to prevent the incident light beam from reaching the edge of the quartz cell E. The emitted fluorescence is observed through the window plug B. Fused quartz (15 $\phi \times 10$ mm) was used as windows for plugs A and B. Plug C, which is not shown in the figure, was located on the opposite side of plug A, and its end was closed with a black rubber stopper, which trapped the excitation light passing through the cell E, preventing its scattering. The window of plug C was made of synthetic sapphire. The holes of window plugs A–C were tapered at the angle of the light beam and were all 5 mm in diameter at their inner end. The optical cell E had dimensions of 13 × 13 × 13 (mm), and had an 8 mm side tube consisting of a piston cylinder device to transport pressure. Pressure was measured with a precision Bourbon-type gauge

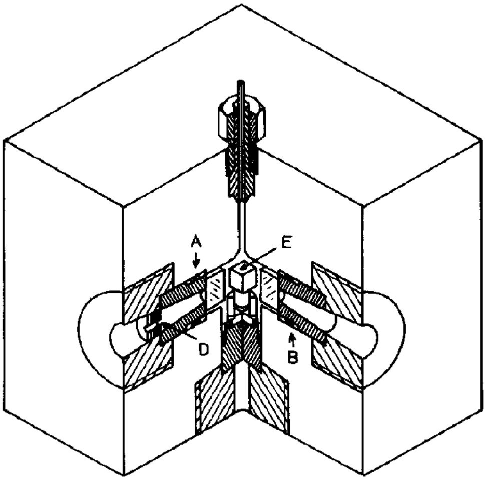

Fig. 1. High-pressure vessel with optical windows. (A, B) Window plugs, (D) orifice, (E) optical cell.

(Naganoseiki). The temperature of the sample chamber was controlled thermostatically to within ± 0.1°C by water circulating around the high-pressure vessel.

The suspension was irradiated at an excitation wavelength of 350 nm, the intensity of emission was measured under various conditions and the degree of fluorescence polarization, $P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$, was determined.

3. Results and discussion

3.1. Aggregation of DNS-PBLG in homogeneous systems and liposomes

Fig. 2 shows the relation between the fluorescence emission maximum of DNS-PBLG (n = 5, C_{12}) and the solvent polarity parameter (E_T value) [4] in various solvents. The emission maximum was very sensitive to the nature of the solvent used and shifted linearly toward longer wavelengths (red shift) with increase in polarity of the solvent. The polarity of the microenvironment around the probe molecules could be estimated from this good correlation. Water and n-hexane, for which the plots deviated largely from the straight line, were poor solvents for DNS-PBLG (n = 5, C_{12}): in these two solvents the labeled PBLG was hardly

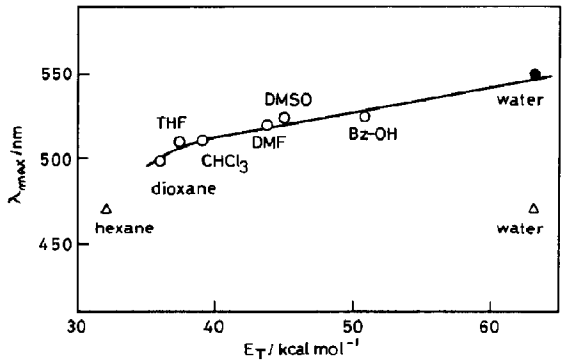

Fig. 2. Emission maximum of fluorescence from DNS-PBLG ($n = 5$, C_{12}) in pure solvents as a function of the solvent polarity (E_T value).

soluble and was present as aggregates ($\lambda_{max} = 470$ nm). However, the λ_{max} of a dilute aqueous solution of a dansylated nonapeptide ester of emerimicin studied by Nagaraj and Balaram [5] lay exactly on the stright line (filled circle), and accordingly values for DNS-PBLGs would also lie on the line if all their dansyl groups were in contact with water.

The emission bands of DNS-PBLG ($n = 5$, C_{12}) in DMPC liposomes at 490 and 550 nm gradually disappeared with increase in the proportion of the peptide (0–2.2 mol%), while the band at 470 nm markedly sharpened. Taking into consideration the good correlation between the emission maximum and solvent polarity (fig. 2), these results suggest that a portion of the DNS-PBLG molecules aggregate in or on the surface of the membranes and that the dansyl groups are surrounded by an apolar environment in this concentration range. Although there is a possibility that the DNS-PBLG molecules were pesent at the outside of the membranes, this was disproved by the results: the fluorescence polarization vs. pressure profiles of two membranes, one treated by gel filtration and the other not, showed similar patterns. The formation of the liposomes containing DNS-PBLGs was confirmed by the fluorescence polarization vs. temperature profiles which showed a clear phase transition (K. Tamura, Y. Higashi and A. Suzuki, unpublished data).

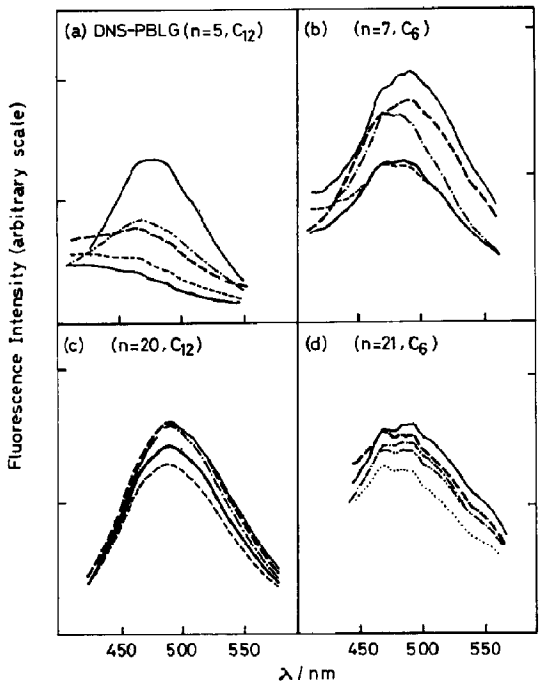

Fig. 3. Effect of KCl on the fluorescence spectra (uncorrected) of DNS-PBLGs (2 mol%) in DMPC liposomes at 30°C and 1 bar. Concentration of KCl: (———) 0.15, (— —) 1.02, (-·-·-) 1.58, (— — —) 2.13, (·····) 2.71, (———) 3.30, (-·-·-) 3.97 mol kg^{-1}. [DNS-PBLG] = 3.7×10^{-5} M.

3.2. Effects of salt on the aggregation of DNS-PBLG in liposomes

On the basis of the finding that DNS-PBLG displays sufficient sensitivity enough to obtain information about the microscopic polarity around it, the fluorescence characteristics of DNS-PBLGs in liposomal bilayers were studied. Fig. 3 illustrates the fluorescence spectra of four kinds of DNS-PBLGs of 2 mol% in DMPC liposomal bilayers when the concentration of KCl was incresed to 3.97 mol kg^{-1} at 25°C and atmospheric pressure [6]. The effect of the salt on the spectra was highly dependent on the degree of polymerization of the peptides and was especially pronounced for the system of DNS-PBLG ($n = 5$, C_{12}) which has a random-coil structure. The observed intensity of fluorescence had a maximum value at 0.15 mol kg^{-1}, the lowest concentration

of KCl used, and was partly quenched at 2.13 and 3.30 mol kg^{-1}, but became stronger again at 3.97 mol kg^{-1}. The effect of added salt was small with DNS-PBLG with a high degree of polymerization. One reason for these results is that peptides c and d in fig. 3 with high degrees of polymerization have helical structures and form relatively regular aggregates in the membranes, whereas peptides a and b with low degrees of polymerization have a random-coil structure and their aggregates disturb the regularity of the membranes. Accordingly, the effect of salt, which weakens the interaction between polar head groups, appeared stronger with peptides a and b.

Fig. 4 shows that the emission maximum (λ_{max}) of the fluorescence of 2 mol% DNS-PBLG ($n = 7$, C_6) in DMPC liposomes decreases monotonically from 490 to 470 nm with increase in salt concentrations; this corresponds to a change in polarity of the microenvironment around the probe from that of dioxane to that of hexane (cf. fig. 2). These results suggest progressive formation of the aggregates of DNS-PBLG with increasing KCl concentration.

The observed fluorescence intensity of DNS-PBLG ($n = 5$, C_{12}) was dependent on the KCl concentration and was minimal at about 2.5 mol kg^{-1} (fig. 5). The decrease in intensity over the concentration range 0–2.5 mol kg^{-1} may be due to self-quenching by aggregation of DNS-PBLG molecules [7], while the increase in intensity above

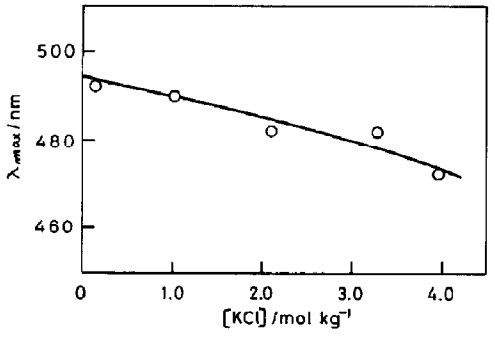

Fig. 4. Emission maximum of fluorescence from 2 mol% DNS-PBLG ($n = 7$, C_6) in DMPC liposomes as a function of KCl concentration at 30°C and 1 bar.

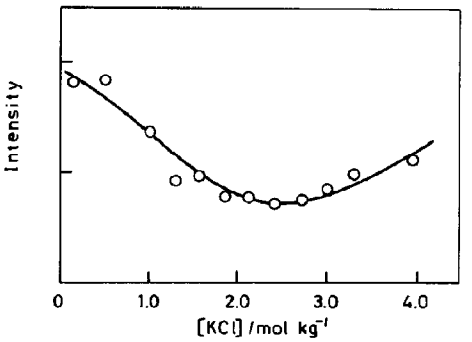

Fig. 5. Fluorescence intensity (arbitrary scale) of 2 mol% DNS-PBLG ($n = 5$, C_{12}) in DMPC liposomes as a function of KCl concentration at 1 bar. [DNS-PBLG] = 3.7×10^{-5} M.

2.5 mol kg^{-1} suggests exposure of the peptide domains of the membranes or collapse of a portion of the membranes. These types of behavior will be explained later in more detail on the basis of data on fluorescence polarization.

Fig. 6 shows the fluorescence polarization of DNS-PBLG ($n = 5$, C_{12}) in DMPC liposomes as a function of the concentration of KCl at various pressures. Fluorescence polarization monitors the mobilities of the environment around the fluorescence probe: a low degree of polarization suggests high mobilities and a high degree of polarization indicates low mobilities. At 46.9°C the liposomal bilayer of DMPC is in the liquid crystal state (a), whereas at 19.5°C it is in the gel state (b). In both systems a maximum of fluorescence polarization was observed; there was a gradual increase to a peak in the liquid crystal state, but a sharp peak in the gel state. All these results can be explained in terms of the difference in mobilities of the lipid and DNS-PBLG molecules. Namely, high mobilities of the lipid molecules at higher temperatures enhance reduction of the interactions between their polar head groups, which are already weakened by the added salt. This results in formation of a peak of fluorescence polarization at lower concentrations of KCl and broadening of the peak.

Fig. 7 shows plots of the fluorescence polarization of all the DNS-PBLGs investigated as functions of the concentration of KCl at 1 and 981 bar, and 19.5°C (in the gel state). The plots of fluorescence polarization for the DNS-PBLGs (*n*

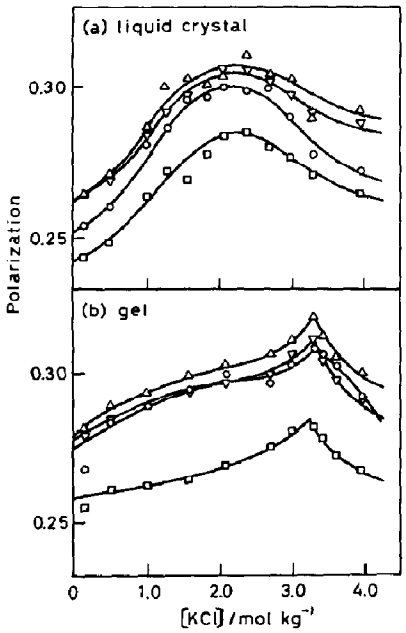

Fig. 6. Effects of salt, temperature and pressure on the fluorescence polarization of 2 mol% DNS-PBLG ($n = 5$, C_{12}) in DMPC liposomes. (a) 46.9°C, (b) 19.5°C. (○) 1, (△) 294, (▽) 588, (□) 981 bar. [DNS-PBLG] = 3.7×10^{-5} M.

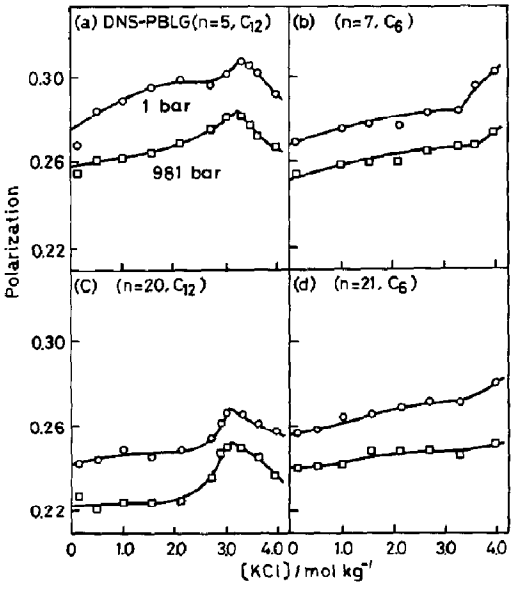

Fig. 7. Fluorescence polarization of four kinds of DNS-PBLGs of 2 mol% in DMPC liposomes as a function of KCl concentration at 1 and 981 bar and 19.5°C (gel). (○) 1, (□) 981 bar. [DNS-PBLG] = 3.7×10^{-5} M.

= 5, C_{12}) and ($n = 20$, C_{12}) showed peaks: the degree of polarization increased rapidly from about 2.0 mol kg^{-1} of KCl, and had maxima at 3 mol kg^{-1} at both 1 and 981 bar. Interestingly, the plots for the DNS-PBLGs ($n = 7$, C_6) and ($n = 21$, C_6) showed no peaks; they began to increase rapidly at about 3.0 mol kg^{-1} at 1 bar, but no increase was apparent at 981 bar. Pressure may retard the phase separation by preventing the decrease in interactions between DMPC molecules induced by salt.

The above results may be explained in terms of schemes A (for the DNS-PBLGs with C_{12}) and B (for DNS-PBLGs with C_6) shown in fig. 8. As the salt concentration is increased, the inter-head group interactions may be weakened, either by binding of the salt to the polar head groups or by increase in the ionic strength, which results in the binding of more water molecules to the surface [8]. Since DNS-PBLGs with C_{12} hydrocarbon form stronger aggregates than those with C_6 due to the van der Waals force between the long hydrocarbons, they are more likely to show phase separation. Therefore, DNS-PBLGs with C_{12} groups aggregate at lower concentrations of KCl than those with C_6. Although portions of the peptides aggregate and form small domains at a KCl concentration of 2 mol kg^{-1}, the aggregation is enhanced in the concentration range 2–3 mol kg^{-1}. At KCl concentrations above 3 mol kg^{-1}, the interactions between the polar head groups of the DMPC membranes become weaker, and this may lead to exposure of the peptide domains on the membrane or collapse of the membranes (scheme A). In contrast, the phase separation of DNS-PBLGs ($n = 7$, C_6) and ($n = 21$, C_6) is accelerated at about 3 mol kg^{-1} of KCl (scheme B). DNS-PBLGs with C_6 groups may have peaks at concentrations above 4 mol kg^{-1}, but this could not be checked since KCl is not soluble at this concentration. Träuble and Eibl [9] found that monovalent cations lower the transition temperature, or fluidize the bilayer structure at a given temperature. From these results, the formation of peaks at 19.5°C might be due to a gel-liquid crystalline phase transition (the phase transition temperature of 2 mol% DNS-PBLG ($n = 5$, C_{12}) in membranes without KCl (T_m) is 22.3°C at 1 bar) (K. Tamura,

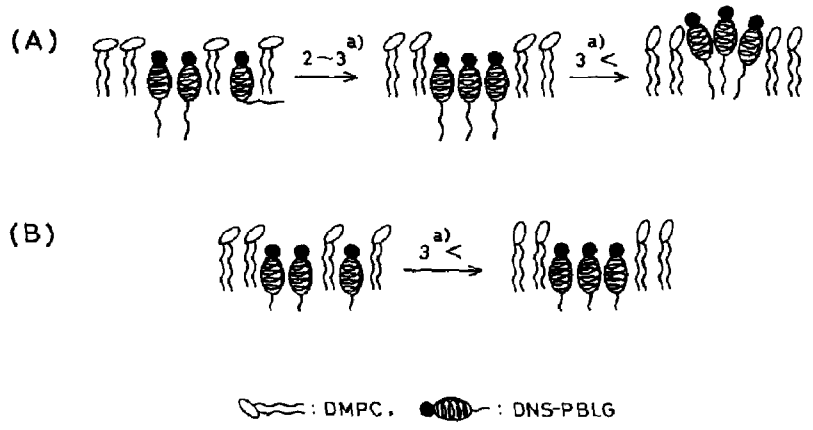

Fig. 8. Aggregation of DNS-PBLGs with C_{12} groups (A) and with C_6 groups (B) induced by KCl. (a) KCl concentration in mol kg^{-1}.

Y. Higashi and A. Suzuki, unpublished data). However, this possibility is disproved by the following facts: (a) the concentration at which the fluorescence polarization is maximal does not change with pressure (T_m increases with pressure: $dT_m/dP = 17.8°C/1000$ bar for 2 mol% DNS-PBLG ($n = 5$, C_{12})) (K. Tamura, Y. Higashi and A. Suzuki, unpublished data) and (b) the liquid crystalline systems also form peaks of polarization (cf. fig. 6a).

In conclusion, a portion of the peptide molecules aggregate in or on the surface of the DMPC liposomal bilayers containing 2 mol% DNS-PBLGs. KCl promoted the phase separation of DNS-PBLGs in DMPC membranes at atmospheric and high pressure. The chain lengths of the hydrocarbons of the peptides had more influence than their degrees of polymerization on aggregation.

References

1 K. Tamura, Y. Higashi, T. Mukai and A. Suzuki, Chem. Phys. Lipids 34 (1984) 301.
2 K. Tamura, Y. Higashi, K. Wazumi and A. Suzuki, Biophys. Chem. 19 (1984) 273.
3 K. Tamura and A. Suzuki, Bull. Fac. Eng. Univ. Tokushima 18 (1981) 13.
4 K. Dimroth, C. Reichardt, T. Siepmann and F. Bohlmann, Ann. Chem. 661 (1963) 1.
5 R. Nagaraj and P. Balaram, Biochem. Biophys. Res. Commun. 89 (1979) 1041.
6 K. Tamura and N. Nii, Chem. Exp. 1 (1986) 33 (in Japanese).
7 K. Iwamoto and J. Sunamoto, Bull. Chem. Soc. Jap. 54 (1981) 399.
8 P.L. Yeagle, Acc. Chem. Rev. 11 (1978) 321.
9 H. Träuble and H. Eibl, Proc. Natl. Acad. Sci. U.S.A. 71 (1974) 214.