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Characteristics of Binding of Zwitterionic Liposomes to Water-Soluble Proteins

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Abstract—The interactions of zwitterionic phospholipids phosphatidylcholine and phosphatidylethanolamine with protein proteinase inhibitors aprotinin and Bowman–Birk soybean proteinase inhibitor have been investigated. An increase in the hydrophobicity of the liposome surface was shown to be an important factor for the formation of proteoliposomes. According to ^{31}P-NMR spectra, incorporation of the proteins into the liposomes does not influence the structural organization of the surface of the liposomes. Increasing the ionic strength does not inhibit the process of proteoliposome formation. Fluorescence assay of the complexes of anthracene-labeled phospholipids with the rhodamine B-labeled protein showed that after the encapsulation into the liposomes, the protein is located inside the particles and between the bilayers. Also, the effect of phospholipids with saturated fatty acid residues on the protein–lipid interaction was studied by differential scanning calorimetry. The results indicate that water-soluble proteins efficiently interact with zwitterionic phospholipids, and the encapsulation of the proteins into the liposomes is provided by electrostatic and hydrophobic forces (in the case of aprotinin) or predominantly by hydrophobic forces (Bowman–Birk soybean proteinase inhibitor).

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Key words: phosphatidylcholine, phosphatidylethanolamine, liposomes, aprotinin, Bowman–Birk soybean proteinase inhibitor, differential scanning calorimetry

In fundamental and applied biochemistry, great attention is being paid to protein–lipid systems, whose structure is stabilized by electrostatic and hydrophobic interactions. This is explained by the fact that without understanding the mechanisms of the interactions between proteins and lipids it is impossible to predict the behavior of protein pharmaceuticals while binding to biological membranes, as well as while incorporating into different kinds of lipid carriers (predominantly liposomes).

In previous works, we demonstrated that the efficiency of the binding of such non-membrane water-soluble proteins as Bowman–Birk soybean proteinase inhibitor (BBI) [1], trypsin [2], and aprotinin (BPTI) [3, 4] to phospholipids was enhanced with increase in the ratio of negatively charged components within the multilamellar vesicles, as well as with lowering pH of the medium from 8.0 to 2.0 and with decrease in the ionic strength. Based on these observations, we concluded that electrostatic forces are the predominant factor in the formation of the protein–lipid complex.

The present work is mainly devoted to the investigation of the mechanisms of interactions of lipids with protein proteinase inhibitors. To analyze forces determining the character of interactions within the protein–lipid system, we studied interactions of BPTI and BBI (p*I* 10.5 and 4.5, respectively) with liposomes composed of zwitterionic phospholipids.

The proteins chosen for the investigation differed in their molecular weight (6.5 kDa for BPTI and 8 kDa for BBI) and secondary structure (BPTI contains two α-helixes and one β-sheet (PDB 1PIT); BBI contains no α-helixes and consists of two β-sheets (PDB 1BBI)). The main structural features of BBI are the following [5, 6]: the absence of a hydrophobic nucleus that is characteristic for the water-soluble proteins including the low-molecular-weight protein proteinase inhibitors of other

Abbreviations: BBI, Bowman–Birk soybean proteinase inhibitor; BPTI, aprotinin; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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families, the presence of two hydrophobic regions that are accessible to the solvent, whose fusion with the formation of the hydrophobic nucleus is presumably prevented by seven disulfide bonds. Thus, BBI takes an intermediate position between typical water-soluble proteins and peripheral membrane proteins. BPTI is a typical water-soluble protein (it has a hydrophobic nucleus) and is often used as a model protein. However, BPTI has a number of specific features: a high content of aromatic residues (four Tyr and four Phe of 58 amino acid residues) and three disulfide bonds (residues 5–55, 14–38, and 30–51) that are responsible for a high thermostability of the protein. In the native state, the protein is resistant to heating up to 90°C. The loss of the disulfide bonds between residues 5–55 or 30–51 decreases the denaturation temperature to 37°C, and the loss of the 14–38 bond decreases the denaturation temperature to 74°C [7].

MATERIALS AND METHODS

Soybean phosphatidylcholine (PC) (Lipoid, China) was used without further purification (the purity as supplied was 98%). Soybean phosphatidylethanolamine (PE) was obtained from a preparation of S-21B (Lipoid) by methanol precipitation from a chloroform solution of the preparation followed by acetone precipitation from diethyl ether solution with subsequent purification using adsorption chromatography on a Silicagel-60 column (Merck, Germany). The purity of the used preparations was estimated qualitatively by TCL and using Dittmer's method as modified by Svetashev and Vaskovsky [8]. The oxidation index of the used phospholipid preparations did not exceed 0.3. The phospholipids exhibited a high content of linoleic (60%), palmitic (20%), and oleic (10%) acids with the exception of S-75-3 (Lipoid), the latter containing mainly residues of saturated acids: stearic (70%) and palmitic (20%). BBI (Sigma, USA) was used without additional treatment. BPTI was obtained from a preparation of Gordox (Gedeon Richter, Hungary) by desalting on a Sephadex G-25 Fine column (Pharmacia, Sweden) and lyophilization. Triton X-114 was from Sigma. Solvents were from Khimmed (Russia).

Distribution of phospholipids in aqueous Triton X-114 solutions. To evaluate the distribution coefficient (K_D) of phospholipids, aqueous solutions of Triton X-114 were used [9]. An 11% aqueous solution of Triton X-114 (0.01 ml), 0.3 ml of an investigated sample in 0.01 M Tris-HCl, pH 8.0, and 0.45 ml of 6% sucrose solution in the same buffer were added into a plastic tube. The concentration of phospholipids in the sample was 3 mg/ml. All solutions were cooled to 0°C. The sample was incubated for 5 min at 0°C (at this temperature, solutions with Triton X-114 are homogeneous), and then 3 min at 30°C (the solution segregates into water and detergent phases). The resulting turbid mixture was centrifuged (7 min, 6000*g*) on a microcentrifuge (Eppendorf, Germany) at room temperature. The concentrations of the phospholipids in the aqueous and organic phases were determined by Dittmer's method as modified by Svetashev and Vaskovsky [8]. Distribution coefficient was calculated using the equation: $K_D = C_T/C_W$, where C_T and C_W are the concentration of lipids in the detergent and water phases, respectively [10].

Incorporation of proteins into liposomes. Empty liposomes as multilamellar vesicles of lipid preparations were obtained as follows: a weight of phospholipids was dissolved in an organic solvent, and then the solvent was removed under vacuum at 45°C. The dried samples were dispersed in 0.001 M HCl (pH 3.0) or in 0.01 M HCl (pH 2.0) to study the interactions with BPTI [3] and BBI [11], respectively, and then freeze–thawed repeatedly (at least 10 times) in liquid nitrogen.

BPTI was encapsulated into the liposomes under the conditions developed earlier [3]: 1 ml of aqueous dispersions of the phospholipid preparations (3 mg/ml, pH 3.0) was mixed with 0.05 ml of BPTI solution (20 mg/ml, pH 3.0). BBI was encapsulated into the liposomes according to the method described earlier [11]: 1 ml of aqueous dispersions of the phospholipid preparations (5 mg/ml, pH 2.0) was mixed with 0.05 ml BBI solution (10 mg/ml, pH 2.0). The samples were incubated for 20 min at 20°C and then centrifuged for 20 min at 500*g*. The sedimented liposomes with the protein were dispersed in 0.05 M Tris-HCl, pH 8.0. Protein content in the supernatant and in the pellet was determined by a modification of the Lowry method [12]. Phospholipid content in the preparations was determined as indicated above [8].

Dimensions of the empty liposomes and the liposomes containing proteins were determined by laser scattering analysis using a MasterSizer device (Malvern Instruments, Great Britain).

Fluorescence assays. Anthracene-labeled PC (PC^{Ant}) in ethanol was synthesized by the method described by de Bony et al. [13]. Ethanol concentrate of PC^{Ant} was added to lipids in chloroform (PC^{Ant} to phospholipid molar ratio 1 : 100) and mixed. The solvents were removed on a rotary evaporator, and then empty liposomes and liposomes with protein were prepared.

Rhodamine B isothiocyanate-labeled BPTI ($BPTI^{RB}$) was obtained by the method described in [14]. The protein was encapsulated into the liposomes as described above ($BPTI^{RB}$ to BPTI molar ratio was 1 : 10). The fluorescence intensity was measured using a Jasco FP-777 fluorimeter (Japan). Excitation wavelength was 358 nm in the case of rhodamine B isothiocyanate and 262 nm for anthracene. For all spectra, corrections were made for the fluorescence of the medium.

^{31}P-NMR spectroscopy. The ^{31}P-NMR spectra of phospholipid aqueous dispersions as multilamellar vesicles were recorded using a DRX500 spectrometer (Bruker, Germany) at frequency $\omega_0/2\pi$ of 202.5 MHz

using a Hahn echo pulse-train [15]. The duration of the 90° pulse was 9 μsec, and the delay between 90 and 180° pulses was 40 μsec. The width of the spectral capture was 60,606 Hz, and the number of experimental points was 2K. During the record of the free induction decay signal, broadband suppression of the ^{31}P-^{1}H dipole interaction was achieved by the WALTZ method. The free induction decay signal was monitored for 17 μsec. The relaxation delay was 4 sec. The number of scans was varied from 512 to 2000 depending on the sample volume (i.e. amount of phospholipids). The free induction decay signal was zero-filled to 4K and multiplied by the Lorentz function (line broadening parameter Lb, 20 Hz). The chemical shifts were determined relative to 85% H_3PO_4 as the external reference. Concentration of the phospholipids for the ^{31}P-NMR spectroscopy analysis was 30 mg/ml. For the investigation of the proteoliposomes in complex with BPTI, the phospholipid to BPTI ratio 3 : 1 was used, which was determined previously for preparations of liposomes with BPTI [3].

Differential scanning calorimetry (DSC). DSC analysis was performed using a DSC 5 device (Perkin-Elmer). The calorimeter was calibrated by the melting points of water and palladium at the heating rate of 2°C/min. An aluminum cell was used as the reference cell. The samples were investigated in the region of 10-130°C. Experimental data were processed using approximations for the molecular mechanics approach. After the calibration of the calorimeter by protein, optimal concentration of BPTI for the investigation was determined (2 mg/ml). Consequently, the initial concentrations of BPTI and phospholipids for the preparation of the liposomes for DSC analysis were 6 and 18 mg/ml, respectively (the optimal ratio phospholipids/BPTI determined for the complexes of liposomes with BPTI [3]).

RESULTS AND DISCUSSION

Properties of empty liposomes prepared from PC/PE mixtures with different PE content. To investigate the interactions of water-soluble proteins (BBI and BPTI) with zwitterionic phospholipids, we used four preparations: PC without additions and in the mixture with PE in the ratio of 67 : 33, 50 : 50, and 33 : 67.

Previously, ^{31}P-NMR spectroscopic analysis demonstrated that PE does not change the bilayer structure of the PC/PE phospholipid aggregates until its content exceeds 67% [16]. The spectrum of the PC/PE dispersion (33 : 67) exhibits a single maximum in the region of 0 ppm. The emergence of this peak could be due to the reduction of the size of the particles yielding aggregates of less than 200 nm [17]. The diameter of the empty liposomes determined by laser scattering for all investigated phospholipid mixtures significantly exceeded this value (Table 1). Thus, changes in the shape of the ^{31}P-NMR spectrum when the

Table 1. Sizes of empty liposomes and proteoliposomes

Composition of lipid preparations, % (w/w)	Mean diameter of liposomes, nm		
	empty	with BPTI	with BBI
PC (100)	610 ± 30	580 ± 30	580 ± 30
PC/PE (67 : 33)	590 ± 30	580 ± 30	600 ± 30
PC/PE (50 : 50)	610 ± 30	580 ± 30	530 ± 230
PC/PE (33 : 67)	540 ± 30	500 ± 25	460 ± 20
PC/PE/LPC (87 : 12 : 1) (non-saturated fatty acid residues)	1250 ± 150*	860 ± 90*	–
PC/PE/LPC (87 : 12 : 1) (saturated fatty acid residues)	2470 ± 200*	3890 ± 250*	–

* At 60°C.

PE content exceeds 67% indicate the presence of the isotropic phase in the dispersion, which agrees with literature data on the investigation of phospholipid mixtures with a significant content of PE [18, 19].

Properties of the surface of the liposomes formed from PC and PC/PE mixtures were investigated by measuring the distribution coefficient of the liposomes in aqueous solutions of Triton X-114. Since this method was suggested for integral membrane proteins [9] and was not employed for phospholipids, the effect of Triton X-114 on the aggregation state and structural organization of phospholipids was studied. Determination of the sizes of aggregates of PC and their mixtures with PE in the absence and in the presence of Triton X-114 under conditions that completely corresponded to the method described for aqueous Triton X-114 solutions [9] demonstrated that the average diameter of the particles for all investigated mixtures increased after the addition of Triton X-114. For example, for the mixture PC/PE (33 : 67), the diameter of the particles increased from 540 to 610 nm. This suggests that at the used concentration, Triton X-114 is incorporated into the vesicles without their breaking. The fact that ^{31}P-NMR spectra of the investigated phospholipid dispersions in the presence of Triton X-114 (the Triton/phospholipid ratio corresponded to that used for determination of K_D) were identical to those without Triton (data not shown) confirmed this conclusion.

According to the data presented in Fig. 1, increase in the PE content in the phospholipid mixture significantly enhances K_D value only in one case: when the PC/PE ratio is 33 : 67. The distribution coefficient K_D determines

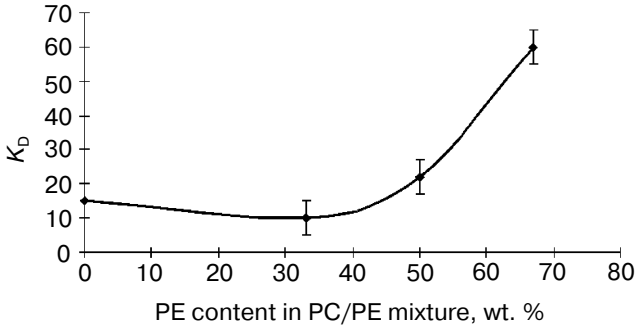

Fig. 1. Distribution coefficient of liposomes prepared from PC/PE mixtures with different PE content in the water/Triton X-114 system.

standard free energy of transport of a compound from one phase to another: $\Delta G_{transport} = -RT\ln K_D$, and in the case of the transport from the aqueous phase to the organic phase, K_D is a measure of the hydrophobicity of the tested compound [20]. Thus, the observed increase in the K_D value (Fig. 1) directly points to the increase in the surface hydrophobicity of the phospholipid aggregates when PE content reaches 67%. Due to this fact, the transition of the PC/PE mixture (33 : 67) from the aqueous phase to the phase of Triton X-114 has an advantage in the free energy over other samples. It is of interest to compare this result with the data on the decrease in the hydration of PE aggregates compared to PC aggregates [21], according to which the hydrophilic–hydrophobic balance of the surface of the mixed phospholipid aggregates shifts towards a higher hydrophobicity with the increase in the PE ratio. Thus, the transition from the bilayer structure in the case of the phospholipid mixtures PC (100), PC/PE (67 : 33), and PC/PE (50 : 50) to the isotropic phase in the case of PC/PE (33 : 67) suggests a decrease in the content of the hydration water bound to the charged parts of the phospholipid molecules, which presumably causes an overall increase in the surface hydrophobicity of the mixed PC/PE (33 : 67) aggregates.

Protein-containing liposomes from PC/PE mixtures. Relative increase in the PE content in the PC/PE mixture enhanced the number of phospholipid molecules per protein molecule in the case of BPTI as well as in the case of BBI (Table 2). Comparing these data with the K_D value of the phospholipid dispersions and their structural organization, it can be concluded that while binding the liposomes from zwitterionic phospholipids with the investigated proteins, one of the crucial factors is the growth of the hydrophobicity of the liposome surface, which grows with increasing PE content. As demonstrated previously, this is explained by a decrease in the hydration of the polar part of the PE molecule compared to PC [22]. The decreased content of the hydration water presumably facilitates the interaction between the polar parts of lipids and the surface of the water-soluble protein.

To estimate the influence of the electrostatic forces on the incorporation of the investigated proteins into the liposomes from zwitterionic phospholipids, the dependence of this process on ionic strength was studied. Increasing the ionic strength in the case of the liposomes from PC/PE dispersions (50 : 50) has virtually no effect on the incorporation of the phospholipids into the proteoliposomes (Fig. 2a). The efficiency of the incorporation of BPTI into the proteoliposomes sharply decreases with the growth in NaCl concentration from 0 to 0.1 M, and subsequent growth in the ionic strength has no effect on the content of BPTI in the liposomes (Fig. 2b). This suggests that together with the hydrophobic forces, the interaction between BPTI and zwitterionic phospholipids is provided by electrostatic forces. In the case of BBI, the

Table 2. Composition of proteoliposomes

Composition of lipid preparations, % (w/w)	Efficiency of incorporation of components into proteoliposomes, % of initial			
	lipids	BPTI	lipids	BBI
PC (100)	33 ± 5	11 ± 2	13 ± 4	14 ± 3
PC/PE (67 : 33)	82 ± 12	16 ± 2	29 ± 7	18 ± 3
PC/PE (50 : 50)	86 ± 15	18 ± 3	80 ± 15	28 ± 6
PC/PE (33 : 67)	90 ± 10	20 ± 2	82 ± 17	20 ± 3
PC/PE/LPC (87 : 12 : 1)	86 ± 13	19 ± 2	–	–
(non-saturated fatty acid residues)	88 ± 12*	42 ± 3*		
PC/PE/LPC (87 : 12 : 1)	83 ± 15	16 ± 1	–	–
(saturated fatty acid residues)	91 ± 11*	32 ± 2*		

* Proteoliposomes were obtained at 60°C.

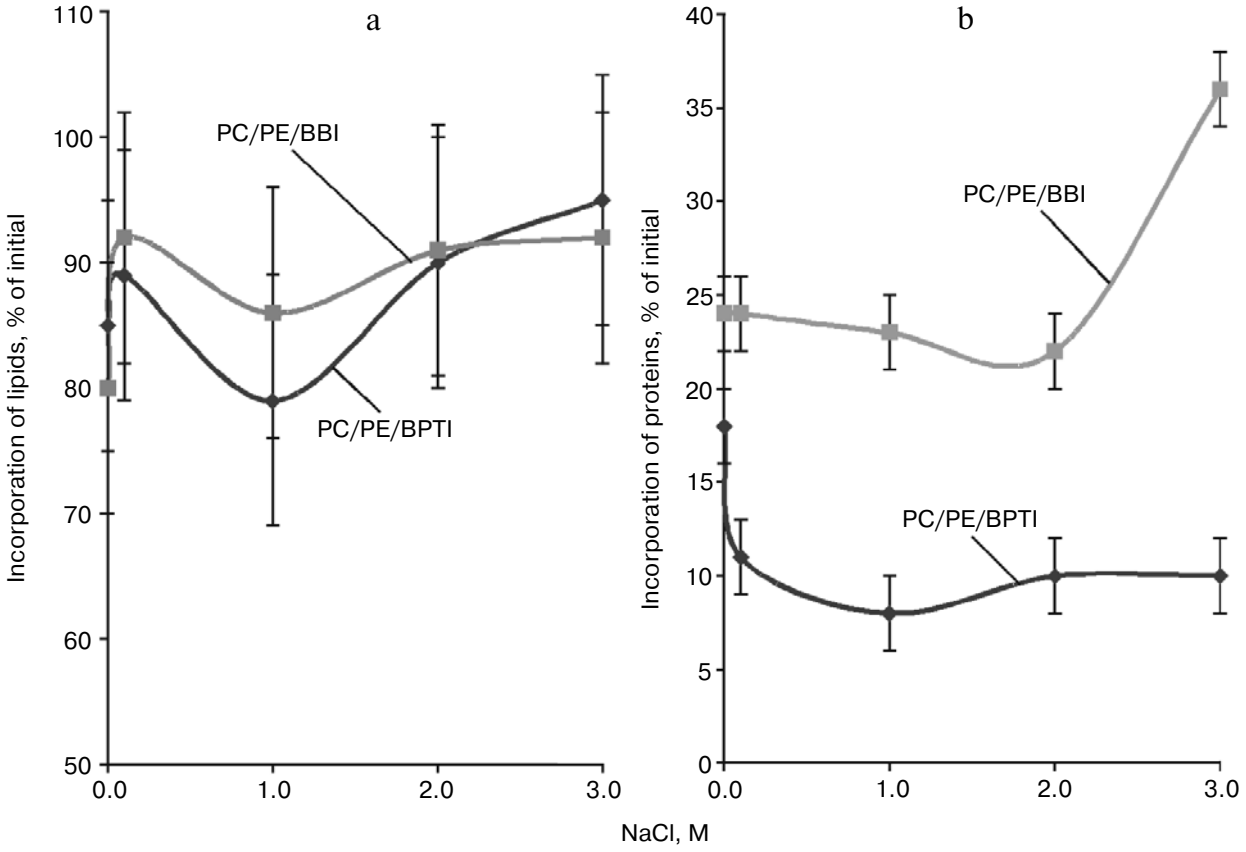

Fig. 2. Efficiency of incorporation of zwitterionic phospholipids (a) and proteins (b) into proteoliposomes prepared from PC/PE (50 : 50) mixture. Dependence on ionic strength of the medium.

efficiency of its incorporation into the liposomes does not depend on the ionic strength until NaCl concentration reaches 3 M. At high ionic strength (3 M), the content of the bound BBI significantly increases. This can be explained by the fact that the growth in the salt concentration increases the thickness of the aqueous layer of the bilayer. Also, the effect of salting out takes place, when high salt content (above 2 M) promotes dehydration of the polar heads of the phospholipids, resulting in the emergence of additional sites for interaction with protein globules [22]. Thus, the presented results indicate the hydrophobic character of interactions between phospholipids and BBI and the mixed (electrostatic and hydrophobic) types of interactions in the case of BPTI.

To test the latter statement, the interactions between BPTI and the phospholipids were studied by the fluorescence method measuring the fluorescence intensity of the liposomes containing anthracene-labeled PC (PC^{Ant}) and rhodamine B-labeled BPTI ($BPTI^{RB}$) (Fig. 3). Considering that the protein and the anthracene label absorb in the same range, corrections were made for the inner filter effect [23]. An increase in the fluorescence intensity during the incorporation of $BPTI^{RB}$ into the liposomes indicates the transition of the protein into a less polar environment (Fig. 3a). At the same time, a decrease in the fluorescence intensity of the anthracene label (Fig. 3b) located in the proximity of the fatty acid chain terminus of the phospholipid molecule indicates that the encapsulation of this water-soluble protein into the liposomes results in its transfer into the hydrophobic region of the membrane. These data agree with the results obtained previously for rhodamine-labeled BBI and anthracene-labeled liposomes [16].

Properties of empty liposomes prepared from mixture of PC/PE/LPC (87 : 12 : 1) containing saturated and nonsaturated fatty acid residues. Proteins usually bind to lipids due to a combination of electrostatic and hydrophobic interactions. The latter is realized mainly by the binding of proteins to the fatty acid residues of lipid molecules. The main parameter affecting these interactions is the presence of the unsaturated bonds in the fatty acid residues of the lipids. To evaluate the effect of the unsaturated fatty acid residues of the phospholipid molecule on their interactions with the water-soluble protein, two mixtures were used that were identical in phospholipid compositions (PC/PE/LPC) but differed in the composition of the fatty acid residues of the phospholipids. However, these mixtures could be compared under conditions when the inves-

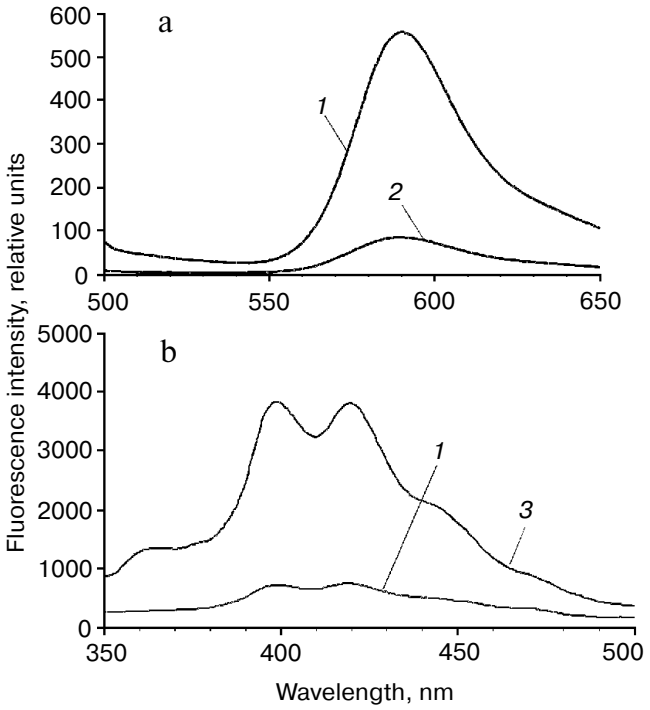

Fig. 3. Fluorescence spectrum of proteoliposomes containing anthracene-labeled PC (PC^{Ant}) and rhodamine B-labeled BPTI ($BPTI^{RB}$): the range of rhodamine B (a) and anthracene (b) labels. *1*) PC^{Ant}/PE (33 : 67) + $BPTI^{RB}$; *2*) $BPTI^{RB}$; *3*) PC^{Ant}/PE (33 : 67).

tigated phospholipids were in the same phase state. So, the chosen phospholipid mixtures as multilamellar vesicles were investigated by ^{31}P-NMR spectroscopy in the temperature range 20-60°C. Analysis of the shape of the spectra presented in Fig. 4 (a-c) showed that the phospholipids with unsaturated fatty acid residues are in L_α phase at 20°C. Increasing the temperature does not affect the shape of the spectrum, but the small maximum in the region of 0 ppm that is responsible for isotropic phase becomes clearer. Comparison of the spectra of PC/PE (67 : 33) and PC/PE/LPC) (87 : 12 : 1) mixtures shows that the effect of PE develops in the case of its significant content (more than 50%), while only 1% LPC disturbs the bilayer structure, resulting in the emergence of the peak in the spectrum corresponding to the isotropic phase.

These results indicate that the mixture of phospholipids with saturated fatty acid residues at 20 and 40°C exists in the gel phase L_β (Fig. 4, e and f) and the transition into the liquid crystal phase L_α occurs at 60°C (Fig. 4g). Under these conditions the spectra of mixtures of phospholipids with different fatty acid residues are identical. Thus, the properties of the two investigated phospholipid mixtures can be compared at 60°C.

Incorporation of BPTI into liposomes from mixtures of zwitterionic phospholipids containing saturated and unsaturated fatty acid residues. According to the spectra presented in Fig. 4, encapsulation of the protein into liposomes from zwitterionic phospholipids does not affect the structure of the phospholipids, which supports the data obtained previously for liposomes from negatively charged phospholipids [16].

The content of the components of the liposomes with BPTI presented in Table 2 suggests that the liposomes composed of phospholipids with unsaturated fatty acid residues bind more protein than the same mixtures of phospholipids with saturated fatty acid residues. These data indicate a significant contribution of hydrophobic forces to BPTI–lipid interaction and agree with the literature data concerning the enhanced hydrophobicity of the unsaturated fatty acid residues compared with the saturated ones [21]. The increase in the protein incorporation into liposomes with increase in temperature also supports the significant contribution of hydrophobic component into the interaction of the water-soluble protein and zwitterionic phospholipids.

In further studies we used differential scanning calorimetry (DSC) to gain information on the behavior of separate components of the protein–lipid system. First, phospholipid dispersions and BPTI were investigated. Heating of the mixture of PC and PE with the unsaturated fatty acid residues in the range of 80-110°C is characterized by an endothermic effect (data not shown). In the same temperature region, the DSC curve for BPTI is exothermic (Fig. 5), and the temperature of denaturation corresponds to the literature data [24] (Table 3). Thus, DSC curves of the protein incorporated into the liposomes from the phospholipids with unsaturated fatty acid residues cannot give information concerning the contribution of separate components into the protein–lipid interaction.

Important data on denaturation of BPTI were obtained during the investigation of the protein by DSC (Fig. 5). BPTI was resistant to thermal denaturation, retaining some parts of its structure after two, three, or even six cycles of heating to 110°C. During the first heating, only partial denaturation occurs, since the DSC curve of the second heating exhibits an additional peak with the maximum at 75°C that, according to the litera-

Table 3. Thermodynamic parameters of phase transitions for PC/PE/LPC (87 : 12 : 1) mixture with saturated fatty acid residues, BPTI, and proteoliposomes with BPTI (25 mM glycine buffer, pH 3.0; heating rate 2°C/min)

System	$T_{max}1$, °C	$T_{max}2$, °C	ΔH, J/g
Phospholipids	57.9 ± 0.5	63.0 ± 0.5	18 ± 2
BPTI	93.5 ± 0.5	–	52 ± 5
Proteoliposomes	52.7 ± 0.5	64.1 ± 0.5	48 ± 5

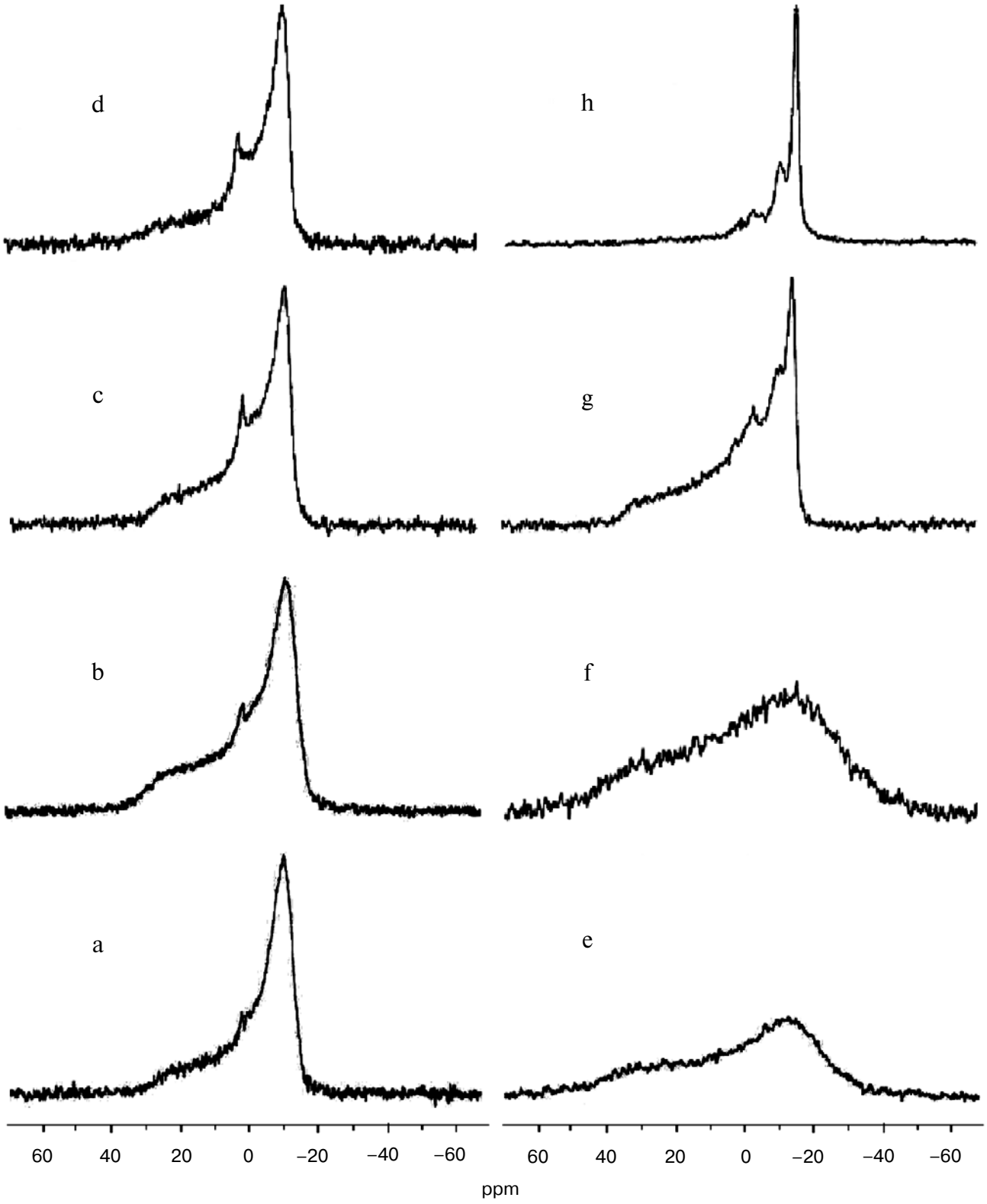

Fig. 4. ^{31}P-NMR spectra of PC/PE/LPC with unsaturated fatty acid residues: a) 20°C; b) 40°C; c) 60°C; d) +BPTI, 60°C. PC/PE/LPC with saturated fatty acid residues: e) 20°C; f) 40°C; g) 60°C; h) +BPTI, 60°C.

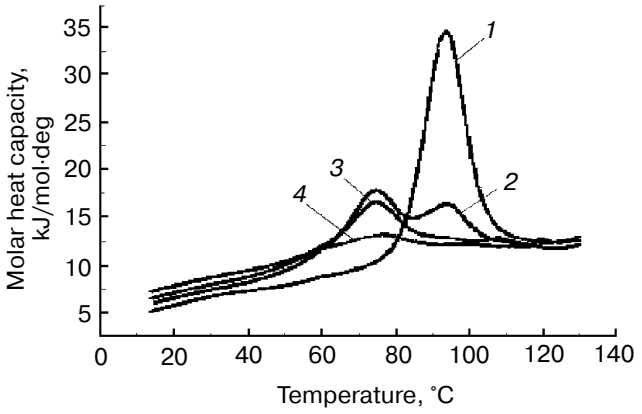

Fig. 5. DSC curves of BPTI: *1-4*) 1st, 2nd, 3rd, and 6th heating, respectively. The sample contained 2 mg/ml of BPTI in 25 mM glycine-HCl buffer, pH 3.0; the heating rate was 2°C/min.

ture [7], corresponds to the breaking of disulfide bond between residues 14 and 38. After the third heating the DSC curve clearly indicates that the sample contains no native protein.

Liposomes from the phospholipids with saturated fatty acids are characterized by an exothermic effect in the range of 50-70°C (dashed line on the left in Fig. 6) corresponding to the transition of the phospholipids with saturated fatty-acid residues from the gel to the liquid crystal state [25]. The complex asymmetric signal with a right shoulder in the range of 50-70°C indicates that the investigated lipid mixture is composed mainly of two components (the content of LPC does not exceed 1%) [26]. As known from the literature, the phase transition temperature of PC (for example, 40°C for dipalmitoyl-PC [27]) is always lower than the phase transition temperature of PE (64°C for dipalmitoyl-PE and 73°C for distearoyl-PE [28]). Thus, in the investigated mixture of soybean phospholipids S-75-3, the low- and the high-temperature peaks can be attributed to PC and PE, respectively. In addition, the DSC results for the lipids with saturated fatty-acid residues support the ^{31}P-NMR data on the existence of the investigated phospholipid mixture in the lamellar liquid crystal phase at 60°C. As it was shown, the presence of PE in the PC mixture increases the elasticity of the bilayer, its susceptibility to deformation, and permeability to water molecules [29].

Subsequent DSC experiments with liposomes containing BPTI were performed for the lipid mixture with saturated fatty acid residues. The DSC curve for the liposomes with the protein is presented in Fig. 6 (solid line), and the thermodynamic parameters of the phase transitions obtained from these curves are presented in Table 3. The curve exhibits one main signal in the range of 50-70°C with an asymmetric region composed of two overlaid signals in the region of phase transition of the lipids. The change in the shape of the exothermic DSC curve after the interaction of the liposomes with the protein (increase in the intensity of the right shoulder yielding a clear maximum) compared to the DSC curve of the liposomes without protein can be explained by the fact that PC and PE molecules interact with BPTI in different ways. The phase transitions of both phospholipids become more pronounced due to their interaction with protein molecules [28]. The phase transition of PE is more noticeable due to the intermolecular lipid–lipid interactions growing as temperature is increased. Such behavior is possible due to the bicomponent composition of the lipid mixture and the formation of unordered clusters with the predominant participation of PE molecules disturbing the ordered bilayer structure as temperature increases [30]. The broadening of the signal in the range of 50-70°C corresponding to the proteoliposomes also suggests that BPTI affects the packing of the phospholipid fatty acid residues, which agrees with the literature data on the study of the interaction between water-soluble proteins and phospholipid bilayers by DSC [31]. The increase in the enthalpy of the proteoliposomes (Table 3) indicates the electrostatic character of the protein–lipid interactions between the positively charges protein molecules and negatively charged lipid molecules [32]. The changes in the phase transition temperature are insignificant, which is related to the nonpolar character of the interactions and to the immersion of the protein into the bilayer, the latter being only partially disturbed and a fraction of lipid molecules being not affected by protein molecules [32]. The main conclusion that can be made based on these data is the following: the presence of the protein controls the conformational structure of the lipids and lateral organization of different lipid components. In turn, the lateral organization of the membrane (lipid clusters) influences the protein and its position in the liposomes.

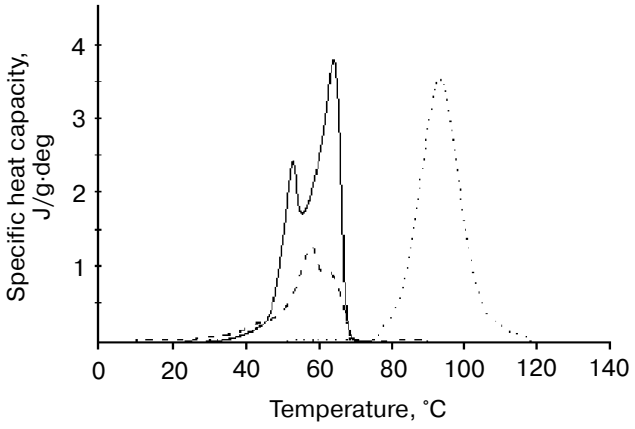

Fig. 6. DSC curves of free BPTI (2 mg/ml, dotted line), free phospholipids with saturated fatty acid residues (18 mg/ml, dashed line), and proteoliposomes with BPTI (protein and phospholipid concentrations were 2 and 18 mg/ml, solid line). Conditions: 25 mM glycine-HCl, pH 3.0; 2°C/min.

The presented results indicate that water-soluble proteins of animal and plant origin having no extended α-helical regions in their structure are capable of interacting with zwitterionic phospholipids. These interactions are related to different forces (electrostatic and hydrophobic). Protein structure is a factor that determines the ratio between these forces. For example, in the case of BPTI containing two α-helixes and one antiparallel β-sheet of two β-folds (PDB 1PIT), the binding is due to both electrostatic (results on the effect of ionic strength) and hydrophobic interactions (data on the fluorescence and binding with the phospholipids containing fatty acid residues of different saturation).

In contrast, in the case of BBI having a unique structure (it contains no α-helixes and is formed by a sequence of two β-sheets (PDB 1BBI)) and containing extended hydrophobic regions in the surface of the globule, the main type of binding is hydrophobic interaction. The character of interaction between BBI and the lipid bilayer of multilamellar vesicles is similar to that for peripheral membrane proteins, such as myelin basic protein [33], cytochrome *c* [34], water-soluble apoproteolipid of myelin [35], apolipophorin III [36], and others.

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