Interaction of recombinant analogs of spider silk proteins 1F9 and 2E12 with phospholipid membranes

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ARTICLE INFO

ABSTRACT

Recombinant analogs of spider dragline silk proteins 1F9 and 2E12 are characterized by numerous repeats consisting of hydrophobic poly-Ala blocks and Gly-rich sequences with a substantial number of positively charged amino acid residues which suggest a pronounced ability to interact with negatively charged phospholipid membranes. Actually both proteins displayed substantial binding affinity towards lipid vesicles formed of acidic lipids as measured by fluorescence correlation spectroscopy (FCS) using rhodamine-labeled conjugates of the proteins. Both proteins did not induce liposome leakage, fusion or breakdown, but were able to bring about liposome aggregation. 1F9 was more active in the induction of liposome aggregation compared to 2E12. Interestingly, 2E12 markedly decreased the rate of calcium-induced liposome fusion. Circular dichroism data showed that binding of the proteins to negatively charged phosphatidylserine liposomes provoked transition from the left-handed helix of polyproline II (PPII) type to β-structures and α-helices. The data suggested predominantly surface location of membrane bound proteins without significant perturbation of their hydrophobic core.

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1. Introduction

Spider dragline silk proteins have attracted much attention due to impressive mechanical properties and promising biomedical applications [1–3]. Spider dragline silk comprises two high molecular weight proteins, spidroin 1 (275 kDa) and spidroin 2 (320 kDa), that exhibit a structure of periodic pattern [4]. The initial repeats, although differing in deletions and inserts, have a general motif containing a hydrophobic poly-Ala (poly-A) segment of 4 to 9 amino acid residues and a more hydrophilic Gly-enriched segment. Dragline silk is hypoallergenic and completely biodegradable [5,6], matrices and films made from recombinant silk exhibits good biocompatibility [7–9]. Silk capsules filled with pharmaceuticals can be used in target therapy [10]. These properties along with the capability of bioresorption open the possibility of different applications in cosmetics and medicine [1,11]. These facts necessitate comprehensive study of the effects of the proteins on mammalian cells and their components.

Previously we described the synthesis of genes of two artificial analogs of spidroin 1 and spidroin 2, the performance of *Pichia pastoris* strains producing the proteins and also transgene plants which express these proteins [12,13]. Fig. 1 shows the sequence of proteins 1F9 (analog of spidroin 1) and 2E12 (analog of spidroin 2) which have molecular weights of 94000 and 113000 Da, respectively. Lyophilized 1F9 and 2E12 can be dissolved in NaSCN and purified by ion exchange chromatography. The proteins have polyproline II-type (PPII) secondary structure in diluted solutions and can form nanofibrils upon the addition of ethanol or vortexing [12]. Synthetic fibers based on 1F9 or 2E12 were shown to have elasticity comparable to that of native draglines and tensile strength close to that of regenerated *N. clavipes* dragline silk [12].

Fig. 1 shows that the hydropathicity index calculated according to Kyte and Doolittle [14] reaches the value of two. Noteworthy, 2E12 has more poly-A repeats in its structure than 1F9, however, at the same time it has more hydrophilic arginine residues (6.84% for 2E12

A

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                        AGQGGYGGLGSQG
AGRGGLGGQGAGAAAAAAGGAGQ---GGLGGQG
          AGQGAGASAAAA-GGAGQGGYGGLGSQG
AGRGGLGGQGAGAVAAAAGGAGQGGYGGLGSQG
AGR---GGQGAGAAAAAA-GGAGQRGYGGLGNQG
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B

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    GPGGYGPGQQGP---GAAAAASA
GRGPGGYGPGQQGPGGSGAAAAAA
  SGPGGYGPGQQGPGGPGAAAAAAA
GRGPGGYGPGQQGPGGSGAAAAAA
GRGPGGYGPGQQGPGGPGAAAAAA
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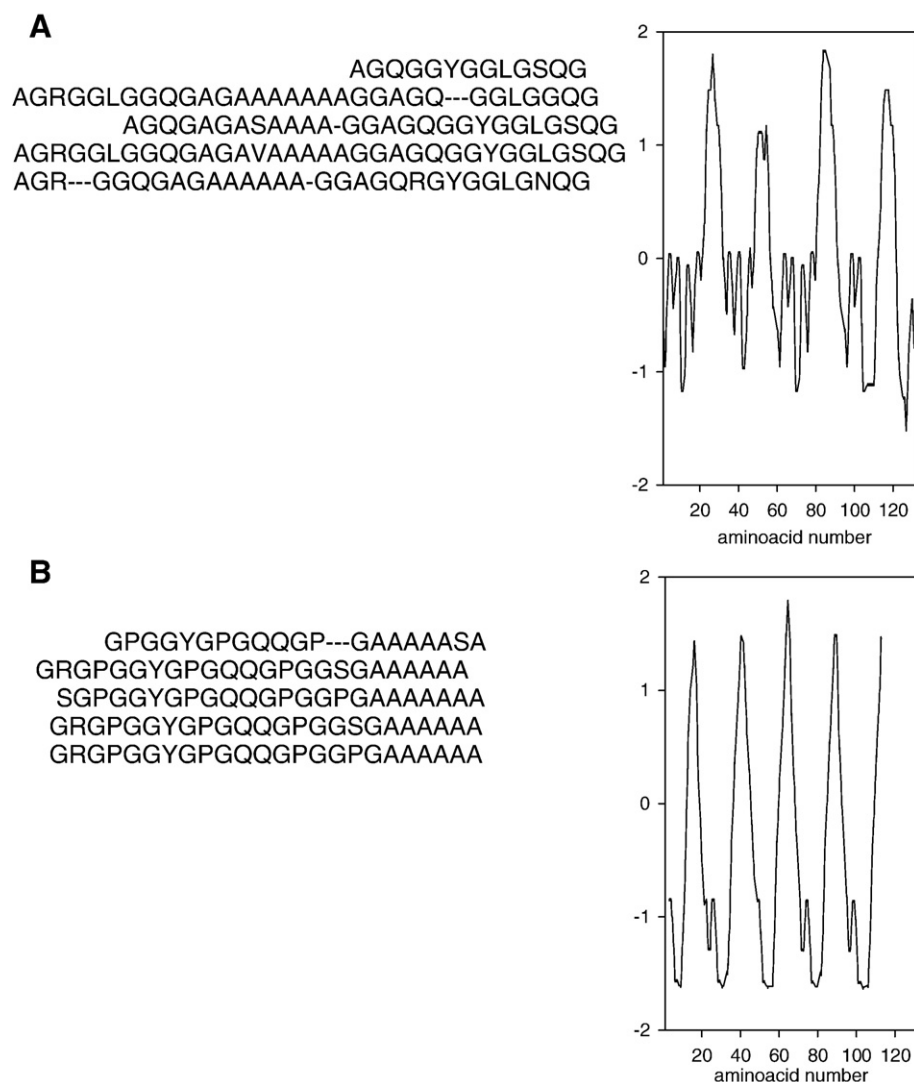
Fig. 1. Aligned amino acid sequences of "building blocks" of 1F9 (A) and 2E12 (B) used for design of the artificial genes structure. 1F9 consisted of 9 blocks of A, 2E12- of 12 blocks of B. Hydrophobicity plots of the proteins according to Kyte and Doolittle [14] are shown on the right.

and 5.35% for 1F9). Therefore, the resulting total hydropathicity index (GRAVY) is lower for 2E12 (−0.414) than for 1F9 (−0.006). It is generally accepted that the value GRAVY for a particular protein exceeding −0.4 suggests the possibility of the protein interaction with membranes [14].

The present work deals with the interaction of 1F9 and 2E12 with artificial lipid membranes. The presence of hydrophobic poly-A repeats in their sequence indicates the possibility of such interactions with a membrane. Besides, the presence of substantial number of positively charged amino-acid residues should facilitate the protein interaction with negatively-charged phospholipid membranes. A method of fluorescence correlation spectroscopy (FCS) was used to study the binding of tetramethylrhodamine-labeled 1F9 and 2E12 to liposomal membranes. It was shown that the proteins did not disrupt liposomes and did not induce their fusion. However, the binding led to liposome aggregation and modulated liposome fusion induced by calcium ions. CD spectra revealed distinct structural rearrangement upon the protein binding to membranes.

2. Materials and methods

Lipids were from Avanti Polar Lipids except for azolectin which was purchased from Sigma (phosphatidylcholine, type IV from soy beans). The approximate phospholipids composition of azolectin was phosphatidyl choline, 29%, phosphatidyl ethanolamine, 30%; phosphatidyl inositol, 26%; phosphatidic acid, 14%; phosphatidyl serine, 1% [15]. Tetramethylrhodamine-5-(and-6)-isothiocyanate (TRITC) was from Molecular Probes, other chemicals were from Sigma-Aldrich.

2.1. Protein expression and purification

Both artificial genes, 1f9 and 2e12, were subcloned into methylotrophic yeast *P. pastoris* by integration into chromosome under the control of promoter AOXI, and proteins 1F9 and 2E12 have been purified using the cation exchange column HiPrepSP Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden) as described in [16] with some modifications. The purified proteins were dialyzed into deionized water, lyophilized and stored at +4 °C until use. To prepare a stock solution, the proteins were dissolved overnight in 6 M guanidine thiocyanate water solution. The protein solution was then centrifuged for 30 min at 12,000 rpm in Eppendorf MiniSpin to remove the insoluble debris and desalted by gel filtration on PD10 column. The concentration of the proteins was determined spectrophotometrically assuming that the concentration of 1 mg/ml corresponded to absorbance of 0.488 (1F9) and 0.676 (2E12) at 280 nm.

2.2. Preperation of rhodamine-labeled spidroin

10 mg of lyophilized 1F9 or 2E12 was dissolved in 6 M guanidine thiocyanate during 60 min and the undissolved debris was removed by centrifugation at 12000 rpm in Eppendorf MiniSpin centrifuge. The protein was passed through PD10 column equilibrated with 100 mM sodium bicarbonate buffer. 1 mg of TRITC was added to 1.5 ml of the protein in sodium bicarbonate and incubated 16 hours at 4 °C in darkness. After centrifugation at 10000 g, the solution was passed through a glass column (5×200 mm) filled with Sephadex G25F equilibrated with distilled water. The ratio of bound rhodamine to the protein was estimated spectrophotometrically to be 1:1.

2.3. Circular dichroism (CD) spectra

CD spectra were recorded on Jasco J-715 (Jasco, Japan) spectropolarimeter equipped with Peltier type temperature control system, model PTC-423 S/L (Jasco, Japan) in the range of 190–250 nm at 25 °C. Spectra were measured in quartz cuvettes with an optical path length of 0.1 cm; protein concentration was 0.1 mg/ml in deionized water. It has been shown in Ref. [17] that liposomes cause very small distortions in CD spectra of water-soluble or membrane proteins.

2.4. Liposome preparation

Liposomes were prepared by evaporation under a stream of nitrogen of a 2% solution of egg yolk phosphatidylcholine, brain phosphatidylserine (all from Avanti Polar Lipids), or azolectin (soy bean phosphatidylcholine, type IV from Sigma) in chloroform followed by hydration with a buffer solution containing 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.0. The mixture was vortexed, passed through a cycle of freezing and thawing, and extruded through 0.1-µm pore size nucleopore polycarbonate membranes using an Avanti Mini-Extruder. FCS measurements (using octylrhodamine B as a marker) showed that the diameter of freshly prepared liposomes was close to 100 nm. The lamellarity of the liposome preparations was checked in a different set of experiments using NBD-labelled phosphatidylethanolamine. Addition of dithionite led to about 50% reduction of NBD fluorescence suggesting predominantly monolamellar character.

2.5. Leakage assay

Liposomes loaded with sulforhodamine (SRB) or carboxyfluorescein (CF) were prepared by extrusion through 100-nm filter (Avanti Mini-Extruder) from appropriate lipid in a suspension containing 50 mM SRB or 100 mM CF titrated with Tris-base. The unloaded CF was then removed by passage through a Sephadex G-50 coarse column using 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.0 as eluting buffer. To measure the rate of SRB or CF efflux, the liposomes were diluted in the same buffer and the fluorescence at 590 nm (excitation at 560 nm) or at 520 nm (excitation at 490 nm) was monitored with a Panorama Fluorat 02 spectrofluorimeter (Lumex, Russia), respectively. At the end of each recording, 0.1% Triton-X100 was added to complete the efflux process.

2.6. Liposome fusion assay

Two types of liposomes were prepared: plain brain phosphatidylserine (Avanti Polar Lipids) liposomes and phosphatidylserine liposomes with the addition of 9 wt.% octadecylrhodamine B (R18, Sigma) according to [18]. The method is based on the concentration quenching of R18 and its de-quenching upon fusion with unlabeled liposomes. Liposomes were prepared by extrusion through 100-nm filter from appropriate lipid suspension containing 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.0 buffer. To measure the rate of fusion,

the liposomes were diluted in the same buffer with the ratio 4:1 (nonlabeled to labeled) and the fluorescence at 590 nm (excitation at 560 nm) was monitored with a Panorama Fluorat 02 spectrofluorimeter (Lumex, Russia). At the end of each recording, 0.1% Triton-X100 was added to read the maximum signal. The measurements were performed at 37 °C.

2.7. Fluorescence correlation spectroscopy (FCS)

The home-made setup was described previously in Ref. [19]. Briefly, excitation of fluorescence and detection utilized an Nd:YAG solid state laser with a 532-nm beam attached to an Olympus IMT-2 epifluorescent inverted microscope equipped with a 40×, NA 1.2 water immersion objective (Carl Zeiss, Jena, Germany). The fluorescence passed through an appropriate dichroic beam splitter and a long-pass filter was imaged onto a 50-µm core fiber coupled to an avalanche photodiode (SPCM-AQR-13-FC, PerkinElmer Optoelectronics, Vaudreuil, Quebec, Canada). The signal from an output was converted to a personal computer with a fast interface card (Flex02-01D/C, Correlator.com, Bridgewater, NJ). The data acquisition time was 30 s. The fluorescence was recorded from the confocal volume located at about 50 µm above the cover glass with 60 µl of the buffer solution added. To calibrate the setup, the FCS signal from a solution of Rhodamine 6G was recorded. Assuming the diffusion coefficient of the dye to be 2.5×10^{-6} cm^2/s, the value of the confocal size $\omega = 0.42$ µm was obtained.

An autocorrelation function which describes the time course of fluorescence fluctuations, associated with statistics of diffusion of several particles around the confocal volume, can be characterized by the parameter τ_d according to Eq. (1) [20,21]

$$G(\tau) = \frac{1}{N} \cdot \left(\frac{1}{1 + \frac{\tau}{\tau_d}} \right) \cdot \left(\frac{1}{\sqrt{1 + \frac{w_0^2 \cdot \tau}{z_0^2 \cdot \tau_d}}} \right) \quad (1)$$

where N is a number of particles, w_0, z_0 are scaling parameters of the confocal volume, and τ_d is average time of a particle dwelling in the confocal volume.

Eq. (2) can be used to describe $G(\tau)$ in the case of protein binding to liposomes [22].

$$G(\tau) = \sum_{i=p,v} A_i g_i(\tau) \quad (2)$$

where $g_i(\tau)$ are autocorrelation functions of free ($g_p(\tau)$) and bound ($g_v(\tau)$) protein which can be described by Eq. (1).

2.8. Electrophysiological study of interaction of recombinant spidroins with planar bilayer lipid membranes (BLM)

BLM were formed from a 2% solution of diphytanoylphosphatidylglycerol (Avanti Polar Lipids) in decane by the brush technique [23] on a 0.5-mm diameter hole in a Teflon partition separating two compartments of a cell containing aqueous solutions of 10 mM MES, 10 mM Tris и 100 mM KCl, pH 7.0. The electrical current was measured with a patch-clamp amplifier (model BC-525C, Warner Instruments, Hamden, CT), digitized by a LabPC 1200 (National Instruments, Austin, TX), and analyzed by a personal computer with the help of WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). The current was low-pass filtered with a cutoff frequency of 100 Hz. Ag AgCl electrodes were placed directly into the cell.

2.9. Optical measurements

Absorption was measured with an Amersham Ultrospec 500/1100 spectrophotometer. Fluorescence was recorded with a Panorama

Fluorat 02 (Lumex, Russia) fluorescence spectrophotometer with excitation and emission slits adjusted to 5 nm.

2.10. Estimation of hydropathicity of proteins

Hydropathicity of proteins was estimated using the grand average of hydropathy (GRAVY) value (ProtParam program; ExPASy proteomics tools, www.expasy.org/tools/protparam.html) as the sum of the hydropathy value of all amino acid residues divided by the length of the amino acid sequence [14].

3. Results

The binding of the proteins to lipid vesicles was studied by FCS using rhodamine-labeled 1F9 and 2E12. Fig. 2A shows autocorrelation functions of TRITC-1F9 (curve 1) alone, after addition of two concentrations of liposomes formed from azolectin (curves 2 and 3) and a control curve for a solution of rhodamine 6G (curve 4).
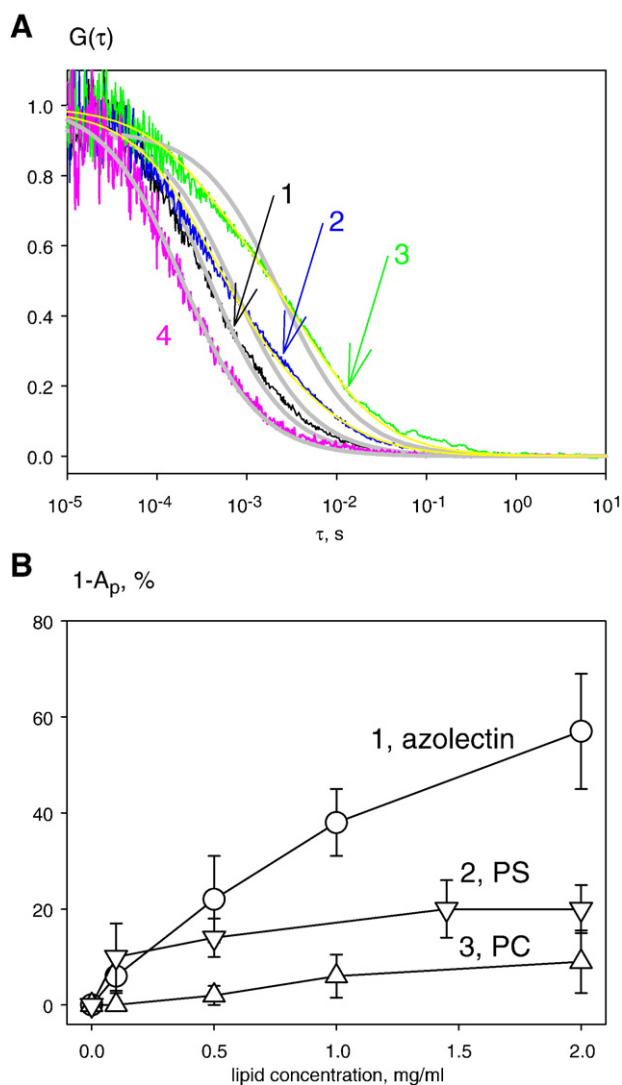

Fig. 2. (A) Normalized autocorrelation functions of $G(\tau)$ of TRITC-1F9 (curve 1, concentration 20 nM), TRITC-1F9 and 0.5 mg/ml azolectin vesicles (curve 2), TRITC-1F9 and 2 mg/ml azolectin vesicles (curve 3), 20 nM of Rhodamine 6G (curve 4). Grey curves are best fits by Eq. (1) with $\tau_d = 185$ μs (curve 4) and 370 μs (curve 1), 730 μs (curve 2), 2350 μs (curve 3). Yellow curves are best fits by Eq. (2) with Ap = 76% (curve 2) and Ap = 43% (curve 3). (B) The dependence of the parameter 1-Ap (characterizing the binding of TRITC-1F9) on the lipid concentration for liposomes made from azolectin (curve 1), phosphatidylserine (curve 2), and phosphatidylcholine (curve 3). The buffer was 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.

Curves 4 and 1 of Fig. 2A can be fitted well by Eq. (1) giving τ_d of 185 μs and 370 μs, respectively. A two-fold increase in τ_d corresponded to a two-fold decrease in the diffusion coefficient (D), as these parameters are inversely proportional to each other [20]. The addition of liposomes shifted the autocorrelation function to the right which reflected the binding of a portion of the protein to liposomes with a characteristic diffusion coefficient of $0.05 \cdot 10^{-6}$ cm^2/s [24]. Noteworthy, Eq. (1) gives only poor fitting of these autocorrelation functions which can be accounted for by a system heterogeneity characterized by the populations of free and bound protein.

Yellow curves in Fig. 2A represent fitting curves for experimental curves 2 and 3 by Eq. (2) which have parameter Ap 76% and 43%, respectively. The bound part of the protein (i.e. 24% and 57%, respectively) increased with the increase in the lipid concentration. The fitting was performed assuming τ_d of free and bound protein as 370 μs and 6 ms. This approach towards the estimation of protein binding to liposomes proved to be consistent with other measurements of protein binding [22].

Fig. 2B shows the dependence of the portion of bound protein (1-Ap) on the concentration of liposomes made from different lipids. TRITC-1F9 bound effectively to azolectin liposomes while the binding was negligible in the case of neutral phosphatidylcholine liposomes (Fig. 2B, curves 1, 3). Azolectin (crude phosphatidylcholine fraction from soy beans) is known to contain considerable portion of negatively charged lipids which apparently strengthen the protein–vesicle interaction. In fact, TRITC-1F9 bound to pure phosphatidylserine (Fig. 2B, curve 2), although the binding was less efficient than in the case of azolectin. It can be speculated that weaker binding of 1F9 to phosphatidylserine than to azolectin was associated with the presence of trace amounts of phospholipids carrying two or more negative charges (i.e. cardiolipin, phosphatidylinositol-di-phosphate, etc.) in the azolectin lipid mixture. In fact, the binding of positively charged peptides to PIP2-containing liposomes was shown to be stronger than to PS-containing liposomes [25].

Fig. 3 shows the dependence of the portion of bound TRITC-2E12 (1-Ap) on the concentration of different liposomes. This protein had higher affinity to membranes and even had the ability to bind to neutral phosphatidylcholine liposomes. These properties can be accounted for by a larger number of hydrophobic polyalanine repeats in 2E12 than in 1F9 owning to shorter initial repeats. It is interesting to note that in contrast to TRITC-1F9, TRITC-2E12 bound to phosphatidylserine more effectively than to azolectin vesicles (Figs. 2 and 3). It can be assumed that there is some specificity in the
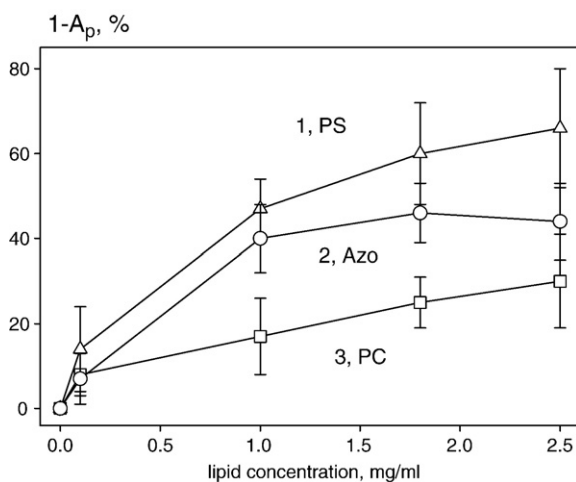

Fig. 3. The dependence of the parameter 1-Ap, characterizing the binding of TRITC-2E12, on the lipid concentration for liposomes made from phosphatidylserine (curve 1), azolectin (curve 2), and phosphatidylcholine (curve 3). The buffer was 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7. TRITC-2E12 concentration was 15 nM.

interaction of the proteins with some components of azolectin which cannot be ascribed to simple electrostatics or hydrophobicity.

The binding of proteins to membrane surface usually induces changes in the conformation of the protein which can be monitored by CD spectra [26–28]. Fig. 4 shows the effect of azolectin and phosphatidylserine liposomes on the CD spectrum of 1F9 (panel A) and 2E12 (panel B). In agreement with our previous data [12], CD spectra of 1F9 and 2E12 in aqueous solution (Fig. 4, solid lines) corresponded to the conformation of polyproline II (PPII) type which is characterized by a minimum at 195 nm and a small maximum at 220 nm. The addition of phosphatidylserine liposomes led to a marked change in the CD spectrum of 1F9 including the change in the sign of the ellipticity in the range of 195–210 nm (short dashed line in Fig. 4A). The

spectrum suggested the appearance of β-sheets and possibly α-helices in the bound protein molecules. In contrast to the effect of phosphatidylserine, the addition of azolectin liposomes led only to distortion of the 1F9 spectra which retained, however, the major features of the spectrum of PPII (long dash line of Fig. 4A). The character of the effects of liposome addition on the CD spectrum of 2E12 was similar to that of 1F9 confirming substantially different effects of azolectin and phosphatidylserine liposomes (Fig. 4B). However, a more pronounced distortion of the CD spectrum upon binding to azolectin liposomes was observed with 2E12 than with 1F9 which may indicate the appearance of supramolecular forms of the 2E12 protein.

The binding of polycations to negatively-charged liposomes is frequently accompanied by liposome aggregation [29]. In agreement with this, the addition of 1F9 and 2E12 to a suspension of phosphatidylserine liposomes led to an increase in the light absorption caused by liposome aggregation (Fig. 5). The effect was absent in the case of neutral liposomes made from phosphatidylcholine and also in the case of azolectin liposomes (Fig. 5). However, 1F9 caused the aggregation of azolectin liposomes at lower ionic strength of the solution (data not shown). The aggregates were large in size (up to hundreds of microns) and can be seen with an optical microscope (data not shown).

Aggregation of liposomes in certain cases is accompanied by their fusion. The process of membrane fusion is very important for cell physiology and special experiments were performed to study the effect of spidroin on liposome fusion. The fusion was monitored by de-quenching of fluorescence of R18-loaded liposomes in a mixture with nonlabeled liposomes as was suggested in [18]. As shown in Fig. 6, 1F9 and 2E12 did not induce fusion of phosphatidylserine liposomes in contrast to the effect of calcium ions which is known to trigger effectively the fusion under these conditions [18]. Interestingly, 2E12 was able to decelerate Ca-induced liposome fusion while 1F9 was not. This observation can be associated with competition between calcium ions and cationic groups of 2E12 for the anionic binding sites on the liposome surface which did not take place in the case of 1F9.

One of the most important parameters of membranes is its integrity, i.e. the ability to maintain the gradient of substances on opposite sides of a membrane. The binding of polycationic proteins frequently leads to leakage of marker molecules from pre-loaded liposomes showing the appearance of defects or pores [30,31]. Fig. 7 displays the effect of 1F9, 2E12, and melittin on the sulforhodamine B (SRB) leakage from azolectin liposomes. In contrast to melittin, a well-known liposome leakage inductor, which provoked a pronounced SRB leakage from azolectin liposomes (curve 3), two spidroins were ineffective in this system suggesting that binding of these proteins to
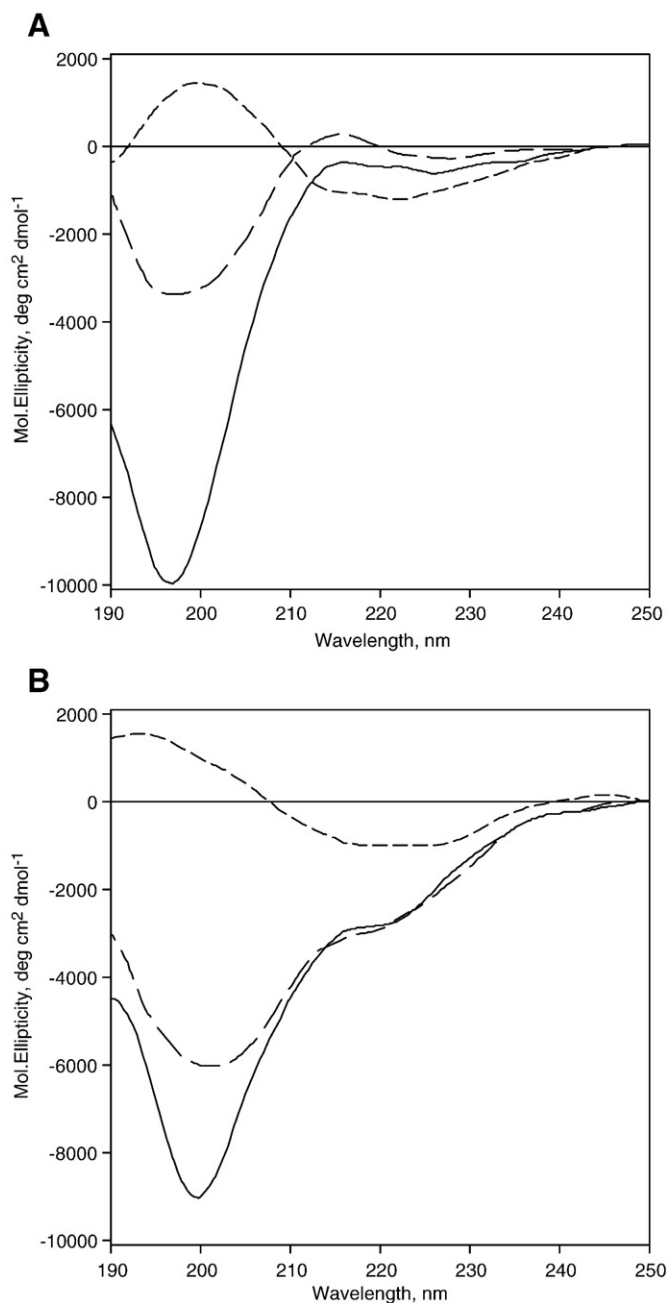

Fig. 4. CD-spectra of solutions of 1F9 (A) and 2E12 (B) in the absence (solid curve) and in the presence of liposomes (dashed curves). Long dashed curve and short dashed curve correspond to the addition of 0.1 mg/ml of azolectin and phosphatidylserine, respectively.
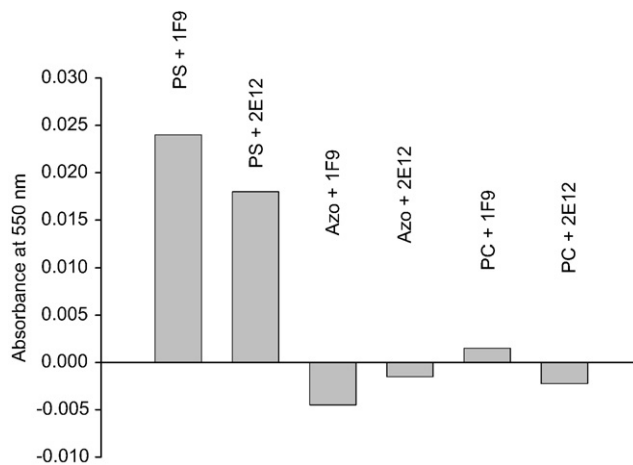

Fig. 5. Aggregation of liposomes of different composition induced by 1F9 and 2E12. The buffer was 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7. Concentration of proteins was 0.1 mg/ml; lipid, 1 mg/ml. The absorbance at 550 nm was read after 40 min of liposomes incubation with the proteins.
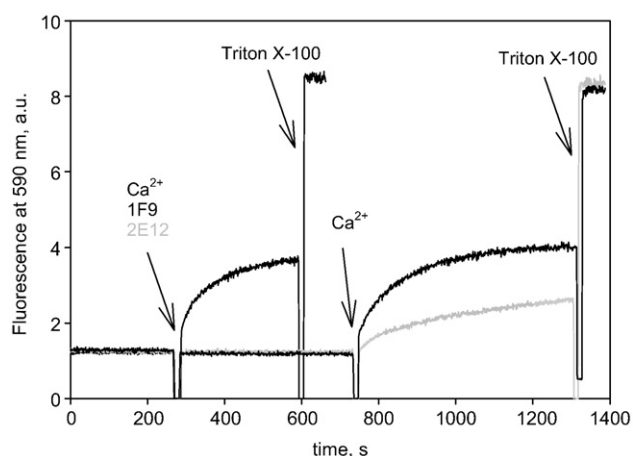
Fig. 6. Effect of 1F9, 2E12, and calcium ions on the fusion of phosphatidylserine liposomes measured by de-quenching of fluorescence of R18. Concentration of lipid in nonlabeled vesicles was 20 μM, concentration of lipid in labeled vesicles was 5 μM. Concentration of 1F9 was 10 μg/ml; 2E12, 5 μg/ml; CaCl₂, 4 mM; Triton X-100, 0.1%. The buffer was 100 mM KCl, 10 mM Tris, 10 mM MES, pH 7.

liposomes did not lead to significant membrane perturbation. The proteins were ineffective in promoting the CF leakage from phosphatidylserine liposomes as well (data not shown) thereby showing that the change in the protein conformation upon lipid binding did not induce membrane leakage.

Alternative method of monitoring permeability defects in membranes consists in electrical current measurements across a planar bilayer lipid membrane. This method allows to detect small-size defects providing permeation of small ions such as potassium or chloride. The membrane was made of negatively-charged lipid diphytanoylphosphatidylglycerol. The experiments showed that 1F9 and 2E12 were inactive in the induction of electrical current up to concentrations of 10 μg/ml (data not shown). The sensitivity of the setup was within a range of several picoamperes. Control experiments showed single channel activity of the channel-forming peptide gramicidin A. These experiments supported the data obtained on liposomes that the proteins were unable to induce conducting defects in lipid membranes.

4. Discussion

Artificial dragline silk proteins 1F9 and 2E12 were shown to bind to lipid membranes and the binding was enhanced by the presence of negatively charged lipids in the membrane. The spidroin binding can be compared to that of another water-soluble protein, α-synuclein, which was studied recently by FCS [32]. In the case of phosphatidyl-
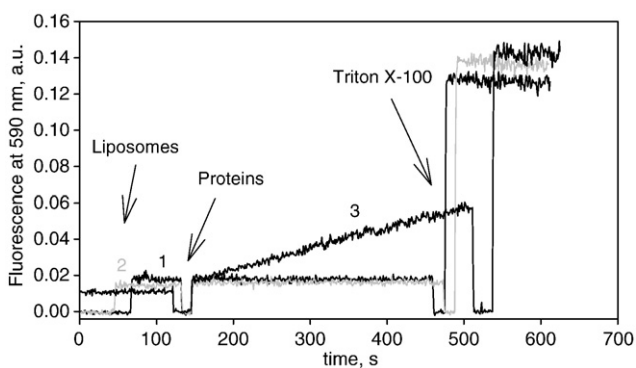

Fig. 7. Sulforhodamine B (SRB) leakage from azolectin liposomes induced by 1F9 (curve 1), 2E12 (curve 2), and melittin (curve 3). Concentrations were: lipid, 5 μg/ml; 1F9, 10 μg/ml; 2E12, 5 μg/ml; melittin 1 μg/ml; Triton X-100, 0.1%. The buffer was 100 mM KCl, 10 mM TRIS, 10 mM MES, pH 7.

choline liposomes, the parameter (1-Ap) for α-synuclein at 1 mM lipid concentration was about 20%. This parameter value corresponded well to that of 2E12 assuming the molecular weight of egg phosphatidylcholine to be 800 Da (Fig. 3). The binding of α-synuclein to negatively-charged phosphatidylserine vesicles was much stronger and the level of 20% binding was reached at 2 nM lipid concentration [32], in contrast to the same level observed at about 0.5 mM lipid concentration in the case of 2E12 (Fig. 3). Therefore, the contribution of electrostatics to the binding of α-synuclein was much higher compared to 2E12, despite the fact that the total hydropathicity index (GRAVY) of α-synuclein (−0.403) is close to that of 2E12 (−0.414). In contrast to 2E12, the binding of 1F9 to phosphatidylcholine liposomes was negligible (Fig. 2) in spite of a higher hydropathicity value (GRAVY = −0.006). This result can be accounted for by somewhat different folding of 1F9 in water solution leading to shielding of hydrophobic regions that prevents direct interaction with membrane lipids. The weak binding of 1F9 and 2E12 to lipid membranes is comparable to that of amphiphilic pluronics copolymers which exhibit significant binding at millimolar lipid concentrations [33]. Pluronics considered also as promising materials for different biomedical applications [34] were shown to inhibit multidrug-resistance protein activity via alteration of membrane environment [35].

In addition to differences in binding to phosphatidylcholine membranes, there were differences in the modulation of Ca-induced fusion of phosphatidylserine liposomes by these two proteins (Fig. 6). Although the binding of the proteins was accompanied by a substantial change in secondary structure of both proteins (Fig. 4), it can be proposed that the binding of 2E12 to a lipid membrane led to penetration of hydrophobic poly-A regions into hydrophobic core of the membrane which hindered membrane fusion presumably by modulation of membrane curvature [36]. Besides, the difference in the modulation of Ca-induced liposome fusion may be due to formation of oligomeric complexes of 2E12 but not of 1F9, which can be suggested from the comparison of CD spectra of these two proteins.

The properties of 1F9 and 2E12 can be compared to that of cytochrome *C* which is known to bind to negatively charged membranes due to the presence of a cluster of positively-charged amino acid residues. The binding of cytochrome *C* leads to liposome aggregation which is not accompanied by liposome fusion and liposome leakage [29]. Except for very low ionic strength conditions, the binding of cytochrome *C* to membranes can be considered as peripheral, i.e. when amino acids locate at the membrane interface without penetration into hydrophobic core of the membrane [37]. By contrast, liposome binding of another α-helical globular protein, lysozyme, can be characterized by amino acids insertion into hydrophobic core which leads to liposome aggregation, fusion and leakage [38,39]. Besides, the binding of lysozyme provoked the formation of nanofibrils [39,40]. 1F9 and 2E12 did not induce liposome leakage but were able to form nanofibrils in aqueous solutions [12].

The fact that 1F9 and 2E12 did not perturb membrane barrier properties is very important in light of the study of the proteins toxicity to cells. Many toxins are water-soluble proteins which are able to bind to membranes, undergo large conformational changes, insert into the membrane core and induce ion fluxes across cellular membranes [41,42]. It can be proposed that inability of 1F9 and 2E12 to insert into hydrophobic core of the membrane can be ascribed to the fact that these proteins have short poly-A clusters while the formation of stable transmembrane α-helix requires the cluster of about 20 hydrophobic amino acids [43]. The absence of liposome leakage is in accord with the data about the usage of spidroin-based matrixes for cultivation of cells of different types [11].

Recently artificial spidroin has been proposed to use for preparation of protein-lipid nano-containers for drug delivery [10]. Spidroin was added to a suspension of multilamellar liposomes and subsequent ethanol treatment led to formation of predominantly proteinaceous containers. The present work showed that this approach should be

very sensitive to lipid composition of liposomes by modulation of the protein binding to membranes. Besides, spidroin was shown to induce liposome aggregation which can lead to a substantial increase in the size of the nano-containers.

Acknowledgements

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