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MEMBRANE-ACTIVE PROPERTIES OF CRAMBIN

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A thionin-like protein with M.M. 5 kDa has been isolated from Crambe kotschyana *seeds. It has been shown that crambin possesses a low lytic action on erythrocytes but inhibits the Ca-ATPase of the sarcoplasmic reticulum of rabbit skeletal muscles. A disordering action of crambin on the structures of both the sarcoplasmic reticulum and liposomes has been established by ESR and microcalorimetry.*

Interest in low-molecular-mass peptides of plant origin is due to the fact that many of them exhibit antibacterial, antimicrobial, and carcinostatic actions. This class of substances includes the thionins, highly basic polypeptides with molecular masses of 5-6 kDa [1-6].

The realization of the toxic effects of thionins apparently takes place at the level of biological membranes. In particular, it has been shown that thionins cause the hemolysis of erythrocytes and activate potential-dependent calcium channels and endogenous phospholipase A_2 [7, 8].

The primary structure and conformation of the molecule of crambin, a thionin isolated from *Crambe abyssinica* seeds and having a number of characteristic features, such as hydrophobicity and neutrality, are known [4]. However, the membranotropic effects of this substance have not hitherto been studied. In the present paper we give information on its isolation and a study of its membrane-active properties.

Various types of grinders are used in the isolation of the majority of the thionins, which is probably necessary for the disruption of the cell wall. In view of this, in the isolation of crambin by aqueous acetone extraction [4] we used a mechanico-chemical treatment leading to a more complete extraction of the components [9].

Analysis by electrophoresis in polyacrylamide gel (PAAG) of the composition of the residue obtained after the dialysis of an aqueous acetone extract of *C. kotschyana* seeds showed the presence of a component making up more than 95% of the protein content and also of two peptides present in trace amounts (Fig. 1*a*). The main component — crambin — was revealed by an intense band at the level of P-thionin from *Pyrularia pubera* (see Fig. 1*a*), which showed a molecular mass of about 5 kDa for it. These results agree with the molecular mass of crambin isolated from *Crambe abyssinica* seeds — 4720 [4]. Additional purification of the polypeptide was achieved by preparative chromatography in a thin layer of cellulose, followed by the elution of an individual spot with 65% aqueous acetone (Fig. 1*b*). The yield of purified crambin was 0.02% of the defatted flour.

The interaction of crambin with membranes was investigated by the ESR method from the influence of the peptide on the dynamic mobility and orientation of certain groups of lipid molecules in the membranes of the sarcoplasmic reticulum. For this purpose we used the probes 5-doxylstearic acid (5-DS) and benzo-γ-carboline (B-γ-c).

In a lipid matrix, the probe 5-DS participates in the collective motion of the lipid molecules, and the information obtained through it corresponds to the level in the bilayer, where its nitroxyl fragment is located; segments of 5-DS can also bend and rotate along a C–C fragment. The B-γ-c molecule is a rigid structure the longitudinal axis of which is oriented along the acyl chains of the lipid molecules.

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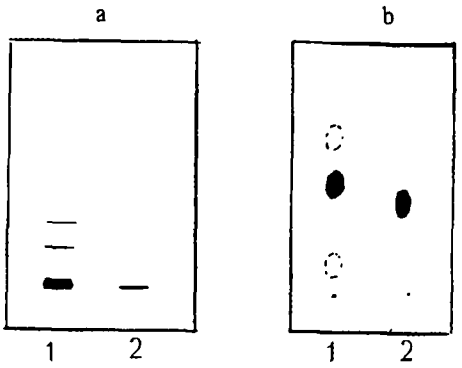

Fig. 1. Electrophoregram (*a*) and thin-layer chromatogram (*b*) of a purified crambin preparation and a marker: *1*) crambin; *2*) P-thionin.

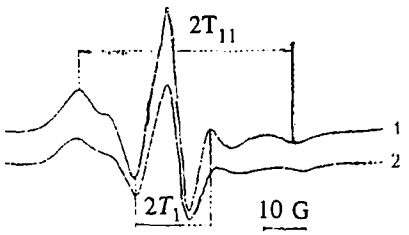

Fig. 2. ESR spectrum of the probe 5-DS in the membranes of the sarcoplasmic reticulum: *1*) control; *2*) in the presence of crambin (214 nmole/mg of protein).

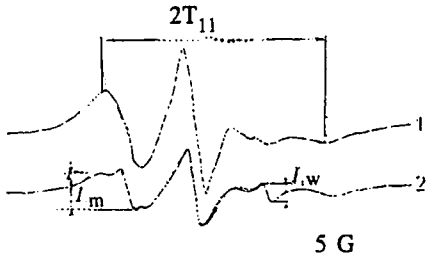

Fig. 3. ESR spectrum of the probe B-γ-c in the membranes of the sarcoplasmic reticulum; *1*) control: *2*) in the presence of crambin (142 nmole/mg of protein).

Figures 2 and 3 give the ESR spectra of 5-DS and B-γ-c in samples of sarcoplasmic reticulum (SR) and show the parameters measured. To evaluate the behavior of 5-DS in the SR we used the order parameter S, a change in which suggests a change in the orientation of the N−O moiety of the nitroxyl radical [10].

To estimate the mobility of the probe B-γ-c in the SR, we used the magnitude of $2T_{11}$, proportional to its mobility and reflecting the fluidity of the membranes [10]. The parameter *W*, quantitatively characterizing the degree of distribution of the probe B-γ-c between the aqueous and lipid phases, was determined as the ratio I_w/I_m, where I_w and I_m are the amplitudes of the signals in the aqueous and membrane phases, respectively (Fig. 3).

As can be seen from Fig. 4, with an increase in the amount of crambin added to a sample of SR containing 5-DS, the value of *S* falls. It is most likely that crambin molecules, on their introduction into the lipid matrix of the SR, lead to a distortion of the mean value of the angle of inclination of the carbon chain of 5-DS relative to the normal to the surface of the bilayer at the level of localization of the nitroxyl fragment, which entails a change in the value of *S*. In this case the mean value of the angle of slope increases, and the value of *S* decreases accordingly. Since 5-DS reflects the collective behavior of the lipid molecules, with a high degree of probability this also relates to them.

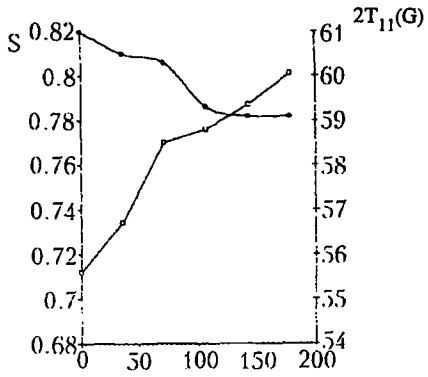

Fig. 4. Dependence of the order parameters S and $2T_{11}$ for the probes 5-DS and B-γ-c localized in SP membranes on the amount of crambin: o) $2T_{11}$(B-γ-c); •) *S*(5-DS).

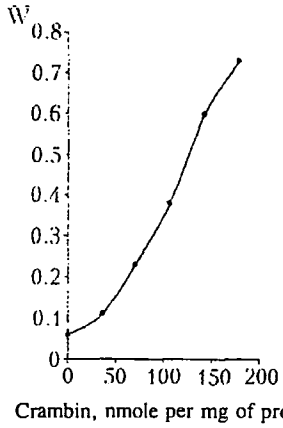

Fig. 5. Dependence of the distribution of the probe B-γ-c between the aqueous and lipid phases in SR membranes (the parameter *W*) on the amount of crambin.

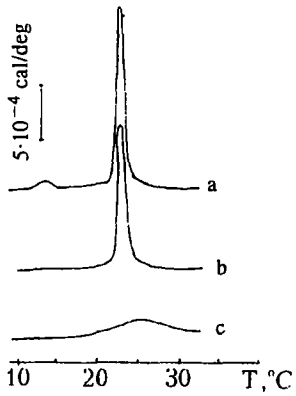

Fig. 6. Thermogram of the melting of the DMPC of multilamellar liposomes in the absence (*a*) and in the presence (*b*, *c*) of crambin. Concentration of DMPC ($3 \cdot 10^{-4}$ M): *1*) control; *2*, *3*) 300 and 600 nmole of crambin, respectively.

For the voluminous molecule B-γ-c an increase in the value of $2T_{11}$ has been found the degree of which depends on the amount of crambin added (see Fig. 4). This shows a decrease in the fluidity of the membrane and corresponds to an increase in the time of rotational mobility of the probe around the selected axis and relative to the normal to the bilayer of the membranes.

When crambin was added to SR samples containing the probe B-γ-c, a displacement of the probe into the aqueous phase was again observed. Figure 5 shows the relative changes in the parameter W in SR samples as a function of the amount of crambin, which indicates the occupancy of a definite volume by the crambin molecules in the lipid matrix of the membranes and the displacement of some of the molecules of the probe into the aqueous phase. The use of this probe enabled us to obtain additional information on the intrusion of crambin molecules into the lipid matrix of membranes.

Thus, the combination of ESR results with the use of different probes unambiguously shows the affinity of the crambin molecules for the lipid matrix of SR membranes and also the fact that the intrusion of crambin molecules leads to a disordering of the lipid matrix over practically the whole profile.

The disordering action of crambin molecules was confirmed unambiguously by the method of differential scanning calorimetry. A thermogram of the melting of multibilayer liposomes from dimyristoylphosphatidylcholine (DMPC) was represented by two endothermic peaks, one of which was small and corresponded to a "pretransition" process. The other, narrower and more intense, peak corresponded to the thermal effect of the phase transition of a hydrated lipid from the pseudocrystalline (gel) state to the liquid-crystalline state at the temperature T_t = 24.2°C [11]. We may note that the process of melting of DMPC multibilayers bears a cooperative nature. The introduction of perturbations into this process may lead to a decrease in cooperativeness, which will be shown in a broadening of the peak, or to a change in temperature of the phase transition, or to other changes in the form of the thermogram.

Figure 6 shows a fragment of a thermogram of multilamellar dispersions of DMPC without crambin (*a*) and with various concentrations of added crambin (*b* and *c*). It can be seen that at a ratio of DMPC to crambin of 5:1 the crambin molecules completely destroy the cooperativeness of melting, as a result of which the peak of this transition disappears completely. This is connected with a disturbance of the packing of the phospholipids in the lipid bilayer.

Thus, it follows from the results obtained by the methods of calorimetry and spin probes that crambin distorts the initial orientation of lipids both in liposomes and in the membranes of the SR, and it also increases the viscosity of the lipid matrix.

It is known that peptides of the thionin group vigorously interact with erythrocytes, which leads to an efflux of hemoglobin, followed by lysis of the cells. The greatest hemolytic activity is possessed by P-thionin, for which a species specificity in relation to erythrocytes has been shown, while an inhibiting influence of Ca^{2+} ions and an activating effect of anions on hemolysis have been investigated. It has been shown that at a concentration of $1.6 \cdot 10^{-6}$ P-thionin causes 35% hemolysis of erythrocytes [12]. The results of a hemolytic study of crambin show that this peptide possesses a low lytic activity and at a concentration 20 times higher causes only 17% hemolysis of erythrocytes.

It was shown earlier that, on interacting with liposomes and biological membranes (erythrocytes), P-thionin causes structural rearrangements in the membranes [6], which may be responsible for its toxic action on the cell. It is assumed that as a result of interaction with the cell membrane there is an activation of the Ca channels, a rise in the concentration of Ca^{2+} in the cell, and an activation of phospholipases, leading in the final account to the destruction of the cell.

At the same time, it is known that inhibition of Ca-ATPase also causes an increase in the concentration of intracellular Ca^{2+} and leads to the so-called programmed death of the cell (apoptosis). Since the transport enzymes are lipid-dependent and react very sensitively to a change in the microenvironment, it may be assumed that such structural modifiers as peptides of the thionin group must change the activity of ATPase. In view of what has been said above, we investigated the influence of crambin on Ca-ATPase.

The results of a study of the influence of crambin on the activity of the Ca-ATPase of the SR showed a dose-dependent nature of its action. As was established, crambin exerts a dual action on the SR Ca-ATPase. Thus, at a ratio of 100-300 nmole/mg an activation of the enzyme was noted. With a further increase in this ratio, an inhibition was observed that reached 60% at a ratio of 1200 nmole/mg.

An activating effect on Ca-ATPase has also been observed for P-thionin [6], but no inhibiting action of P-thionin on transport enzymes has been reported.

The results obtained can apparently be explained from the aspect of a change in the structural organization of the lipid skeleton of the membrane. This is shown, in particular, by the experiments that we conducted with spin probes having different localizations in the membranes of the sarcoplasmic reticulum. The activating influence of crambin on Ca-ATPase at low concentrations may be the result of the disordering action of the peptide on membranes that we have noted. However, with

an increase in the concentration of crambin the predominating effect is, apparently, a decrease in the fluidity of the membranes as a result of the occupancy of a definite volume by the crambin molecules, which, in the final account leads to an inhibition of the transport of the enzyme as a result of the restriction of its conformational mobility.

On the whole, it must be mentioned that crambin, a hydrophobic protein of the thionin group, causes a disturbance in the structural organization of membranes a possible consequence of which is a change in the functional activity of the cells. It is known that the mechanism of the interaction of the highly active water-soluble protein P-thionin with biological and model membranes is based on electrostatic interaction [12], while for crambin, apparently, the determining efect is hydrophobic. It is possible that the differences in hemolytic activity and action on Ca-ATPase found between crambin and P-thionin are determined by the physicochemical characteristics of the peptides and, as a consequence by the nature of their interaction with membranes.

EXPERIMENTAL

Isolation of Crambin. Seeds of *C. kotschyana* were gathered in July in the environs of the town of Angren and the village of Isuduruk. The seeds were frozen in liquid nitrogen, ground, and defatted by steeping with diethyl ether in the cold. The flour was extracted with 65% aqueous acetone at a ratio of 1:5 in a D-109 disintegrator (Dezintegrator NPO, Tallin, Estonia) at room temperature in the flow-through regime three times at a rotor speed of 130-140 rps and a feed of the pulp raw material at the rate of 30-35 g/min. Then the extract was subjected to ultrasonic treatment in an ultrasonic bath (Retsch URG, FRG) at an ultrasonic power of 110 W (in 5-min bursts, the total time of treatment being 30 min). Extraction was carried out in a magnetic mixer with ice cooling for 3 h. The extract was separated by centrifuging with cooling. The supernatant liquid was dialyzed. The precipitate that formed during dialysis was freeze-dried and used in the subsequent investigations.

Electrophoresis was carried out, after the residue had been incubated in 1% Na-DDS, on 15% PAAG plates in the presence of 0.1% Na-DDS, and the plates were fixed in 10% TCA and stained with Coomasie R-250. P-Thionin was used as a marker.

Chromatography in a thin layer of FND cellulose (Filtrak, GDR) was conducted on 20 × 20 cm plates in the butanol–pyridine–acetic acid–water (15:10:3:12) system. The spots were revealed with 3% ninhydrin in acetone at 100°C for 5 min. In the preparative purification of crambin the edge of the plate was stained, with the conditions of analytical chromatography being preserved. Elution of the individual spots in TLC was achieved with 65% aqueous acetone. The crambin preparation obtained was freeze-dried. Solutions of crambin in DMSO were used in the experiments. The equivalent amount of DMSO was added to the control samples.

Fragments of sarcoplasmic reticulum were isolated from rabbit skeletal muscles by Ritov's method [13]. The ATPase activity of the SR was evaluated from the increase in the amount of inorganic phosphorus, P_i, in an incubation volume of 0.5 ml at 37°C in 10 min. The incubation medium for the SR contained 25 mM tris-HCl, pH 7.5, 100 mM KCl, 2.5 mM $MgCl_2$, and 1 mM ATP. Inorganic phosphorus was determined by Panusz' method [14]. ATP activity was expressed in μmole P_i calculated to mg of protein/min. Protein contents were determined by the biuret reaction [15].

The hemolytic activity of crambin was determined spectrophotometrically at a wavelength of 540 nm on the basis of the efflux of hemoglobin from erythrocytes resuspended in a medium containing 140 mM NaCl, 1 mM EDTA, and 10 mM tris-citrate buffer, pH 7.2. The optical density corresponding to 100% hemolysis was determined after the lysis of control samples of erythrocytes in distilled water.

ESR spectra were obtained on a Bruker radiospectrometer (FRG) at a modulation amplitude not greater than 1 G and an irradiation power of 20 dB.

An alcoholic solution of the probe (not more than 10^{-4} M) was added to a sample of membrane SR.

A suspension of multilamellar liposomes for the calorimetric measurements was prepared from DMPC (Sigma, USA) as described in [16]. The thermodynamic parameters of the phase transitions of lipid dispersions were determined in a DASM-4 differential adiabatic scanning microcalorimeter at a scanning rate of 1°C/min. Error ±0.2°C. Transition temperatures were determined from the positions of the maxima of the corresponding peaks.

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