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Incorporation of the antimicrobial protein seminalplasmin into lipid bilayer membranes

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Abstract. The interaction between seminalplasmin, an antimicrobial protein from bull semen, and lipid bilayers has been investigated. The fluorescence of the single tryptophan residue of the protein was measured. In the presence of phosphatidylcholine or phosphatidic acid bilayer vesicles the fluoresence maximum was shifted to shorter wavelengths, indicating transfer of the tryptophan to a more apolar environment. Circular dichroism spectra show an increased α-helical content for the protein in the presence of lipid. Quenching experiments clearly show the incorporation of the protein with the tryptophan localized near the bilayer surface. The shift of the tryptophan fluorescence emission was used to monitor the lipid phase transition in phosphatidylcholine membranes.

Key words: Lipid-protein interaction, seminalplasmin, antimicrobial protein, membrane structure, fluorescence

Introduction

Seminalplasmin, an antimicrobial protein from bull semen was shown to inhibit the growth of Gram-positive and Gram-negative bacteria as well as yeast cells (Reddy and Bhargava 1979). In all cases investigated so far seminalplasmin specifically interfered with RNA-synthesis (Venkov and Scheit 1984; Scheit and Zimmer 1984). In order to be effective it obviously needs to penetrate the cell membrane. The aim of the present paper is to demonstrate that seminalplasmin is able to incorporate into the bilayer membranes of both negatively charged and neutral lipids.

This highly basic protein has an isoelectric point of 9.8 and contains 48 amino acids ($M_r = 6{,}385$). It probably contains two α-helical domains and has six lysine and five arginine residues distributed almost homogeneously over the peptide chain (Theil and Scheit 1983a). Because of a similar random distribution of the hydrophobic residues this protein is highly water soluble and does not aggregate in aqueous solution, as demonstrated by equilibrium sedimentation (Theil and Scheit 1983b).

From biophysical investigations we know that basic peptides such as poly-lysine may adsorb to negatively charged bilayer surfaces and may induce a phase separation of rigidified peptide bound lipids within a fluid matrix (Hartmann and Galla 1978). The antibiotic peptide polymyxin B is anchored within the bilayer membrane by its hydrophobic tail, which is linked to the basic decapeptide part. The combined hydrophobic and electrostatic interaction leads to a cooperative domain formation of negatively charged lipids bound to the integrated polymyxin (Sixl and Galla 1979; Sixl and Galla 1981; Sixl and Galla 1982). Very recently, polymyxin was also found to incorporate into neutral phosphatidylcholine membranes and to slightly increase the gel-to-liquid crystalline phase transition temperature (Mushayakarara and Levin 1984).

Another example of a highly water soluble basic protein is melittin, the bee venom peptide with clustered lysine and arginine residues at its carbonyl terminal and with clustered hydrophobic residues. This protein also incorporates into bilayer membranes, increasing its α-helical content from 7% in solution to 70% (Vogel et al. 1983). Dilatometric measurements showed that there was a large increase in the volume of the phosphatidylcholine membrane after melittin incorporation (Posch et al. 1983).

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Abbreviations: TEMPOL 2,2,6,6-Tetramethyl-4-hydroxy-piperidine-1-oxyl; DMPC 1,2-Dimyristoylphosphatidylcholine; DMPA 1,2-Dimyristoylphosphatidic acid; SL 5 2-(3-Carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinoxyl; SL 12 2-(10-Carboxydecyl)-4,4-dimethyl-2-hexyl-3-oxazolinoxyl

We have investigated the interaction of seminalplasmin with bilayer membranes in order to elucidate the process of its membrane penetration. Fluorescence from the single tryptophan residue and circular dichroism measurements have been used to monitor the incorporation of the protein. Fluorescence quenching experiments with membrane bound and water soluble quencher molecules rule out pure adsorption onto the bilayer surface and favour the idea of integration into the membrane.

Materials and methods

Seminalplasmin was purified from frozen bull semen by the method of Theil and Scheit (1983b). The final step of the isolation procedure included preparative high performance liquid chromatography on a reversed phase column. The purity of the protein was checked by amino acid analysis. The molar extinction coefficient was $\varepsilon = 6{,}383$ at $\lambda = 280$ nm, corresponding to the single tryptophan residue.

Large unilamellar vesicles were prepared according to the method of Szoka and Paphadjopoulos (1978). 17 mg of lipid was dissolved in 1 ml of a chloroform/buffer 2:1 v/v mixture; the buffer solution was 10 m*M* *Tris*/HCl at the appropriate pH. After short ultrasonication the solvent was evaporated under vacuum on a rotatory evaporator and the remaining aqueous lipid dispersion was diluted with 5 ml of buffer solution. Vesicles were checked by negative stain electron microscopy and were found to range in size from 80 to 100 nm. Seminalplasmin was added to the vesicle dispersion and incubated for 30 min at a temperature above the phase transition temperature. These dispersions were used for fluorescence and circular dichroism measurements. Longer incubation times did not affect the fluorescence spectra any further.

For quenching experiments TEMPOL was added to the lipid-protein dispersion at the quoted concentration. Fatty acid spinlabels were incorporated into the membrane by adding the appropriate amount to the lipid solution in chloroform during the preparation of the large unilamellar vesicles.

Vesicles were centrifuged and assayed for seminalplasmin content by measuring the fluorescence intensity of the resuspended pellet in comparison to that of the supernatant. Vesicles used in our experiments contained between 50% and 60% of the added protein. Cooling of the vesicles below the phase transition temperature did not change the protein content within the vesicles.

Fluorescence spectra were taken with a Perkin Elmer MPF3 spectrometer. Irradiation of the tryptophan fluorescence was performed at 280 nm. Circular dichroism spectra were taken with a Cary spectrometer.

Results

Fluorescence measurements

Fluorescence spectra of seminalplasmin in the absence and in the presence of phosphatidylcholine vesicles are shown in Fig. 1. The fluorescence emission with a maximum at 350 nm in the absence of lipid is characteristic of a tryptophan containing protein in aqueous solution and is very similar to the corresponding spectrum for melittin (Mollay et al. 1976). Addition of phosphatidylcholine vesicles up to a molar lipid-protein ratio of $X_{LP} = 180$ leads to a continuous shift of the fluorescence maximum to 338 nm. Further increase in the lipid concentration does not change the fluorescence spectrum any further. Such a blue shift in the tryptophan fluorescence suggests transfer of the indole ring from a polar to a nonpolar environment, which in our experiment is the apolar bilayer membrane.

The blue shift of the emission maximum was used to titrate a protein solution with different lipids; the results are summarized in Fig. 2. Dimyristoylphosphatidylcholine induces a blue shift by about 12 nm. At 33 °C, that is above the lipid phase transition temperature, a molar lipid-protein ratio of 180 is necessary to obtain a maximal shift (Fig. 2a). Negatively charged dimyristoylphosphatidic acid (Fig. 2b) causes a somewhat smaller but comparable blue shift of the tryptophan fluorescence. The final value is reached at a lower lipid concentration $X_{LP} \sim 30$, independent of both pH and ionic strength. However, the maximal shift attainable decreases with decreasing pH and increasing

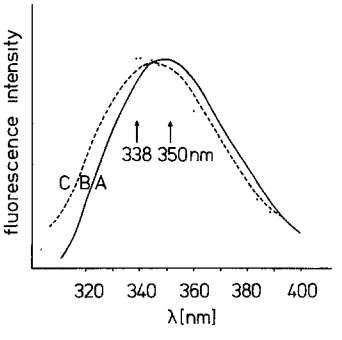

Fig. 1. Fluorescence spectra of seminalplasmin in the absence and presence of preformed dimyristoylphosphatidylcholine vesicles. The temperature of 33 °C is well above the lipid phase transition temperature $T_t = 23{,}5$ °C. *A:* aqueous protein solution; *B:* molar lipid/protein ratio 50:1; *C:* molar lipid/protein ratio 200:1

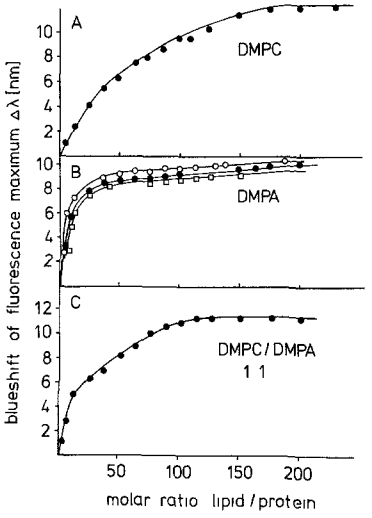

Fig. 2A–C. Blueshift of the fluorescence maximum of seminalplasmin in the presence of neutral dimyristoylphosphatidylcholine (**A**) and negatively charged dimyristoylphosphatidic acid (**B**) in varying lipid protein ratios. The symbols in B are: ○–○–○, borate/boric acid buffer, pH 9.0, ionic strength $I = 0.03\,M$; □–□–□ pH 9.0, $I = 0.15\,M$; ●–●–● pH 5 in pure water. Part (**C**) represents a 1:1 mol/mol mixture of dimyristoylphosphatidylcholine and dimyristoylphosphatidic acid at pH 9.0 and $I = 0.03\,M$. The temperature was kept above the corresponding lipid phase transition temperature. Curve *A* was taken at 33 °C, curves *B* and *C* were at 54 °C

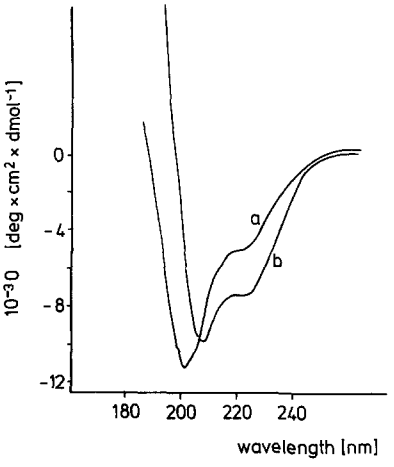

Fig. 3. Circular dichroism spectra of seminalplasmin. *a*: in 10 m*M Tris*-buffer at pH 7.4; *b*: in the presence of dimyristoylphosphatidylcholine vesicles at 24 °C; lipid/protein molar ratio 100:1

ionic strength due to a weakening of the electrostatic interaction. Spectra were taken above the lipid phase transition temperature at 54 °C. At this temperature the protein was not denatured during the time required for the measurement; as confirmed by circular dichroism measurements.

Mixed bilayer vesicles of DMPA and DMPC in a 1:1 molar ratio were also used to titrate a protein solution, this leads to a biphasic binding curve (Fig. 2c). Phosphatidylcholine and phosphatidic acid are known to be only poorly miscible, thus they form vesicles that are composed of enriched PC and PA domains (Galla and Sackman 1975a). At low lipid content seminalplasmin preferentially incorporates into DMPA domains; binding to DMPC domains occurs only at higher lipid content and leads to the further decrease in λ_{max} between $X_{LP} = 20$ and $X_{LP} = 100$. All the protein is bound at $X_{LP} = 100$ in the mixed membrane compared to $X_{LP} = 180$ in DMPC-vesicles. This clearly shows that the electrostatic interaction facilitates the incorporation of seminalplasmin into the bilayer membranes.

Circular dichroism

An α-helix content of seminalplasmin of about 28% was estimated from the amino acid sequence data (Theil and Scheit 1983b); the circular dicroism spectrum of seminalplasmin in aqueous solution (Fig. 3) suggests a somewhat lower helix content. Heating the protein solution to 70 °C for 1 h does not change the spectrum. However, addition of phospholipids strongly affects the circular dichroism spectra; the changes observed are consistent with a small but significant increase in α-helix content upon addition of phospholipids. These results are again comparable to results obtained on the incorporation of melittin into bilayer vesicles (Vogel 1981).

Quenching of the tryptophan fluorescence

In addition to their normal use for EPR-experiments, spinlabels are excellent quenchers of fluorescence (Luisetti et al. 1979) and may be used to locate a fluorophore within the bilayer membrane. We used both the water soluble spinlabel TEMPOL, which preferentially quenches fluorescence of fluorophores near the lipid-water interface or in the aqueous phase, and spinlabeled fatty acids to quench membrane incorporated fluorophores. Using fatty acids that carry the nitroxide-group at different positions along the chain, it is also possible to locate the depth of a fluorophore in the hydrophobic part of the bilayer (Luisetti et al 1979).

In Fig. 4a we used two fatty acid spinlabels, SL5 and SL12, with the paramagnetic nitroxide group at carbon 5 and 12 respectively. Tryptophan fluorescence was quenched at 37 °C in DMPC membranes containing 1 Mol% seminalplasmin. The Stern-Volmer relationship $I_0/I - 1 = k_Q \cdot c \cdot \tau_0$ (k_Q = quench-

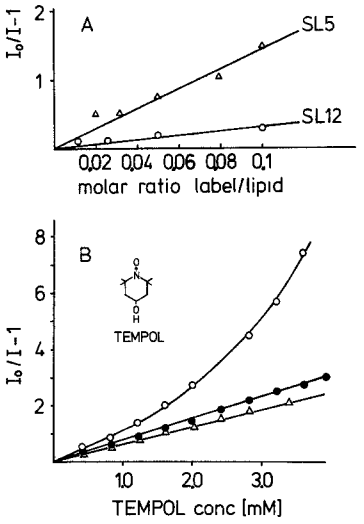

Fig. 4 A and B. Fluorescence quenching of seminalplasmin added to dimyristoylphosphatidylcholine vesicles in a lipid/protein molar ratio of 100:1. **A** Membrane dissolved spin-labeled stearic acid carrying the nitroxide group at carbon 5 (SL5) or at carbon 12 (SL12) were used as quencher molecules. The slope of the intensity ratio $(I_0/I-1)$ versus quencher concentration within the lipid bilayer is a measure of the quenching efficiency. **B** A water soluble quencher, TEMPOL, was used to quench the tryptophan fluorescence from the water phase. ○–○–○ seminalplasmin in the absence of lipid vesicles; ●–●–● presence of dimyristoylphosphatidylcholine vesicles, lipid/protein ratio 100:1; △–△–△ presence of dimyristoylphosphatidic acid vesicles at pH 5.0, lipid/protein ratio 100:1

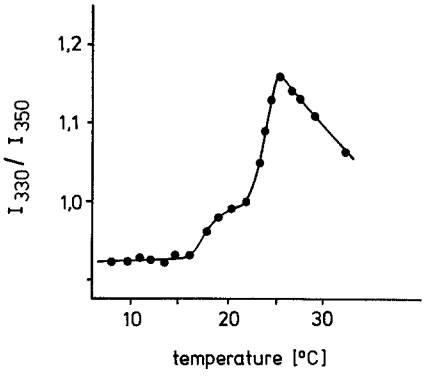

Fig. 5. The intensity ratio I_{330}/I_{350} of the seminalplasmin fluorescence measured at 330 and 350 nm is given with increasing temperature. An increased ratio is characteristic of a larger shift in the fluorescence maximum to lower wavelength and thus is characteristic of a stronger lipid-protein interaction. A maximum was obtained near the lipid phase transition temperature of the DMPC-vesicles. The molar lipid/protein ratio is again 100:1

ing constant, c = concentration, I_0 = fluorescence intensity in the absence of quencher, I = fluorescence intensity in the presence of quencher, τ_0 = lifetime of the fluorophore), is fulfilled. The quenching efficiency is greater for SL5 compared with SL12, which implies that the vertical location of the tryptophan residue is near the lipid headgroup region. The same interpretation holds for the TEMPOL-label, which is highly water soluble. Label dissolved in the bilayer membrane could not be determined by EPR-spectroscopy. There is considerable quenching of protein fluorescence in DMPC and slightly less in DMPA bilayer membranes, demonstrating the reduced accessibility of the chromophore in a negatively charged membrane. Quenching of the protein fluoresence in aqueous solution is considerably more effective and does not follow the Stern/Volmer equation. The experiments clearly show the protection of the tryptophan residue against quenching from the water phase in the presence of lipids and its accessibility by a quencher incorporated into the bilayer membrane but located near to the glycerol backbone.

The effect of lipid phase transition on seminalplasmin incorporation

The wavelength shift of tryptophan fluorescence was used to follow the incorporation of seminalplasmin into the lipid bilayer in a temperature range where the lipid phase transition occurs. The fluorescence intensity ratio, I_{330}/I_{350}, was used to monitor the incorporation after vesicle addition (Fig. 5). At temperatures below the pre-transition temperature, T_p, almost no wavelength shift was observed. A slight increase in I_{330}/I_{350} occurs at the pre-transition temperature whereas the main shift leading to an increased I_{330}/I_{350} ratio occurs at the lipid main-phase-transition temperature, T_t. Above T_t the ratio decreases which may be due to a temperature-dependent movement of the protein towards the bilayer surface. Such a decrease in I_{330}/I_{350} was also observed with seminalplasmin incorporated into egg-lecithin, which has no phase transition in the observed temperature range. This decrease with temperature above T_t is fully reversible whereas the step at T_t is not. Upon lowering the temperature below T_t it takes several hours for the value of I_{330}/I_{350} to recover from $I_{330}/I_{350} = 1.1$ to the starting value at $T < T_t$. The kinetics of the incorporation and/or extrusion will be reported elsewhere.

Discussion

The results presented here clearly demonstrate the incorporation of seminalplasmin into lipid bilayer membranes and yield the basic information necessary to investigate the penetration of the protein

through lipid bilayer membranes. The aim of our present study was to investigate the lipid-protein interaction at low protein concentrations, where membrane lysis or membrane disruption do not occur. Such a cell disrupting activity is a characteristic feature of amphiphatic peptides such as the bee venom peptide melittin (Williams and Bell 1972), which is structurally similar to seminalplasmin.

However, such cell disruption may not be the only biologically relevant process that affects cellular functions. Very recently a strong interference of mellittin with intracellular Ca^{2+}-dependent enzymatic activities was reported by Gietzen and Bader (1984). Melittin was found to be one of the most potent substances presently available for antagonizing the calmodulin induced activation of Ca^{2+}-transport ATPase. Such experiments suggest that small amphiphatic proteins may interfer with biochemical events through processes other than cell disruption. Thus it seems important to investigate their incorporation into and especially their penetration through lipid bilayer membranes. This has been done in the past using melittin as a model system for lipid-protein interactions. However, most of these studies reported experimental artefacts due to phospholipase A_2 contamination (Dasseux et al. 1984).

Here we have focused attention on the lipid-protein interaction of another amphiphatic protein, the antimicrobial protein, seminalplasmin, from bull semen. Seminalplasmin inhibits the growth of E. coli and inhibits RNA synthesis at a concentration of 25 μg/ml (Scheit and Zimmer 1984). To induce these effects the protein has to permeate the cell membrane barrier.

Our experiments were performed with protein purified by preparative HPLC on a nucleosil C 18 reversed phase column. The purity was checked by amino acid analysis. We used low protein concentrations with respect to the lipid to prevent severe disruption of the bilayer membrane structure. This was clearly demonstrated by measuring the thermotropic phase transition and the order degree of the lipid bilayer using the spin label technique. Protein concentrations of less than 10 mmol per mole of lipid increased the transition temperature by about 1 °C and increased the lipid order below and above the phase transition temperature (Schulze and Galla, unpublished work).

The transfer of a chromophore from a polar to a nonpolar medium is often accompanied by a blue-shift of the fluorescence maximum. Seminalplasmin contains a tryptophan residue which allows one to follow the incorporation of the protein into preformed unilamellar lipid vesicles by observing the shift of the fluorescence maximum from $\lambda = 350$ nm to $\lambda = 338$ nm after incubation with lipid vesicles. This clearly demonstrates the interaction with the lipid bilayer. Fluorescence quenching experiments using water soluble and lipid soluble paramagnetic quencher substances show the incorporation of the protein into the hydrophobic lipid bilayer with the tryptophan residue located near the glycerol backbone region. A preferential interaction with negatively charged lipids was found. The protein conformation changes to a more α-helical structure after incorporation into the bilayer membrane.

One outstanding result is the temperature dependence of the lipid-protein interaction follwed by the wavelength shift of the tryptophan fluorescence. Below the pre-transition temperature there is almost no change in the fluorescence maximum measured by the intensity ratio I_{330}/I_{350}. However, this ratio increases at the pre-transition temperature and even more drastic changes occur at the main transition temperature. Above the lipid phase transition temperature the fluorescence at 330 nm decreases with respect to the 350 nm value. We centrifuged the peptide containing vesicles and assayed the supernatant for free protein and the pellet for incorporated proteins by fluorescence measurements. Within the incubation time of 30 min about 60% of the added peptide was found in the pellet at $T > 25$ °C whereas about 50% was found at $T < 15$ °C. The fluorescence maximum was only slightly shifted at low temperatures even though 50% of the protein is membrane associated; this may be explained if a tryptophan is located near the membrane surface below the pre-transition temperature. The same is true above the lipid main transition temperature. The ratio I_{330}/I_{350} decreased with increasing temperature at $T > 25$ °C in DMPC-bilayer membranes. The same temperature dependence was found in egg-lecithin between 10° and 30 °C where egg-lecithin has no lipid phase transition. Obviously the protein is most deeply incorporated into the hydrophobic part of the lipid bilayer exactly at the lipid phase transition temperature.

These results may be interpreted by a preferential incorporation into the boundaries between fluid and crystalline domains that coexist at the phase transition temperature (Marsh et al. 1976). The amount of phase boundary lipid is maximal at the phase transition temperature.

Another interesting temperature range for DMPC-membranes is between 15° and 20 °C. This lipid phase between the pre- and the main phase transition temperature, called the $P_{\beta'}$-phase, is characterized by the so-called ripple structure (Rüppel et al. 1982). In a very recent paper by Kapitza et al. (1984) it was demonstrated that glycophorin, the integral membrane protein isolated from red blood

cells preferentially incorporated into defect structures of the $P_{\beta'}$-phase. In agreement with these findings we interpret the increased fluorescence shift in the $P_{\beta'}$-phase as being due to a protein fraction that is more deeply buried in the defects of the rippled phase.

To summarize, our data show the importance of defect structures whether they are present as phase boundary lipids at the phase transition temperature or between the pre- and the main transition temperature for the incorporation and probably also for the penetration of protein into or through lipid bilayer membranes.

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