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Evidence for membrane affinity of the C-terminal domain of bovine milk PP3 component

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Abstract

Component PP3 is a phosphoglycoprotein isolated from bovine milk with unknown biological function, which displays in its C-terminal region a basic amphipathic α-helix, a feature often involved in membrane association. According to that, the behaviour of PP3 and of a synthetic peptide from the C-terminal domain (residues 113–135) was investigated in lipid environment. Conductance measurements indicated that the peptide was able to associate and form channels in planar lipid bilayers composed of neutral or charged phospholipids. Electrostatic interactions seemed to promote voltage-dependant channel formation but this was not absolutely required since the pore-forming ability of the 113–135 C-terminal peptide was also detected with the zwitterionic lipid bilayer. Additionally, a spectroscopic study using circular dichroism argues that the peptide adopts an α-helical conformation in interaction with neutral or charged micelles. Thus, the conducting aggregates in bilayers might be composed of a bundle of peptides in helical conformation. Besides, similar conductance measurements performed with the whole PP3 protein did not induce any channel fluctuations. However, with the latter, an early breakdown of the bilayers occurred, a finding that can be tentatively explained by a massive incorporation of PP3. In the light of the present results, it could be inferred that PP3 membrane attachment may be achieved by oligomerization of the C-terminal amphipathic helical region.

Keywords: Milk; PP3 component; Planar bilayer; Amphipathic helix; Channel

1. Introduction

Component PP3 is a 135 amino acid phosphorylated glycoprotein isolated from bovine milk (for review, see [1]). Highly homologous proteins are also found in milk of other species, such as camel [2], llama [3], ovine and caprine milk [4,5], but, intriguingly, not in human milk [4]. Immunological studies with antibodies raised against highly purified PP3 have shown that this protein is present in whey and in the milk fat globule membrane [4].

The PP3 cDNA sequence [6,7] reveals about 56% similarity with a gene coding for a glycosylation-dependent adhesion molecule, GlyCAM-1, in mice [8] and rats [9]. GlyCAM-1 is expressed in peripheral and mesenteric lymph nodes as well as in lung and mammary gland [8], whereas PP3 expression is only detected in the mammary gland of lactating cows [6]. In the lymph nodes, GlyCAM-1 is indirectly involved

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in the immune response by acting as an endothelial cell surface ligand for L-selectin, a lymphocyte homing receptor, whose function requires the presence of sulphate and sialic acid [10]. Since sulphation has not been demonstrated in the mammary gland [9], the exact function of PP3 in vivo still remains to be highlighted.

Beside non-resolved functional properties, structural data are becoming available to dissect the functional domains of the protein. Posttranslational analysis [11] demonstrated that the N-terminal domain of the mature component PP3 contains five phosphorylation sites (serines 29, 34, 38, 40, 46), two O-linked and one N-linked glycosylation sites (threonines 16 and 86, asparagine 77), modifications largely conserved for PP3 and GlyCAM-1 (Fig. 1). In addition, a peptide mimicking the C-terminal 17 residues of PP3 displayed a strong propensity to interact with anionic lipid monolayers [12] and adopted a basic amphipathic helical conformation in membrane-like environments [13]. A recent NMR study further demonstrated that this helical propensity can be extended to the 38-mer C-terminal fragment (residues 98–135) with a possible bend near residue 117 [14]. According to primary structure analysis, this amphipathic helical C-terminus is also shared, although less conserved, by the homologous proteins of the GlyCAM-1 family [8,14].

Since a basic amphipathic helix is a structural motif shared by numerous cytotoxic peptides known to perturb cell membranes [15,16], we investigated further the interaction of component PP3 with membranes. Thus, the ability of component PP3 to incorporate into planar lipid bilayers was addressed by means of conductance measurements. The same experiments were also carried out with a 23-mer synthetic peptide corresponding to the 113–135 C-terminal part of component PP3 to evaluate if this region would be the promoter of membrane interaction. This fragment was also studied by circular dichroism (CD) spectroscopy to determine its conformation in a lipid environment.

2. Materials and methods

2.1. Purification

Component PP3 was extracted from the bovine milk proteose-peptone fraction by affinity chromatography as described previously [12].

The peptide corresponding to fragment 113–135 of PP3 (sequence NTVKETIKYLKSLFSHAFEVVKT) was synthesized by the solid phase technique on a Synergy 432 peptide synthesizer (Perkin-Elmer) using the standard cycle for the 9-fluorenylmethoxycarbonyl strategy. Purification of the peptide was carried out by C_{18} reversed-phase high performance liquid chromatography. The purified product was checked for sequence by amino acid analysis and mass spectrometry.

2.2. CD measurements

Measurements were carried out on a CD6 dichrograph (Jobin Yvon, Longjumeau, France) at room temperature. The instrument was routinely calibrated with an aqueous solution of (+)-10-camphorsulphonic acid. Cells with path lengths of 0.5 and 1 cm were used for the different experiments.

Peptide concentrations were typically 150–300 μM as determined by the absorbance of tyrosine at 275 nm using an extinction coefficient of 1420 cm^{-1} M^{-1} [17]. The micellar solutions of L-α-lysophosphatidylcholine (LPC) and sodium dodecyl sulphate (SDS) were prepared by dissolving the desired amount of detergent in 10 mM phosphate buffer (pH 6.5) and the resulting solutions were directly used for sample preparations. Three to five scans were averaged and corrected for baseline. Ellipticity is reported as $[\theta]$, the mean residue molar ellipticity (in deg·cm^2 $dmol^{-1}$ unit). The pH of the mixture was controlled after addition of the peptide.

2.3. Conductance assays in planar lipid bilayers

Measurement compartments were symmetric glass cells containing each 2 ml of electrolyte solutions (1 M KCl, 10 mM HEPES, pH 7.4) separated by a 10 μm thick Teflon film. The bilayer composition was either a mixture of palmitoyloleoylphosphatidylcholine/dioleoylphosphatidylethanolamine (POPC/DOPE, 2:1, w/w) or palmitoyloleoylphosphatidylcholine/dioleoylphosphatidylethanolamine/dioleoylphosphatidylglycerol (POPC/DOPE/DOPG, 2:1:1, w/w) purchased from Avanti Polar Lipids (Alabaster, AL, USA). Five to 10 μl of this lipid solution (0.5%

v/v in hexane) were spread on the top of the electrolyte solution. Planar lipid bilayers were formed by apposition of two lipid monolayers over a 150 μm diameter hole made in the Teflon film pretreated with hexadecane/hexane (1:40, v/v) to generate the hydrophobic environment required for bilayer formation [18]. The latter was monitored by measurement of capacitance responses and then the bare membrane was tested under voltage for control before peptide or protein addition.

The current fluctuations were amplified using a BLM 120 amplifier (Bio-Logic, Claix, France), filtered with an eight-pole Bessel filter AF180 (Bio-Logic) and digitally stored (DAT or CD system, Bio-Logic) before computer analysis (Satori v3.1 software from Intracell, Royston, UK).

3. Results and discussion

In order to measure the conformational propensity of the amphipathic peptide corresponding to the C-terminal domain of PP3, we performed circular dichroism experiments in different environments close to physiological conditions (i.e. buffered aqueous solutions or membrane-like environments). Then, the ability of the peptide to interact with membranes was tested using incorporation assays in planar lipid bilayers.

3.1. Circular dichroism

The secondary structure of the 23-mer peptide was investigated by CD measurements. In aqueous solution, the interpretation of CD spectra showed that the peptide is randomly structured (Fig. 2). The conformational study was also carried out in two other media: in electrically neutral LPC micelles and in negatively charged SDS micelles. In both cases, the CD spectra display two bands with negative ellipticity centred at 222 and 208 nm and one positive band centred at 190 nm, characteristics of an α-helical conformation. The transconformation of the peptide from random coil to helix appears to be independent of the net charge of detergent since the helical conformation is also observed in zwitterionic LPC micelles (Fig. 2A). These results are in line with a recent NMR study which demonstrated that the 98–135 C-terminal fragment of PP3 displays an α-helical conformation in the presence of lipids [14].

From numerical data, it is possible to estimate the helical content of a peptide from the ratio $[\theta]_{222}/[\theta]_{max}$ where $[\theta]_{222}$ is the ellipticity at 222 nm and $[\theta]_{max} = -39\,500 \times [(1-2.57/\text{number of residues})]$ [19]. For a 23-residue peptide, $[\theta]_{max} = -35\,086$ deg·cm^2 dmol^{-1}. Based on this approximation, the helical content of the 113–135 peptide in a lipid environment is about 50%. By NMR, the amount of helical structure observed for the 38-residue peptide is larger than 80% [14]. However, it is quite difficult to compare these two results since the length of the two peptides is too different and other parameters, such as the pH and the nature of the lipid/detergent, also differ between the two studies. Moreover, the presence of aromatic residues (especially Tyr) and more generally distortions in helical structure often lead to an underestimation of the helical content using CD spectroscopy by altering the ellipticity at 222 nm [20].

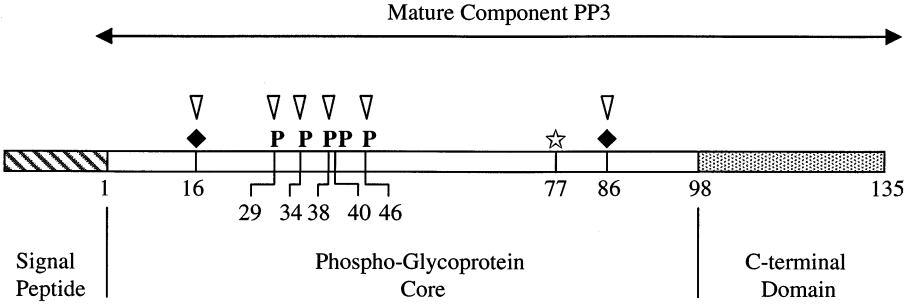

Fig. 1. Schematic representation of the primary structure of bovine component PP3 (accession No. in SwissProt databank: P80195). O-Linked phosphate groups are denoted P. O-Linked carbohydrate groups are symbolized with ♦ and the *N*-carbohydrate group with ☆. Arrows indicate the positions of posttranslational modifications conserved for bovine PP3 and murine GlyCAM-1.

3.2. Conductance measurements

Single-channel experiments were carried out at room temperature in artificial membranes with either component PP3 or a peptide corresponding to its

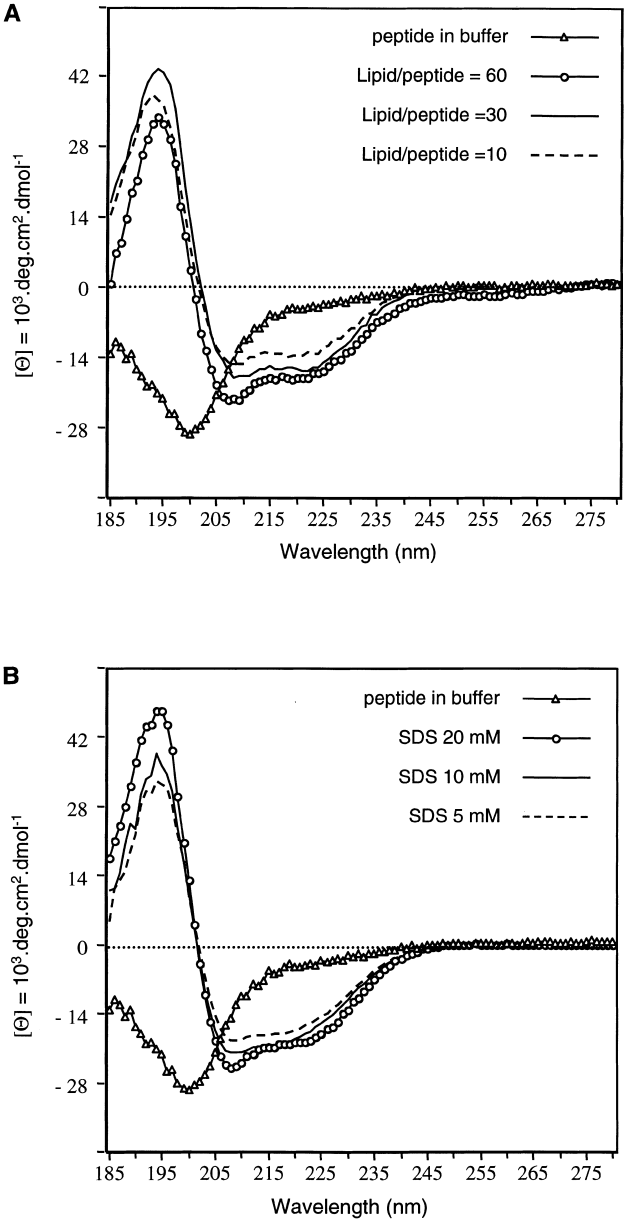

Fig. 2. Circular dichroism spectra of the 113–135 C-terminal fragment of bovine component PP3 in mixtures of (A) lysophosphatidylcholine at different lipid to peptide molar ratio or (B) at different concentrations of SDS. In both cases, the buffer was a 10 mM sodium phosphate buffer, pH 6.5. The experiments were carried out at room temperature. [Θ] is mean residue ellipticity.

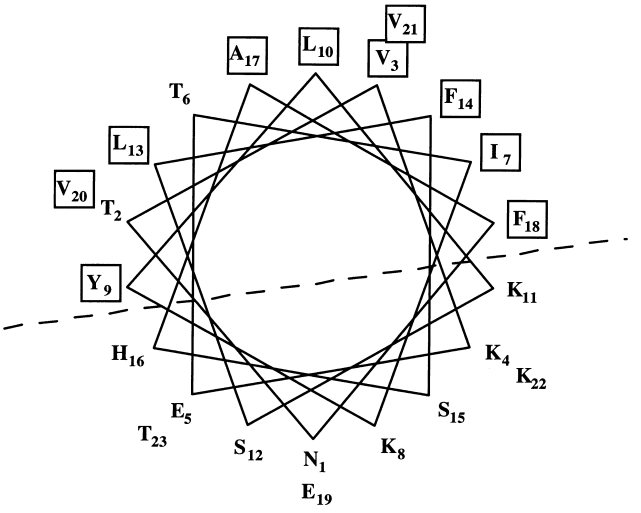

Fig. 3. Helical wheel representation of the 113–135 C-terminal fragment of bovine component PP3 (sequence NTVKETIKYLKSLFSHAFEVVKT). The hydrophobic residues are boxed.

113–135 C-terminal part. After bilayer formation and control, the protein or its C-terminal fragment was added to the conductance measurement cells. The subphase was stirred for 1 h to reach equilibrium of partition between the bulk solution and the membrane. Then, transmembrane potential differences were applied in order to analyse the pore-forming properties of the protein/peptide.

3.2.1. Component PP3

With PP3, no change of conductance of the zwitterionic lipid bilayer was observed even for high aqueous protein concentrations (up to 10^{-6} M). Channel formation was neither promoted by the addition of the protein in a detergent solution (2% (v/v) octylpolyoxyethylene (octylPOE)) nor by the addition of charged lipids such as DOPG (25%) to the lipid mixture forming the bilayer. Nevertheless, the rupture of the bilayer containing negatively charged lipids occurs at lower voltages, around 80–100 mV, instead of around 200 mV for a bare membrane. Just before disruption, bilayer perturbations were observed but no typical behaviour was recorded. This easier breakdown of the bilayer might be the result of a massive incorporation of the protein since PP3 has been shown to form spontaneously large and stable aggregates in milk and in aqueous solutions [11]. The mean diameter of these aggregates was es-

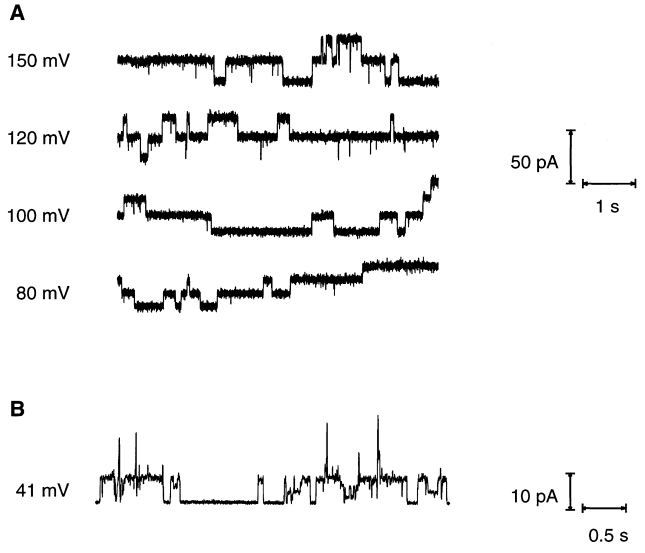

Fig. 4. Single-channel currents induced by the 113–135 C-terminal fragment of bovine component PP3 into Montal-Mueller bilayers at different voltages. These fluctuations were recorded with a peptide concentration of 20 nM. Electrolyte solutions were symmetrical 1 M KCl, 10 mM HEPES (pH 7.4). Experimental conditions: bilayers made of (A) POPC/DOPE (2:1); (B) POPC/DOPE/DOPG (2:1:1).

timated by light scattering experiments to be 24 ± 6 nm (unpublished result). The monomeric form of the protein, generated only in drastic conditions such as 8 M urea, was not tested in this study. However, the fact that the oligomeric form of PP3 is incorporated into planar lipid bilayers and strongly permeabilized them is well correlated with the ability of component PP3 to insert into phospholipid monolayers even at high initial pressure [12].

3.2.2. C-Terminal peptide

The choice of the C-terminal peptide was directed by two factors: (1) the peptide should preserve the same structural characteristics as the 98–135 C-terminal domain, i.e. the same mean charge ratio and the same hydrophobic/hydrophilic sectors according to the helical wheel projection (Fig. 3); (2) the length of the peptide (presumed in helical conformation) should allow a correct match with the bilayer thickness [21]. The 119–135 C-terminal fragment used in previous studies [12,13] was tested but no activity was detected. A longer peptide corresponding to the 113–135 C-terminal fragment of PP3 was thus synthesized.

In aqueous solutions, the 113–135 peptide (final peptide concentration in the bath in the range 10^{-8}–5×10^{-7} M) induced lysis of the bilayer. This effect was observed whatever the lipids used for bilayer formation and after applying several voltage ramps to promote peptide/bilayer interaction. It is likely that the peptide is greatly adsorbed at the bilayer/water interface at rest and then is incorporated massively under an applied electric field to irreversibly destabilize the bilayer.

The behaviour was significantly different when the peptide was solubilized in 2% octylPOE solution. In this case (with a peptide concentration in the bath of 2×10^{-8} M), discrete current fluctuations were recorded in planar lipid bilayers upon applying transmembrane voltages (Fig. 4). OctylPOE is a neutral detergent that probably limits the amount of peptide adsorbed at the membrane interface, thus allowing to characterize a pore-forming activity. In addition, it was shown previously that octylPOE has no permeabilizing effect on the bilayer at the concentration used here [22].

Analysis of current fluctuations pointed out a slightly different behaviour depending on the lipid mixture used to form the bilayer. Conductances measured in POPC/DOPE bilayers demonstrate a multilevel channel with two mainly observed conductances of 155 and 775 pS. Only the higher conductance level of 775 pS is seen in Fig. 4A and there are many identical conducting bundles which open and shut independently. These two conductance values are reminiscent of the two first conductance values displayed by alamethicin, a natural peptaibol with a pronounced pore-forming propensity (see, for example, [23]). Based on the conductance value, this suggests that conducting aggregates made of an assembly of peptides corresponding to the C-terminal domain of component PP3 would consist of a limited number of monomers. To estimate the number of helices involved in conducting pore formation, single-channel conductances can be compared to those induced by the peptaibol alamethicin [24,25]. With this natural peptide, first conductance levels of 20, 200 and 900 pS were observed and the first level of 20 pS was tentatively attributed to a trimeric bundle of alamethicin helices [24]. In our work, the two main conductance values could be compared to the values of the second and third open levels which might be associated with bundles consisting of four to six PP3 C-terminal helices.

In planar lipid bilayers with anionic phospholipids (DOPG), the lower conductance level of 175 pS was mainly observed (Fig. 4B). In these conditions, the voltage necessary to induce current fluctuations was greatly decreased as compared with zwitterionic bilayers and the single-channel events were not as well resolved as in POPC/DOPE bilayers. With these charged lipids, electrostatic attractions are enhanced between the anionic head group of the lipids and the highly basic peptide (p*I* 9.40). These interactions promote an important adsorption of the peptide at the bilayer/water interface thus favouring destabilization of the bilayer. After a short time of voltage application, the membrane was strongly permeabilized, often leading to a complete breakdown of the planar bilayer.

Two alternative mechanisms were described in detailed steps to explain the permeabilization of the membrane by amphipathic α-helical peptides [16]. First, in the barrel-stave mechanism, peptides bind to the membrane with an α-helical conformation. Transmembrane channels are then formed via a bundle of α-helical peptides such that their hydrophobic faces interact with the lipid core whereas the hydrophilic surfaces point inward, making an aqueous pore. In the alternate 'carpet' model, the α-helical peptides are in contact with the lipid head groups and cover the membrane in a carpet-like manner. In this case, the peptide/membrane interaction is driven by electrostatic interactions and the peptide does not need to adopt a canonical conformation. In this mechanism, the membrane-adsorbed peptide concentration is locally high and allows a kind of micellization of the membranous structure.

In this study, the peptide mimicking the C-terminal domain of PP3 adopts an α-helical structure whatever the charge of the membrane environment and displays an ability to induce well resolved current fluctuations in planar lipid bilayers. According to these observations, it can be postulated that in the mechanism of channel formation, bundles of PP3 C-terminal peptides are formed according to the previously described barrel-stave model by association of a reduced number of monomers. All together this argues that the peptide has an intrinsic tendency for helical folding and membrane interaction. Regarding the PP3 properties, it suggests that the 23-residue C-terminal part would allow anchoring to a yet undefined membrane structure.

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