Purification and reconstitution into liposomes of an integral membrane protein conferring immunity to colicin A

Vincent Geli *, Martine Knibiehler, Alain Bernadac and Claude Lazdunski

Centre de Biochimie et de Biologie Moléculaire du C N R S., Marseille, France

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1. SUMMARY

The immunity protein to colicin A protects producing cells from the action of this pore-forming toxin. It is located into the cytoplasmic membrane. This protein has been 'tagged' with an epitope from the colicin A protein for which a monoclonal antibody is available. The fusion protein (named VL1) has been purified after extraction from the membrane in two steps using a chromatofocusing and an immunoadsorbant chromatography. The purified protein has then been reconstituted into lipid vesicles.

2. INTRODUCTION

The plasmids which encode pore-forming colicins also direct the synthesis of a protein conferring upon its host strain the property of being insensitive (immune) to these toxins. The immunity protein for colicin A (designated Cai, for colicin A immunity) belongs to this class of polypeptides. It is encoded by a gene located in the intercistronic region between the colicin (caa) and lysis (cal) genes which form an operon on pColA [1,2]. Moreover, it is constitutively expressed at a low level but this expression is turned off when the transcription of the cca-cal operon is derepressed [1]. This protein is a quantitatively minor membrane protein and it can only be slightly overexpressed under the control of a strong promoter and because of a limited capacity for insertion into the inner membrane, the excess protein is rapidly degraded by proteases [3]. The topology of the 178 amino acid Cai polypeptide chain [4] within the cytoplasmic membrane, has recently been determined and site-directed mutagenesis has been used to try to define the polypeptide regions important for the function of Cai [5]. To understand how Cai can prevent the formation or the opening of ion channels formed by the COOH-terminal domain of colicin A [6], a useful approach would be to reconstitute an in vitro system. Cai inserted into ions charged lipid vesicles should prevent the release of ions by preventing the formation of channels by externally added colicin A. A necessary first step is therefore to purify Cai and reconstitute it into liposomes.
In this study, we describe a purification technique which has taken advantage of a foreign epitope used both as a 'tag' for assaying the degree of purification and as an antigen for immuno-adsorbant chromatography. We previously demonstrated that this epitope did not alter the function of Cai [3]. Conditions were then established to reconstitute the recombinant protein into lipid vesicles.

3. MATERIALS AND METHODS

3.1. Bacterial strains, plasmids, growth conditions

The bacterial host strain W3110 and plasmid pVL1 have been described previously [3]. The synthesis of the protein VL1 was induced by adding 300 ng/ml of mitomycin C to cultures grown in LB medium to an OD600 of 1.

3.2. Immunoadsorbant column

The monoclonal antibody 1C11 (IgG 2b class) has been previously described [7]. An immunoadsorbant column was made by covalently coupling this monoclonal antibody to cyanogen bromide activated Sepharose 4B (Pharmacia) as previously described [8].

3.3. Electron microscopy

Liposomes, deposited on grids, were first incubated for 60 min with the monoclonal antibody 1C11 (undiluted supernatant of hybridoma culture), then 30 min with a rabbit anti-mouse IgG serum (1/500 dilution) and then 30 min with gold coated protein A (1/25 dilution). After labelling, the grids were positive-negative stained with uranyl acetate (0.1%) in methyl cellulose (0.8%) [9].

3.4. SDS-polyacrylamide gel electrophoresis and immunoblotting

Protein analysis was carried out on 12.5% polyacrylamide gels. The electrophoretic transfer of proteins to nitrocellulose and their immunological detection were carried out as described [3].

4. RESULTS AND DISCUSSION

4.1. Structure of the VL1 protein

The amino-terminal region of Cai (12 residues) is directed to the cytoplasm and is not necessary for its protective function [3]. We have thus been able to replace these 12 residues with a soluble polypeptide tail of 178 residues from the NH2-terminal domain of colicin A [3]. The protein constructed contained the first 178 residues of colicin A with 3 additional amino acids fused to amino acid 13 of Cai. This 347 residue protein, designated VL1, could be induced in the presence of mitomycin C and detected in immunoblot analysis using a monoclonal antibody (1C11) directed against the NH2-terminal region of colicin A [3,7]. It contained 3 tryptophan and 16 tyrosine residues which gives a theoretical extinction coefficient of 8.36 (E 1% = 8.36). Due to its high content in basic residues (17 Lys and 16 Arg as compared to 8 Asp and 9 Glu residues) it has a net charge of +16.

4.2. Purification of the VL1 protein

A culture of W3110 (pVL1) [3] was induced for 3 h in the presence of mitomycin C (300 ng/ml). After harvesting, the cells (1 liter) were disrupted by lysozyme-EDTA treatment and osmotic shock as described [10]. The total membrane fraction was obtained by centrifugation at 105,000 × g for 1 h (Rotor 60 Ti, Beckman). The membrane pellet was manually homogenized in 8 ml of cold buffer containing 75 mM Tris-Acetate, 1% Triton X-100, 1.5 mM ethylenediamine tetracetate (EDTA), 0.5 mM N-ethyl maleimide (NEM), 0.2 mM phenylmethyl sulfonyl fluoride (PMSF) at pH 9.3. Final homogenization was performed by (two × 20 seconds) sonication in ice. The homogenate was immediately diluted ten fold with the buffer described above but devoid of Triton X-100 such that the final concentration of detergent was 0.1%. After centrifugation (20,000 × g for 30 min), the supernatant was immediately applied to a chromatofocusing column (MonoP, Pharmacia). Isoelectric focusing was described, by Mankowich et al., as the first step of purification of the colicin Ia immunity protein [11]. The starting buffer was at pH 9.3 and the elution buffer (PBE, Pharmacia)
was at pH 6 (containing also 0.1% Triton X-100, 0.5 mM NEM, 0.2 mM PMSF). In the self-forming pH gradient obtained, the protein was eluted and fractions containing VL1 were detected by immunoblot analysis (Fig. 1). Due to the presence of Triton X-100 the elution profile at 280 nm could not be exploited. One of the uses of this chromatographic step was to remove proteases which otherwise rapidly digested VL1 (data not shown). The pooled fractions containing VL1 were dialyzed against 10 mM phosphate buffered saline (0.15 M NaCl, pH 7) (PBS) containing 0.1% Triton X-100. This solution was then slowly percolated through an immunoadsorbant column containing the monoclonal antibody 1C11. The column was washed with 3 volumes of PBS, 0.1% TX-100 and then eluted with 0.2 M HCl-Glycine containing 0.1% TX-100 at pH 2.2. As shown in Fig. 2, the protein VL1 was eluted in the first acid fractions which were immediately neutralized by the addition of 0.75 M Tris-Base. A routine yield of about 0.5 mg of purified VL1 per liter of culture, was obtained.

4.4. Reconstitution of VL1 into lipid vesicles

Phosphatidyl choline (5 mg/ml) from Soybean or phosphatidylethanolamine, phosphatidylglycerol and cardiolipin (PE/PG/cardiolipid with a molar ratio of 70:25:5) in 2% polyoxyethylene (POE) was added to the pooled fractions eluted from the affinity chromatography column and the mixture was extensively dialysed against 4 x 6 liters of a buffer containing 20 mM Tris-HCl, 1 mM EDTA, pH 7.8. Two techniques were used to demonstrate the reconstitution. First, the liposome solution was applied to a 5–35% sucrose gradient and centrifuged (rotor SW41, Beckman) at 280 000 × g for 36 hours. The gradient was eluted and for each fraction, after chloroform extraction, the presence of lipid was detected by thin layer chromatography (chloroform 65/MEOH 25/water 4/acetic acid 2) and molybdate staining. In these fractions, the presence of the VL1 protein was analyzed by dot blot detection using the monoclonal antibody 1C11 (Fig. 3).

The presence of VL1 in the lipid vesicles was also demonstrated by electron microscopy. The
Fig. 3. Reconstitution of the VL1 protein into liposomes. Fraction 20 corresponds to the bottom of the 5-35% sucrose gradient. After thin layer chromatography, the distribution of the phospholipids (♦) was quantified by measuring the area of the spot revealed with the molybdate staining. VL1 (♦) was quantified by scanning nitrocellulose paper after dot blot analysis.

Fig. 4. Immunogold labelling of VL1 reconstituted into the liposomes. Original magnification ×120000. (a) Negative staining of the VL1 liposome solution using uranyl acetate. (b) Immunogold-labeling of the VL1 protein with the monoclonal antibody 1C11. (c) Same as b using a monoclonal antibody (lgG 2B class) specific for rabbit endoplasmic reticulum.

fact that the hydrophilic tail could be labelled from the outside of the vesicles (Fig. 4) indicated that at least some of the VL1 polypeptides had the reverse orientation as compared to that in vivo (the NH2-terminal region is cytoplasmic, [3]). Since in the process that we used for liposome formation, a random insertion would be expected, about 50% of the polypeptide chains should have the proper orientation.

To conclude, we have now purified a functional hybrid protein conferring immunity to colicin A [3]. This protein has been successfully reconstituted into lipid vesicles.

At alkaline pH, we did not find any specific binding of radiolabelled colicin A to reconstituted lipid vesicles. It has been previously demonstrated that the equivalent surface pressure in liposomes is too high to allow penetration of colicin A [12]. This was considered as a possible reason for the lack of specific interaction. One thus tried at acidic pH, a condition promoting high extent of colicin A binding to compressed lipid monolayers [12,13] or to liposomes [14]. One then observed binding of radiolabelled colicin A to VL1 containing vesicles but not to a larger extent than to control vesicles devoid of VL1 protein. The signal/background ratio was then eventually too small to allow detection of any significant difference. There are other possibilities to explain the lack of specific binding of colicin A to VL1-containing vesicles. For example, a significant percentage of the VL1 polypeptide in vesicles has an opposite orientation to that in the cells since their N-terminal region is accessible to the monoclonal antibody 1C11. One cannot rule out the hypothesis that 100% of the VL1 molecules have this same orientation, some bias existing during the reconstitution process. The lack of membrane potential in the vesicles is also a difference with the in vivo situation. All these parameters must be carefully studied. This is now possible with the system described in this work and we are continuing our efforts since we feel that this simple system should be helpful in determining the mechanism of action of the immunity protein.

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