Volume 329, number 1,2, 25–28 FEBS 12852 August 1993

A rapid and efficient purification method for recombinant annexin V for biophysical studies

A. Burger, R. Berendes, D. Voges, R. Huber and P. Demange

Max-Planck-Institute für Biochemie, D-82152 Martinsried, Germany

Received 25 June 1993

Annexin V binds in a calcium-dependent manner to acidic phospholipids and exhibits ion channel activity in vitro. We are investigating mutants of annexin V by single channel measurements, X-ray crystallography and electron microscopy in order to understand the structure–function relationships of the ion channel activity. We describe here a method to obtain very pure recombinant annexin V required for such studies. The initial step is the mild opening of the bacterial cells by an osmotic shock. In the purification procedure, use is made of the reversible calcium-mediated binding of annexin V to liposomes. In the last purification step the protein is subjected to ion-exchange chromatography and elutes as a single peak free of any detectable contaminants.

Annexin; Ion channel; Protein purification; Crystallization; Patch clamp; Electron microscopy

1. INTRODUCTION

Annexin V is a member of the annexin family of proteins, which share the common property of binding to acidic phospholipids in a calcium-dependent manner [1–4]. More than ten members of this family have been described. They are distinct from the 'EF-hand' family of proteins [5]. Annexins have been identified in higher and lower eukaryotes and constitute a significant amount of the cellular protein in a variety of cell types [3]. Their biochemical and immunological properties are closely related. Their sequence is composed of four (in the case of annexin VI eight) repeats, which are homologous [6]. The N-termini are diverse in sequence and different in length and can also be altered by alternative splicing [7,8]. The in vivo role of the annexins is still unclear. It has been proposed that they are involved in anti-coagulation [9], anti-inflammatory processes [10], participate in cell replication or differentiation [11] and exocytosis [12]. Annexins catalyze membrane fusion in vitro and bind to components of the cytoskeleton [13,14]. Annexin V and VII form voltage-gated ion channels in vitro [15–18]. We have presented the three-dimensional structure of annexin V from human placenta at a resolution of 2.0 Å [19,20]. The molecule, which is almost entirely α-helical, has an overall flat, slightly curved shape, with two faces: a convex and a concave one. The calcium binding sites are at the convex face, whereas the N-terminus is located at the concave face. Each of the four repeats folds into a compact domain, consisting of five α-helices. The domains are arranged in a planar, cyclic array, surrounding a hydrophilic pore with charged residues and water molecules with a well-defined electron density. We suggested that the hydrophilic pore is the ion pathway [19,20]. We are studying the ion channel activity of human annexin V to get an insight into the structure–function relationships. We proposed that the annexin V molecule disturbs the membrane by electroporation, thereby making it ion permeable in an unspecific manner [21], while the selectivity for calcium resides in the protein moiety. Our approach is by site-specific mutagenesis and characterization of the mutated protein by X-ray-crystallography, electron microscopy, spectroscopy and electrophysiological single channel measurements. For these biophysical methods, very pure protein is required. We describe here a simple, short and reliable method for obtaining pure recombinant annexin V, as judged by silver-stained SDS-PAGE and HPLC-profile analysis. The most important improvement is the avoidance of the otherwise inevitable co-purification of other factors by the mild opening of the bacterial cells.

Correspondence address: A. Burger, Max-Planck-Institute für Biochemie, D-82152 Martinsried, Germany. Fax (49) (89) 8578-3516.

Abbreviations: DNase, desoxyribonuclease; EDTA, ethylene diamine tetraacetic acid; *E. coli*, *Escherichia coli*; HPLC, high-pressure liquid chromatography; IPTG, isopropylthiogalactoside; PAGE, polyacrylamide gel-electrophoresis; PMSF, phenylmethylsulfonyl fluoride; RNase, ribonuclease; SDS, sodium dodecyl sulfate; v/v, volume per volume; w/v, weight per volume.

2. MATERIALS AND METHODS

The expression vector, pTRC99A-PP4 [22], and the *E. coli* strain, W3110, were a kind gift of the Behring Institutes, Marburg. Bactotryptone and yeast extract were from Difco, MI, USA. Pepstatin A, brain extract type III, bis-Tris and ampicillin were from Sigma, Deisenhofen. IPTG was from Gerbu, Gaiberg. Lysozyme, RNase (bovine

pancreas) and DNase I were from Boehringer-Mannheim, Mannheim. The column material DEAE–Sepharose was from Pharmacia, Freiburg. All other reagents were from Merck, Darmstadt in the p.A. purity grade.

2.1. *Purification of recombinant annexin V*

An overnight culture of *E. coli* W3110, transformed with pTRC99A-PP4 grown at 33°C in LB medium with 50 μg/ml ampicillin is diluted 5-fold into fresh LB medium. When the OD (optical density) at 600 nm has reached a value of 1.5–2, IPTG is added to a final concentration of 1 mM. After 2–4 h growth, the bacterial cells are harvested by centrifugation (5,000 × *g*, 15 min, 4°C). The cells are resuspended in spheroblast buffer (0.5 mM EDTA, 750 mM Sucrose, 200 mM Tris, pH 8.0) and a solution of lysozyme in spheroblast buffer (10 mg/ml) is added to a final concentration of 1 mg/ml. Immediately the 7-fold volume of Spheroblast buffer, diluted 1:1 with water, is poured into the lyzozyme solution and the suspension is incubated on ice for 30 min under gentle shaking. The spheroblasts are collected by centrifugation (30 min, 14,000 × *g*, 4°C) and resuspended in ultracentrifugation buffer (2 mM EDTA, 5 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml RNase (bovine pancreas), 0.1 mg/ml DNase I, 2 mM PMSF, 0.5 μg/ml pepstatin A, 0.1% (w/v) Triton X-100, 20 mM Tris, pH 8.0). The bacterial cells are opened by osmotic shock. To remove the cell debris the suspension is centrifuged at 100,000 × *g* overnight.

Lyophilized brain extract containing 80–85% phosphatidylserine is dissolved in a mixture of chloroform/methanol, 2:1, and dried down under a stream of nitrogen. It is redissolved in ether and dried down again. The phospholipid mixture is resuspended by vortexing in liposome buffer (100 mM NaCl, 3 mM $MgCl_2$, 20 mM Tris, pH 8.0) and sonicated for 15 min. The supernatant of the ultracentrifugation is carefully removed and combined with the liposomes. Then the solution is adjusted to an excess of 5 mM $CaCl_2$ and stirred for 30 min on ice. The mixture is ultracentrifuged for 45 min at 40,000 × *g* and 4°C, the supernatant is removed and the pellet washed with a solution of 5 mM $CaCl_2$, 100 mM NaCl, 3 mM $MgCl_2$, 20 mM Tris, pH 8.0 and centrifuged (30 min, 50,000 × *g*, 4°C). The pellet is resuspended in 10 mM EDTA, 100 mM NaCl, 3 mM $MgCl_2$, 20 mM Tris, pH 8.0, and the liposomes are removed by centrifugation for 1 h at 50,000 × *g* and 4°C. The supernatant is then dialyzed against 20 mM bis-Tris, pH 6.0, 0.02% (w/v) sodium azide. If there is any precipitate, consisting of degraded protein, it is removed by centrifugation (20,000 × *g*, 30 min, 4°C). The protein solution is loaded on a DEAE–Sepharose column (Diameter 14 mm, bed height 150 mm) and washed with at least 3 column vols. of 20 mM bis-Tris, pH 6.0. Then the protein is eluted with a linear NaCl gradient (0–200 mM) at a flow rate of about 0.4 ml/min (see Fig. 1 for the elution profile). Annexin V elutes as a single peak between 80 and 90 mM NaCl. The yield is 1–5 mg/l of *E. coli* culture. The purity of the protein is tested by SDS-PAGE and HPLC-profile analysis. Fig. 2 shows a 12% SDS-polyacrylamide gel [23], which has been silver-stained [24]. The purified protein also elutes as a single peak from a HPLC-column (see Fig. 3).

2.2. *HPLC analysis*

The HPLC system was composed of two pumps (Model 420, Kontron instrument) and a spectromonitor variable wavelength UV detector (Spectroflow 757, Kratos Analytical Instrument) linked to a recorder (Model BD41, Kipp & Zonen). HPLC analysis was performed on a C4 reverse-phase column (Nucleosil 250*4 5 μm; Macherey-Nagel) eluted with a linear gradient 30–70% (v/v) acetonitrile (Lichrosolv gradient grade; Merck, Darmstadt) in 0.1% (w/v) trifluoroacetic acid (Uvasol; Merck, Darmstadt) for 60 min at a flow rate of 1 ml/min. Detection was measured at 280 nm.

3. RESULTS AND DISCUSSION

There exist a variety of reports on the purification of members of the annexin family, most of them describing the purification of the proteins from eukaryotic cells, a few dealing with recombinant annexins (see for example [25–28]). No purification procedure has been published so far yielding annexin which is pure on a silver-stained SDS-gel. The staining of proteins with silver salts is 100-to 1000-fold more sensitive than the staining with Coomassie blue. We report here a purification procedure which uses the reversible calcium-mediated binding of annexin V to liposomes, modified from previous approaches [27,29]. Our expression system yields soluble annexin V and no inclusion bodies. The first and very important step is the mild opening of the bacterial cells by an osmotic shock. Bacterial cell rupture by ultrasonication or French press followed by purification steps, as described above did not yield pure protein. Annexin V bound a variety of impurities very tightly. These were not identified. The addition of detergents (lysophosphatidylcholine, sodium desoxycholate, SDS), denaturing agents (urea) or high concentrations of NaCl and chaotropic agents (KI) did not improve the situation (data not shown).

When the cells were opened by mechanical shearing forces, the recombinant annexin V was not pyrogen free and still contained lipopolysaccharides (J. Roemisch, personal communication), which could indicate that components of the outer cell wall stick to annexin V. In order to avoid this, we prepared spheroblasts, which still have the inner cell wall intact. By the following

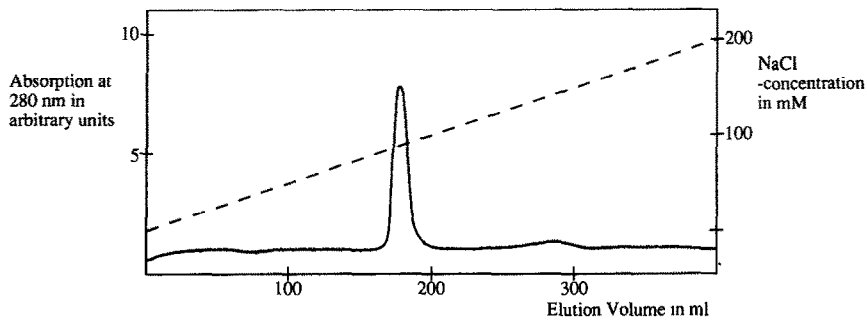

Fig 1. Elution profile of annexin V from a DEAE–Sepharose column with a linear NaCl gradient (dotted line) from 0–200 mM. The single peak eluting between 80 and 90 mM NaCl contains annexin V and was analyzed by SDS-PAGE (see Fig. 2).

centrifugation step, soluble components were removed. The isolated spheroblasts were then resuspended in a buffer containing no sucrose, and were thereby opened by the osmotic shock. This mild opening should also ensure that only large membrane fragments are produced. Tiny fragments, which cannot be removed by the following centrifugation, were largely avoided [30]. All components interacting with the inner cell membrane were spun down during the ultracentrifugation, a step which probably diminished the yield but was inevitable if a high grade of purity was to be obtained. Afterwards, liposomes of bovine brain extract type III were prepared, which has a high content of phosphatidylserine essential for annexin V membrane binding. The reversible calcium binding step served as an affinity step and very selectively removed most of the other proteins as can be seen in Fig. 2. These liposomes were prepared from bovine brain extract, because a pure annexin V preparation was not achieved when using *E. coli* membranes in this affinity step.

At this step the protein is not yet pure. In some preparations other protein bands were still detectable (not seen in Fig. 2), and also the ratio of the absorbance at 260 nm to 280 nm was only about 1:1. The remaining contaminants were all removed in the last purification step with anion-exchange chromatography at pH 6.0, about one pH unit above the isoelectric point (4.8) of annexin V. Most contaminants present did not bind to the column and were found in the flow through. Other contaminants remained bound to the column and eluted at much higher NaCl concentrations.

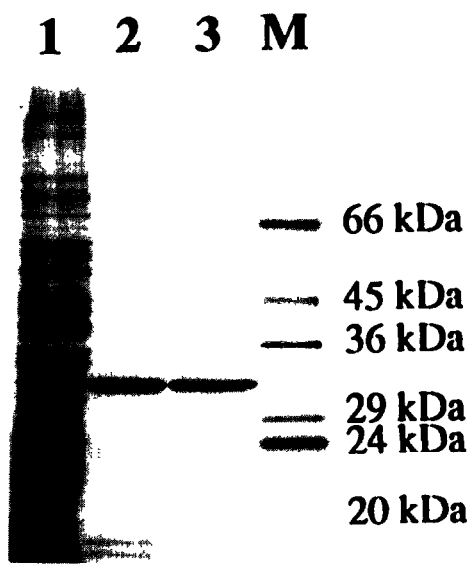

Fig. 2. Silver-stained 12% SDS-polyacrylamide gel of the various purification steps. The very faint protein bands with low molecular weight in lanes 2 and 3 are proteolytically degraded products of annexin V. Lane M, Marker Dalton VII-L (Sigma, Deisenhofen) with the indicated molecular masses (1 μg). Lane 1, cell extract after the ultracentrifugation step (diluted 1:20); lane 2, supernatant after the treatment of liposomes with EDTA (1.2 μg protein); lane 3, annexin V, eluted from DEAE–Sepharose (see Fig. 1) (1.2 μg protein).

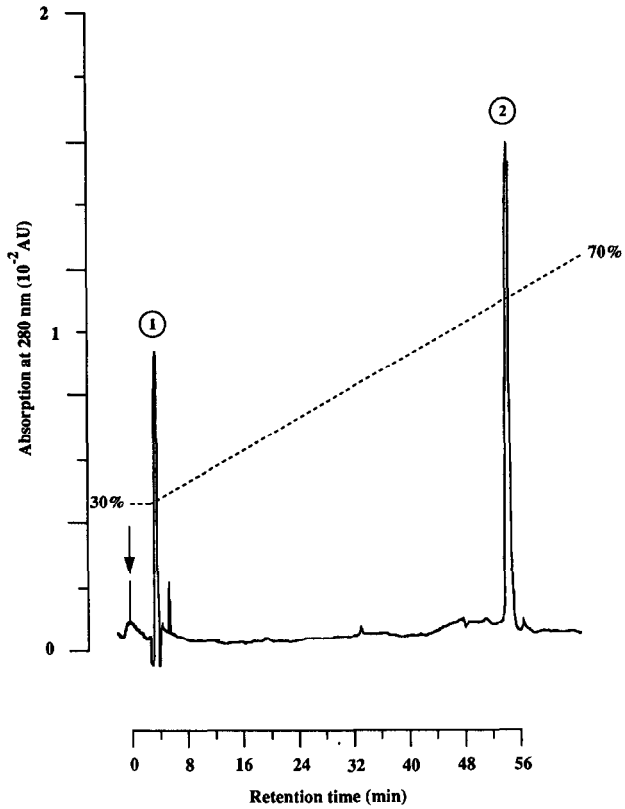

Fig. 3. HPLC analysis on a reverse-phase C4 column of annexin V (AU, arbitrary units). Peak 1 corresponds to the dead volume of the column and peak 2 to highly purified annexin V. The arrow indicates the starting point and the dashed line shows the acetonitrile gradient.

By this method we reached reasonable yields, which are sufficient for our further studies. The purity is further underlined by the crystallization of the mutant proteins by the vapour diffusion method (Berendes et al., submitted for publication; Burger et al., manuscript in preparation). We obtained crystals, which diffract X-rays beyond 2.0 Å resolution. Also two-dimensional crystallization has been successful and the protein was very active in single channel measurements (Berendes et al., submitted for publication; Burger et al., manuscript in preparation; Voges et al., manuscript in preparation).

In summary, we can obtain very pure annexin V by opening the bacterial cells by osmotic shock and removal of the components of the *E. coli* cell wall. The affinity step of the calcium-mediated binding to liposomes is a fast and efficient way to remove nearly all other proteins. The last chromatography step ensures the removal of further contaminants, yielding very pure material suitable for biophysical studies.

Acknowledgements: We thank Drs. J. Römisch, E. Paques, U. Grundmann, and G. Zettlmeißl, Behring Institutes, Marburg, for assistance, and for the expression vector pTRC99A-PP4 and the *E. coli* strain W3110. Furthermore, we thank Kai Matuschewski for valuable assistance.

REFERENCES

[1] Klee, C.B. (1988) Biochemistry 27, 6645–6653.
[2] Smith, V.L., Kaetzel, M.A. and Dedman, J.R. (1990) Cell Reg. 1, 165–172.
[3] Moss, S.E., Edwards, H.C. and Crumpton, M.J. (1991) in: Novel Calcium-binding proteins (Heizmann, C.W., ed.) Springer-Verlag, Berlin.
[4] Huber, R., Berendes, R., Burger, A., Luecke, H. and Karshikov, A. (1992) Behring Inst. Mitt. 91, 107–125.
[5] Kretsinger, R.H. (1980) Crit. Rev. Biochem. 8, 119–174.
[6] Barton, G.J., Newman, R.H., Freemont, P.S. and Crumpton, M.J. (1991) Eur. J. Biochem. 198, 749–760.
[7] Magendzo, K., Shirvan, A., Cultraro, C., Srivastava, M., Pollard, H.B. and Burns, A.L. (1991) J. Biol. Chem. 266, 3228–3232.
[8] Towle, C.A., Weissbach, L. and Treadwell, B.V. (1992). Biochim. Biophys. Acta 1131, 223–226.
[9] Römisch, J., Schorlemmer, U., Fickenscher, K., Paques, E.P. and Heimburger, N. (1990) Thromb. Res. 60, 355–366.
[10] Goulding, N.J. and Guyre, P.M. (1992) Immunol. Today 13, 295–297.
[11] Schlaepfer, D.D. and Haigler, H.T. (1990) J. Cell Biol. 111, 229–238.
[12] Creutz, C.E. (1992) Science 258, 924–931.
[13] Gerke, V. and Weber, K. (1984) EMBO J. 3, 227–233.
[14] Cheney, R.E. and Willard, M.B. (1989) J. Biol. Chem. 264, 18068–18075.
[15] Burns, A.L., Magendzo, K., Shirvan, A., Srivastava, M., Rojas, E., Alijani, M.R. and Pollard, H.B. (1989) Proc. Natl. Acad. Sci. USA 86, 3798–3802.
[16] Pollard, H.B. and Rojas, E. (1988) Proc. Natl. Acad. Sci. USA 85, 2974–2978.
[17] Pollard, H.B., Burns, A.L. and Rojas, E. (1990) J. Membr. Bi 117, 101–112.
[18] Rojas, E., Pollard, H.B., Haigler, H.T., Parra, C. and Burns, A.L. (1990) J. Biol. Chem. 265, 21207–21215.
[19] Huber, R., Römisch, J. and Paques, E. (1990) EMBO J. 9, 3867–3974.
[20] Huber, R., Berendes, R., Burger, A., Schneider, M., Karshikov, A., Luecke, H., Romisch, J. and Paques, E. (1992) J. Mol. Biol. 223, 683–704.
[21] Karshikov, A., Berendes, R., Burger, A., Cavalie, A., Lux, H.-D. and Huber, R. (1992) Eur. Biophys. J. 20, 337–344.
[23] Lämmli, U.K. (1970) Nature 227, 680–685.
[24] Schoenle, E.J., Adams, L.D. and Sammons, D.W. (1984) J. Biol. Chem. 259, 12112–12116. Sammons, D.W., Adams, L.D. and Nishizawa, E.E. (1981) Electrophoresis 2, 135–141.
[25] Gerke, V. and Weber, K. (1985) J. Biol. Chem. 260, 1688–1695.
[26] Römisch, J. and Heimburger, N. (1990) Biol. Chem. Hoppe-Seyler 371, 383–388.
[27] Kaplan, R., Jaye, M., Burgess, W.H., Schlaepfer, D.D. and Haigler, H.T. (1988) J. Biol. Chem. 263, 8037–8043.
[28] Thiel, C., Weber, K. and Gerke, V. (1991) J. Biol. Chem. 266, 14732–14739.
[29] Smallwood, M., Keen, J.N. and Bowles, D.J. (1990) Biochem. J. 270, 157–161.
[30] Schnaitman, C.A. (1984) in: Manual of Methods for General Bacteriology (Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, W.A., Krieg, N.R. and Phillips, G.B. eds.) pp. 52–61, American Society for Microbiology, Washington, DC, USA.