Volume 233, number 1, 148–152 FEB 05912 June 1988

High-performance liquid chromatography of the main polypeptide (MP26) of lens fiber plasma membranes solubilized with *n*-octyl β-D-glucopyranoside

S. Manenti, I. Dunia, M. le Maire°, and E.L. Benedetti

Institut Jacques Monod, Centre National de la Recherche Scientifique (CNRS), Université Paris VII, 75251 Paris Cédex 05 and °*Centre de Génétique Moléculaire, Laboratoire du Centre National de la Recherche Scientifique Associé à l'Université Paris VI, 91190 Gif-sur-Yvette, France*

Received 7 April 1988

The main polypeptide isolated from lens fiber membrane has been solubilized in octyl glucoside and studied by gel filtration in high-performance liquid chromatography (HPLC). The combination of $S_{20,w}$ value obtained from analytical ultracentrifugation and Stokes radius determined by HPLC of the soluble fraction indicates that more than 90% of the protein is monomeric. The solubilization of the protein seems to be dependent upon the presence of the NH_2 and COOH terminal sequences, since proteolytic degradation of MP26 which removes these terminal sequences is less soluble than the uncleaved polypeptide. Moreover, there is a higher amount of oligomer after proteolysis. Fatty acid analysis by gas chromatography shows that the insoluble membrane fraction from both cortical and nuclear fibers comprises a special class of long (C22) saturated fatty acids (behenic acid).

Membrane protein; Solubilization; Gel chromatography; Monomer

1. INTRODUCTION

Differentiation of epithelial cells into cortical fibers in the lens, is characterized by the biosynthesis of an intrinsic polypeptide of 28 kDa (called MP26), representing more than 50% of the total membrane proteins, and assembled in junctional domains. The molecular model of MP26 based on amino acid analysis and cDNA cloning [1] conceives the existence of six transmembrane α-helical domains and the presence of the NH_2 and COOH terminal segments exposed at the cytoplasmic side of the membrane. It is remarkable that among these α-helices, one of them displays an amphipathic nature which might generate a potential domain for the hydrophilic transmembrane channel. More recent experiments of Horwitz and Bok [2] on MP26 solubilized in octyl glucoside tend to prove that this polypeptide comprises 50% of α-helical domains and about 20% of β-sheets. A number of experiments have shown that MP26 in reconstituted proteoliposomes [3] or black film [4] forms a transmembrane channel. Further information concerning the type of structure that MP26 and its natural proteolytic derivative (MP22) adopt in the lipid bilayer clearly indicates that this polypeptide in reconstituted proteoliposomes forms tetragonal oligomers either randomly distributed or clustered in geometrical arrays [5]. Moreover, the exposed domains of the reconstituted oligomers are potential sites of membrane to membrane interaction. It remains to be established whether the MP26 and MP22 reconstituted oligomers resulting from the reassociation of single copies of this polypeptide solubilized in the octyl glucoside, or reinsertion of non-disassembled oligomers. Preliminary results by ultracentrifugation ruled out that the par-

Correspondence address: E.L. Benedetti, Institut Jacques Monod Centre National de la Recherche Scientifique, Université Paris VII, 75251 Paris Cédex 05, France

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

ticulate membrane domains found by freeze fracture experiments of the reconstituted proteoliposomes were merely remnants of junctional domains insoluble in the octyl glucoside [5]. The present paper refers to the study of solubilized MP26 and MP22 in octyl glucoside, by high-performance liquid chromatography (HPLC). Moreover, analysis of the lipid composition of the octyl glucoside soluble and insoluble fractions has been carried out. It is shown that a high proportion of the two polypeptides is solubilized by octyl glucoside in its monomeric form. V8 proteolysis of NH_2 and COOH terminal sequences yields a soluble fraction which contains a higher amount of oligomeric form. Moreover, the lipid composition of the octyl glucoside insoluble membrane material is characterized by a high content of long saturated fatty acid chains which might trap the protein tightly in a rather insoluble and solid bilayer.

2. MATERIALS AND METHODS

Purification of MP26 was performed as described in [5]. Briefly, a membranous fraction, called U-Na fraction, was obtained by repeated treatments of the fibers with 6 M urea and 0.1 N NaOH. Solubilization of MP26 from this fraction was performed with a 10% octyl glucoside solution, in Tris-HCl buffer (50 mM Tris, 100 mM NaCl, 1 mM azide, pH 7.3). The octyl glucoside soluble (called Csol. and Nsol., respectively, for cortex and nucleus fibers) and insoluble (Cins. and Nins.) fractions were separated by centrifugation at 120000 × g, for 1 h. The supernatant contained 70–80% of the proteins of the membranous fraction [5].

2.1. *Fatty acid analysis*

Fatty acid analysis was performed on the cortex and nucleus soluble and insoluble fractions, as described below. Lipids were extracted according to Bligh and Dyer [6], based on a separation between an aqueous and an organic (chloroform) phase. Lipids were then conserved in a toluene-ethanol solution (4:1, v/v) at 4°C. We used the Carreau and Dubacq [7] method, consisting of a treatment with sodium methylate, to obtain methylic esters of the total lipidic extract. These esters were extracted with pentane, and conserved in chloroform. We used capillary gas chromatography (Girdell chromatography) for the analysis of these samples, with a 25 m × 0.25 mm column. Helium was used as the vector gas and the temperature was 185°C.

2.2. *Soluble fraction analysis*

Soluble fraction analysis by HPLC was processed on a TSK 3000 LKB column, of 60 cm length, used with a Millipore Waters model 510 pump system. Isocratic elution was performed with Tris-HCl buffer containing 2.5% octyl glucoside. The flow rate was 0.75 ml/min and 400 μl of each sample were injected for the different experiments. Samples corresponding to the different peaks were collected, and analyzed by electrophoresis, after precipitation of proteins with 10% trichloroacetic acid (TCA). This was done incubating 1–1.5 ml of the soluble fractions with 10% TCA for 10 min, followed by centrifugation for 15 min at 10000 × g. The pellets were resuspended in electrophoresis sample buffer (50 μl) and neutralized with 1 N KOH. Electrophoresis was performed according to Laemmli [8] in 15% polyacrylamide gels. The gels were stained with silver. The same experiments were performed on a proteolyzed fraction of MP26, referred to as solubilized V8 fraction (V8 sol.). This fraction was obtained by treating the C U-Na fraction with V8 protease, as described in [9], and by solubilizing the resulting membrane fraction. SDS-PAGE analysis of the protease-treated membrane fraction shows the presence of MP21, very similar to MP22, the natural proteolytic derivative of MP26 [5,9].

The calibration of the TSK 3000 SW HPLC column was performed with water-soluble proteins of known Stokes radius as previously described [10,11]. The molecular mass determination of the protein-detergent complex (M*) is based upon the following equation [12]:

$$M^* = \frac{s6\pi\eta RsN}{1 - \bar{V}^*\varrho} \quad (1)$$

where s is the sedimentation coefficient measured with a Beckman model E analytical ultracentrifuge equipped with a photoelectric scanner [5], η is the viscosity coefficient of the solution, Rs the Stokes radius obtained from HPLC column calibration, N is the Avogadro's number and $1 - \bar{V}^*\varrho$ is the buoyancy of the complex.

$$\bar{V}^* = \bar{V}_p + \delta\bar{V}_d/1 + \delta \quad (2)$$

where $\bar{V}_p$ is the partial specific volume of the protein calculated here to be 0.735 cm^3/g from the amino acid sequence [1] and $\bar{V}_d$ the partial specific volume of octyl glucoside (0.859 cm^3/g). δ is the detergent binding ratio in g/g of protein. It may be noted that the possible presence of a few phospholipids which may remain bound to the protein after solubilization does not affect the molecular mass determination, since their partial volumes are very close to 1 cm^3/g [13]. The molecular mass of the protein part of the complex (M_p) is calculated from:

$$M^* = M_p(1 + \delta) \quad (3)$$

3. RESULTS AND DISCUSSION

The elution profile of the octyl glucoside solubilized cortical membrane fraction when the absorption is measured at 280 nm, is composed of three main peaks called P1, P2 and P3 (fig.1a). In some experiments, a variable amount of insoluble material is eluted in the void volume (P0). When the supernatant of the centrifugation at 100000 × g prior to HPLC analysis was very carefully removed, P0 was not present in the elution profile (see fig.1c,d). P3 contains about 80% of the absorption, P2 about 15% and P1 about 3%. Hence we assume that P3 contains almost exclusively protein moiety. This assumption is reinforced by fur-

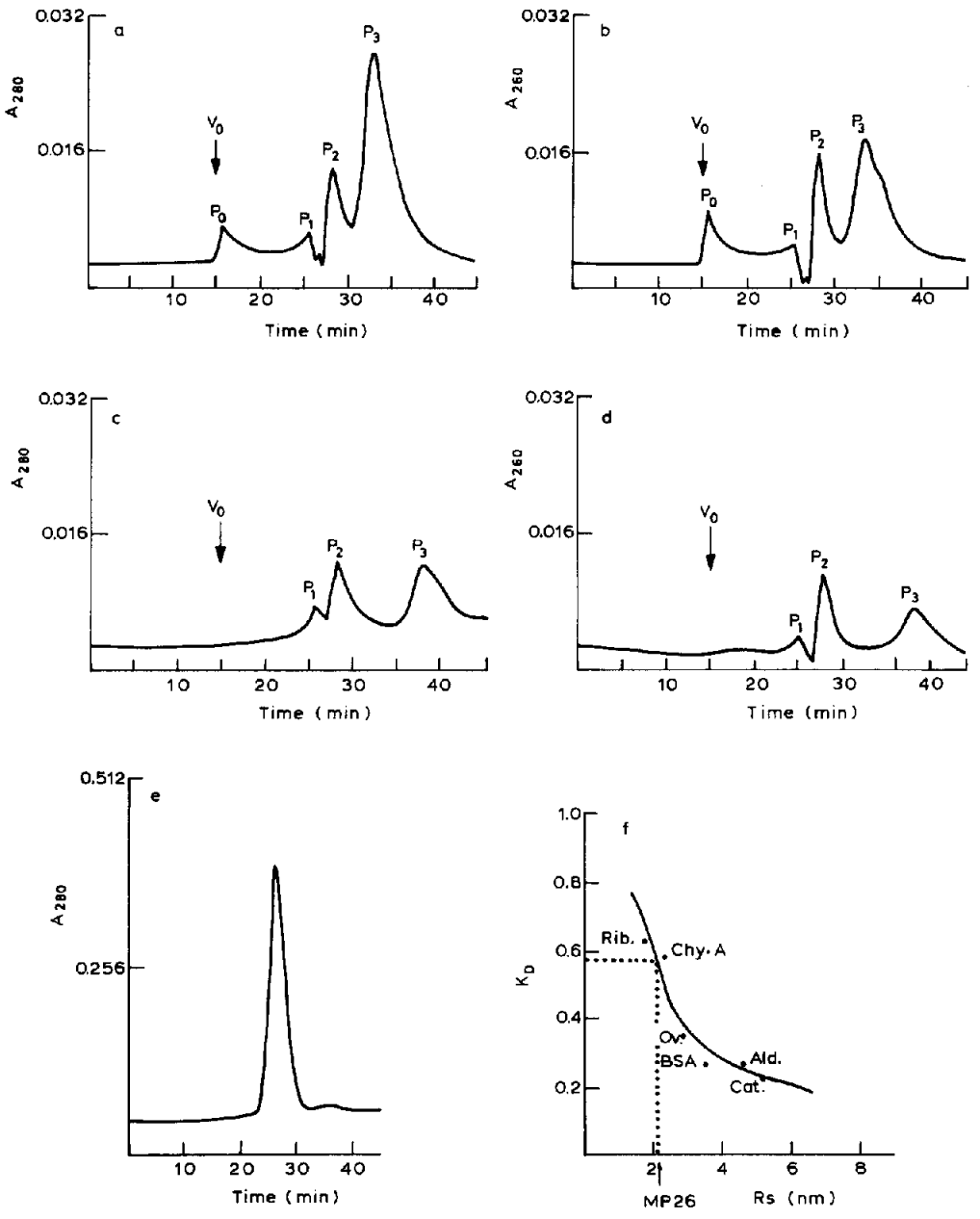

Fig.1. (a and b) HPLC elution profiles of cortex soluble fractions at 280 nm (a) and 260 nm (b). (c and d) HPLC elution profiles of cortex soluble fractions obtained from a previously proteolyzed (V8 protease) membranous solution. (e) HPLC elution profile of an azolectin solution (4 mg/ml) solubilized in 2.5% octyl glucoside. (f) Calibration curve of HPLC column with known Stokes radius proteins.

ther analysis of the fraction using a 260 nm wavelength (fig.1b). In this case, P3 and P2 intensities decrease in a proportion indicating absorption by proteins. Conversely, P2 increases, indicating its non-proteic nature, probably due to a high lipid and detergent content. We confirmed this hypothesis by injecting into the column a solution of azolectin (a mixture of various lipids) solubilized with octyl glucoside (fig.1e), and showing that the main peak of this profile (mixed

micelles) possessed the same retention time as P2 in fig.1a. These data lead to the conclusion that P3 contains more than 90% of the total protein content. Peak 3 in fig.1b (A_{260}) shows a small shoulder in its profile with a retention time of 35 min whose non-proteic nature is difficult to assess. The calibration of the column with water-soluble proteins of known Stokes radius (fig.1f) gave an Rs value of 2.05 nm for the protein detergent complex present in P3. This Rs value can be used in conjunction with analytical ultracentrifugation data to estimate the molecular mass of the protein present in P3.

We recently established that the cortex solubilized fraction contained a main species with a sedimentation coefficient of 4.6 S [5]. We estimated the detergent bound to the protein to be about 1 g/g of protein by comparison with the binding of other non-ionic detergents to strongly hydrophobic membrane proteins [14], and found a molecular mass of 27 kDa for the protein without the bound detergent. Since the MP26 molecular mass, calculated from cDNA cloning and amino acid analysis, is 28.2 kDa we conclude that P3 is composed of the monomeric form of the protein. It is important to realise that in this case, the estimated amount of bound detergent does not affect the conclusion that P3 contains the monomer of MP26. If instead of 1 g/g, one uses 0.5 g/g or 1.5 g/g in eqns 1–3, the M_p value becomes respectively 32 or 23.5 kDa. Peak 1 has a Stokes radius of 3.2 nm, suggesting that it contains an oligomeric form of MP26, probably the dimer.

Fig.1c and d shows the elution profiles of a V8 proteolyzed fraction of MP26. Although it is difficult to estimate the total amount of the proteolytic derivatives of MP26 which is actually solubilized by octyl glucoside, the proteic content of this fraction is clearly lower than the preceding one (cf. fig.1a and c). Furthermore, P3 in fig.1c is not as conspicuous as the one of monomeric MP26 prior to proteolysis (70–80% instead of more than 90%). This indicates that the partial removal of the NH_2 and COOH segments by V8 protease [9] increases both the insolubility of the polypeptide and its tendency to aggregate into oligomers (P1). This may also suggest that the degradation product of MP26 can establish preferential interactions with a molecular class of lipids, insoluble in the detergent.

SDS-PAGE performed on a cortical membrane solution fraction (fig.2) demonstrates that the major amount of MP26/MP22 is found in P3 (lane 4). The fact that the protein profile of P1 (lane 2) is characterized only by the presence of MP26 may suggest that this polypeptide is in its dimeric form, as was previously proposed. The protein profile of P2 (lane 3) shows the presence of a little amount of both MP26 and MP22. Temperature-dependent aggregation in a reducing environment is a well established feature of MP26. Slight aggregation also seems to occur in the precipitation process with TCA that we used, and may explain the presence of a small amount of higher molecular mass components in fig.2. In fact, Western blotting has shown that high molecular mass components are labelled by monospecific antibody against MP26/MP22 [5].

In the previous study [5], we took into consideration the hypothesis that the particulate entities visualized by freeze fracture experiments of reconstituted proteoliposomes were the result of the reassociation in the bilayer of MP26 and/or MP22 monomers. The data presented in this paper are consistent with this assumption, since they show that the majority of MP26 and MP22 is monomeric. Furthermore, the monomeric form of MP26 is likely to be essentially associated with detergent, only few lipids remaining bound after chromatography, since the detergent concentra-

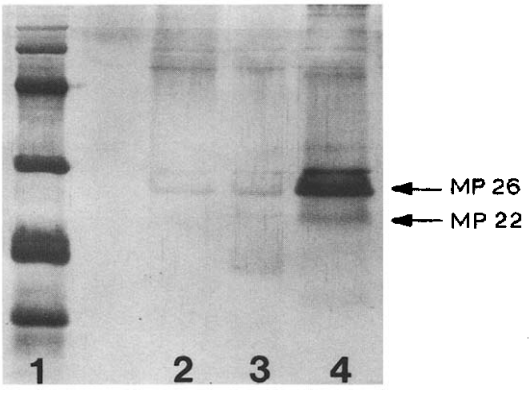

Fig.2. SDS-PAGE patterns of P1, P2, and P3 separated by HPLC from the cortical soluble fraction (see fig.1a). Lanes: 1, molecular mass markers of 92.5, 66.2, 45, 31, 21.5 and 14.4 kDa; 2, SDS-PAGE profile of P1; 3, SDS-PAGE profile of P2; 4, SDS-PAGE profile of P3. Experimental conditions permit a direct quantitative comparison between these profiles.

Table 1

Fatty acid analysis of the octyl glucoside soluble and insoluble fractions obtained from the cortical and nuclear fiber plasma membranes

Fatty acid chains (% of the total fatty acid content)	Samples			
	Nucleus soluble fraction	Nucleus insoluble fraction	Cortical soluble fraction	Cortical insoluble fraction
Myristic acid (C14:0)	3.0	–	3.1	–
Palmitic acid (C16:0)	24.0	16.9	24.7	21.7
Stearic acid (C18:0)	10.0	11.3	7.3	18.0
Oleic acid (C18:1)	42.9	20.2	42.9	40.3
Arachidic acid (C20:0)	–	6.5	0.2	–
Behenic acid (C22:0)	2.5	23.2	1.3	11.7
% of saturated fatty acids	39.5	57.9	36.6	51.4

tions used are very high [15]. However, we must take into consideration that at least in the octyl glucoside insoluble fraction, the lipid-protein or the protein-protein interactions may be sufficiently strong to hinder the detergent action. In other work [5,16], we presume that MP26 and its proteolytic derivative may be comprised in the natural lens fiber membrane in different domains, where specific classes of lipids are tightly associated with the protein. Probably this is the case of the membrane organization in aging lens fibers. We have in fact demonstrated that the insoluble membrane fraction comprises long chain saturated fatty acids (see table 1). It is remarkable that behenic acid (C22:0) is more concentrated in the nuclear insoluble fraction than in the cortical one. From these results we cannot yet correlate the polar character of MP26 and of MP22, and the specific classes of lipids, which might be associated with the different structural patterns that we have previously studied, in particular the orthogonal one. Further reconstitution experiments are needed with proteoliposomes containing the partially delipidated MP26/MP22 and well defined classes of lipids.

Acknowledgements: We are deeply grateful to Professor P. Mazliak, Dr P. Nicolas and Mr G. Bernardi for helping us with the lipid-gas chromatography, HPLC and analytical ultracentrifugation experiments. We want to thank them also for valuable advice and suggestions during our work. This work was supported by Institut National de la Santé et de la Recherche Médicale (INSERM), France, grant 509935 and Fondation de la Recherche Médicale, France.

REFERENCES

[1] Gorin, M.B., Yancey, S.B., Clive, J., Revel, J.P. and Horwitz, J. (1984) Cell 39, 49–59.
[2] Horwitz, J. and Bok, D. (1987) Biochemistry 26, 8092–8098.
[3] Gooden, M., Rintoul, D., Takekama, M. and Takemoto, L. (1984) Biochem. Biophys. Res. Commun. 128, 993–999.
[4] Zampighi, G.A., Hall, J.E. and Kreman, M. (1985) Proc. Natl. Acad. Sci. USA 82, 8468–8472.
[5] Dunia, I., Manenti, S., Rousselet, A. and Benedetti, E.L. (1987) J. Cell Biol. 105, 1679–1689.
[6] Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911–917.
[7] Carreau, J.P. and Dubacq, J.D. (1978) Chromatogr. 151, 384–390.
[8] Laemmli, U.K. (1970) Nature 227, 680–685.
[9] Do Ngoc, L., Paroutaud, P., Dunia, I., Benedetti, E.L. and Hoebeke, J. (1985) FEBS Lett. 181, 74–77.
[10] Le Maire, M., Aggerbeck, L.P., Monteilhet, C., Andersen, J.P. and Moller, J.V. (1986) Anal. Biochem. 154, 525–535.
[11] Le Maire, M., Ghazi, A., Moller, J.V. and Aggerbeck, L.P. (1987) Biochem. J. 243, 399–404.
[12] Tanford, C., Nozaki, Y., Reynolds, J.A. and Makino, S. (1974) Biochemistry 13, 2369–2376.
[13] Moller, J.V., Le Maire, M. and Andersen, J.P. (1986) in: Progress in Protein-Lipid Interactions (Watts, A. and De Pont, J.J.H.H.M. eds) vol.2, chapter 5, pp.147–196, Elsevier, Amsterdam, New York.
[14] Le Maire, M., Kwee, S., Andersen, J.P. and Moller, J.V. (1983) Eur. J. Biochem. 129, 525–532.
[15] Le Maire, M., Lind, K.E., Jorgensen, K.E., Roigaard, H. and Moller, J.V. (1978) J. Biol. Chem. 253, 7051–7060.
[16] Dunia, I., Do Ngoc, L., Manenti, S. and Benedetti, E.L. (1985) Curr. Eye Res. 4, 1219–1234.