Isolation of mitochondrial porin from *Neurospora crassa*

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1. INTRODUCTION

Mitochondrial porin forms channels in the outer mitochondrial membrane which allows the passage of molecules with diameters up to ~20 Å [1–6]. We have described the purification of this protein [7] from purified outer membranes by differential extraction with detergents and anionic exchange chromatography. The purified porin displayed an app. M_r of 31 000 as judged by polyacrylamide gel electrophoresis. The isolated protein could be inserted into artificial lipid bilayers in an asymmetric fashion producing voltage-dependent channels for anions and cations [3,4,7]. Mitochondrial porin bears a number of similarities to the porins of the outer membrane of Gram-negative bacteria [8,9].

A detailed investigation of this interesting protein is hampered by the fact that only small quantities can be prepared when the isolation procedure starts with purified outer mitochondrial membrane. Therefore we have developed a method for a rapid isolation which gives pure, active porin, in high yield. This procedure entails chromatography of detergent solubilized mitochondrial protein on hydroxyapatite and celite. The latter procedure was introduced for the isolation of the phosphate carrier from animal mitochondria [10]. The porin purified by this procedure shows a single band upon SDS gel electrophoresis (M_r = 31 000). Upon isoelectric focusing it displays two bands with pI-values of 7.7 and 7.8. It is functionally active after insertion into lipid bilayers.

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2. MATERIALS AND METHODS

2.1. *Growth of* Neurospora crassa *and preparation of mitochondria*

Neurospora crassa hyphae (wild type 74 A) were grown on Vogel's minimal medium [11] in the presence of 2% sucrose [12]. Disruption of cells and isolation of mitochondria in the presence of 0.5 mM phenylmethyl sulfonyl fluoride were carried out as described [13].

2.2. *Isolation of mitochondrial porin*

A mitochondrial pellet (4–6 mg protein) was lysed with 0.7 ml of buffer A containing 2.5% Genapol X-100 from Farbwerke Hoechst, 50 mM KCl, 10 mM KP_i, 1 mM EDTA, 10 mM Tris–HCl (pH 7.0). After incubation for 30 min at 4°C, the solution was centrifuged at 4°C for 15 min at 27 000 × *g*. The supernatant was applied to a Pasteur pipette which was filled with 0.6 g of dry hydroxyapatite (Bio-Gel HTP) from BioRad. When the solution had entered the column, further buffer was applied and 0.7 ml of the eluate was collected. This was transferred to a Pasteur pipette containing 0.6 g of a dry mixture of equal weights of hydroxyapatite and celite (Celite 535) from Roth. Elution was performed with buffer A until 0.7 ml had been collected. The eluate containing porin with only minor contaminating proteins. For further purification, the solution was dialysed three times for 6 h in Visking tubing 18/32 (Union Carbide) against 20 vol. of 1% Genapol X-100, 5 mM Tris–HCl (pH 7.5) and subjected to chromatography on DEAE-cellulose (Whatman). The column (19 × 1

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cm) was equilibrated with 1% Genapol X-100, 5 mM Tris–HCl (pH 7.5) and eluted with the same buffer at a flow rate of 10 ml/h. The protein peak which appeared with the buffer front was collected.

For the preparation of larger amounts of porin, two different procedures were used. To prepare porin from up to 50 mg of mitochondria, the mitochondria were extracted as described above and the first two chromatographic steps were performed in 10 Pasteur pipettes used in parallel. The final chromatography over DEAE-cellulose was then carried out with the combined eluates on the same column as described above. This procedure is rapid and avoids proteolysis which has been observed when the initial steps last longer. In order to prepare porin in amounts up to 15 mg, larger columns were used. Mitochondria (1.2 g protein) were dissolved in 130 ml buffer A and chromatographed on columns containing 400 g hydroxyapatite or hydroxyapatite–celite mixture (diameter 3 cm) and over a DEAE-cellulose column of 40 × 3 cm. The yield was 3.75 mg porin.

2.3. *Other methods*

Protein was precipitated from detergent containing solutions by adding 1/10 vol. of 3 M trichloroacetic acid (TCA) and 1/3 vol. of methanol, conditions which prevent precipitation of the detergent. After 1 h at 4°C, the samples were centrifuged and the pellet subjected to further analysis. Gel electrophoresis was performed on vertical slab gels (17.5% acrylamide) according to [14]. Isoelectric focusing was carried out according to [15], with the exception that 6 M urea was included in gel buffers. The samples which were applied on the gels contained 1% Genapol X-100, 5 mM Tris–HCl (pH 7.5). Immunoprecipitation was carried out as in [7].

3. RESULTS AND DISCUSSION

Treatment of whole mitochondria with the nonionic detergent Genapol X-100 leads to solubilisation of most mitochondrial proteins. When the solution was passed through dry hydroxyapatite, the majority of the proteins were adsorbed (fig.1, lanes 2,3). The major protein in the eluate is the ADP,ATP-carrier. The latter protein could be removed by chromatography on celite [10]. The eluate, after passage through a mixture of hydroxyapatite and celite, contained mostly enriched mitochondrial porin (fig.1, lane 4). The minor contaminations, which are in the M_r range from 32 000–43 000, were removed by chromatography on DEAE-cellulose. A single band with M_r 31 000 was seen after SDS gel electrophoresis (fig.1, lane 5). It displayed the same app. M_r as porin isolated from purified outer mitochondrial membranes (fig.1, lane 6). When the protein was precipitated before electrophoresis with 0.3 M TCA for 1 h at room temperature or 15 min at 56°C, the SDS gels displayed an additional protein band with an app. M_r of 25 000 (fig.1, lane 7). This band became stronger upon prolonged exposure to TCA and was absent after precipitation with acetone, or with TCA for 1 h at 4°C. This indicates the presence of an acid labile peptide bond in porin.

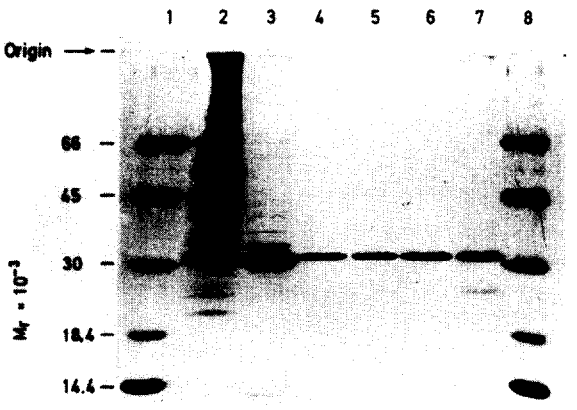

Fig.1. Isolation of mitochondrial porin. Protein was precipitated from the detergent containing fractions with 0.3 M TCA at 4°C, and redissolved in SDS containing buffer [14]. (1,8) M_r standards; (2) total mitochondrial proteins, after solubilisation in Genapol X-100; (3) eluate from HTP-column; (4) eluate from HTP/celite-column; (5) eluate from DEAE-cellulose; (6) porin isolated from purified outer mitochondrial membranes; (7) as in (5), but precipitation with 0.3 M TCA at 56°C for 15 min.

The yield of the purified protein was in the range of 0.36–0.40% of total mitochondrial protein (table 1). Quantitative immunoprecipitation has indicated that the percentage of porin in isolated whole mitochondria is 0.4% [7]. Thus the isolation

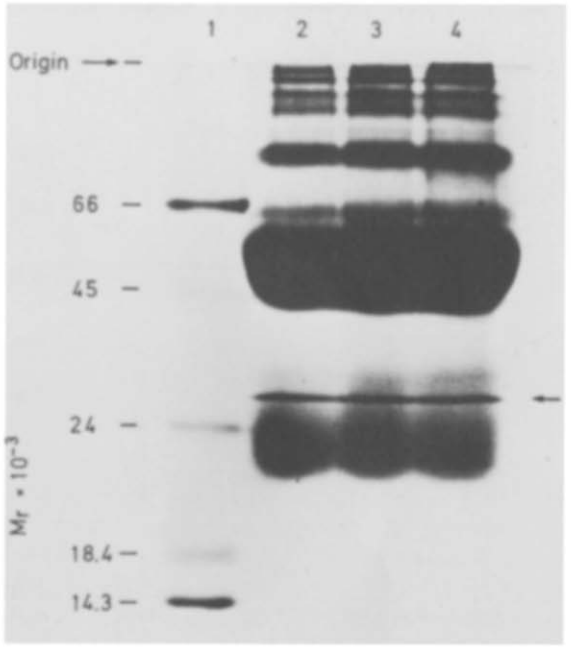

Fig.2. Immunoprecipitation from the various fractions of the isolation procedure with antibodies against porin isolated from purified outer membranes. The detergent containing solutions (2.5% Genapol X-100) were diluted with equal vols of 0.6 M KCl before immunoprecipitation. The washed immunoprecipitates were analysed by SDS gel electrophoresis and the gels stained with Coomassie Blue. The amounts of antibodies used were determined to be sufficient for complete immunoprecipitation. (1) M_r markers; (2) immunoprecipitation from whole mitochondria (1.9 mg protein); (3) immunoprecipitation from the eluate from HTP (93 μg protein); (4) immunoprecipitation from the eluate from HTP/celite (9 μg protein). The arrow indicates the position of purified porin.

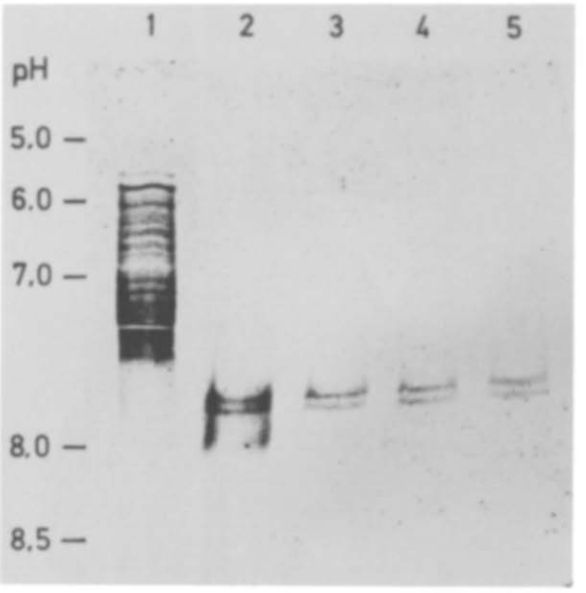

Fig.3. Isoelectric focusing of isolated porin. The various fractions of the isolation procedure were subjected to isoelectric focusing in the presence of 6 M urea. Protein bands were stained with Coomassie Blue. (1) whole mitochondria dissolved in Genapol X-100; (2) eluate from HTP; (3) eluate from HTP/celite; (4) eluate from DEAE-cellulose; (5) porin isolated from purified outer membrane.

procedure described here results in an excellent yield of porin.

In order to verify the identity of the purified protein, immunoprecipitation was performed with antibodies against porin isolated from purified outer mitochondrial membranes. Fig.2 shows that this antibody reacts with the porin isolated by the procedure described above. Immunoprecipitation after the various steps of this procedure yields always the same amount of porin as judged from the intensity of the stained band after analysis of the immunoprecipitation on SDS gels.

Isoelectric focusing of porin isolated from purified outer mitochondrial membranes reveals two bands with isoelectric points of 7.7 and 7.8 (fig.3, lane 5). The same was seen with porin isolated from whole mitochondria (fig.3, lane 4). The majority of mitochondrial proteins have a lower isoelectric point (fig.3, lane 1). The ADP,ATP-carrier, the major protein in the hydroxyapatite eluate (see fig.1, lanes 2,3), forms a broad band on the basic side of the porin.

Porin purified from *Neurospora* outer mitochondrial membranes spontaneously inserts into artificial lipid bilayers [7]. The porin isolated by the procedure described here shows the same behaviour. Microscopic conductance measurements show a stepwise increase of membrane conductance which represents the formation of channels in the bilayer (fig.4). The specific activities (specific

Table 1

Purification of mitochondrial porin

Step	Total Protein (mg)	% of Total Mitochondrial Protein	Purification* factor
Mitochondria solubilized with Genapol X-100	56	100	1
Eluate from HTP	3.2	5.8	17
Eluate from HTP/celite	0.24	0.43	233
Eluate from DEAE-cellulose	0.21	0.38	263

* Assuming a 100% yield as indicated by quantitative immunoprecipitation

Isolation was carried out by dissolving the mitochondria in 7 ml detergent containing buffer and filtering over 10 Pasteur pipettes in parallel.

conductance related to porin concentration in the aqueous phase) and the single channel conductance of the porins prepared by the two methods were the same. Furthermore, porin from whole mitochondria shows the same asymmetric membrane current response as described for porin from outer membrane in [7].

We conclude that the simple and rapid procedure described in this communication allows the preparation of large quantities of functional mitochondrial porin.

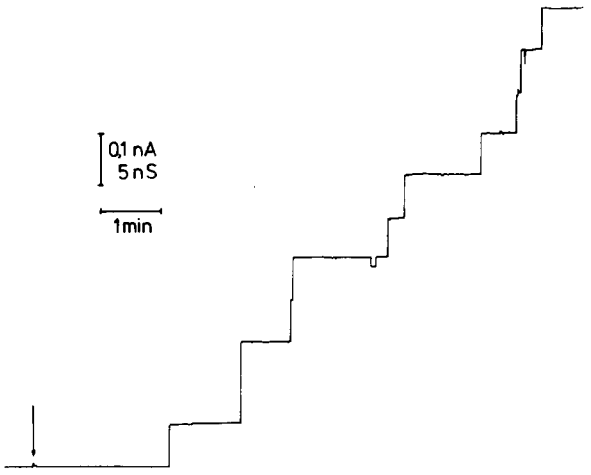

Fig.4. Stepwise increase of membrane conductance after addition of porin to an artificial bilayer system. A membrane was formed from asolectin/*n*-decane. The aqueous phase contained 1 M KCl, pH 6.0. Temperature was 25°C. Purified porin in Genapol X-100 was added to one chamber such that the final protein concentration was 5 ng/ml and the final detergent concentration 100 ng/ml. The voltage applied was 20 mV positive at the cis-side (where the protein was added). The arrow indicates the time at which protein was added.

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