Purification of a protein having pore forming activity from the rat liver mitochondrial outer membrane

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A protein with pore-forming activity has been isolated from the outer membrane of rat liver mitochondria. The purification involves sucrose gradient centrifugation, differential centrifugation in the presence of Triton X-100, and DEAE–Sepharose and CM–Sepharose chromatography. The yield of the purified protein was approx. 2% of the total outer membrane proteins. The protein, when inserted into soya bean phospholipid vesicles, increases the [^{3}H]sucrose permeability of the vesicles but had no effect on the permeability of high-molecular-weight [^{14}C]dextran (M_r 70000). The protein is very active, since as little as 3–4 μg of protein per mg of phospholipid is required for the complete release of [^{3}H]sucrose from the vesicles. Sucrose diffusion channels could not be reconstituted with other membrane proteins such as rat liver cytochrome oxidase or cytochrome b_5. Purified pore protein revealed a single band of apparent M_r 30000 when resolved by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This polypeptide could be further resolved by isoelectric focusing into a major (pI 7.9) and two relatively minor (pI 7.6 and 7.2) components. Proteolytic mapping with V8 proteinase from *Staphylococcus aureus* suggests that these probably represent a single component showing charge heterogeneity. The reason for the charge heterogeneity is not known. The amino acid composition of the protein revealed 47.8% polar amino acids with a relatively high lysine content.

The mitochondrial outer membrane contains pore structures which allow the passage of low molecular weight substance from the cytosol to the inter membrane space (Werkheiser & Bartley, 1957; O'Brien & Brierley, 1975; Pfaff *et al.*, 1965; Wojtczak & Zaluska, 1969; Parsons *et al.*, 1966; Mannella & Bonner, 1975; Zalman *et al.*, 1980). A recent report by Zalman *et al.* (1980) describes the isolation of a peptide (M_r 30000) from the outer membrane of mung bean mitochondria, which is able to reconstitute pore structures in phospholipid vesicles. Resolution of this preparation on SDS gel electrophoresis showed, however, the presence of a second diffuse band in the region of 30000 daltons (Zalman *et al.*, 1980). Furthermore, this preparation exhibited a much lower pore-forming activity than did a crude extract from the outer membrane. Thus, it is not clear from these experiments if the 30000-dalton peptide can alone form the pore structure, or if additional peptides are required. The size of the functional oligomeric pore

structure has been estimated to be approx. 110000 daltons (Colombini, 1980). In order to obtain more detailed information on the physical properties of the pore, it is thus necessary to have available a more highly purified preparation of the protein in its active form. We report here a method by which this can be achieved with the outer membranes from rat liver mitochondria. Some of the physical characteristics of this highly purified and active polypeptide are also described.

Materials and methods

[6,6'(*n*)-^{3}H]Sucrose (1–5 mCi/mmol) was purchased from The Radiochemical Centre, Amersham. [*carboxyl*-^{14}C]Dextran (M_r 70000) (specific radioactivity 1.1 mCi/g) was purchased from New England Nuclear. V8 proteinase from *Staphylococcus aureus*, CM–Sepharose, DEAE–Sepharose and Sepharose-4B were all bought from Pharmacia, Sweden. Ampholines were from LKB. All other chemicals used in this study were reagent grade and were obtained from commercial sources.

Abbreviation used: SDS, sodium dodecyl sulphate.

Purification of pore protein

Rat liver mitochondria were isolated from Sprague–Dawley rats according to Johnson & Lardy (1967). The outer membranes were prepared from freshly isolated mitochondria by the method of Sandri et al. (1978). After sucrose gradient centrifugation the outer membranes were collected from the interface between 1.12 M- and 0.45 M-sucrose, diluted in 3 vol. of distilled water, and pelleted at 105000 g for 45 min. The outer membrane pellets were then extracted with 2% (w/v) Triton X-100/1 M-NaCl essentially as described by Zalman et al. (1980). The supernatant (2.2 ml), referred to as the Triton/NaCl soluble fraction, was obtained after 1 h centrifugation at 130000 g. This fraction was dialysed against 1 litre of 10 mM-Tris/HCl (pH 7.0)/0.05% Triton X-100 overnight at 0–4 °C. The dialysate was chromatographed on a DEAE–Sepharose column (0.7 cm × 9 cm) and equilibrated with 10 mM-Tris/HCl (pH 7.0)/0.05% Triton X-100. It was eluted with the same buffer at a rate of 0.17 ml/min. The pore protein, as determined by pore-forming activity, was eluted in the void volume (approx. 6.5 ml) together with Triton X-100. The first 13 fractions (0.5 ml per fraction) were pooled and applied to a CM–Sepharose column which had been equilibrated with 10 mM-sodium phosphate (pH 7.0)/0.05% Triton X-100. The column (0.7 cm × 5 cm) was eluted with the equilibration buffer at a rate of 0.17 ml/min. The pore protein was eluted together with Triton X-100 in the first 15 fractions (0.5 ml per fraction).

Pore forming activity was assayed essentially as described by Zalman et al. (1980). The only change was that the length of the Sepharose-4B column was increased to 25 cm to achieve a better separation of [^{14}C]dextran-containing vesicles and free [^{14}C]-dextran.

SDS/polyacrylamide-gel electrophoresis was carried out essentially as described by Laemmli (1970) using 12.5% acrylamide gels. Two-dimensional electrophoresis was run according to O'Farrell et al. (1977) in a non-equilibrium electrophoresis system. Peptide mapping was run according to Cleveland et al. (1977). Protein samples (20–40 μg) were digested with 4 μg of V8 proteinase enzyme from Staphylococcus aureus for 30 min at room temperature. The digestion products were separated on a 18% SDS/polyacrylamide-gel (Laemmli, 1970). The amino acid composition of pore protein was kindly determined by Dr. Hans Jörnvall, Karolinska Institute, Stockholm.

Other procedures

Cytochrome oxidase (Wielburski et al., 1982) and cytochrome b_5 (Elhammer et al., 1978) were purified according to published methods. Protein was determined by the biuret procedure (Gornall et al., 1949),
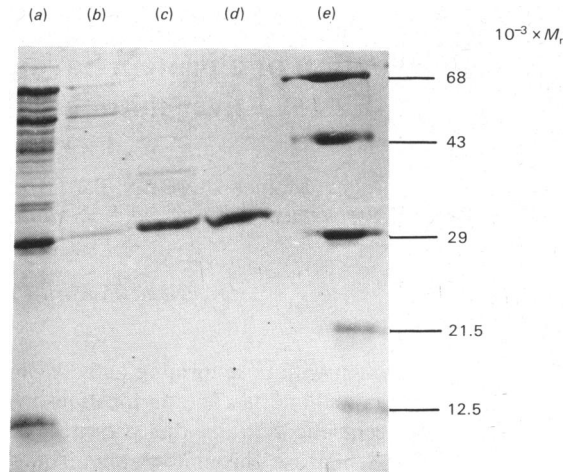

Fig. 1. SDS/polyacrylamide-gel electrophoresis of various fractions from the purification of pore protein

The electrophoresis was run on a 12.5% gel as described in the Materials and methods section. The fractions electrophoresed were: (a) outer mitochondrial membranes (50 μg); (b) Triton/NaCl soluble fraction (10 μg); (c) DEAE–Sepharose fraction (10 μg); (d) CM-Sepharose fraction (purified pore protein) (8 μg); (e) standard proteins (5 μg each). The standard proteins were: albumin (M_r 68000), ovalbumin (43000) carbonic anhydrase (29000), trypsin inhibitor (21500) and cytochrome c (12500).

or according to the method of Petterson (1977) when Triton X-100 was present in the samples.

Results

Purification of pore protein

A protein with pore forming activity was isolated from rat liver mitochondrial outer membranes. The initial step involves the isolation of highly purified outer membranes (Sandri et al., 1978). The outer membrane preparations used contained less than 3% of contaminating inner membranes (Sandri et al., 1978; Gellerfors & Lindén, 1981) and revealed on SDS/polyacrylamide-gel electrophoresis four heavily stained bands and several minor components (Fig. 1, lane a). Rat liver outer membranes were extracted with 2% Triton X-100 and the resulting pellet was solubilized in 2% Triton X-100/1 M-NaCl (Fig. 1, lane b). Further purification of pore protein was achieved by DEAE–Sepharose chromatography, during which most of the contaminating proteins, including the three main components of the outer membranes, were removed (Fig. 1, lane c). To remove the final contaminants, fractions 1–13 from the DEAE column were pooled and rechromato-

Table 1. *Protein yield and pore forming activity of various fractions obtained during the purification of pore protein*
Part (5 µg) of each fraction was reconstituted with 1.4 mg of soya bean phospholipid vesicles in the presence of [³H]sucrose and [¹⁴C]dextran (M_r 70000) using 1.8×10^5 c.p.m. of each isotope in the reconstitution mixture. After separation on a Sepharose 4B column the radioactivity associated with the vesicles were determined. Since [¹⁴C]dextran could not readily diffuse out the ratio of [¹⁴C]dextran and [³H]sucrose was taken as a measure of activity.

	Protein		Pore forming activity		
Fraction	(mg)	(%)	[³H]sucrose (c.p.m.)	[¹⁴C]dextran (c.p.m.)	¹⁴C/³H
Mitochondria	570	—	—	—	—
Outer membranes	9.3	100	862	2525	2.9
Triton X-100 insoluble fraction	6.7	72	559	1844	3.3
Triton X-100/NaCl soluble fraction	2.3	25	391	2414	6.2
DEAE–Sepharose	0.24	2.6	278	4663	16.8
CM–Sepharose	0.19	2.0	55	1875	34.0
Control (no protein)	—	—	956	1455	1.5

graphed on a CM–Sepharose column. The pooled fractions from the CM–Sepharose column revealed a single band on SDS/polyacrylamide-gel electrophoresis (Fig. 1, lane *d*).

Characterization of purified pore protein

The isolated pore protein migrates as a single band on SDS/polyacrylamide gels, with an apparent M_r of 30000. No minor Coomassie Blue-staining polypeptides were detected when as much as 20 µg of the isolated protein was subjected to electrophoresis.

Table 1 summarizes the protein yields and the pore forming activities of various fractions obtained during the purification of pore protein. The yield of the purified peptide was approx. 0.2% starting from mitochondria or 2% starting from outer membranes. The pore forming activity of each fraction increased during the purification from 2.9 (outer membranes) to a maximal value of 34.0. A quantitative estimate of purification based on the pore-forming activity was not, however, possible to obtain, since a non-linear relationship between the pore-forming activity and the amount of pore protein was observed (Fig. 2). The non-linearity was expected, since the initial rates of [³H]sucrose release could not be measured. Maximal release of [³H]sucrose (95%) was observed with 5 µg of pore protein per 1.4 mg of phospholipid. Thus, these experimental conditions must result in a minimum of one pore structure per vesicle.

The specificity of the purified pore protein in producing diffusion channels was studied (Table 2). Neither purified rat liver cytochrome b_5 nor cytochrome oxidase exhibited pore forming activity, suggesting that the formation of diffusion channels is a unique property of pore protein. Since pore protein is isolated in the presence of Triton X-100, the effects of 0.1% Triton X-100 in the reconstitution
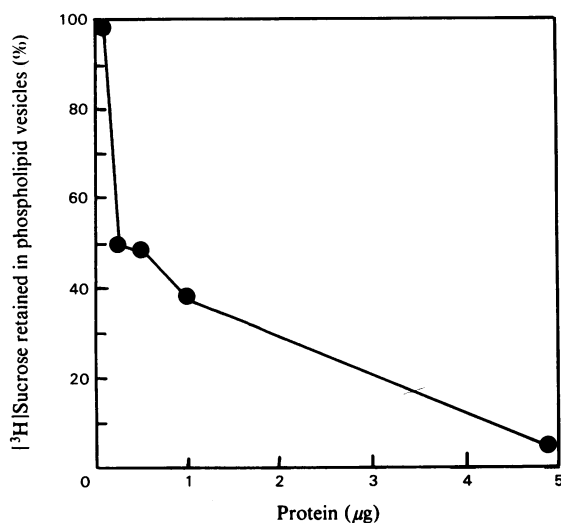

Fig. 2. *Titration of pore-forming activity with purified pore protein*
Soya bean phospholipid vesicles (1.4 mg) were reconstituted either without (control, 100%) or with increasing amounts of purified pore protein in the presence of [³H]sucrose. After gel filtration on a Sepharose 4B column the amount of [³H]sucrose still retained inside the vesicles was determined. No [¹⁴C]dextran was used in this experiment.

system was also assayed. No significant permeability changes were observed. However, in the presence of 1% SDS (results not shown) no vesicles could be isolated containing either [³H]sucrose or [¹⁴C]dextran.

To investigate further the purity of the pore protein, it was subjected to two-dimensional gel electrophoresis according to O'Farrell *et al.* (1977).

Table 2. *Pore-forming activity of various purified mitochondrial membrane proteins*

Purified protein (5 μg of each) was used in the reconstitution assay as described in the Materials and methods section and the legend to Table 1.

Preparation	Activity $^{14}C/^3H$
Vesicles	1.1
Vesicles plus purified pore protein	30.2
Vesicles plus purified rat liver cytochrome oxidase	2.1
Vesicles plus purified rat liver cytochrome b_5	1.4
Vesicles plus 0.1% Triton X-100	2.7

Fig. 4. *Peptide map analysis of the two main protein components (A and B) separated by two-dimensional gel electrophoresis*

Components A (pI 7.9) and B (pI 7.6) which were separated by two-dimensional gel electrophoresis were cut out and subjected to digestion for 30 min by V8 proteinase from *Staphylococcus aureus* according to Cleveland *et al.* (1977). The digestion products were separated on a 18% SDS polyacrylamide gel. (a) V8 enzyme; (b) component A plus V8; (c) component A plus B (2 μg each); (d) component B plus V8.
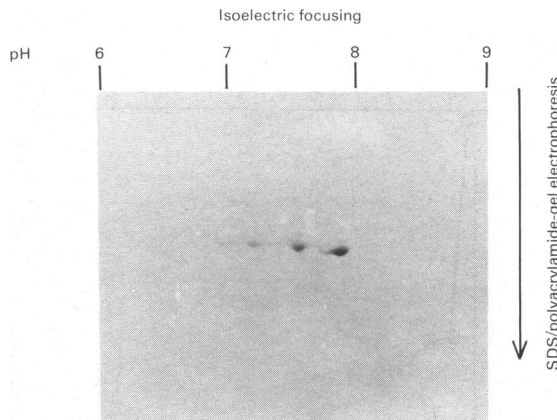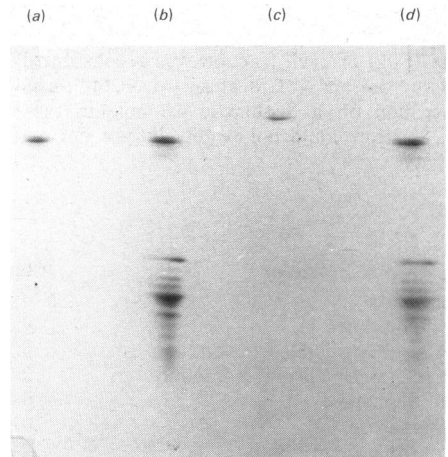

Fig. 3. *Two-dimensional gel electrophoresis of purified pore protein*

In the first dimension 20 μg of pore protein was run on an isoelectric focusing gel system O'Farrell *et al.* (1977) for 2000 V·h using Ampholines in the pH range of 3.5–10. The gel was then transferred to an ordinary 12.5% SDS/polyacrylamide gel and electrophoresed in the second dimension.

As can be seen in Fig. 3, three polypeptides with isoelectric points of 7.9, 7.6 and 7.2, respectively, were separated in the first dimension using a non-equilibrium pH gradient system (O'Farrell *et al.*, 1977). These polypeptides showed decreasing staining intensity towards the acid side of the isoelectric focusing gel. Whether these three components are distinct proteins differing only in their isoelectric point or whether they represent only one protein showing charge heterogeneity (O'Farrell, 1975), could not be determined by this kind of experiment. To test these two possibilities, the two most intensely stained protein spots, A (pI = 7.9) and B (pI = 7.6), were cut from the gel and digested by the proteolytic enzyme V8 from *Staphylococcus aureus* (Cleveland *et al.*, 1977). Fig. 4 shows that the peptide maps from component A and B are identical,

Table 3. *Amino acid composition of purified pore protein from rat liver mitochondria*

N.D., not determined

Amino acid	Composition (mol %)
Lys	9.44
His	1.35
Arg	2.27
Asx	11.61
Thr	8.96
Ser	5.83
Glx	8.29
Pro	2.51
Gly	11.65
Ala	7.38
Cys	N.D.
Val	5.93
Met	0.59
Ile	3.65
Leu	9.97
Tyr	4.41
Phe	6.16
Trp	N.D.

indicating that they are most likely the same protein component, which, for unknown reasons, exhibits charge heterogeneity.

The amino acid composition of pore protein

(Table 3) reveals a relatively high polarity index (Capaldi & Vanderkooi, 1972) of 47.8%, considering its hydrophobic properties. No amino sugars were detected during the amino acid analysis, suggesting that this is not a glycoprotein (Colombini, 1980). The protein also contains a relatively high lysine content, which is in keeping with the pI values determined for the isolated protein (see above).

Discussion

The aim of the present study was to isolate and characterize the pore protein from the outer membrane of mammalian mitochondria. Recent studies by Zalman et al. (1980) have led to the isolation of a pore protein from plants. The pore protein from both plants (Zalman et al., 1980; Mannella & Bonner, 1975) and mammalian mitochondria exhibit an apparent molecular weight of approx. 30 000 daltons when resolved on SDS/polyacrylamide-gel electrophoresis. These results suggest that the pore structure might be relatively well conserved. Striking similarities between the mitochondrial outer membrane pore protein and the bacterial porins (Nakae, 1976; Chen et al., 1982) also seem to exist (Colombini, 1980).

The method described in the present paper yields a highly purified and active pore protein from rat liver. It is entirely free from the minor components which are associated with the plant protein (compare Fig. 1 of the present manuscript and Fig. 2, lane 6 of Zalman et al., 1980). The high degree of purity is a prerequisite if structural and physical properties of the protein are to be determined. Furthermore, the rat liver outer membrane polypeptide is highly active in producing sucrose diffusion channels when reconstituted with phospholipid vesicles. Thus, only the 30 000-dalton polypeptide is required for the formation of such channels. This conclusion was not obvious from the studies on the mung bean protein, since the latter polypeptide lost its ability to form diffusion channels during the final stage of its purification (Zalman et al., 1980). It was not determined if the decrease in pore-forming activity was due to inactivation of the polypeptide or to a loss of an additional polypeptide required for the diffusion channel. The present study strongly indicates that the former is probably the case.

Although the rat liver pore protein exhibits but a single band on SDS/polyacrylamide-gel electrophoresis, we are consistently able to resolve this fraction into three polypeptides with different isoelectric points. The two major polypeptides which were separated by isoelectric focusing were shown to have identical V8 proteinase digestion patterns, indicating that the pore protein is a single polypeptide exhibiting charge heterogeneity. The relevance of the charge heterogeneity is not yet understood. We have considered the possibility that it could be due to physiological alterations in the protein, for example via phosphorylation or glycosylation (O'Farrell, 1975; Colombini, 1980). However, studies in this laboratory (M. Lindén & P. Gellerfors, unpublished observations), were unable to detect phosphorylation of pore protein isolated from rat hepatocytes phosphorylated in vitro. Furthermore, no sugar moiety was detected during analysis of the amino acid composition. Presently, the most reasonable explanation is that the charge heterogeneity is an artifact arising during the isoelectric focusing step. Charge heterogeneity of purified proteins is frequently observed (O'Farrell, 1975).

A possible heterogeneity of the channel is, however, suggested by the findings of Colombini (1980), that the outer membrane voltage-dependent anion-selective channel-forming complex (Colombini, 1979) (M_r 110000) contains a glycoprotein which binds concanavalin A. As indicated above, the glycoprotein component appears to be different from the 30 000-dalton polypeptide isolated in the present study. The porins from Gram-negative bacteria, which are similar in many ways to the outer membrane pore proteins of mitochondria (for comparison see Table 1, Colombini, 1980), have been shown to be comprised of three different peptides in the M_r range of 30 000 (Nakae, 1976; Hancock et al., 1979; Chen et al., 1982). These polypeptides appear to impart different ion selectivities to the membrane (Luckey & Nikaido, 1980; Heuzenroeder & Reeves, 1980). By analogy with bacterial porins, the function of the glycoprotein component in the outer mitochondrial membrane (Colombini, 1980), might be to regulate the ion selectivity of the channel formed by the 30 000 dalton protein or to direct the insertion of the pore protein into the outer membrane. Thus, further work on the structure and the functional role of the 30 000 dalton polypeptide, as well as a closer search for the glycoprotein component, is warranted.

After completion of the present manuscript two reports have appeared which describe pore proteins from the mitochondrial outer membranes of rat (Roos et al., 1982) and Neurospora (Freitag et al., 1982). Both preparations form voltage-dependent pore structures and exhibit molecular properties similar to those described here.

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