PORE PROTEIN AND THE HEXOKINASE-BINDING PROTEIN FROM THE OUTER MEMBRANE OF RAT LIVER MITOCHONDRIA ARE IDENTICAL

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1. Introduction

Hexokinase of tumor cells [1] and certain normal tissues [2] are bound to the outer membrane of the mitochondrion. This binding is, at least in part, responsible for increased aerobic glycolysis, which characterizes rapidly growing tumor cells [3]. The rat liver outer membrane protein responsible for binding of hexokinase has been isolated and partially characterized [4]. The app. M_r reported for this peptide (31 000) is nearly identical to that of the outer membrane pore protein [5], purified and characterized in [6]. Since SDS electrophoresis of outer membranes reveals a single major band in the 31 000 M_r region, we have investigated the possibility that pore protein and the hexokinase-binding protein are identical. These results indicate that this is the case.

2. Materials and methods

Pore protein [6] and the hexokinase-binding protein [4] were isolated as described. The hexokinase-binding preparation used was purified to the stage termed '1 × reconstituted vesicles' [4]. Partial proteolysis and peptide map analysis were done as in [7], using 18% acrylamide gels for the separation of peptide fragments. SDS–PAGE [8] and two-dimensional gel electrophoresis [9] were done as described. Pore activity was assayed essentially as in [5] with the modification in [6]. Protein was determined according to [10].

Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; HBP, hexokinase-binding protein [4]

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[6,6′(*n*)-^{3}H]Sucrose (1–5 Ci/mmol) was purchased from The Radiochemical Center (Amersham). [*carboxyl*-^{14}C]Dextran (M_r 70 000) with spec. act. 1.1 mCi/g was from New England Nuclear. *Staphylococcus aureus* V8 protease was purchased from Pharmacia (Uppsala). Chymotrypsin, soy bean lecithin and octyl-β-D-glucopyranoside were all purchased from Sigma. Ampholines were from LKB (Stockholm).

3. Results and discussion

Fig.1 shows SDS–polyacrylamide gel electrophoresis of purified pore protein [6] and a hexokinase-binding protein preparation isolated as in [4]. The hexokinase-binding protein preparation (lane 1) consists of a major polypeptide and several minor components. The major polypeptide (the component binding hexokinase [4]) and pore protein (lane 2) exhibited identical mobilities (M_r 30 000) on SDS–PAGE.

Hexokinase-binding protein and pore protein were subjected to proteolytic digestion and peptide map analysis [7]. Purified hexokinase-binding protein and pore protein were electrophoresed as in fig.1, cut from the gels and digested with V8 protease from *Staphylococcus aureus* (fig.2A) or with chymotrypsin (fig.2B). Pore protein and the hexokinase-binding protein exhibited identical peptide patterns after digestion with V8 or chymotrypsin (fig.2). Identical partial digestion patterns were obtained for both proteins upon varying chymotrypsin concentration (fig.2B). This provides strong proof that the same digestion sites are found in both pore protein and the hexokinase-binding protein.

The hexokinase-binding protein and pore protein were also subjected to two-dimensional gel electro-

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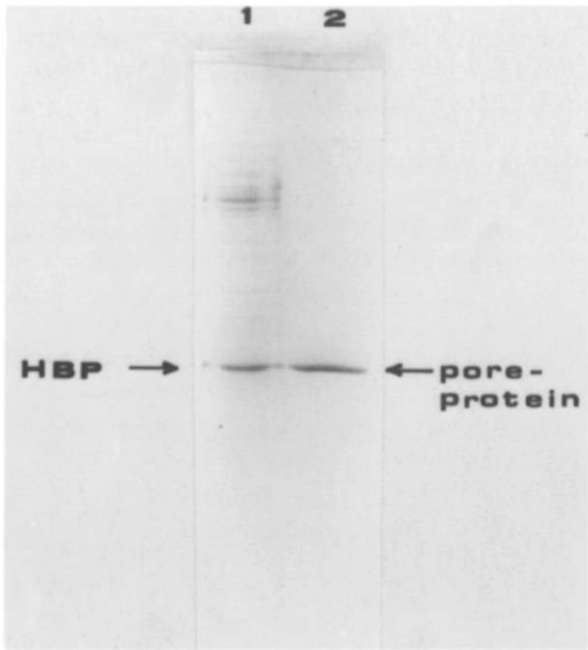

Fig.1. SDS–polyacrylamide gel electrophoresis of a hexokinase-binding protein preparation and purified pore protein. The hexokinase-binding protein and pore protein were isolated as in section 2. About 7 μg of each protein were co-electrophoresed on a 12.5% SDS gel as in [8]. The following standard proteins were used to calibrate the gel (M_r): albumin (68 000); ovalbumin (48 000); carbonic anhydrase (29 000); trypsin inhibitor (21 500); cytochrome *c* (12 500) (not shown).

phoreses [9], either separately (fig.3A,B) or as a mixture (fig.3C). In agreement with [6], purified pore protein was separated into 3 peptides of identical M_r-value but with different pI-values (fig.2A). The major component and two minor components exhibited pI-values of 7.9, 7.6 and 7.2, respectively. The reason for the observed charge-heterogeneity of purified pore protein is not known, although this phenomenon is frequently observed [11]. The hexokinase-binding protein was also resolved into 3 peptides with isoelectric points identical to those of pore protein (fig.3B). That hexo-

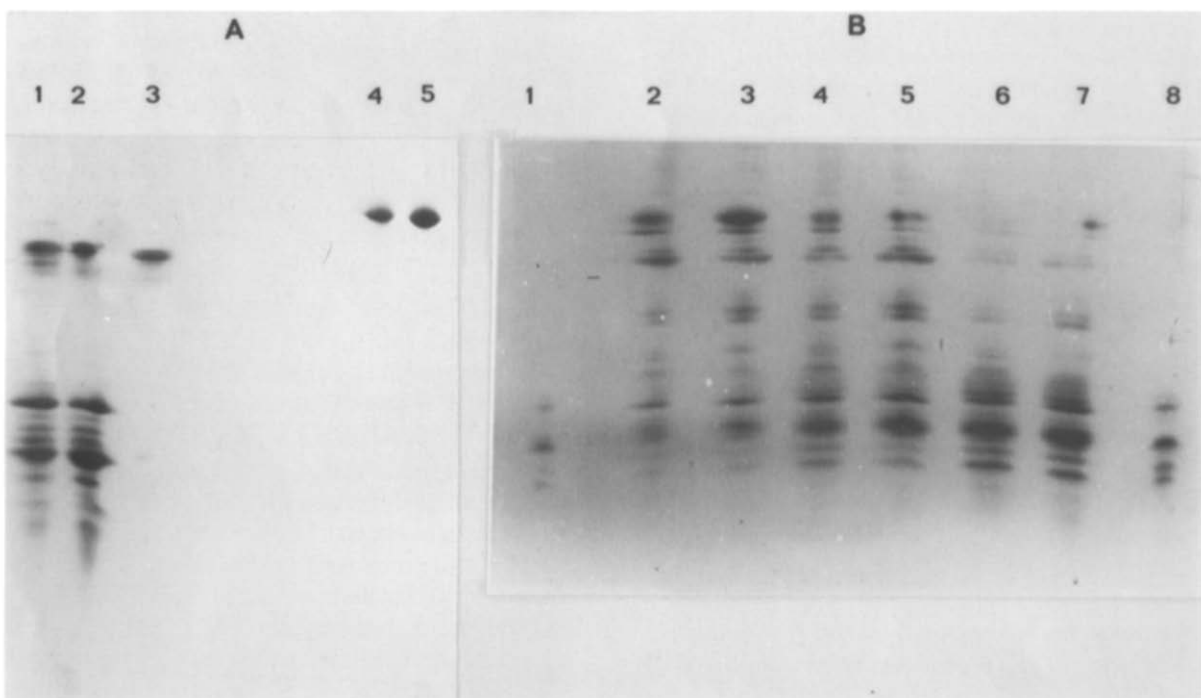

Fig.2. Peptide map analysis of the hexokinase-binding protein and pore protein. The hexokinase-binding protein and purified pore protein were electrophoresed as in fig.1. The polypeptide bands were cut from the gel and digested with: (A) V8 protease from *Staphylococcus aureus* (4 μg); or (B) chymotrypsin (1–4 μg) according to [7]. After 20 min of digestion the peptide fragments were separated on a 18% SDS polyacrylamide gel. (A1) Hexokinase-binding protein + V8; (A2) pore protein + V8; (A3) V8; (A4) hexokinase-binding protein; (A5) pore protein. (B1) Chymotrypsin (1 μg); (B2,4,6) hexokinase-binding protein plus 1 μg, 2 μg and 4 μg chymotrypsin, respectively; (B3,5,7) pore protein + 1 μg, 2 μg and 4 μg chymotrypsin, respectively; (B8) chymotrypsin (4 μg).

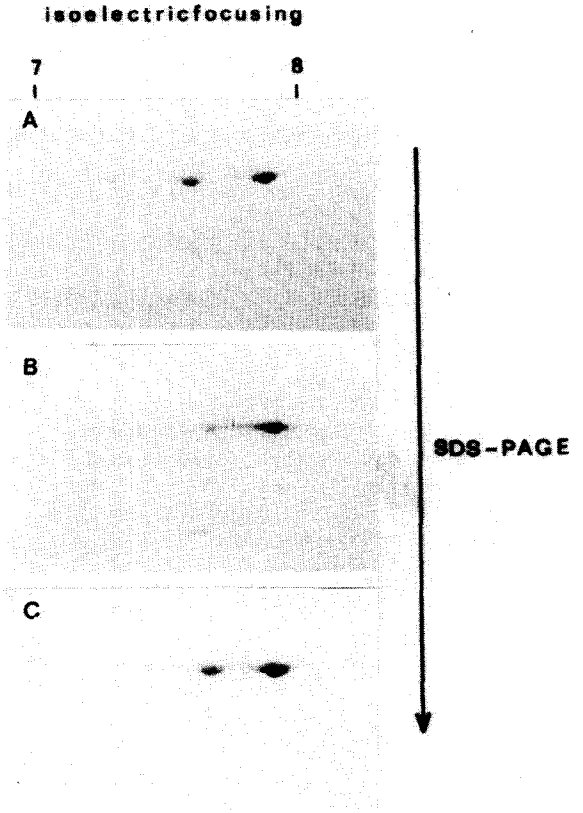

Fig.3. Two-dimensional electrophoretic analyses of the hexokinase-binding protein and pore protein. The hexokinase-binding protein and pore protein were analyzed by two-dimensional gel electrophoresis as in [9]: (A) pore-protein (10 μg); (B) hexokinase-binding protein (50 μg); (C) pore-protein (10 μg) + the hexokinase-binding protein (50 μg).

kinase-binding protein and pore protein are identical is further supported by co-electrophoreses of a mixture of the two (fig.3C).

Finally, the ability of the hexokinase-binding protein preparation to form pores or aqueous channels when reconstituted with lipid vesicles was tested. This was measured as passive diffusion of [^{3}H]sucrose out of the vesicles [5]. [^{14}C]Dextran (M_r 70 000) was used as an internal standard, since vesicles are virtually impermeable to dextran both in the presence or absence of added pore protein [6] or of hexokinase-binding protein (table 1). A decrease in the ratio of [^{3}H]sucrose/[^{14}C]dextran was taken as a measure of pore activity [5,6]. Table 1 shows that the hexokinase-binding protein exhibited a pore activity of 5.4. This agrees well with the value of 6.2 observed for partially purified pore protein having about the same degree of purity as the hexokinase-binding preparation used. Furthermore, the pore activity of the hexokinase-binding protein preparation is significantly higher than that observed for purified cytochrome oxidase or cytochrome b_5 [6], two membrane proteins with no known pore activity. This suggests that the observed pore activity is not due to non-specific leakage, but rather is specifically mediated by the hexokinase-binding protein. Furthermore, no pore activity was observed in the presence of 0.1% octylglucopyranoside, which is approximately the detergent concentration contributed by the hexokinase-binding protein during reconstitution with lipid vesicles.

We propose that the pore protein and the hexo-

Table 1
Pore activity (cpm) of the isolated hexokinase-binding protein

Additions	Saccharide retained in vesicles		^{3}H/^{14}C
	[^{3}H]Sucrose	[^{14}C]Dextran	
Vesicles	956	1455	1.5
Vesicles + hexokinase-binding protein[a]	178	961	5.4
Vesicles + partially purified pore protein	391	2414	6.2
Vesicles + purified pore protein	85	1562	18.4
Vesicles + 0.1% octyl-β-D-glucopyranoside[b]	1588	2679	1.7

[a] The hexokinase-binding protein preparation used is purified to the '1 × reconstituted state' (see [4] and section 2)
[b] The amount of detergent contributed by the hexokinase-binding protein preparation

kinase-binding protein of the outer mitochondrial membrane of rat liver are most likely identical proteins since:

(1) They exhibit the same app. M_r-values (30 000) on SDS–PAGE;

(2) They show identical peptide patterns after digestion with either *Staphylococcus aureus* V8 protease or chymotrypsin;

(3) They co-electrophorese in a two-dimensional system and exhibit identical isoelectric points;

(4) The hexokinase-binding protein preparation is able to form aqueous pores similar to those formed by outer membrane pore protein [6].

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