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Cross-linking analysis of yeast mitochondrial outer membrane

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By enrichment of contact sites between the two mitochondrial boundary membranes it has been shown that this fraction contained a high activity of glutathione transferase and hexokinase which was bound to the outer membrane pore protein (Ohlendieck, K. et al. (1986) Biochim. Biophys. Acta 860, 672–689). Therefore, an interaction between the three proteins in the contact sites has been suggested. Cross-linking experiments with isolated outer membrane of yeast mitochondria show that glutathione transferase and the pore protein are already associated in the free outer membrane. Porin appeared to adopt four different oligomeric complexes in the membrane, including interactions with a 14 kDa polypeptide, which has glutathione transferase activity. The latter polypeptide could be phosphorylated by intrinsic or extrinsic protein kinases, while the porin itself was not phosphorylated. Yeast hexokinase, when bound to the outer membrane, was able to cross-link to the pore protein.

Introduction

The outer membrane of mitochondria has been isolated from several sources including animals [2], plants [3] and lower eukaryotes [4]. Isolated yeast outer membrane has been shown to form sealed, right-side-out vesicles [5]. Its major components include a 29 kilodalton (kDa) polypeptide, which is a pore-forming protein [6,7] and other polypeptides with relative molecular masses of 70 kDa, 45 kDa, 33 kDa, and 14 kDa. It has been established that the stainable polypeptide band at 45 kDa consists of two immunologically distinct proteins, one of which is exposed at the inner surface and the other at the outer surface of the outer membrane [5]. Excluding the 14 kDa protein, no specific functions have yet been ascribed to these polypeptides. Morgenstern et al. [8] identified the 14 kDa polypeptide in liver microsomes as glutathione transferase and reported that the same enzyme was present in the outer mitochondrial membrane. In the preceding investigation [1], we obtained the same results for the outer membrane following isolation and characterization of the contact sites between the two boundary membranes of rat liver mitochondria. The outer membrane in the contact region appears to have different properties compared to the pure outer membrane: (1) specific activity of glutathione transferase is elevated and (2) the outer membrane pore has a 5-fold higher capacity to bind hexokinase. We consider interactions between these two proteins possible because electron microscopy sug-

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Abbreviations: DMSO, dimethylsulfoxide; DTSP, dithiobis (succinimidylpropionate).

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gests the formation of contacts in rather limited areas of the outer membrane [9]. We, therefore, attempted to analyze whether these enzymes are already present as preformed complexes in the pure outer membrane, or if the complexes are created by contact formation.

The enhanced binding of hexokinase to the pore at the contact sites suggests that these sites are important in creating a microcompartment between peripheral bound kinases and the ATP/ADP translocating system in the inner boundary membrane [10]. However, it is not known if hexokinase can also bind to porin outside the contact regions or if the enzyme can bind unspecifically to other outer membrane proteins.

In addition, it was of interest to analyze the structure of the pore which binds hexokinase. When incorporated into black lipid bilayer membranes, the pore protein can adopt two conformations of different conductance depending on the applied voltage [11–13]. Physiologically, an increase in negative surface charge by phosphorylation [14] can affect the intrinsic membrane potential across the outer membrane and, thereby, modulate pore conductance [11,13]. Likewise, it may also result in desorption of negatively charged hexokinase [10,15]. Structural changes of the pore (resulting from charge alterations) may, therefore, include different states of porin oligomer formation.

Using cross-linking methods, we have analyzed the interactions of porin monomers with other porin monomers and with integral and peripheral proteins (such as hexokinase) in isolated outer membranes from yeast mitochondria. Compared to the liver, the outer membrane from yeast contains a lower number of integral proteins and therefore, allows a better interpretation of the results from cross-linking experiments.

Materials and Methods

Preparation of outer membrane from yeast mitochondria. The membrane fraction was isolated essentially according to Riezman et al. [5], except that the Percoll gradient centrifugation step was omitted.

Binding of yeast hexokinase to the outer membrane. 10 U yeast hexokinase (Boehringer-Mannheim, Germany) were incubated with 200 µl isolated outer membrane (3 mg protein/ml) for 20 min at ice temperature in 1 ml of 0.25 M sucrose, 10 mM Hepes (pH 7.4), 10 mM glucose, 10 mM $MgCl_2$. The samples were centrifuged for 4 min in a tabletop centrifuge at 8800 × *g*. The supernatant and pellet were separated and used for enzymatic testing and cross-linking experiments.

Preparation of antibodies. Antibodies active against the 14 kDa, 29 kDa, 70 kDa proteins and the mixed 45 kDa protein bands were raised in rabbits by injection of protein electroeluted from polyacrylamide gel slices as previously described [16]. Monoclonal antibody reactive against the internal 45 kDa protein was that described by Riezman et al. [5]. Antibodies against yeast hexokinase were induced in rabbits by subcutaneous injection of the isolated enzyme mixed with Freund's complete adjuvant.

Cross-linking with dithiobis(succinimidylpropionate) (DTSP): Mitochondrial outer membrane (approx. 4 mg protein per ml) was diluted with an equal volume of 0.1 M triethanolamine buffer (pH 8). Cross-linking was performed at room temperature for 4 min using 10–150 µg/ml DTSP (dissolved to 4 mg/ml in DMSO). The reaction was stopped by adding 50 µl of 1 M ammonium acetate/ml reaction mixture, to inactivate the unreacted DTSP [17,18]. The membranes were collected by centrifugation through a 3 ml cushion of 0.625 M sucrose at 165 000 × *g* for 3 h [19]. The pellet was resuspended in 50 µl 20 mM Hepes-KOH (pH 7.4) and subjected to electrophoretic analysis.

Phosphorylation of outer membrane proteins. Either 50 µl untreated outer membrane or 80 µl cross-linked membrane were used for phosphorylation. Membranes were centrifuged off and suspended in 20 mM Hepes-KOH (pH 7.4) until a protein concentration of approx. 2,5 mg/ml was attained. The phosphorylation was performed at 4°C for 15 min. The assay mixture contained 5 mM $MgCl_2$, 2 mM [γ-^{32}P]ATP (0.1 mCi) and, when indicated, 25 µl cAMP-dependent protein kinase (active subunit from bovine heart, 40 mU/mg). The reaction was stopped by the addition of EDTA at a final concentration of 5 mM.

SDS-polyacrylamide gel electrophoreiss. Outer membrane polypeptides were dissociated by in-

cubation at room temperature for 15–30 min in 6% SDS. The solubilized proteins were separated on either 10% or 12.5% acrylamide gels using the system of Douglas and Butow [20] with minor modifications. For two-dimensional electrophoresis, the samples were first run on tube gels and soaked for 15 min in electrophoresis buffer containing 10% (v/v) 2-mercaptoethanol (this treatment cleaved the cross-linked polypeptides by reducing the disulfide bond in the DTSP molecule). The tube gels were subsequently embedded in 1% agarose over slab gels containing the same acrylamide concentration as in the tube gel and were run at constant voltage for 4 h.

Electrotransfer and immunodecoration. Proteins were transferred from slab gels to nitrocellulose filters as previously described [21,22]. The transferred proteins were stained with Ponceau-S solution (Serva, F.R.G.), destained, incubated with specific antibodies, followed by peroxidase-conjugated Protein-A. The peroxidase reaction was performed in a mixture of 9 ml 4-chloro-1-naphthol (0.3% in methanol), 141 ml 0.14 M NaCl buffered with 10 mM phosphate, pH 7.0, and 60 μl 35% H_2O_2.

Glutathione transferase assay. The activity of glutathione transferase was determined according to Habig et al. [23].

Autoradiography. Dried gels or nitrocelluose filters were exposed to Kodak NS-2T or XS-5 films.

Results

Effect of DTSP on proteins of the outer membrane

In isolated yeast mitochondrial outer membranes, DTSP (0–200 μg/ml) reacted mainly with four polypeptides of 45 kDa, 33 kDa, 29 kDa, and 14 kDa (Fig. 1). As the DTSP was increased, the amount of these four polypeptides in the sample decreased, while the relative concentration of high molecular weight species (larger than 70 kDa) increased. In subsequent experiments, a concentration of DTSP between 100 and 150 μg/ml was used.

Analysis of the cross-linked species by two-dimensional electrophoresis and immunodecoration

In this symmetric, two-dimensional electrophoretic system, the mobility of an oligomer produced by cross-linking can be determined from the vertical positioning of protein spots below the diagonal. The horizontal positions of polypeptides indicate the mobilities of monomers released from a given cross-linked species. Estimation of the molecular size was based on the mobilities of the major polypeptides in the outer membrane seen in the diagonal line (Fig. 2). Under our reaction conditions, the polypetide at 45 kDa formed an apparent dimer. The 29 kDa protein formed complexes of 60 kDa, 96 kDa, 43 kDa and 72 kDa. As expected, all of the stained protein spots of the second dimensional gel that lay on the line horizontal to the monomeric porin reacted with monospecific antiporin antibody (Fig. 2B). Since the spots at vertical positions corresponding to 60 kDa and 96 kDa were unique to their axes, i.e. no other spots of either higher or lower monomeric

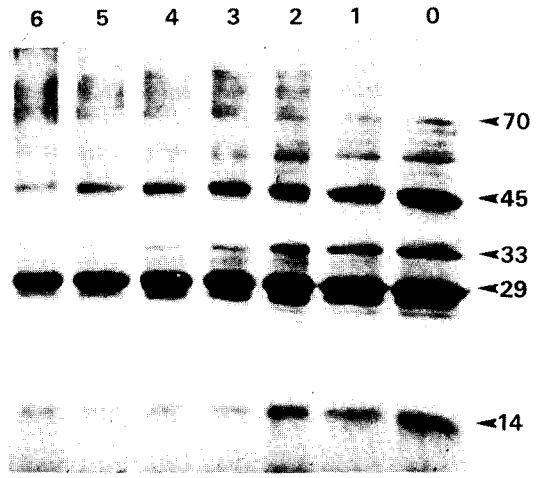

Fig. 1. Effect of increasing DTSP concentrations on outer membrane proteins. Outer membrane (4 mg/ml) treated with different concentrations of DTSP for 4 min at room temperature was run on a 12.5% polyacrylamide gel in the presence of SDS as described in Methods. Lane 0 to 6 correspond to increasing DTSP concentrations: untreated outer membrane (0), 10 μg/ml (1), 30 μg/ml (2), 50 μg/ml (3), 75 μg/ml (4), 100 μg/ml (5), 150 μg/ml (6). Cross-linking can be seen in this one dimensional separation as a decrease in staining intensity of the major outer membrane polypeptides (marked by arrows and the corresponding molecular mass in kDa) and a concomitant increase in amount of high molecular weight material above 70 kDa. In the subsequent figures a cross-linker concentration as in lanes 5 and 6 was used.

species lined up vertically with them (Fig. 2A), we infer that these represent, respectively, cross-linked dimers and trimers of porin. The additional complexes of 43 kDa and 72 kDa probably result from cross-linking of porin with other polypeptides.

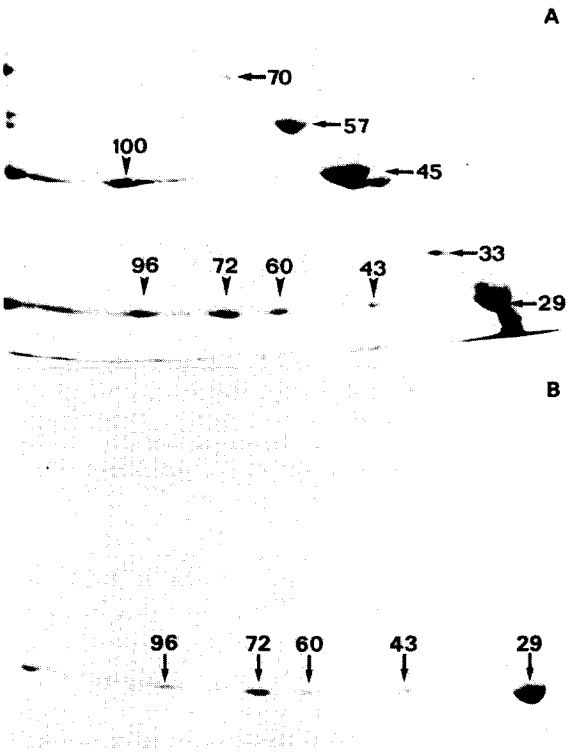

Fig. 2. Two-dimensional analysis of cross-linked oligomers in yeast mitochondrial outer membranes. Isolated outer membrane was treated with 100 μg/ml DTSP and was run on a 10% polyacrylamdie tube gel (from left to right in this and all other figures). Following cleavage of DTSP with 2-mercaptoethanol, the tube gel was overlaid on a 10% slab gel, and the sample subjected to electrophoresis in the second dimension (top to bottom). The numbers along the diagonal indicate the apparent molecular weights of the monomeric forms of five major outer membrane components. The numbers below the diagonal give the estimated molecular masses of the observed cross-linked products (in kDa). The calculation of the molecular masses of these oligomers was based on the known values of the corresponding monomers in the diagonal. (A) Two-dimensional gel transferred to nitrocellulose sheet and stained with Ponceau-S. (B) The same nitrocellulose sheet destained and subsequently decorated with antibodies against the 29 kDa polypeptide. The antibodies were visualised by reaction of protein-A conjugated peroxidase showing that the 29 kDa polypeptide is present in four different oligomeric states.

To determine whether only one or both of the 45 kDa protein species were cross-linked by DTSP, cross-linked proteins were separated by two-dimensional electrophoresis and transferred to nitrocellulose sheets in tandem. Both sheets, stained with Ponceau S after transfer, showed spots corresponding to monomeric and dimeric 45 kDa protein. After the sheets were destained, one sheet was reacted with rabbit anti-45 kDa (reactive with both protein species) and the other with monoclonal anti-45 kDa (reactive only with the internal 45 kDa protein). The position of the bound antibodies demonstrated that the monoclonal antibody recognized only the antigen present on the diagonal (Fig. 3C), whereas the polyclonal rabbit antibody bound to the antigen corresponding to both a 45 kDa monomer and a 100 kDa dimer (Fig. 3B). This result indicated that only the 45 kDa protein on the external face of the outer membrane reacted with DTSP under the conditions used here. Furthermore, the results suggested that the additional porin complex of 72 kDa is not composed of the 45 kDa and a porin monomer because no spot of the 45 kDa polypeptide lines up vertically with the 72 kDa oligomer.

Besides creation of defined oligomers, cross-linking usually resulted in the formation of further protein complexes which could not be classified as species of defined molecular weight. These were complexes remaining on top of the first gel and the oligomers with a molecular mass higher than 200 kDa (Fig. 2).

Phosphorylation of outer membrane proteins

The isolated outer membrane was incubated with [γ-^{32}P]ATP either in the presence or absence of the active subunit of cAMP-dependent protein kinase from heart. Several polypeptides of the outer membrane became phosphorylated even in the absence of the exogenous protein kinase, suggesting that there was a protein kinase intrinsic to the outer membrane preparation. Under both conditions the 14 kDa protein was the most prominent phosphorylated protein (Fig. 4), whereas the 29-kDa polypeptide was not phosphorylated under either set of conditions. After cross-linking with DTSP, the 14 kDa polypeptide could still be phosphorylated, while the phosphorylation of the

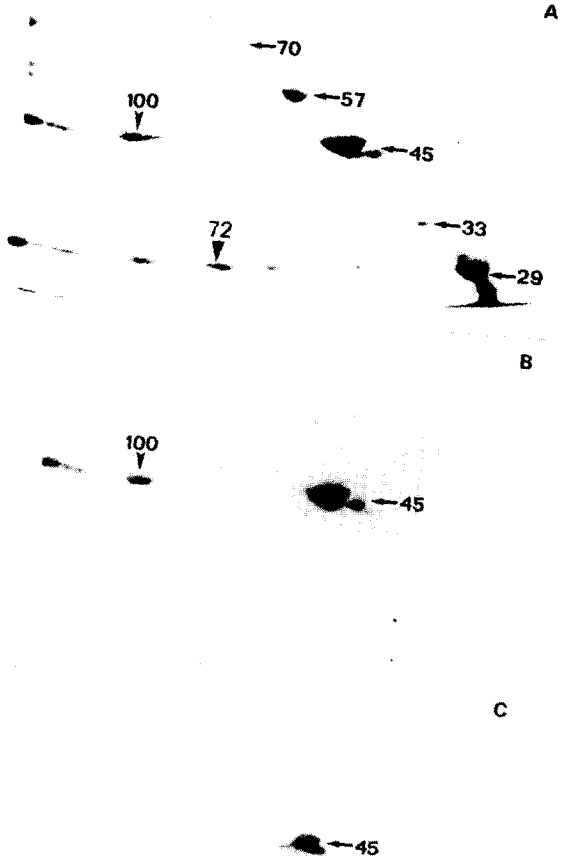

Fig. 3. Two-dimensional analysis of cross-linked polypeptides in yeast outer mitochondrial membrane. (A) shows all proteins transferred to a nitrocellulose sheet stained with Ponceau-S. The molecular weights of monomers and oligomers are indicated as in Fig. 2A. Two nitrocellulose sheets as in (A) were decorated with antibodies against the two different 45 kDa polypeptides; (B) polyclonal antibody reactive with both 45 kDa proteins, (C) monoclonal antibody against the 45 kDa protein located at the inner surface of the outer membrane.

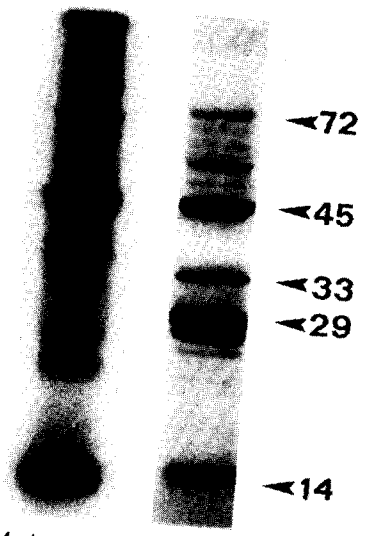

Fig. 4. Autoradiography of phosphorylated yeast outer membrane components. Isolated mitochondrial outer membrane from yeast was phosphorylated in the presence of [γ-^{32}P]ATP as described in Methods without addition of protein kinase. The polypeptides of the outer membrane were separated on a 12.5% polyacrylamide gel in the presence of SDS. The right lane shows protein staining, the left lane autoradiography.

45 kDa polypeptide band seemed to be reduced (Fig. 5).

Identification of heterooligomers

The phosphorylation of the 14 kDa polypeptide was used to identify the protein in the hetero-oligomers, because probing the gel with antibodies against this polypeptide resulted also in un-specific labeling. The 14 kDa protein could only be observed when a lower % acrylamide gel was used (Fig. 5). When such a gel was run, 14 kDa phosphor-protein spots in the autoradiography (Fig. 5B) aligned horizontally with the two porin spots, as described above, corresponding to 43 kDa and 72 kDa. These 14 kDa spots represent the 14 kDa polypeptide because they align with the 14 kDa protein in the second dimension, interact with 14 kDa specific antiserum (data not shown), and can be phosphorylated. Thus, the 43 kDa spot apparently represents a heterodimer of the 14 kDa and 29 kDa proteins, whereas the 72 kDa is presumably a heterotrimer containing one 14 kDa polypeptide and two 29 kDa subunits. The additional 30 kDa spot, which aligned with the 14 kDa polypeptide in the second dimension, most likely represents a homologous dimer of this protein.

Glutathione transferase activity in the outer membranes of yeast mitochondria

The phosphorylated polypeptide of 14 kDa in the liver outer membrane was identified as glutathione transferase by specific antibodies against this enzyme [8]. Activity of this enzyme is present in the outer membrane of yeast

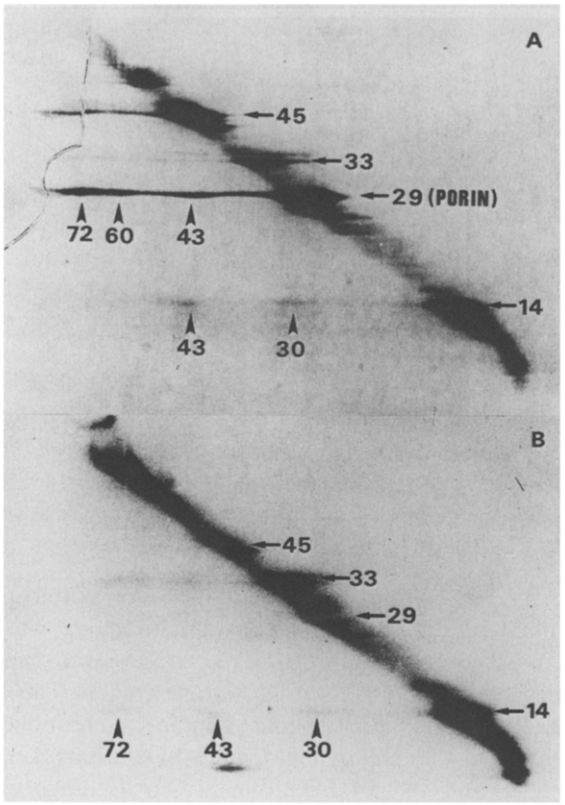

Fig. 5. Two-dimensional analysis of cross-linked and phosphorylated mitochondrial outer membrane. Isolated mitochondrial outer membrane was cross-linked as in Fig. 2 and afterwards phosphorylated by external cAMP-dependent protein kinase in the presence of [γ-^{32}P]ATP. The polypeptides were separated in two dimensions on 12.5% polyacrylamide gels in the presence of SDS. (A) shows the gel stained for proteins with Coomassie blue, (B) shows the autoradiography of the same gel. As in Figs. 2 and 3 the numbers give the apparent molecular masses of the monomers and the oligomers, respectively, in kDa.

mitochondria, although the specific activity is lower compared to that of liver (Table I).

Binding of hexokinase to the outer mitochondrial membrane

We observed that yeast hexokinase could bind to the outer membrane of yeast mitochondria as previously described for mammalian tissues. Under the conditions used, 8 to 10 mU hexokinase per mg of the isolated outer membrane was bound. The enzyme recovered in the outer membrane pellet could be visualized by probing with specific antiserum. It migrated in the SDS-polyacrylamide

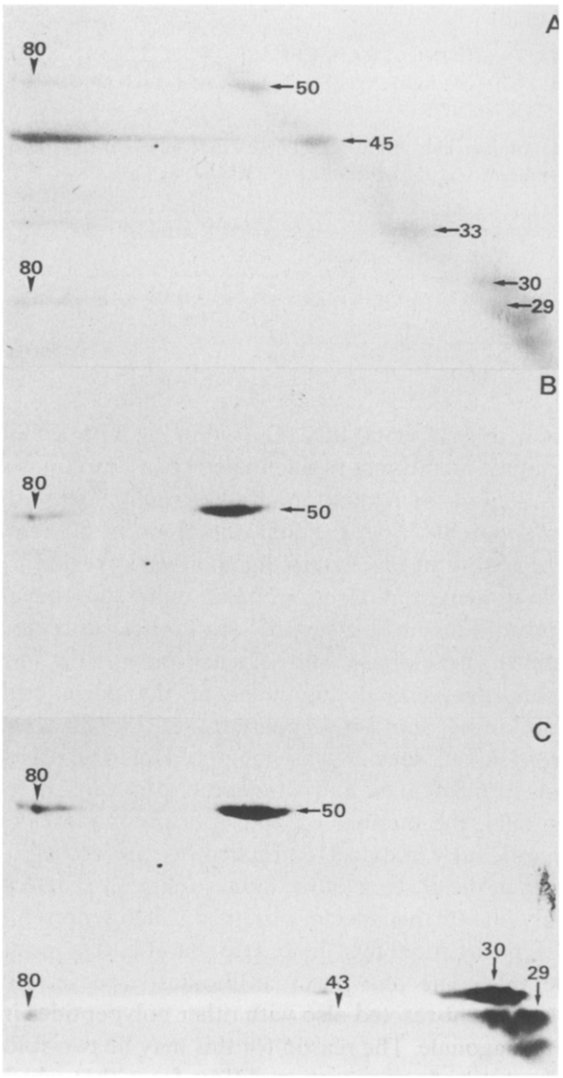

Fig. 6. Two-dimensional analysis of cross-linked outer membrane after binding of hexokinase. Hexokinase was bound to the outer mitochondrial membrane and cross-linked as in Fig. 2. The polypeptides were separated in two dimensions on 10% polyacrylamide gels in the presence of SDS. (A) Two-dimensional gel transferred to a nitrocellulose sheet and stained with Ponceau-S. The molecular masses of monomers and oligomers are indicated as in Figs. 2A. (B) Same nitrocellulose sheet destained and decorated with antibodies against yeast hexokinase, showing a monomer and a high molecular mass oligomer of hexokinase. (C) Same nitrocellulose sheet redecorated with antibody against porin. Several polypeptides are decorated in the diagonal; in addition there is a protein spot corresponding to a heterologous dimer of 29 kDa polypeptide and hexokinase. The reasons for the decoration of proteins other than the 29 kDa polypeptide in this experiment are not clear.

TABLE I

GLUTATHIONE TRANSFERASE ACTIVITY IN THE OUTER MEMBRANES OF YEAST AND LIVER MITOCHONDRIA

Glutathione transferase activity was determined in the outer membrane fractions according to Habig et al. [23].

Outer membrane of	Enzyme activity (mU/mg)
Yeast	23.3
Liver	119 ± 10.8

gel with a M_r of 50 kDa. Cross-linking with DTSP of outer membrane preincubated with hexokinase, resulted in the formation of a heterooligomer with an apparent M_r in the first dimension of 80 kDa. The nature of this heterooligomer was revealed by two-dimensional electrophoresis and subsequent immunodecoration with antibodies directed against hexokinase and against porin (Fig. 6). From the vertical alignment of the porin and hexokinase spot, it appeared that the 80 kDa cross-linked species was composed of one molecule of hexokinase and a monomer of porin. Interestingly, the number of other porin complexes is significantly reduced compared to the cross-linking in the absence of hexokinase (Fig. 2). There is only one further species observed which represents the porin monomer linked to the 14 kDa polypeptide. The anti-porin antibodies used in this experiment reacted also with other polypeptides in the diagonale. The reason for this may be two-fold. The antibody preparation differs from that which was used in the experiment shown in Fig. 2. The 30 kDa polypeptides which additionally reacted with the antibody was present in this outer membrane preparation in a higher concentration as was routinely observed. In addition to the immunodecoration, the identification of the porin was possible from the relative mobility compared to the known molecular weight of the major polypeptides of the outer membrane.

Discussion

Treatment of isolated yeast mitochondrial outer membrane with the reagent DTSP results in the formation of cross-linked oligomers composed of both identical and nonidentical monomeric proteins. The observed cross-linked species and their presumed compositions are summarized in Table II. The major homologous oligomers identified were the 45 kDa dimer, the 29 kDa dimer and trimer and the 14 kDa dimer. Our findings of dimers and trimers of the 29 kDa porin correlates well with the apparent oligomeric functional form of the molecules as reconstituted in liposomes and Triton X-100 micelles [24].

TABLE II

OBSERVED CROSS-LINKED OLIGOMERS AND THEIR PRESUMED COMPOSITION OF IDENTICAL AND NON-IDENTICAL MONOMERIC PROTEINS IN THE OUTER MITOCHONDRIAL MEMBRANE OF YEAST

HK, hexokinase.

M_r oligomers	M_r monomers				Inferred structure
	14 kDa	29 kDa (porin)	45 kDa	50 kDa (HK)	
30 kDa	(+)	–	–	–	$(14)_2$
43 kDa	(+)	(+)	–	–	$(14)\cdot(29)$
60 kDa	–	(+)	–	–	$(29)_2$
72 kDa	(+)	(+)	–	–	$(14)\cdot(29)_2$
80 kDa	–	(+)	–	(+)	$(29)\cdot(50)$
90 kDa	–	(+)	–	–	$(29)_3$
100 kDa	–	–	(+)	–	$(45)_2$

This is the first report that other integral proteins of the outer membrane can be cross-linked with porin. The fact that such proteins can be bridged by DTSP, which has a 1.2 nm spacer, suggests that porin associates and perhaps even interacts functionally with these polypeptides. The identifiable heterologous cross-linked species of this type appear to be (14 kDa monomer)-(29 kDa monomer) and (14 kDa monomer)-(29 kDa dimer). It is noteworthy that, despite the apparent ability of the 14 kDa protein to form a homologous dimer, we did not find species corresponding to (14 kDa dimer)-(29 kDa monomer or dimer). The 14 kDa polypeptide in microsomes represents the glutathione transferase [8] as well as in the outer membrane of liver mitochondria. This has been shown by reaction with specific antibodies against this enzyme [8,1]. The 14 kDa polypeptide in yeast has the same properties: (1) it is exposed to the

outer surface of the outer membrane [5], (2) is susceptible to proteolysis, and (3) it can be phosphorylated. Furthermore, yeast outer membrane contains glutathione transferase activity as was observed in the liver. It therefore, can be concluded that the 14 kDa polypeptide, also present in yeast outer membrane, represents glutathione transferase. Because glutathione transferase and the porin were found to be activated in the contacts between the boundary membranes, an interaction between the two proteins has been suggested [1]. The cross-linking experiments show that these two proteins are already associated in the outer membrane outside the contact regions. If our results truly reflect the in vivo distribution of proteins in the mitochondrial outer membrane, then we are left to consider the funcitonal significance of the 14 kDa-29 kDa association. Although the pores formed by the 29 kDa protein are voltage-dependent and anion-selective in black membranes, we do not know how the pores might be regulated in vivo. An exciting possibility, although speculative, is that the 14 kDa protein could either regulate porin oligomerization-dissociation or modulate pore activity, with phosphorylation contributing to this mechanism.

It has previously been shown that liver porin binds hexokinase [25,26]. Our results indicate that this also holds true for yeast porin. In addition, the observation that hexokinase is not crosslinked to other mitochondrial outer membrane proteins suggests that hexokinase interacts exclusively with porin among the outer membrane proteins. From our electron microscopic observations with gold-labelled hexokinase in the liver, it also appears that hexokinase prefernetially binds to porin in the outer membrane at sites of contact with the inner boundary membrane [27]. In agreement with this observation, the isolated contact membrane fraction has a higher capacity for hexokinase binding than the outer membrane fraction not associated with contact sites [1]. Since both membrane fractions contained porin protein, this finding suggests that the porin may assume different functional or conformational states in different regions of the outer membrane. Indeed, single-channel conductance measurements of the purified porin, reconstituted into planar bilayers, indicate that this molecule can form at least two diffierent kinds of pores [13]. This functional difference might reflect differences in the oligomeric state of the porin molecules.

The fact that we could not link hexokinase to the porin oligomeric form does not necessarily imply a preferential binding in vivo of the enzyme to the presumptive porin monomer. It is possible, for example, that cross-linking of hexokinase to one porin molecule can suppress the subsequent formation of porin-porin dimers. That hexokinase might bind to the porin oligomers agrees with the observation of a significant reduction of porin oligomeric forms upon cross-linking in the presence of hexokinase. The observation that the porin monomer linked to the glutathione transferase is still detectable in these experiments suggests that hexokinase might not bind to this molecular species of the pore. Finally, although yeast hexokinase has been reported to form dimers [28], in this study we did not observe the homologous cross-linking of this protein.

Previous studies have suggested that one must interpret the results of a cross-linking study with caution [29]. Cross-linking alone cannot determine the oligomeric conformation of the active molecule in the native membrane. However, it supports the assumption of different molecular porin species present in the outer membrane and the close association of the pore with glutathione transferase and hexokinase [1].

Acknowledgements

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