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The outer membrane of yeast mitochondria: isolation of outside-out sealed vesicles

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The yeast mitochondrial outer membrane was isolated and 10 of its major polypeptides were identified (mol. wts. 109, 70, 57, 45, 45, 42, 33, 29, 25 and 14 kd). The membrane has no major polypeptide in common with either mitochondrial inner membrane or rough microsomes. Protease treatment and immunochemical techniques showed that virtually all of the isolated outer membrane vesicles are sealed and display the same surface orientation as in the intact mitochondrion.
Key words: outer membrane/mitochondria/vesicles/yeast

Introduction

The yeast mitochondrion can be fractionated into four compartments: the matrix space containing 'soluble' enzymes of the citric acid cycle, the inner membrane containing the respiratory chain, an intermembrane space with cytochrome *c* peroxidase, adenylate kinase and flavocytochrome b_2 lactate dehydrogenase, and the outer membrane containing kynurenine hydroxylase (Daum *et al.*, 1982) and a non-specific pore (Colombini, 1979).

Most mitochondrial proteins are encoded in the nucleus, synthesized as larger precursors on cytoplasmic polysomes and can be post-translationally imported into mitochondria in an energy-dependent fashion (Neupert and Schatz, 1981). In order to understand this biogenetic pathway, it will be essential to define the role of the mitochondrial outer membrane in recognizing and transporting precursors of mitochondrial proteins.

Mitochondrial outer membrane has been previously isolated from several sources including animals (Sottocasa *et al.*, 1967), plants (Mannella and Bonner, 1975) and lower eukaryotes (Neupert and Ludwig, 1971); however, the array of proteins which are particular to this membrane has not been identified nor shown to be exclusively localized to the mitochondrial outer membrane. In fact, it has been claimed that several of the mitochondrial outer membrane polypeptides are shared with other cellular membranes (Shore, 1979; Bendayan and Shore, 1982) and that the mitochondrial outer membrane may be specifically associated with other cellular organelles (Franke and Kartenbeck, 1971). Moreover, it has remained open whether isolated mitochondrial outer membrane vesicles have the same (outside-out) or opposite (inside-out) 'sidedness' as the outer membrane in carefully isolated mitochondria or whether outer membrane vesicles are sealed. This information is essential for studying initial steps of mitochondrial protein import with isolated outer membrane.

We show here that the yeast mitochondrial outer membrane can be isolated as outside-out, sealed vesicles containing at least 10 unique polypeptides. We cannot detect any similarity in polypeptide composition between the outer membrane and other cellular membranes, such as the endoplasmic reticulum or the mitochondrial inner membrane. In addition, using proteolysis we have been able to derive information concerning the orientation of some of the major polypeptides in the outer membrane.

Results

If a crude preparation of mitochondrial outer membrane is subjected to sucrose gradient centrifugation, typical outer membrane markers (kynurenine hydroxylase and the 29-kd protein) form two discrete peaks (Figure 1). One peak (peak I) equilibrates at a density of 1.13 g/ml, is essentially free of inner membrane markers and contains purified outer membrane. The other peak (peak II) equilibrates with the residual inner membrane markers (cytochrome *c* oxidase and subunit I of ubiquinone-cytochrome *c* reductase) at 1.21 g/ml, and probably represents outer membrane still attached to inner membrane. The relative contribution of this dense fraction to total protein and outer membrane markers varies considerably among different experiments.

In a separate experiment, crude outer membrane was purified through a shallower gradient, equal volume aliquots of the gradient fractions were dissociated with SDS, subjected to electrophoresis and the gel was stained with Coomassie Blue (Figure 2A). Several major protein bands (arrows) co-fractionate exactly with the 29-kd protein (heavy arrow); their apparent molecular sizes are 14, 25, 33, 42, 45, 57, 70 and 109 kd (see also Figure 3). None of these proteins can be removed from the membrane by EDTA, high salt or chaotropic agents such as 0.7 M NaBr (not shown). Five of these polypeptides (those with apparent molecular sizes of 14, 29, 33, 45 and 70 kd) react strongly with an antiserum raised against total outer membrane (Figure 2B). These data suggest that all of these proteins are outer membrane proteins. In agreement with the data of Figure 1, the two major outer membrane antigens (the 29- and 45-kd proteins) can also be detected in the dense portion of the gradient. The additional bands seen in fractions 3 – 5 of panels A and B are not detectable in the peak of purified outer membrane (fractions 11 – 14) and are thus probably not outer membrane constituents.

Figure 3 shows that the polypeptide compositions of mitochondrial outer membrane (OM), mitochondrial inner membrane (IM) and rough microsomes (RM) are very different from one another. In particular, none of the nine major protein bands of the outer membrane (Figure 2) is found in either of the other two fractions except to the extent accounted for by cross-contamination. Even though some of these nine protein bands appear to be shared by the inner and outer membrane (see for example the 33-kd band, Figures 2 and 3), these

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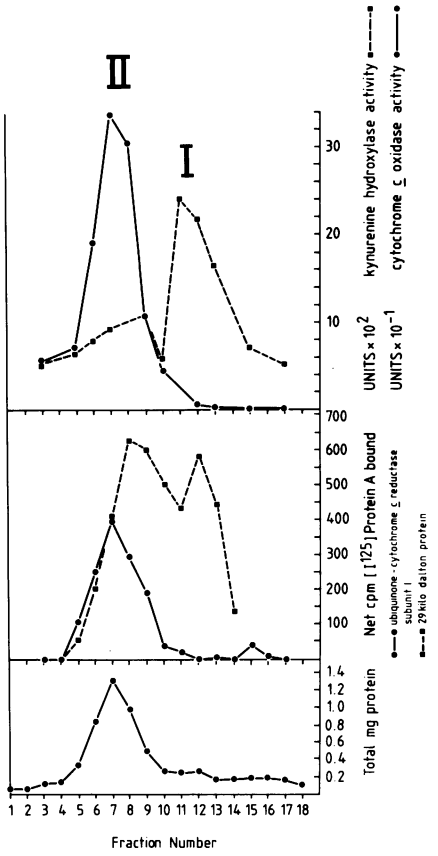

Fig. 1. Purification of mitochondrial outer membrane on a sucrose gradient. Crude outer membrane was layered onto a linear gradient (0.75 – 1.7 M sucrose in buffer B) and centrifuged for 12 h at 95 000 g_{max}. Eighteen 2 ml fractions were collected and assayed for protein content and for kynurenine hydroxylase and cytochrome *c* oxidase activities. Sedimentation was from right to left. Total enzyme activity recovered in each fraction is shown in the top panel. The specific activities of kynurenine hydroxylase and cytochrome *c* oxidase in their peak fractions were 0.136 U/mg and 16.84 U/mg, respectively. 10 μl of each gradient fraction was dissociated in SDS, separated by SDS-gel electrophoresis, transferred to nitrocellulose filters and incubated with antisera specific for either the 29-kd protein or the subunit I of ubiquinone-cytochrome *c* reductase. Antibody-antigen complexes were decorated with [^{125}I]protein A and the bands were visualized by autoradiography, excised and counted. The net c.p.m. ^{125}I recovered in each band are shown in the middle panel. Total protein content in each fraction is depicted in the bottom panel. Fractions 11 – 13 were pooled to obtain purified outer membrane. In this experiment, 15% of the mitochondrial protein was recovered as crude outer membrane.

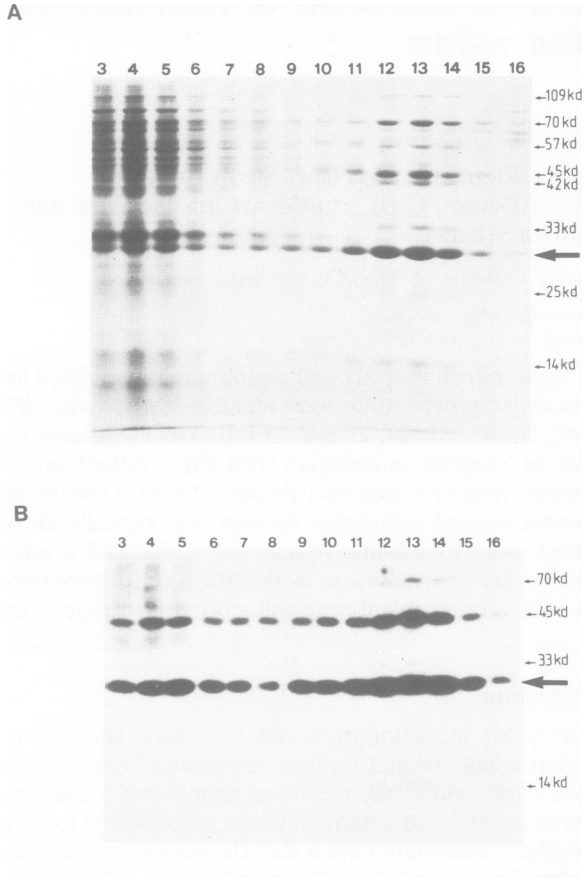

Fig. 2. Eight major polypeptides co-fractionate exactly with the 29-kd outer membrane protein. Crude outer membrane was layered onto a linear sucrose gradient (0.85 – 1.6 M sucrose) and centrifuged for 12 h at 95 000 g_{max}. Eighteen 2 ml fractions were collected and analyzed for stainable proteins and for immunoreactivity towards an antiserum raised against total outer membrane. Sedimentation was from right to left. **(A)** Equal volume aliquots of each gradient fraction were dissociated in SDS and separated on a 12.5% acrylamide gel. The gel was stained with Coomassie blue, destained and photographed. The sample loaded in fraction 13 corresponds to 15 μg protein. **(B)** Equal volume aliquots of each gradient fraction were dissociated in SDS, separated on a 12.5% acrylamide gel, transferred to a nitrocellulose filter and decorated with anti-outer membrane antiserum followed by [^{125}I]protein A. A photograph of an autoradiogram is shown. The apparent mol. wts. of the major outer membrane proteins are indicated in the right margin. In this experiment, 7.4% of the mitochondrial protein was recovered as crude outer membrane.

proteins are different as judged by immunodecoration with anti-outer membrane serum (not shown). We conclude that these nine proteins are *bona fide* components of the mitochondrial outer membrane.

Table I lists relative concentrations of five different marker proteins in the three fractions used in Figure 3 and the mitochondria from which the inner and outer membranes were prepared. These data were derived by immunodecoration as described in Materials and methods and permit several conclusions. First, the mitochondria prepared using Percoll are not significantly contaminated with microsomal membranes or cytoplasmic ribosomes, since they do not react with antisera raised against the major endoplasmic reticulum glycoproteins of dog pancreas or against yeast cytoplasmic ribosomes. Second, the rough microsome fraction does not contain appreciable concentrations of mitochondrial membrane markers. Third, the enrichments of kynurenine hydroxylase and 29-kd protein in the outer membrane fraction are similar; the fact they they are not identical probably reflects a loss of kynurenine hydroxylase activity during isolation. Fourth, the outer membrane is not significantly contaminated by microsomes or inner membrane. Finally, the enrichment of kynurenine hydroxylase and the 29-kd protein in the outer membrane fraction implies that the outer membrane constitutes between 8 and 16% of the mitochondrial protein. Using this figure and our recovery of 0.8% of the mitochondrial protein as outer membrane, we can calculate a total yield of 5 – 10%. In spite of this low recovery, all data reported above are consistent with the view that the recovered outer membrane is representative of the total outer membrane.

Characterization of monoclonal antibodies against outer membrane components

We have isolated several hybridoma lines which secrete antibodies against outer membrane proteins. The specificity

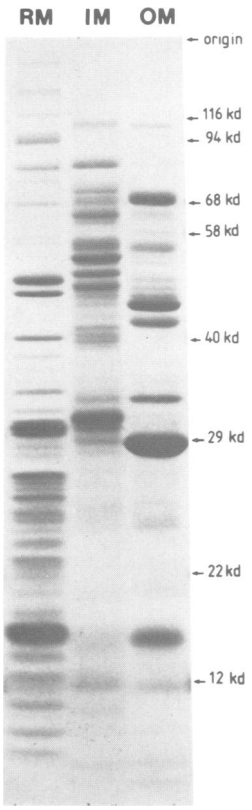

Fig. 3. The pattern of stainable protein in the mitochondrial outer membrane is unique. 35 μg of gradient-purified RM, IM and OM were subjected to electrophoresis on an SDS-polyacrylamide gel containing a linear (10 – 15% w/v) gradient of acrylamide. The gel was stained with Coomassie blue, destained and photographed. The mobilities of mol. wt. standards are indicated by arrows on the right side of the figure.

Table I. Relative concentrations of markers in subcellular fractions

Marker	Fraction			
	Mitochondria	Inner membrane	Outer membrane	Rough microsomes
Subunit I of cytochrome bc_1 complex	1.0	2.6	<0.05	<0.05
29-kd outer membrane protein	1.0	0.86	12.0	<0.05
Dog pancreas microsomal glycoprotein	<0.05	<0.05	<0.05	1.0
30-kd ribosomal protein	<0.05	<0.05	<0.05	1.0
Kynurenine hydroxylase	1.0	0.43	5.6	0.06

The relative specific content of the first four marker proteins in the fractions was estimated by immunodecoration based on standard curves as described in Materials and methods. The relative specific activity of kynurenine hydroxylase was determined from enzyme assays. Relative specific activities were normalized to 1.0 in the mitochondria or in the rough microsomes as indicated.

of three of these antibodies is demonstrated in Figure 4. Antibodies reactive against the 70-kd, 45-kd and 14-kd proteins react strongly with these proteins in isolated outer membranes but not with purified rough microsomes or appreciably with

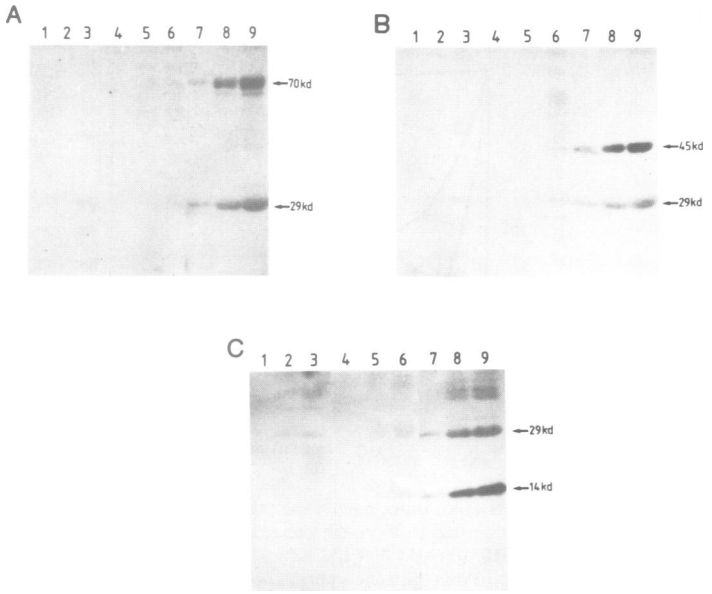

Fig. 4. Monoclonal antibodies reactive against the 70-kd, 45-kd and 14-kd proteins react only with outer membrane. Gradient-purified samples of rough microsomes **(lanes 1 – 3)**, mitochondrial inner membrane **(lanes 4 – 6)** and mitochondrial outer membranes **(lanes 7 – 9)** were subjected to immunodecoration with monoclonal antibodies (ascites fluid of mice which had been injected with the appropriate hybridoma cells). For each sample, 5 μg **(lanes 1, 4, 7)**, 20 μg **(lanes 2, 5, 8)** and 40 μg **(lanes 3, 6, 9)** of protein was applied to the gel. Antibodies reactive with the 70-kd protein **(panel A)**, the 45-kd protein **(panel B)** and the 14-kd protein **(panel C)** were used. A photograph of the autoradiogram is shown.

purified inner membrane.

Unexpectedly, all of the monoclonal antibodies also appear to react with the 29-kd protein. Control experiments without added monoclonal antibody demonstrated that this reaction is an artefact of immunodecoration: the 29-kd protein binds avidly to some radioactive component of our iodinated probe preparations.

Comparing immunodecoration results obtained with monoclonal antibodies and those obtained with the rabbit antiserum raised against whole outer membrane led us to suspect that there are two 45-kd proteins. To investigate this possibility, we treated isolated outer membranes with different amounts of trypsin, and then evaluated the disappearance of the 45-kd band by staining the gel and by immunodecoration with either a monoclonal antibody or rabbit antiserum. As demonstrated in Figure 5, more than half of the 45-kd band, as detected by staining or by decoration with the rabbit antiserum, is removed by trypsin; however, none of the 45-kd outer membrane protein which reacts with the monoclonal antibody is removed by the protease. The simplest explanation is that there are two 45-kd proteins. Both of them are recognized by the rabbit antiserum, but only one is recognized by the monoclonal antibody; that latter protein does not have a trypsin cleavage site exposed outside the isolated membrane.

Orientation of outer membrane vesicles

To study the sidedness of outer membrane vesicles and the orientation of individual outer membrane proteins, isolated mitochondria, 'mitoplasts' (mitochondria whose outer membrane had been ruptured osmotically to expose the inner surface of the outer membrane) and outer membrane were treated with trypsin or papain. The membranes were then re-

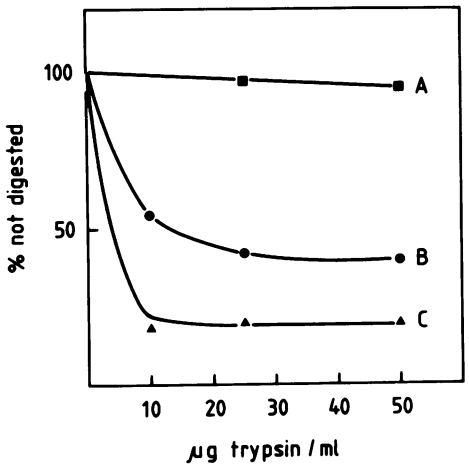

Fig. 5. There are two 45-kd outer membrane proteins. Isolated outer membrane vesicles were adjusted to a protein concentration of 1 mg/ml in 20 mM HEPES-KOH, 100 mM NaCl, 2 mM $MgCl_2$, pH 7.4, and incubated at 23°C for 10 min with the indicated concentrations of trypsin. Proteolysis was stopped by the addition of cold 10% trichloroacetic acid. The precipitates were collected, resuspended and dissociated by boiling in SDS. Aliquots were subjected to SDS-polyacrylamide gel electrophoresis. Proteins on one gel were fixed, stained with Coomassie blue and photographed. The 45-kd protein was quantified by densitometry of the photographic negative (**curve C**). Proteins in unfixed gels were transferred to nitrocellulose filters and decorated with anti-45-kd monoclonal antibody followed by [^{125}I]rabbit anti-mouse immunoglobulin (**curve A**) or with rabbit antiserum raised against isolated outer membrane followed by [^{125}I]protein A (**curve B**). For **curve A**, the 45-kd decoration was quantified by densitometry of the autoradiogram. For **curve B**, the 45-kd bands located by autoradiography were excised from the filter and counted directly for ^{125}I.

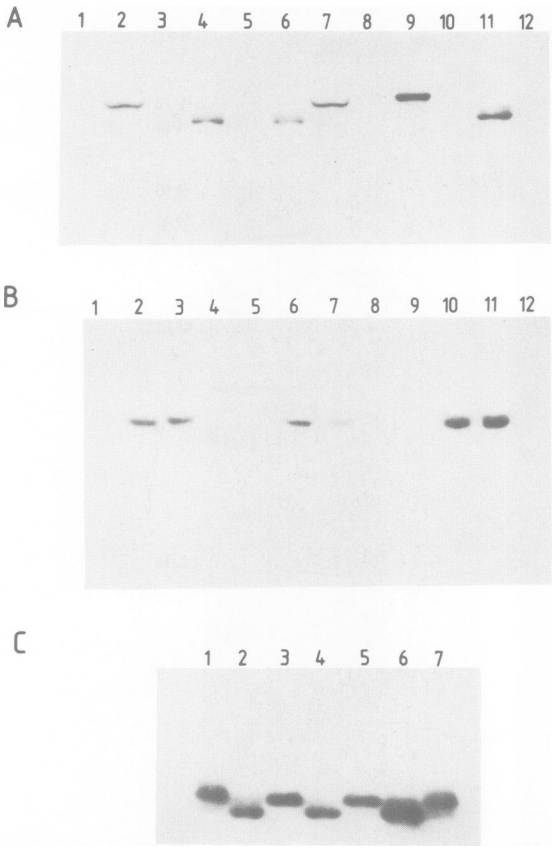

Fig. 6. Outer membrane vesicles are outside-out. Mitochondria, 'mitoplasts' and outer membrane were incubated at 2, 2 and 1 mg protein/ml, respectively, in 0.6 M mannitol, 20 mM HEPES-KOH, pH 7.4, in the presence or absence of 10 µg/ml trypsin (**panels A** and **B**) or 25 µg/ml papain (**panel C**) for 10 min at 0°C. The proteases were inhibited (100 µg/ml soybean trypsin inhibitor and 1 mM tosyllysylchloromethylketone for trypsin, 2 mM diisopropylfluorophosphate for papain) and the membranes were separated from soluble components by centrifugation. Supernatant fractions and pellets were dissociated with 3% SDS and analyzed by immunodecoration using monoclonal antibodies and [^{125}I]rabbit anti-mouse immunoglobulin. Photographs of the autoradiograms are shown. **Panel A** (decoration with monoclonal antibody against 70-kd protein): **lanes 1** and **2**, supernatant and pellet from untreated mitochondria; **lanes 3** and **4**, pellet and supernatant from treated mitochondria; **lane 5**, pellet from treated 'mitoplasts'; **lane 6**, supernatant from treated mitochondria (same as **lane 4**); **lane 7** pellet from untreated 'mitoplasts'; **lane 8**, supernatant from untreated 'mitoplasts'; **lane 9**, pellet from untreated outer membrane; **lane 10**, pellet from treated outer membrane; **lane 11**, supernatant from treated outer membrane; **lane 12**, supernatant from untreated outer membrane. **Panel B** (decorated with monoclonal antibody against 45-kd protein): **lanes 1, 4, 5, 8, 9, 12** are supernatants, **lanes 2, 3, 6, 7, 10** and **11** pellets. **Lanes 1–4** mitochondria, **lanes 5–8** 'mitoplasts', **lanes 9–12** outer membranes. **Lanes 3, 4, 7, 8, 11** and **12** were trypsin-treated, the others not. **Panel C** (decoration with monoclonal antibody against 14-kd protein): only pellets are shown. Even numbers are papain-treated samples; odd numbers are untreated samples. **Lanes 1, 2** and **7** outer membrane, **lanes 3** and **4** mitochondria, **lanes 5** and **6** 'mitoplasts'.

isolated by centrifugation and their polypeptide compositions were analyzed by immunodecoration with rabbit antisera or with monoclonal antibodies (Figure 6). The results of these experiments can be summarized as follows.

(a) In agreement with previous observations, the 29-kd protein is inaccessible to trypsin or papain in mitochondria, 'mitoplasts' and in isolated outer membrane (data not shown). This result suggests that the 29-kd protein is firmly embedded in the lipid bilayer, consistent with previous reports (Freitag *et al.*, 1982; Gasser and Schatz, 1983).

(b) The 70-kd protein is very sensitive to proteolysis in mitochondria, 'mitoplasts' and in isolated outer membranes. Indeed, it is lost if no phenylmethylsulphonyl fluoride (PMSF) is included during isolation of the outer membrane. Upon treatment of mitochondria, 'mitoplasts' or outer membrane with trypsin at 0°C, a 60-kd fragment of this protein is released into the supernatant (Figure 6, panel A). Thus, a major portion of the 70-kd protein appears to protrude from the mitochondrial surface.

(c) The 14-kd protein is cleaved by papain in mitochondria, 'mitoplasts' and outer membrane to yield a membrane-associated 12-kd fragment (Figure 6, panel C). At least part of the 14-kd protein is thus exposed on the cytoplasmic face of the outer membrane.

(d) The results with one of the two 45-kd proteins are of particular importance. The 45-kd protein which reacts with the monoclonal antibody is trypsin-sensitive in 'mitoplasts', but not in mitochondria or in outer membrane (Figure 5 and Figure 6, panel B). This observation indicates that this 45-kd protein is only accessible to trypsin when the inner surface of the outer membrane is exposed. We conclude not only that this 45-kd protein is exposed to the intermembrane space in the intact mitochondrion, but that virtually all of our isolated outer membrane is in the form of outside-out, sealed vesicles. We present additional evidence to support this claim below.

Isolated outer membrane vesicles are sealed

Isolated outer membrane prepared by swelling, shrinking and sonication is frequently contaminated with intermembrane space enzymes (Ernster and Kuylenstierna, 1970). We have taken advantage of this property by showing that the flavocytochrome b_2 (L-lactate: cytochrome *c* reductase)

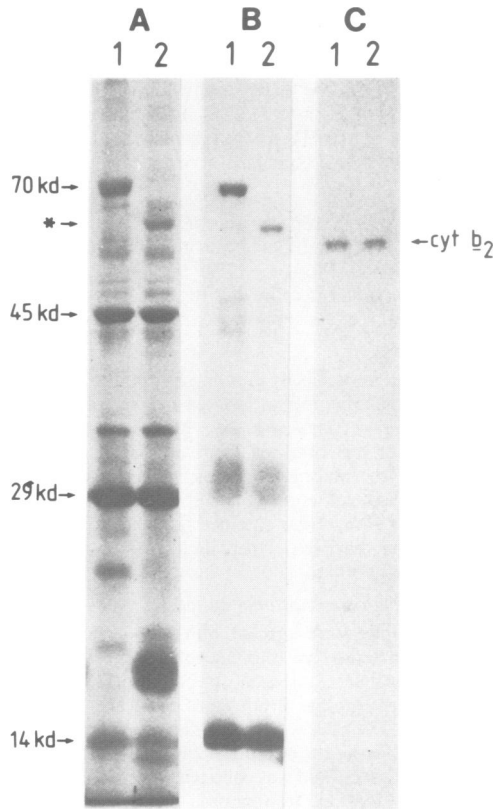

Fig. 7. Cytochrome b_2 present in gradient-purified outer membrane vesicles is inaccessible to externally-added trypsin. Gradient-purified outer membranes from yeast mitochondria were adjusted to 1 mg protein/ml in 0.6 M sorbitol. Equal volumes were incubated for 10 min on ice with no additions (**lanes 1**) or with 10 μg trypsin/ml (**lanes 2**). Trypsin was inhibited as described in Figure 5 and samples were analyzed by SDS-gel electrophoresis and staining with Coomassie Blue (**Panel A**) or immunodecoration with monoclonal antibodies against the 70-kd and the 14-kd proteins (**Panel B**) or with a rabbit antiserum against flavocytochrome b_2 lactate dehydrogenase (**Panel C**). Antibody-antigen complexes were reacted with [^{125}I]protein A or -IgG as described in the text and visualized by autoradiography. The latency of the flavocytochrome b_2 lactate dehydrogenase (cyt b_2) was measured in outer membrane vesicles using lactate as substrate and cytochrome *c* as electron acceptor. Stimulation of enzyme activity by the addition of Triton X-100 (final concentration 0.01%) to untreated outer membranes was 22.5-fold, and the corresponding stimulation in trypsin-treated outer membranes was 21-fold (96% and 95% latent activity, respectively). When ferricyanide was used as electron acceptor, the enzyme activity was not influenced by Triton X-100. The apparent molecular sizes and positions of the major outer membrane proteins are indicated on the left. The asterisk marks the position of the 60-kd fragment which is proteolytically derived from the 70-kd protein.

which contaminates the outer membrane is trapped inside sealed vesicles. This point was established in two ways. First, flavocytochrome b_2 lactate dehydrogenase activity with cytochrome *c* as an electron acceptor [cytochrome *c* cannot cross the outer membrane pore (Ernster and Schatz, 1981)] is increased 22.5-fold by the addition of 0.01% Triton X-100 whereas its activity with ferricyanide as an electron acceptor (which should freely diffuse across the outer membrane pore) is not affected by the detergent. Assuming that all vesicles trap flavocytochrome b_2 lactate dehydrogenase, this latency indicates that at least 95% of the vesicles are sealed. Second, the trapped enzyme is not digested by externally-added trypsin, whereas all of the externally-located 70-kd protein is cleaved from the membrane (Figure 7). If Triton X-100 is added during protease digestion, all of the trapped flavocytochrome b_2 lactate dehydrogenase is digested (not shown).

Therefore, we conclude that virtually all of our isolated mitochondrial outer membrane is in the form of outside-out, sealed vesicles. We can rule out the presence of substantial amounts of either membrane sheets or inside-out vesicles because of the demonstration that virtually none of the internal 45-kd protein (recognized by the monoclonal antibody and trypsin-sensitive in mitoplasts) is cleaved by trypsin treatment of the outer membrane (Figure 5 and Figure 6, panel B) and because the activity of the trapped flavocytochrome b_2 lactate dehydrogenase is latent and resistant to trypsin digestion under conditions where the 70-kd protein on the outer face of the outer membrane is completely converted to the 60-kd fragment.

Discussion

The mitochondrial outer membrane is the key to understanding how the mitochondrion interacts with the rest of the cell. It must play an important role in the selection and import of precursors of mitochondrial proteins, in interactions with the cytoskeleton and other organelles and in the response of mitochondria to stimuli. These interactions can only be examined successfully *in vitro* if outer membrane can be isolated in a state that resembles that *in vivo*. It should above all display the same 'sidedness' as in mitochondria, exposing the same variety of 'receptors' to the outside as does the mitochondrion to the cytoplasm. Moreover, only sealed vesicles are useful for investigating transport phenomena.

Previous studies of the mitochondrial outer membrane have been primarily enzymatic and morphological (Ernster and Schatz, 1981) and have, in general, been limited to defining the presence or absence of different enzymatic activities. The membrane has been shown to contain an antimycin-insensitive NADH-cytochrome *c* reductase and kynurenine hydroxylase, as well as a non-specific pore (Ernster and Kuylenstierna, 1970; Colombini, 1979). While the non-specific pore is possibly the 29-kd protein (Mihara *et al.*, 1982), no other major polypeptide of the yeast mitochondrial outer membrane has yet been ascribed a specific function. To determine the relatedness of yeast mitochondrial outer membrane to other cellular membranes and to characterize the state of the outer membrane vesicles, we decided first to purify and biochemically analyze the yeast mitochondrial outer membrane.

Our isolation procedure has enabled us to isolate mitochondrial outer membrane largely free from other membranes. We have identified 10 proteins which seem to be specific to this membrane. Contrary to the data published for rat liver mitochondrial outer membrane (Shore, 1979; Bendayan and Shore, 1982), we have been unable to find any prominent yeast mitochondrial outer membrane protein that is also present in either microsomes or mitochondrial inner membrane. Of course, small amounts of outer membrane proteins can be detected in other fractions, but in all cases they are present in the same proportions indicating simple contamination of these fractions with outer membrane. These data imply that the mitochondrial outer membrane is composed of unique polypeptides, probably reflecting its unique biogenesis (Freitag *et al.*, 1982; Gasser and Schatz, 1983) and unique functions.

One problem with our outer membrane isolation procedure is the low yield: at best ~10% of the total outer membrane is recovered. This recovery may reflect the presence of contact points between the inner and outer membranes (Hacken-

brock, 1968) which prevent their complete separation. Nonetheless, the isolated outer membrane should be representative of the total outer membrane, since the ratios of the different outer membrane proteins to each other (by electrotransfer and immunodecoration) are the same in isolated outer membrane and mitochondria (not shown).

The outer membrane vesicles are also representative of the native outer membrane in their 'sidedness' (see Results). They should be useful for assaying functions of the mitochondrial outer membrane which require proper orientation of its components, such as protein import or binding of the outer membrane to other cellular structures. In the accompanying paper we use these outer membrane vesicles to identify a receptor-like activity which specifically binds cytoplasmically-synthesized precursors of mitochondrial proteins (Riezman *et al.*, 1983).

Materials and methods

Growth of cells and subcellular fractionation

Wild-type cells of *Saccharomyces cerevisiae* strain D273-10B (ATCC 25657) were grown, harvested and converted to spheroplasts and homogenized as described (Daum *et al.*, 1982) in 3 volumes of ice-cold buffer A (0.6 M sorbitol, 20 mM Tricine-KOH, 10 mM KCl, 1 mM dithiothreitol (DTT), 1 mM PMSF pH 8.0). The homogenate was centrifuged at 1400 g_{max} for 5 min, the pellet resuspended in buffer A, recentrifuged as above and the combined supernatants from the first two spins were pooled and spun at 9600 g_{max} for 10 min. The supernatant ('post-mitochondrial supernant') was saved for the subsequent isolation of microsomes (see below). The pellet, consisting of crude mitochondria, was resuspended in a minimal volume of buffer A, layered over a discontinuous gradient of 10 ml 40% Percoll® (Pharmacia) in buffer A and 40 ml of 20% Percoll in buffer A and centrifuged at 105 000 g_{max} for 20 min. The band at the interface of the two Percoll layers was collected, resuspended, diluted with at least 20 volumes of buffer A and centrifuged at 1400 g_{max} for 5 min to remove large aggregates. The supernatant was centrifuged at 17 200 g_{max} for 15 min and the resulting pellet of mitochondria was washed once with buffer A by resuspension and centrifugation as above.

Mitochondria were swollen, shrunken and sonicated as described by Sottocasa *et al.* (1967) except that 1 mM PMSF was included throughout. After sonication the mixture was centrifuged at 27 000 g_{max} for 15 min. The pellet was resuspended in buffer B (5 mM HEPES-KOH, 10 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, pH 7.4). This resuspended pellet and the supernatant fraction from the same centrifugation were separately centrifuged at 140 000 g_{max} for 1 h. The pellet obtained from the resuspended pellet is the crude inner membrane; the pellet obtained from the supernatant is the crude outer membrane. The relative amount of mitochondrial protein recovered as crude outer membrane varies from experiment to experiment (7.4%, 15% and 20% in three separate isolations).

The post-mitochondrial supernatant (see above) was centrifuged at 16 000 g_{max} for 10 min to remove residual mitochondria and the supernatant was layered over a discontinuous sucrose gradient (1 volume of 1.3 M sucrose and 3 volumes of 1.8 M sucrose, both in buffer B) and spun at 100 000 g_{max} for at least 5 h. The band at the interface between the two sucrose layers was resuspended, diluted with buffer B and centrifuged at 100 000 g_{max} for 1 h to pellet the crude rough microsomes.

The crude membrane fractions were each resuspended in a small volume of buffer B and layered over linear sucrose gradients in buffer B (the concentrations of sucrose are indicated in the figure legends) and centrifuged for 14–16 h at 95 000 g_{max}. Fractions of 1–2 ml were collected from the bottom by puncturing the gradient tubes. Fractions corresponding to the peak activity of cytochrome *c* oxidase in the gradient of crude inner membrane, or to the peak activity of kynurenine hydroxylase from the gradient of crude outer membrane, were pooled, diluted with buffer B and centrifuged for at least 1 h at 100 000 g_{max} to sediment membranes. Membranes from the peak protein fractions of the crude microsome gradient were also pooled and repelleted. The pooled fractions were designated inner membrane (IM), outer membrane (OM) and rough microsomes (RM). Gradient-purified membrane fractions were resuspended in buffer B, frozen in liquid nitrogen and stored at −80°C. The yield of gradient-purified outer membrane was usually ~0.8% of the starting mitochondrial protein which agrees well with that found for *Neurospora crassa* mitochondria (Neupert and Ludwig, 1971).

In some experiments not requiring isolation of outer membrane, mitochondria were prepared as described (Daum *et al.*, 1982).

Protein estimation

Fractions to be analyzed for protein content were dissolved directly in 0.1 N NaOH and 1% SDS, or first precipitated in 10% w/v trichloroacetic acid for at least 30 min at 0–4°C. The pellets were washed once with 95% ethanol and dissolved in 0.1 N NaOH and 1% SDS and suitable aliquots were assayed according to Lowry *et al.* (1951), using crystalline bovine serum albumin in 0.01 N NaOH, 0.1% SDS as a standard.

Enzyme assays

Assays for cytochrome *c* oxidase (Mason *et al.*, 1973), kynurenine hydroxylase (Bandlow, 1972) and flavocytochrome b_2 [lactate:cytochrome *c* reductase] (Appleby and Morton, 1959) were performed as described previously. One enzyme unit is defined as the amount of enzyme converting 1 μmol of substrate/min under the specified assay conditions.

Preparation of antisera

Antisera were raised in rabbits by injecting membrane fractions or subunits of mitochondrial protein complexes isolated by preparative SDS-polyacrylamide gel electrophoresis (Nelson *et al.*, 1973). Antiserum against yeast cytoplasmic ribosomes was obtained from T. Mason (University of Massachusetts-Amherst). The major immunoreactive polypeptide has an apparent molecular size of 30 kd. Antiserum against the major endoplasmic reticulum glycoproteins of dog pancreas was obtained from D. Meyer (EMBL, Heidelberg). The major cross-reactive polypeptide in yeast microsomes has an apparent molecular size of 30 kd.

Preparation and testing of monoclonal antibodies

BALB/cJ female mice were injected with 100 μg purified mitochondrial outer membrane, then boosted with 100 μg outer membrane 8, 36 and 47 days later. Three days after the last injection, spleen cells were harvested and fused with FO myeloma cells (de St. Groth and Scheidegger, 1980) as described (Stähli *et al.*, 1980). FO cells were kindly provided by G. Köhler (Basel). Hybridomas were grown in the peritoneal cavity of BALB/cJ mice and the antibody-containing ascites fluid was collected as described (McKearn, 1980).

Outer membrane was electrophoresed on SDS-polyacrylamide gels, transferred to nitrocellulose and cut vertically into strips. Culture supernatants were tested for specific antibody production by immunodecoration using these outer membrane test strips.

Protein electrophoresis and immunodecoration

Electrophoresis of proteins in polyacrylamide gels containing SDS was performed as described by Douglas and Butow (1976) except that in most cases no acrylamide gradient was used. For immunodecoration, proteins were transferred electrophoretically from unfixed SDS-polyacrylamide gels to nitrocellulose sheets and individual antigens on the sheets were detected by incubation with specific antisera followed by [^{125}I]protein A, or with monoclonal mouse antibodies followed by [^{125}I]rabbit anti-mouse IgG, essentially by the procedure of Towbin *et al.* (1979) as modified by Rott and Nelson (1981). Apparent mol. wts. on SDS-polyacrylamide gels were calibrated with the following mol. wt. standards: *Escherichia coli* β-galactosidase (116 kd), phosphorylase *b* (94 kd), bovine serum albumin (68 kd), bovine liver catalase (58 kd), rabbit muscle aldolase (40 kd), human erythrocyte carbonic anhydrase (29 kd), soybean trypsin inhibitor (22 kd), and yeast cytochrome *c* (12 kd).

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