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Biochemistry

In vitro synthesis and integration into mitochondria of porin, a major protein of the outer mitochondrial membrane of *Saccharomyces cerevisiae*

(detection of porin activity/immunoprecipitation by specific antiserum/uncleaved signal sequence/posttranslational integration)

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ABSTRACT **We have isolated an outer mitochondrial membrane (OMM) fraction from baker's yeast, *Saccharomyces cerevisiae*, that possesses porin activity and contains a major polypeptide of 29,000 daltons. By analogy to similar data for an OMM fraction from rat liver and mung bean [Zalman, L. S., Nikaido, N. & Kagawa, Y. (1980) *J. Biol. Chem.* 255, 1771–1774], the 29,000-dalton polypeptide of the isolated yeast OMM fraction has been tentatively identified as porin. Evidence to substantiate this identification was provided by the finding that both the porin activity and the 29,000-dalton polypeptide were entirely resistant when the OMM fraction was exposed to trypsin digestion, with the 29,000-dalton polypeptide being virtually the only polypeptide in the OMM fraction to be unaffected by trypsin digestion. There was no protection when trypsin digestion was carried out in the presence of detergent. Using monospecific antibodies, we have shown that yeast porin is apparently not synthesized as a larger precursor in a cell-free translation system. *In vitro*-synthesized porin could not be integrated into dog pancreas microsomal vesicles or into an isolated OMM fraction from yeast, either co- or posttranslationally. *In vitro*-synthesized porin, however, could be integrated posttranslationally into whole isolated mitochondria. This membrane specificity suggests that integration does not proceed by unassisted partitioning. The integration of porin into whole mitochondria occurred with fidelity by the criterion of its resistance to trypsin. Morever, integration was not inhibited in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone whereas translocation into the mitochondrial matrix of the *in vitro*-synthesized γ subunit of F_1-ATPase was inhibited.**

The biogenesis of the outer mitochondrial membrane (OMM) is of interest because of the potentially dual origin of its integral membrane proteins (1). Theoretically, integral membrane proteins that are dependent for their asymmetric integration on a translocation system (1) could be integrated directly into the OMM, by using the OMM translocation system, or else they could be integrated into the rough endoplasmic reticulum (RER), by using the translocation system of that membrane, and subsequently be "sorted" into the OMM (1) via transient connections (or else via vesicles) between the ER and the OMM.

Recently, Zalman *et al.* (2) characterized an integral membrane protein of 30,000 daltons, a major polypeptide in OMM preparations of rat liver and mung bean (*Phaseolus aureus*), suggested that it forms channels for the nonspecific passage of low molecular weight hydrophilic solutes, and called it "porin" by analogy to proteins of similar function present on the outer membrane of Gram-negative bacteria.

In this paper, we demonstrate that an isolated OMM fraction of yeast also possesses porin activity and that it contains a major polypeptide of 29,000 daltons, tentatively designated as porin. Using monospecific antibodies raised in rabbits against porin, we have investigated its cell-free synthesis and its mode of integration into mitochondria.

METHODS

Isolation of Mitochondria. *Saccharomyces cerevisiae* strain D273-10B (ATCC 24657) was grown to midlogarithmic phase in a rich medium (3). To prepare spheroplasts, cells (8 g) were incubated in 20 ml of 0.1 M Tris·H_2SO_4, pH 9.3/3 mM dithiothreitol at 29°C for 5 min, collected by centrifugation, washed by two cycles of suspension/centrifugation with 1.5 M sorbitol/10 mM Tris·HCl (pH 7.4), resuspended in 13 ml of the same solution, and incubated with 11.5 mg of Zymolyase 60,000 at 29°C for 30 min. The resulting spheroplasts were collected and washed by two cycles of suspension/centrifugation in 1.5 M sorbitol/10 mM Tris·HCl, pH 7.4. The washed spheroplasts were suspended in 5 ml of 0.65 M sorbitol/10 mM Tris·HCl, pH 7.4, in the presence of 15 ml of glass beads (0.1 mm in diameter) and homogenized in a polypropylene centrifuge tube fitted with a motor-driven Teflon pestle. The cell lysates were centrifuged at 1,100 × *g* for 5 min to remove the glass beads and unbroken cells. The sorbitol concentration of the supernatant was adjusted to 1.3–1.5 M, and the resulting mixture was centrifuged at 3,000 × *g* for 5 min. The resulting supernatant was then centrifuged at 17,000 × *g* for 15 min to obtain a pellet of crude mitochondria. The crude mitochondrial pellet was suspended in 1.5 ml of 1.5 M sorbitol/10 mM Tris·HCl, pH 7.4; 0.3-ml aliquots of this suspension were layered on 5 ml of a linear 40–65% (wt/vol) sucrose gradient in 10 mM Tris·HCl (pH 7.4); six gradients were centrifuged in a swinging bucket rotor at 180,000 × *g* for 2 hr. Mitochondria banded at about 50% (wt/vol) sucrose (density, about 1.19) and were collected with a Pasteur pipette in a total volume of about 6 ml. After dilution with 2 vol of 1.0 M sorbitol/10 mM Tris·HCl, pH 7.4, the purified mitochondria were sedimented at 17,000 × *g* for 15 min and subsequently used for the preparation of outer and inner mitochondrial membranes or for integration of *in vitro*-synthesized porin (see below).

Isolation of Outer and Inner Mitochondrial Membranes. Purified mitochondria obtained from 8 g of cells were suspended in 10 ml of 20 mM potassium phosphate buffer (pH 7.5) by homogenization in a glass tube fitted with a Teflon pestle. After incubation for 30 min in an ice bath, the mitochondrial lysate was centrifuged at 2,800 × *g* for 10 min. The pellet was subjected to two more cycles of hypotonic lysis and centrifugation. The pellets were saved for the preparation of an inner

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Abbreviations: ER and RER, endoplasmic reticulum and rough ER; OMM, outer mitochondrial membrane; PRS, postribosomal supernatant; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

mitochondrial membrane fraction according to published procedures (4). The supernatants were combined and centrifuged at 105,000 × *g* for 30 min to obtain a crude OMM fraction. The crude OMM fraction was suspended in 0.5 ml of 20 mM potassium phosphate buffer (pH 7.5); this suspension was then layered over a discontinuous sucrose gradient consisting of 2 ml of 35% (wt/wt), 1.5 ml of 25% (wt/wt), and 1 ml of 10% (wt/wt) sucrose in 20 mM potassium phosphate buffer (pH 7.5) and, following centrifugation in a swinging bucket rotor at 220,000 × *g* for 60 min, OMM were recovered from the interphase between the 25% and 35% sucrose layers. After dilution with 3 vol of 10 mM Tris·HCl (pH 7.4), OMM were sedimented at 105,000 × *g* for 60 min and subsequently resuspended in 10 mM Tris·HCl (pH 7.4) to a final concentration of 2 mg of protein/ml.

Preparation of Immunoselected Antibodies. Antibodies were raised in rabbit against the major polypeptide (M_r, 29,000) of the purified OMM fraction (see Fig. 1, lane 4) excised from a $NaDodSO_4$/polyacrylamide slab gel as described in ref. 5. An IgG fraction was purified by affinity chromatography (6) of the antiserum on Sepharose 4B conjugated to the $NaDodSO_4$-denatured purified OMM fraction. An IgG fraction from preimmune serum was prepared by protein A-Sepharose affinity chromatography (7).

Assay for Porin Activity of the OMM Fraction. The assay was carried out exactly as described by Zalman *et al.* (2) using [^{14}C]sucrose and [^{3}H]dextran. Porin activity was expressed as saccharide retention ratio ([^{3}H]dextran/[^{14}C]sucrose). The $^{3}H/^{14}C$ ratio of the reaction mixture was normalized to 1.0 (2).

Cell-Free Translation and Integration of Porin into Mitochondria. Yeast total RNA was translated either in a wheat germ S-23 extract (8) or in a staphylococcal nuclease-treated rabbit reticulocyte lysate (9) in the presence of [^{35}S]methionine. Translation was carried out for 90 min at 29°C (5). After translation, the mixtures were cooled to 0–4°C and centrifuged in a Beckman Airfuge at 135,000 × *g* for 60 min, yielding a postribosomal supernatant (PRS); 2.0 M sucrose was added to the PRS to adjust it to a final sucrose concentration of 0.35 M. Indicated amounts (see Fig. 4) of purified mitochondria (see above) were added to the adjusted PRS and the mixture was incubated at 25°C for 60 min. The mixture was then cooled to 0–4°C and centrifuged at 10,000 × *g* for 12 min to yield a pellet (mitochondria) and a supernatant. Where specified, the mixture was incubated with trypsin (250 μg/ml) at 4°C for 60 min prior to the centrifugation step.

In vitro-synthesized porin was isolated by immunoprecipitation in the presence of 0.4% $NaDodSO_4$/2% Triton X-100 (10) using the immunoselected IgG fraction. The immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (using 15% polyacrylamide slab gels) and subsequent fluorography (11) of the stained and dried gels. Unlabeled porin, used for competition experiments, was eluted from excised bands of $NaDodSO_4$/polyacrylamide slab gels after electrophoresis of the purified OMM fraction.

Other Methods. NADPH–cytochrome *c* reductase (12) and succinate–cytochrome *c* reductase (13) activities were assayed by published methods. Protein was determined by the Lowry method (14) using bovine serum albumin as a standard.

Materials. Molecular weight markers (bovine serum albumin, chicken ovalbumin, rabbit muscle lactic dehydrogenase, bovine pancreas α-chymotrypsinogen A, soybean trypsin inhibitor, sperm whale myoglobin, and horse heart cytochrome *c*) and Trasylol were purchased from Sigma. L-[^{35}S]Methionine (specific activity, 1,084 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) and ^{125}I were obtained from New England Nuclear. [^{3}H]Dextran (mean M_r, 70,000; 174 mCi/g) and [^{14}C]sucrose (5.1 mCi/mmol) were from Amersham. Zymolyase 60,000 was from Seikagaku Kogyo (Tokyo). Asolectin (total soybean phospholipids) was isolated as described in ref. 15. The sources of other materials are described elsewhere (5).

RESULTS

Characterization of Porin in Yeast OMM. When purified yeast mitochondria were fractionated as described in *Methods*, about 2% of the total mitochondrial protein was recovered in the purified OMM fraction. This yield was similar to that of OMM from rat liver mitochondria (16). The activity of succinate–cytochrome *c* reductase, a marker of inner mitochondrial membrane, in the OMM and inner mitochondrial membrane fractions indicated that the extent of contamination of the former by the latter was 0.93%. Determination of the activity of NADPH–cytochrome *c* reductase, a widely used marker of microsomes, showed that the extent of contamination of the OMM fraction by microsomes was as high as 12%, but it is possible that this enzyme is constitutive to OMM like NADH–cytochrome b_5 reductase, which is intrinsic not only to the ER but also to the OMM and some other cellular membranes (17, 18).

For the objectives of our present studies, the most pertinent data with regard to a "marker" activity for our yeast OMM fraction were obtained by using the porin assay of Zalman *et al.* (2) (Table 1). This assay measures the retention of radioactively labeled large ([^{3}H]dextran, mean M_r, 70,000) versus small ([^{14}C]sucrose) molecules that had both been trapped into "hybrid" vesicles during sonication of lipids (soybean asolectins) with OMM and subsequent separation of these hybrid vesicles by sieving through a Sepharose 4B column. The data show that our OMM fraction from yeast has porin activity (Table 1). In a control in which the OMM fraction was omitted during sonication, the [^{3}H]dextran/[^{14}C]sucrose ratio in the isolated *pure* asolectin vesicles was identical to that in the incubation mixture. In contrast, when various amounts of OMM were added during sonication, the [^{3}H]dextran/[^{14}C]sucrose ratio in the isolated *hybrid* vesicles [presumably consisting of asolectin and OMM (2)] was proportionally higher with higher amounts of OMM (Table 1) due to efflux of sucrose (via the putative pores) and retention of dextran. The porin activity of our yeast OMM preparation is in the same order of magnitude as that reported by Zalman *et al.* (2) for their preparation of OMM from mung bean and rat liver.

Another revealing feature of our yeast OMM fraction was the predominance of a 29,000-dalton band in its $NaDodSO_4$/polyacrylamide gel electrophoresis banding pattern (Fig. 1, lanes 4 and 5). A similar prominence of a 30,000-dalton band was observed in the OMM fraction of mung bean and rat liver and it was this polypeptide that, after partial purification, was proposed to represent the porin activity (2).

Table 1. Removal of [^{14}C]sucrose from vesicles reconstituted with yeast OMM

	Incubation mixture		Isolated vesicles		
Addition	[^{3}H]-Dextran, cpm	[^{14}C]-Sucrose, cpm	[^{3}H]-Dextran, cpm	[^{14}C]-Sucrose, cpm	$^{3}H/^{14}C$
None	22,100	36,000	8,340	13,100	1.04
OMM					
0.5 μg	23,100	35,700	6,570	5,200	1.93
2.0 μg	24,300	35,700	6,290	3,300	2.80
10.0 μg	24,100	35,100	4,870	1,710	4.13

Results are normalized so that the $^{3}H/^{14}C$ ratio of the reaction mixture would be equal to 1.0.

To further characterize the porin activity of our yeast OMM fraction we treated it with trypsin and followed the progress of digestion both by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 2*A*) and by the porin activity assay (Fig. 2*B*). The results showed that the 29,000-dalton yeast porin was both physically (Fig. 2*A*, lanes 3–5) and functionally (Fig. 2*B*, lanes 3–5) resistant to trypsin digestion. It should be noted that the 29,000-dalton polypeptide was virtually the only polypeptide of the OMM fraction that was resistant to trypsin digestion [Fig. 2*A*, compare lane 2 (untreated) with lanes 3–5 (trypsin treated)]. This, together with the complete retention of porin activity (compare corresponding lanes in Fig. 2*B*), provides additional circumstantial evidence for the 29,000-dalton polypeptide being porin. The resistance to trypsin digestion was abolished only after detergent solubilization of the OMM fraction (Fig. 2*A*, lane 6).

Cell-Free Synthesis and Posttranslational Integration of Porin into Mitochondria. Many integral membrane proteins are synthesized as larger precursors with a transient signal sequence that is cleaved during or following integration into one of the translocation-competent membranes (1). To examine whether this is the case for porin, we translated total yeast RNA in a wheat germ cell-free system (which is free of translocation-competent membranes), then isolated porin from among the total translation products by immunoprecipitation with our monospecific antiporin IgG, and analyzed the immunoprecipitated product by $NaDodSO_4$/polyacrylamide gel electrophoresis and subsequent fluorography. We obtained a single major polypeptide of 29,000 daltons (Fig. 3, lane 3) that is indistinguishable in electrophoretic mobility from authentic porin (lane 2). That this band was specifically precipitated and therefore was newly synthesized yeast porin was shown by competition with unlabeled porin (lane 4) or by replacing immune IgG with preimmune IgG (lane 5). Thus, based on its electrophoretic mobility, it appears that yeast porin is not synthesized as a larger precursor.

The next question was: into which of two translocation-competent membranes, RER or OMM (1), can porin be integrated? To answer this question, we first examined isolated dog pancreas microsomal membranes and the yeast OMM fraction for their capacity for *in vitro* integration of porin. To this end, either of these two membrane fractions was added to a rabbit reticulocyte cell-free system at the beginning of translation or after translation was completed. In neither instance did we observe correct asymmetric membrane integration, at least by the criterion of resistance to trypsin digestion: in contrast to the trypsin resistance of native porin in the isolated OMM fraction (see Fig. 2, lanes 3–5), the *in vitro*-synthesized porin was not made trypsin resistant, whether incubated with dog pancreas microsomal membranes or with yeast OMM, cotranslationally or posttranslationally (data not shown).

These data then suggested that a putative signal sequence

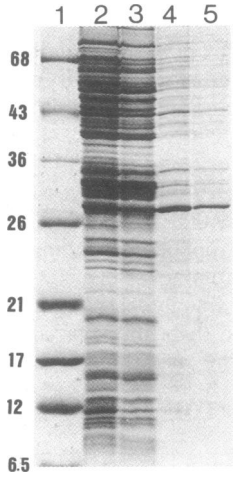

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoretic analysis of purified mitochondria and of inner and outer mitochondrial membrane fractions. Aliquots of mitochondria and submitochondrial fractions were electrophoresed in a 15% polyacrylamide slab gel containing $NaDodSO_4$. The gel was stained with Coomassie brilliant blue. Lanes: 1, molecular weight markers (from top to bottom, bovine serum albumin, chicken ovalbumin, rabbit muscle lactic dehydrogenase, bovine pancreas α-chymotrypsinogen A, soybean trypsin inhibitor, sperm whale myoglobin, bovine heart cytochrome *c*, Trasylol; numbers to the left indicate $M_r \times 10^{-3}$); 2, total mitochondria, 133 μg of protein; 3, inner mitochondrial membrane, 113 μg of protein; 4 and 5, OMM, 37.5 and 12.5 μg of protein, respectively.

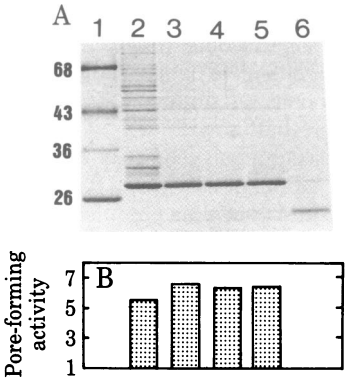

FIG. 2. Resistance of porin and of porin activity to trypsin digestion. Aliquots (120 μl) of purified OMM in 50 mM Tris·HCl (pH 7.4) were incubated with trypsin at 0, 50, 100, and 200 μg/ml (*A*, lanes 2–5) at 25°C for 60 min. Then, 125 units of Trasylol was added to inhibit trypsin. After dilution with 2.5 vol of 50 mM sodium phosphate buffer (pH 7.5) and centrifugation at 105,000 × *g* for 60 min, membranes were suspended in water and aliquots were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis (*A*) or for porin activity (*B*). (*A*) A slab gel stained with Coomassie brilliant blue. Lanes: 1, molecular weight (numbers to the left × 10^3) markers; 2–5, 5, 15.0, 13.4, 11.9, and 11.1 μg of proteins were applied, respectively; 6, as in lane 5 except that the digestion mixture included 1% Triton X-100. (*B*) Porin activity of trypsin-digested OMM. Lanes 2–5 correspond to those in *A*; 10, 8.9, 8.0, and 7.4 μg of membrane protein, respectively, were used. Activities are expressed as normalized $^3H/^{14}C$ ratios per 10 μg of membrane proteins.

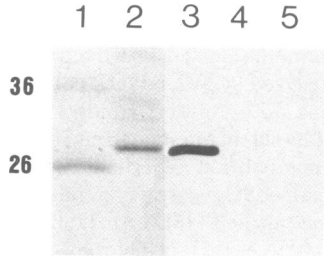

FIG. 3. *In vitro* synthesis of porin. Yeast total RNA was incubated at 29°C for 90 min in a wheat germ cell-free protein-synthesizing system containing [^{35}S]methionine. Aliquots (30 μl, 5.3 × 10^6 cpm) of the translation mixture were subjected to immunoprecipitation with preimmune or anti-porin IgGs. The immunoprecipitates were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis. The radioactive bands were detected by fluorography (lanes 3–5) of a Coomassie brilliant blue-stained and dried slab gel (lanes 1 and 2). Lanes: 1, molecular weight (numbers to the left × 10^3) markers; 2, 20 μg of OMM; 3, immunoprecipitate with anti-porin IgG; 4, as in lane 3, except that excess unlabeled porin was present during the immunoprecipitation; 5, immunoprecipitate with preimmune IgG.

of porin is certainly not addressed to the RER translocation system because dog pancreas microsomal membranes have been shown to decode RER-targeted signal sequences of yeast polypeptides (19, 20). On the other hand, the inability of our OMM fraction to decode a signal sequence targeted to the OMM translocation system may have resulted from loss or inactivation of this activity during subfractionation of mitochondria. We therefore carried out integration experiments with whole mitochondria. A posttranslational PRS of reticulocyte lysate was incubated with isolated mitochondria for 60 min at 25°C. Mitochondria were then reisolated by centrifugation and the amounts of porin in the sedimented mitochondria and in the supernatant were determined by immunoprecipitation (Fig. 4). It is clear that a major fraction of the porin originally present in the PRS (lane 1) sedimented with the mitochondria (lane 3), whereas a minor fraction remained in the postmitochondrial supernatant (lane 2). Quantitation of these results by measuring the radioactivity in the excised porin bands of lanes 1–3 showed that 76% of the porin cosedimented with the mitochondria.

Next, we examined whether this cosedimenting porin was indeed integrated into mitochondria by using trypsin as a probe. If integration had occurred with fidelity, one would expect the cosedimenting porin to be trypsin resistant, as was demonstrated for native porin in the OMM fraction (see Fig. 2, lanes 3–5). The experimental protocol to test this was as above except that the posttranslational incubation with mitochondria was followed by incubation with trypsin prior to the centrifugation step. We found that the porin cosedimenting with mitochondria was protected from trypsin digestion (Fig. 4, lane 5) whereas all of the porin in the supernatant was degraded (Fig. 4, lane 4). Quantitation of the trypsin-protected porin showed that it amounted to 83% of the cosedimenting porin. The fraction of cosedimenting porin that was degraded probably represented adventitiously bound nonintegrated porin. The overall integration efficiency was calculated to be 63%.

We compared the ability of isolated mitochondria for porin integration with their previously demonstrated (21) capacity to translocate the newly synthesized precursor of the γ subunit of F_1-ATPase into the mitochondrial matrix. To this end, after immunoprecipitation of porin (Fig. 4), we carried out immunoprecipitations of the same fractions with an antiserum raised against the γ subunit of F_1-ATPase (Fig. 5). Quantitation of the data showed that 77% of the γ-subunit precursor was processed to mature size (lane 3) and that 74% of the processed γ subunit was resistant to trypsin digestion, amounting to an overall translocation efficiency of 57%. The latter is thus in the same range as the overall efficiency to integrate porin.

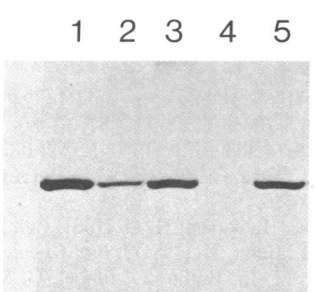

FIG. 4. Posttranslational integration of *in vitro*-synthesized porin into isolated mitochondria. After translation of total yeast RNA in the presence of [^{35}S]methionine in a reticulocyte cell-free protein synthesizing system, a PRS was prepared. The PRS was divided into three aliquots (200 μl each). Two aliquots were incubated with purified mitochondria (330 μg of protein) at 25°C for 60 min. Subsequently, one incubation mixture (lanes 2 and 3) was stored in an ice bath, and the other (lanes 4 and 5) was incubated with trypsin (250 μg/ml) at 4°C for 60 min and then treated with soybean trypsin inhibitor (500 μg/ml). These mixtures were then centrifuged to separate supernatants (lanes 2 and 4) and mitochondria (lanes 3 and 5). These fractions and the nonincubated aliquot of the PRS (lane 1) were then boiled in $NaDodSO_4$ and subjected to immunoprecipitation with antiporin IgG. The immunoprecipitates were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and subsequent fluorography.

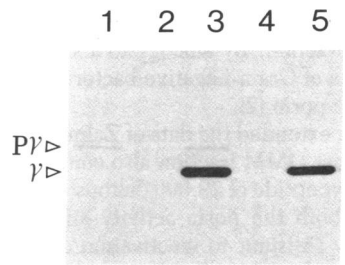

FIG. 5. Posttranslational translocation of the *in vitro*-synthesized γ subunit of F_1-ATPase into mitochondria. After immunoprecipitation with antiporin IgG, the remaining "immunosupernatants" of Fig. 4 were incubated for 2 hr at 25°C with 10 μg (wet weight) of protein A-Sepharose to remove the remaining IgG and then subjected to immunoprecipitation with the immunoselected anti-γ subunit of F_1-ATPase IgG (5). Lanes correspond to those in Fig. 4. The small amount of pre-γ subunit (Pγ) in lane 1 is due to accidental loss during the second immunoprecipitation.

Finally, we investigated the effect of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on both the integration of porin (Fig. 6*A*) and the translocation of the γ subunit of F_1-ATPase (Fig. 6*B*). Trypsin was again used as a probe to assay integration and translocation. CCCP had no effect on the integration of porin (Fig. 6*A*; compare lanes m, CCCP versus control) whereas it strongly inhibited translocation of the γ subunit into the mitochondrial matrix (Fig. 6*B*). Similar observations—i.e., no effect of CCCP on translocation across the outer membrane but strong inhibition of translocation into the matrix—have been reported for a number of cytoplasmically synthesized mitochondrial proteins (22).

DISCUSSION

Zalman *et al.* (2) have demonstrated the existence of a pore-forming activity in the OMM of rat liver and of mung bean. They have tentatively identified this activity as a 30,000-dalton poly-

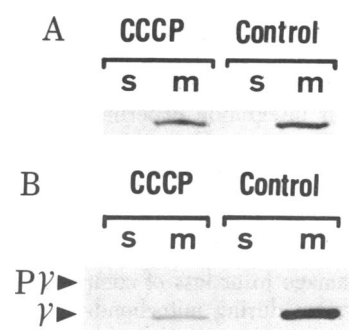

FIG. 6. CCCP does not affect integration of porin (*A*) into mitochondria but inhibits translocation of the γ subunit of F_1-ATPase (*B*) into mitochondria. Assays were identical to those in Fig. 4 except that incubation of the PRSs with mitochondria was in the absence (control) or presence of 13 μM CCCP. Lanes: s, material immunoprecipitated from trypsin-treated supernatants (corresponding to lane 4 in Fig. 4); m, material immunoprecipitated from trypsin-treated mitochondria (corresponding to lane 5 in Fig. 4). The data for the γ subunit of F_1-ATPase were obtained by immunoprecipitation with the immunoselected anti-γ subunit of F_1-ATPase IgG.

peptide that constituted the predominant polypeptide in their isolated OMM fraction. By analogy to a similar activity in the outer membrane of Gram-negative bacteria, they have termed this polypeptide porin (2).

We have now extended the data of Zalman *et al.* to yeast. We found that a yeast OMM fraction also contained porin activity and a major polypeptide of 29,000 daltons. Moreover, we demonstrated that both the porin activity and the 29,000-dalton band were fully resistant to inactivation and degradation, respectively, when the OMM fraction was digested with trypsin. The fact that the 29,000-dalton polypeptide was virtually the only polypeptide in the OMM fraction that remained entirely unaffected by trypsin digestion provided additional circumstantial evidence that the 29,000-dalton polypeptide represents yeast OMM porin. We have also characterized the early biosynthetic form of porin synthesized *in vitro* and, furthermore, have investigated the intracellular site and mode of integration of *in vitro*-synthesized porin into membranes.

We found that the form of porin synthesized in a cell-free system (presumably representing the primary translation product) is indistinguishable in molecular weight from mature porin. Thus, porin belongs to the growing list of membrane proteins that are synthesized without a transient (cleaved) signal sequence. However, we believe that porin contains at least one (uncleaved) signal sequence. Because porin functions as a hydrophilic channel in the membrane, it can be assumed to span the membrane. Thus, translocation of at least one specific segment of the porin polypeptide chain across the membrane would be required to achieve such an orientation. The asymmetric integration of porin would therefore be dependent on a translocation system. If porin were to span the membrane several times, additional "topogenic" sequences (either signal and stop-transfer sequences or insertion sequences; see ref. 1 for definitions) would be required to "stitch" the polypeptide properly into the membrane (1).

Theoretically, one of two distinct cellular translocation systems might be able to decode the signal sequence of porin, either that of the RER or that of the OMM (1). In the first case, integration would be coupled to translation and would require subsequent "sorting" of porin from its site of integration to the OMM (1). In the latter case, integration would be posttranslational and would of course not require any subsequent sorting.

Our data show that integration of porin proceeds directly into mitochondria and not into the RER. By the criterion of resistance to trypsin digestion, the newly synthesized porin was integrated with fidelity. Furthermore, as expected from the properties of the putative OMM translocation system, integration of porin proceeded posttranslationally (1). It should be noted, however, that our integration experiments were carried out with whole mitochondria and we assume that porin was integrated exclusively into the OMM although we have not attempted to provide direct evidence on this point. It is interesting to note that the isolated OMM fraction was not competent to integrate porin. The reasons for this incompetence might be several (e.g., damage to or loss of components of the OMM translocation system during mitochondrial fractionation) and remain to be investigated. This together with our finding that dog pancreas microsomal membranes also were incompetent to integrate porin strongly supports our conjecture that the integration of porin into mitochondria is a membrane-specific process that is dependent on a specific translocation system and that does not occur merely by an unassisted or spontaneous partitioning into a membrane.

The existence of two distinct mitochondrial translocation systems for import of cytoplasmically synthesized proteins, one for import into the intermembrane space and the other for import into the matrix, has been postulated (1). In support of this postulate, it has been shown (22) that CCCP can be used to distinguish between these two systems. Our results here confirm and amplify these observations by two more examples: CCCP did not affect the OMM translocation system, because it did not inhibit the integration of porin; however, CCCP did inhibit the translocation of the γ subunit of F_1-ATPase, which requires translocation into the matrix.

Finally, it should be noted that another integral membrane protein of the OMM, albeit of unidentified function, has recently been shown to be synthesized by free ribosomes (23), suggesting that it also may require the OMM—and not the RER—translocation system for its integration.

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