Biosynthesis of Mitochondrial Porin and Insertion into the Outer Mitochondrial Membrane of *Neurospora crassa*

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Mitochondrial porin, the major protein of the outer mitochondrial membrane is synthesized by free cytoplasmic polysomes. The apparent molecular weight of the porin synthesized in homologous or heterologous cell-free systems is the same as that of the mature porin. Transfer *in vitro* of mitochondrial porin from the cytosolic fraction into the outer membrane of mitochondria could be demonstrated. Before membrane insertion, mitochondrial porin is highly sensitive to added proteinase; afterwards it is strongly protected. Binding of the precursor form to mitochondria occurs at 4 °C and appears to precede insertion into the membrane. Unlike transfer of many precursor proteins into or across the inner mitochondrial membrane, assembly of the porin is not dependent on an electrical potential across the inner membrane.

The vast majority of mitochondrial proteins are synthesized outside the mitochondria on cytoplasmic ribosomes. For a number of proteins the transfer into the mitochondria has been investigated and in all cases was found to be a post-translational process [1]. While proteins of the inner membrane [2−10], the matrix space [11−16] and the intermembrane space [17−19] have been studied, information on a well-defined protein of the outer mitochondrial membrane has been lacking. The assembly of such proteins into the outer membrane poses several intriguing questions. For example, the outer mitochondrial membrane, in contrast to the inner membrane, is in direct contact to the cytosolic space and to the cytoplasmic ribosomes. Direct insertion of nascent polypeptide chains into the membrane is therefore possible. Given this, does the mechanism of assembly of the outer membrane proteins resemble other mitochondrial proteins? Do they use the same mitochondrial machinery, have the same requirements and so on? A further question concerns the membrane choice that membrane proteins made on cytoplasmic ribosomes must make: what directs a protein of the outer mitochondrial membrane to this membrane and why does it not insert into the membrane of the endoplasmic reticulum or the plasma membrane?

To begin to answer these and other questions concerning the assembly of mitochondrial outer membrane proteins we have studied the synthesis and membrane insertion of the most abundant protein of the outer mitochondrial membrane, the mitochondrial porin. This protein, which is tightly integrated into the outer membrane, forms a pore that allows the unspecific passage of molecules with molecular weights below 2000−6000 [20]. Porin has been isolated from purified outer mitochondrial membranes from *Neurospora* and has a subunit M_r of 31000. It can be inserted into artificial lipid bilayers where it forms characteristic voltage-dependent channels [20−22].

In this communication we report that the mitochondrial porin is made on free polysomes and that precursor synthesized *in vitro* in heterologous or homologous cell-free systems has the same apparent molecular weight, determined by electrophoresis on dodecyl-sulfate-containing gels, as the mature protein. Insertion of newly made porin into outer membrane can be observed *in vitro*. In contrast to a number of proteins which are transferred into the inner mitochondrial membrane [23], transfer of the mitochondrial porin is not dependent on the mitochondrial transmembrane electrical potential.

MATERIALS AND METHODS

Growth of Neurospora and Isolation of Mitochondria

Neurospora crassa hyphae (wild type 74A) were grown on Vogel's minimal medium [24] supplemented with 2 % sucrose. When indicated, the culture medium contained a reduced concentration of sulfate (0.08 mM) and 0.5 mCi/l [^{35}S]-sulfate (800 Ci/mol, New England Nuclear, Boston, MA). For labelling of cells with [^{3}H]leucine, 0.5 mCi/l [^{3}H]-leucine (40−60 Ci/mmol, New England Nuclear, Boston, MA) were added after 13 h growth and cells were grown for a further hour. Mitochondria were isolated from *Neurospora* spheroplasts [4].

Immunoprecipitation

Specific antibodies against the mitochondrial outer membrane porin were prepared as reported previously [22]. Immunoprecipitation from reticulocyte lysates was carried out using Sepharose-bound protein A [4]. Mitochondria were lysed with Triton X-100 and immunoprecipitation was performed with formaldehyde-fixed *Staphylococcus aureus* cells, which had been washed as described by Crowlesmith et al. [25]. 50 mg of the pelleted cells, 100 µl of antiserum and 1 ml of the lysed mitochondrial solution were mixed and shaken for 45 min at 4 °C. Washing and dissociation of the immunocomplex was done as described previously [4].

Isolation of Membrane-Free and Membrane-bound Polysomes

Immediately after adding 0.1 g cycloheximide/l culture medium, *Neurospora* cells were harvested on an ice-filled

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Abbreviations. PheMeSO$_2$F, phenylmethanesulfonyl fluoride; ClPh₂C(CN)₂, carbonyl cyanide m-chlorophenylhydrazone.

Enzymes. Proteinase K (EC 3.4.21.14).

Büchner funnel. The hyphae were washed with ice-cold water and with a buffer containing 0.1 M ammonium chloride, 10 mM magnesium chloride, 15 mM Tris/HCl, pH 8.4. The hyphae were then homogenized for 1 min in a 2.5-fold volume (with respect to wet weight) of 0.88 M sucrose, 0.2 M ammonium chloride, 20 mM magnesium chloride, 0.05% heparin, 30 mM Tris/HCl, pH 8.4 using a Waring blender. The suspension was passed twice through a grindmill [26]. Centrifugation for 6 min at $8000 \times g$ was performed twice to remove unbroken cells and cell walls. Mitochondria were isolated from the resulting supernatant by centrifugation for 12 min at $18000 \times g$. The mitochondrial pellet was resuspended in 0.44 M sucrose, 0.1 M NH_4Cl, 10 mM $MgCl_2$, 15 min Tris/HCl pH 8.4. The suspension was centrifuged for 12 min at $18000 \times g$ to obtain the mitochondrial pellet. Membrane-bound and membrane-free polysomes were isolated from the postmitochondrial supernant according to the procedure of Scheele et al. [27].

Protein Synthesis in vitro
and Protein Transfer into Mitochondria

Protein synthesis in a cell-free system from *Neurospora* was performed as described [17]. Preparation of reticulocyte lysates, translation of *Neurospora* RNA in the presence of [^{35}S]methionine, and the transfer of proteins into isolated mitochondria were carried out as reported previously [4]. For protease digestion experiments, samples were incubated with 100 µg proteinase K per ml for 45 min at 4 °C and proteolysis was stopped by the addition of 0.5 mM (final concentration) phenylmethylsulfonyl fluoride.

Isolation of Nucleic Acids

The nucleic acids of membrane-bound and membrane-free polysomes and of polysomes associated with mitochondria were extracted according to the method of Ullrich et al. [28] with modifications described by Payvar and Schimke [29]. Mitochondria and membrane-bound polysomes were resuspended in 0.1 M NH_4Cl, 10 mM Tris/HCl, pH 8.4, membrane-free polysomes in a medium containing 0.1 M NH_4Cl, 50 mM EDTA 0.6 M KCl, 30 mM Tris/HCl, pH 8.4 [30].

For extraction, the samples were added to the guanidinium thiocyanate extraction-medium [28] so that 5% of the total volume consisted of polysomal suspension. After homogenization in a siliconized potter, the samples were centrifuged for 30 min at $48000 \times g$ at 21 °C in order to sediment insoluble material. Cesium chloride centrifugation was performed according to Glisin et al. [31] using a Beckman Ti 70 rotor at $140000 \times g$ for 21 h at 21 °C. The nucleic acid pellets obtained by cesium chloride centrifugation were dissolved in 10 mM Tris/HCl, 5 mM EDTA pH 7.5 and heated to 50 °C for 10 min. The solutions were adjusted to 0.2 M potassium acetate, pH 5.0, and nucleic acids were precipitated with ethanol overnight at −20 °C. The precipitates were collected by centrifugation (20 min $10000 \times g$, −20 °C), washed twice with 66% ethanol, dried under vacuum, and redissolved in water to a final concentration of 300 A_{260nm} units/ml.

Fractionation of Mitochondria with Digitonin

A 1% stock solution of digitonin C from Merck, Darmstadt (recrystallized from ethanol) in sucrose/EDTA/Tris buffer (0.44 M sucrose, 1 mM EDTA, 0.03 M Tris/HCl, pH 7.6) was prepared by sonication until the solution became clear.

This solution was cooled to 4 °C and was used within 30 min. Transfer of proteins was performed employing mitochondria from cells labelled with [^{3}H]leucine. After 45 min transfer *in vitro*, mitochondria were pelleted (0.9 mg protein) and were resuspended in 0.1 ml of sucrose/EDTA/Tris buffer. Then 0.1 ml of sucrose/EDTA/Tris buffer containing 0−0.72 mg digitonin was added. After incubation for 1 min at 4 °C, the suspension was diluted with 1.2 ml of the sucrose/EDTA/Tris buffer and layered on 3.6 ml 1.0 M sucrose, 1 mM EDTA, 30 mM Tris/HCl, pH 7.6 in Beckman SW 65 tubes. Centrifugation was carried out for 45 min at $70000 \times g$. Under these conditions the outer membrane, which was fragmented and displaced from mitochondria by digitonin, stays on top of the 1.0 M sucrose layer [32]. The pellets were lysed with Triton-containing buffer, porin and ADP/ATP carrier were immunoprecipitated and the immunoprecipitates analysed by dodecylsulfate gel electrophoresis. ^{3}H and ^{35}S radioactivities in the protein peaks were determined after slicing the gels [17].

RESULTS

Newly Synthesized Porin Has the Same Apparent Molecular Weight as Mature Porin

Neurospora RNA was translated in a reticulocyte lysate in the presence of [^{35}S]methionine, and then a postribosomal supernatant was prepared. Immunoprecipitation of mitochondrial porin was carried out with antibodies against porin purified from the outer mitochondrial membrane. The mature porin was immunoprecipitated from mitochondria isolated from ^{35}S-labelled *Neurospora* cells. Immunoprecipitates were analyzed by electrophoresis in the presence of dodecylsulfate, and by autoradiography. The mature porin and the newly synthesized porin displayed the same apparent electrophoretic mobility (Fig. 1). When a mixture of immunoprecipitated mature porin and porin synthesized *in vitro* was analyzed by gel electrophoresis, one single band was seen. Protein synthesis *in vitro* was also carried out in a cell-free homologous system from *Neurospora*. Also in this case, *in vitro*, the product identified by immunoprecipitation had the same apparent molecular weight as the mature protein (data not shown).

Labelled formyl-methionyl-transfer RNA has been employed, to demonstrate synthesis of precursor proteins with an uncleaved amino terminus [5, 6, 33 − 35]. However, in case of porin, due to the small amounts synthesized (about 0.008% of total radioactivity in a reticulocyte lysate), sufficient label could not be incorporated into the precursor protein. On the other hand, artificial processing of mitochondrial precursor proteins in the reticulocyte lysate has not been observed for any of the 30 or more proteins which have been investigated. Therefore, it is unlikely that the detected porin does not have its original size. Moreover, porin synthesized *in vitro* inserts into the outer mitochondrial membrane (see below), displaying the behaviour of a bona fide precursor.

Mitochondrial Porin Is Synthesized on Free Polysomes

In order to determine whether the mRNA coding for the porin is associated with membrane-bound or with free polysomes, RNA was isolated from these fractions [30]. Translation of these RNAs in reticulocyte lysates followed by gel electrophoretic and autoradiographic analysis of the [^{35}S]-methionine-labelled products showed that the mRNA from
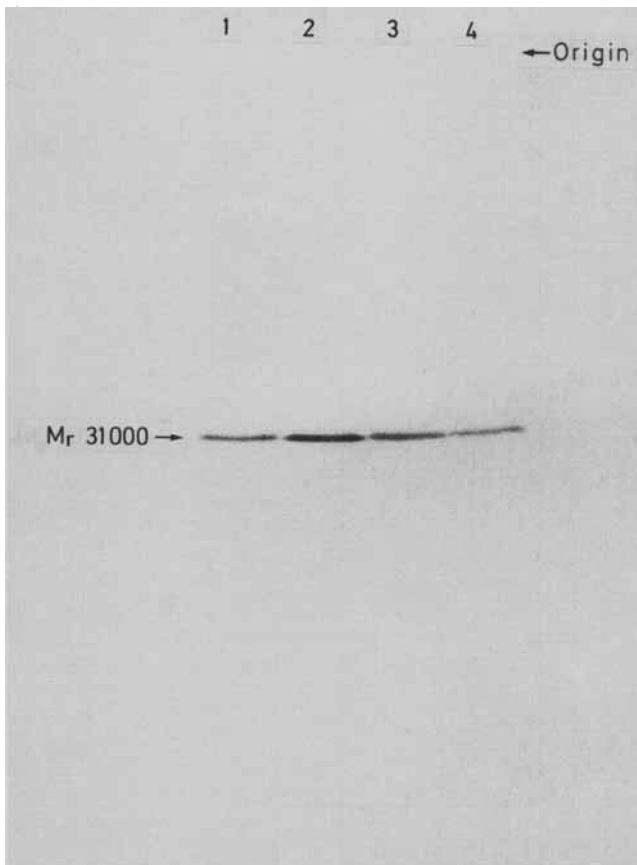
Fig.1. *Biosynthesis of mitochondrial porin in a reticulocyte lysate.* A reticulocyte lysate was programmed with *Neurospora crassa* mRNA and [^{35}S]methionine was incorporated for 60 min at 25 °C. Phenylmethylsulfonyl fluoride and *p*-hydroxymercuribenzoate were added to a final concentration of 0.5 mM, each. After centrifugation for 60 min at 166000 × g, the post-ribosomal supernatant was adjusted to 1 % Triton X-100, 0.3 M KCl; then immunoprecipitation was carried out. The mature protein was immunoprecipitated from Triton X-100 lysed mitochondria isolated from *Neurospora* cells grown in the presence of [^{35}S]sulfate. Analysis of the immunoprecipitates was performed by gel electrophoresis in the presence of dodecylsulfate and subsequent autoradiography. (1) Mature porin from mitochondria; (2) porin synthesized *in vitro*; (3) mixture of (1) and (2) (equal amounts of radioactivity); (4) porin synthesized *in vitro* was bound to antibody and agarose-linked protein A; then the immunocomplex was separated by centrifugation and resuspended in 1 ml 1 % Triton, 0.3 M KCl containing 3 μg isolated porin. The suspension was shaken at 4 °C for 40 min. Then the agarose beads were pelleted and analysed like samples 1 — 3

the two ribosomal fractions specified strongly different patterns of proteins (Fig. 2). When these translation products were subjected to immunoprecipitation with antibodies against the mitochondrial porin, newly made porin was found to be synthesized only under the direction of RNA from free polysomes but not from membrane-bound polysomes (Fig. 2). RNA extracted from mitochondria which were prepared in the presence of cycloheximide stimulated the synthesis of mitochondrial porin in the reticulocyte lysate, but to a lower degree than corresponding amounts of RNA from free polysomes. Since mitochondria-bound ribosomes make up only some 5 % of free ribosomes, the total amount of porin-specific message associated with these ribosomes is very small. Apparently, free ribosomes are the major site of synthesis of porin. Nevertheless, it may be that nascent porin can already bind to recognition sites on the mitochondria.

Porin Synthesized in vitro Is Transferred into the Outer Membrane of Isolated Mitochondria

Mitochondrial porin was synthesized in a reticulocyte lysate and the postribosomal supernatant was prepared. Isolated mitochondria were resuspended in the supernatant and incubated at 25 °C. After various times of incubation, aliquots were cooled to 0 °C. One half of each sample was immediately subjected to centrifugation to separate mitochondria. The other half was incubated with proteinase K, and mitochondria were then separated by centrifugation. The porin was immunoprecipitated from mitochondria and supernatants in the presence of 1 % Triton and 0.3 M KCl. Porin became associated with the mitochondrial fraction within 20 min (Fig. 3). The porin in the mitochondria was protease-resistant, as is mature assembled porin [22]. The precursor in the supernatant was completely digested by proteinase K.

This suggests that porin is transferred into mitochondria. The protease resistance indicates that it is inserted into the outer membrane and not merely attached to it. The transfer of porin *in vitro* appears to be rather efficient since after 20 min incubation no more precursor was detected in the supernatant.

Fig. 4 A gives a quantitative estimation of the protease resistant form after transfer into mitochondria. Virtually all the porin transferred at 25 °C was in protease-protected form at any time of the transfer. Apparently, there was no precursor bound to the surface of the mitochondria, either specifically or unspecifically.

With other mitochondrial precursor proteins, binding to specific sites on the mitochondrial surface has been demonstrated. In most cases specific binding could only be observed after the further steps of the assembly reaction were blocked [7,23,36]. To demonstrate binding of the porin, transfer *in vitro* was carried out at low temperature. After incubation at 4 °C for 3 — 10 min porin was found associated with mitochondria. But in contrast to incubation at 25 °C, the porin was largely sensitive to added protease (Fig. 4 B). This suggests that also in the case of porin, binding to the mitochondria precedes the insertion into the membrane, and that insertion of porin into the membrane occurs more rapidly than binding at 25 °C.

In order to determine whether mitochondrial porin was actually inserted into the outer mitochondrial membrane, mitochondria were subfractionated after transfer *in vitro*. Upon treatment with increasing amounts of digitonin, which leads to preferential solubilization of the outer membrane [32,37], mature ^{3}H-labelled porin was removed from the mitochondria at lower digitonin/protein ratios than ^{3}H-labelled ADP/ATP carrier, which served as a marker for the inner membrane. ^{35}S-labelled porin transferred *in vitro* behaved like the mature porin, and differently from both ^{3}H-labelled and ^{35}S-labelled ADP/ATP carrier (Fig. 5). This shows that the porin is selectively inserted into the outer membrane *in vitro*.

Transfer of Porin in vitro Is not Dependent on Energization of Mitochondria

The transfer of most cytoplasmically synthesized mitochondrial proteins into mitochondria is dependent on an electrical potential across the inner mitochondrial membrane [23]. Transfer into mitochondria of apocytochrome *c*, a protein which is not translocated across or inserted into the inner membrane, is not dependent on energization. It was therefore
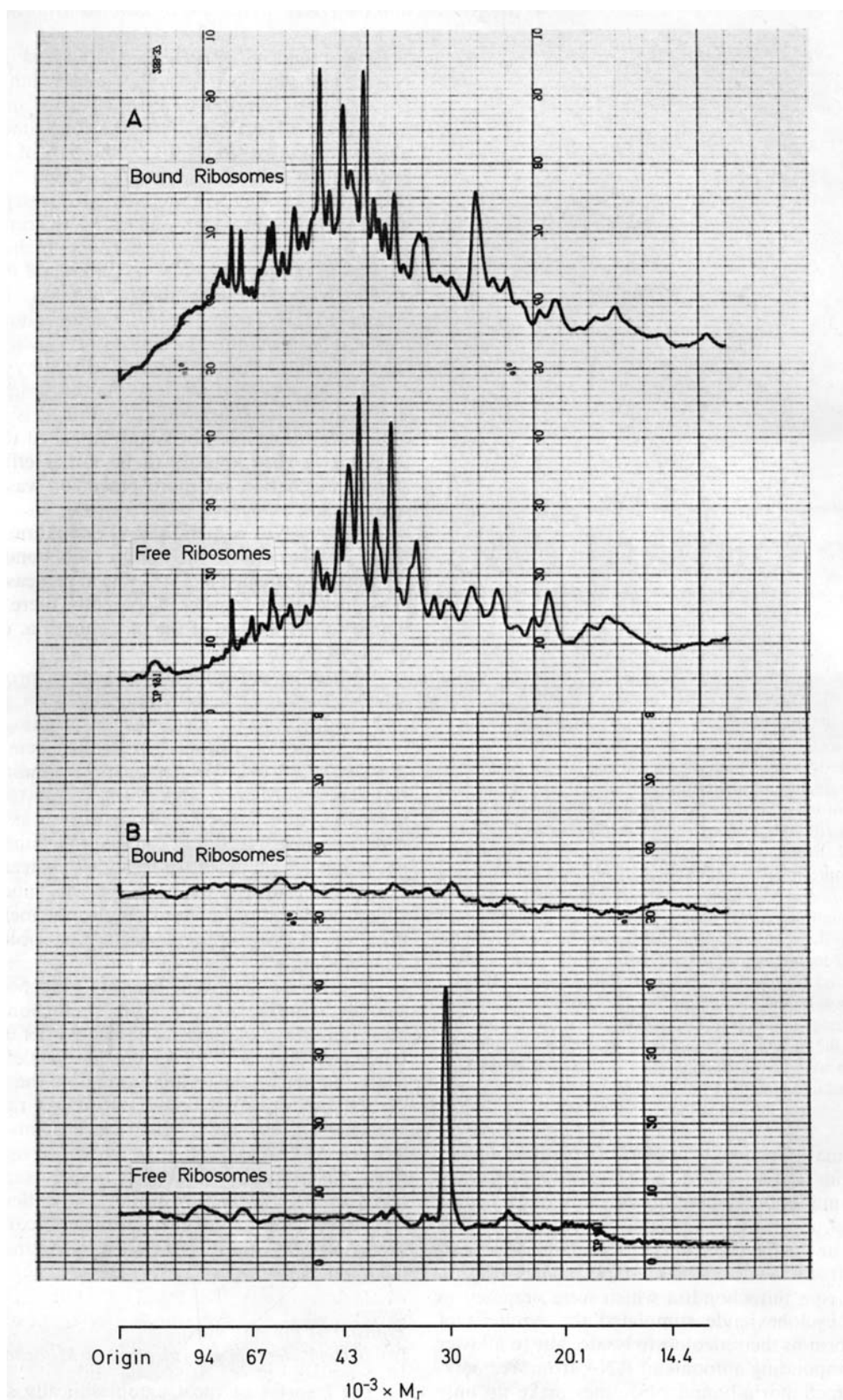
Fig. 2. *Synthesis of mitochondrial porin on free polysomes.* Free and membrane-bound polysomes were isolated from *Neurospora* cells, and their RNA was extracted and used to program reticulocyte lysates. After protein synthesis in the presence of [^{35}S]methionine for 60 min, the post-ribosomal supernatants were prepared. Aliquots of 10 µl were analysed by gel electrophoresis and autoradiography. Aliquots of 500 µl of the same incubation were subjected to immunoprecipitation. The precipitates were analysed by electrophoresis and autoradiography. The autoradiographs were subjected to densitometry at 546 nm. (A) Total translation products; (B) immunoprecipitation of mitochondrial porin
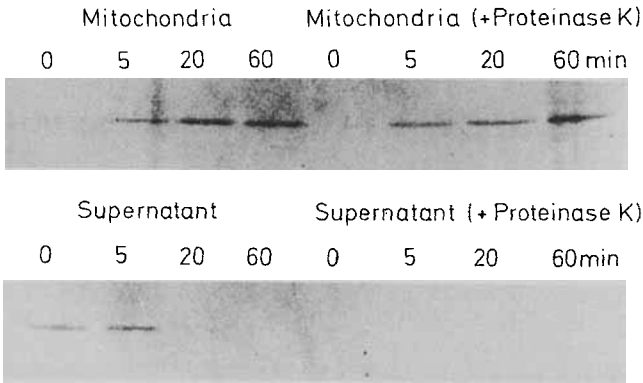
Mitochondria   Mitochondria (+Proteinase K)

0   5   20   60   0   5   20   60 min

Supernatant   Supernatant (+ Proteinase K)

0   5   20   60   0   5   20   60min

Fig. 3. *Transfer into mitochondria of porin synthesized* in vitro. After protein synthesis *in vitro* in the presence of [^{35}S]methionine, a reticulocyte lysate was centrifuged to remove the ribosomes. It was then incubated with mitochondria isolated from *Neurospora* spheroplasts at 25 °C. At the times indicated, aliquots of the suspension were withdrawn. One half of each sample was immediately centrifuged to separate mitochondria and supernatant. The other half was cooled to 4 °C and incubated with proteinase K for 45 min; then mitochondria and supernatant were separated by centrifugation. The mitochondrial pellets were lysed with 1 % Triton X-100, 0.3 M KCl, 10 mM Tris/HCl, pH 7.5 and the supernatants were adjusted to 1 % Triton and 0.3 M KCl. Then immunoprecipitation, gel electrophoresis and autoradiography were performed
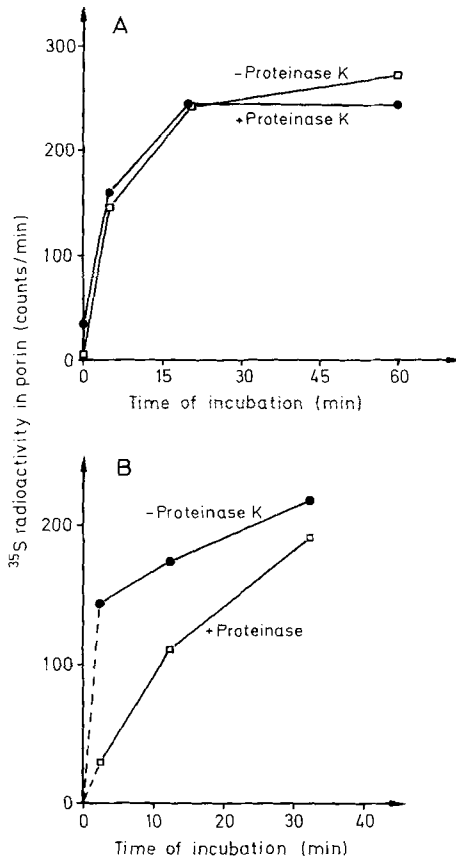
A

B

Fig. 4. *Binding of porin to mitochondria can be discriminated from membrane insertion by lowering the temperature of the transfer system* in vitro. Incubation of mitochondria with a supernatant from a reticulocyte lysate containing porin synthesized *in vitro* was performed with two samples. Sample A was incubated at 25 °C, sample B at 4 °C. After incubation for various time periods, mitochondria were isolated by centrifugation, lysed with Triton X-100, and immunoprecipitation of the porin was performed. Immunoprecipitates were subjected to electrophoresis on horizontal slab gels. Gels were sliced and the radioactivity in the porin peak was determined. (●——●) Without protease treatment; (□——□) with protease treatment
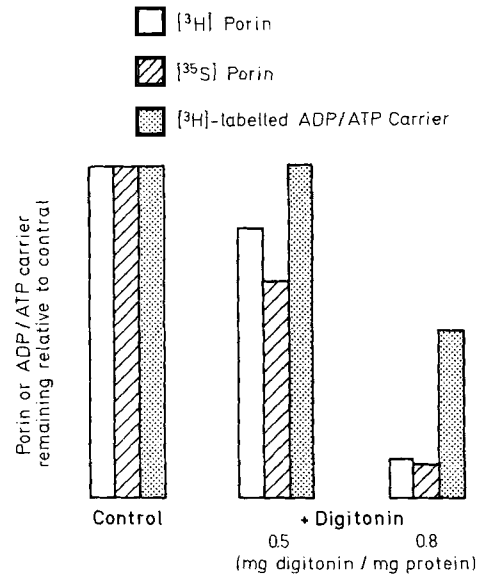
□ [^{3}H] Porin

▨ [^{35}S] Porin

▦ [^{3}H]-labelled ADP/ATP Carrier

Control   + Digitonin

0.5   0.8

(mg digitonin / mg protein)

Fig. 5. *Insertion of mitochondrial porin* in vitro *into the outer mitochondrial membrane*. Transfer *in vitro* of ^{35}S-labelled mitochondrial proteins was performed employing mitochondria from cells labelled with [^{3}H]leucine. After transfer, the mitochondria were re-isolated and treated with increasing concentrations of digitonin (see Materials and Methods). Then the mitochondrial suspensions were layered on cushions containing 1.0 M sucrose which held back the separated outer membrane. After centrifugation, the mitochondria in the pellet were lysed with Triton, and porin and ADP/ATP carrier were immunoprecipitated from aliquots. Immunoprecipitates were subjected to electrophoresis on horizontal gels and the ^{3}H and ^{35}S radioactivities in the peaks corresponding to porin and ADP/ATP carrier were determined. The amounts of [^{3}H]porin, [^{35}S]porin, and ^{3}H-labelled ADP/ATP carrier in the control were normalized to unity and the radioactivities in the immunoprecipitates from the digitonin-treated mitochondria expressed as the fractional values

of interest to determine whether a protein of the outer mitochondrial membrane needs an energized inner membrane for membrane insertion. The protonophore carbonylcyanide *m*-chlorophenylhydrazone, the ionophore valinomycin, and a combination of oligomycin and antimycin A were found to block transport and processing of ATPase subunit 9 and of the ADP/ATP carrier [23]. No inhibition of transfer into a protease-resistant location was seen with the mitochondrial porin, when these inhibitors were applied to the transfer system *in vitro*. Obviously, an electrical potential across the inner mitochondrial membrane is not required for membrane insertion of the mitochondrial porin.

DISCUSSION

The mitochondrial porin, an intrinsic protein of the outer mitochondrial membrane, is synthesized on cytoplasmic ribosomes *in vitro*. This is in agreement with earlier studies on whole *Neurospora* cells [38]. After synthesis the porin is transferred into the outer mitochondrial membrane in a post-translational process according to the following criteria. First, it is translated by free polysomes; very little RNA coding for porin was found associated with mitochondria. Second, transfer could be observed in a cell-free system in which synthesis and membrane insertion were separated in time and space.

As judged from its apparent molecular weight on dodecyl-sulfate containing gels, the precursor form of the mito-

chondrial porin does not have an additional sequence. In this respect, mitochondrial porin is related to the ADP/ATP carrier [34], cytochrome *c* [35] and subunit VI of the cytochrome bc_1 complex (M. Teintze, M. Slaughter, H. Weiss, and W. Neupert, unpublished results); it differs from some 30 other mitochondrial precursor proteins on which extensions of apparent molecular weight of about 400—7000 have been found [1—19]. It is interesting in this context that a posttranslational transfer of precursor proteins synthesized on free ribosomes into the endoplasmic reticulum membrane and into the plasma membrane has been observed. These precursors do not have polypeptide extensions (for review see [39]). Posttranslational insertion of a protein into a membrane apparently does not require the cleavage of an extra sequence. The data presented show that insertion in the cell-free system occurs selectively into the outer mitochondrial membrane. Further support for correct membrane choice *in vitro* comes from the observations that porin synthesized *in vitro* is not inserted into isolated plasma membranes from human fibroblasts, but is inserted into mitochondria from rat liver and yeast (H. Freitag and W. Neupert, unpublished). Transfer and processing of other *Neurospora* precursor proteins into rat liver and yeast mitochondria has been observed before [23,40].

Binding of precursors to receptors on the surface of the mitochondria has been identified as a first step in the transfer process [4,7,36]. With the porin, no such intermediate form could be detected when transfer was performed *in vitro* at 25°C. At 4°C, however, part of the porin which became associated with mitochondria was protease-sensitive. This indicates that also in this case binding to a receptor may occur before the protein is integrated into the outer membrane.

Membrane insertion of the porin is not dependent on the energization of mitochondria. The porin shares this independence with cytochrome *c* and differs from all other mitochondrial proteins studied so far [4,7,23,41]. One can speculate that a membrane potential is not required for proteins which do not have to be inserted into or translocated across the inner mitochondrial membrane in the course of their assembly. As we have already noted before [7], more than one pathway appears to exist for the assembly of the different mitochondrial proteins.

Recently, Shore et al. [42] have described the synthesis of a protein associated with the outer mitochondrial membrane of rat liver by free polysomes and its transfer to mitochondrial membranes *in vitro*. These data would support a posttranslational mechanism of transfer of outer membrane proteins.

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