Import Pathways of Precursor Proteins into Mitochondria: Multiple Receptor Sites Are Followed by a Common Membrane Insertion Site

Rupert Pfaller, Heinrich F. Steger, Joachim Rassow, Nikolaus Pfanner, and Walter Neupert
Institut für Physiologische Chemie der Universität München, D-8000 München 2, Federal Republic of Germany

**Abstract.** The precursor of porin, a mitochondrial outer membrane protein, competes for the import of precursors destined for the three other mitochondrial compartments, including the Fe/S protein of the bc1-complex (intermembrane space), the ADP/ATP carrier (inner membrane), subunit 9 of the F0-ATPase (inner membrane), and subunit β of the F1-ATPase (matrix). Competition occurs at the level of a common site at which precursors are inserted into the outer membrane. Protease-sensitive binding sites, which act before the common insertion site, appear to be responsible for the specificity and selectivity of mitochondrial protein uptake. We suggest that distinct receptor proteins on the mitochondrial surface specifically recognize precursor proteins and transfer them to a general insertion protein component (GIP) in the outer membrane. Beyond GIP, the import pathways diverge, either to the outer membrane or to translocation contact–sites, and then subsequently to the other mitochondrial compartments.

**NUCLEAR-coded mitochondrial proteins are synthesized as precursor proteins on cytosolic polysomes and are subsequently imported into mitochondria** (for review see Pfanner and Neupert, 1987a; Nicholson and Loon, 1986). Most precursors contain positively charged peptide extensions (presequences) at their amino terminus. The presequences and the amino-terminal portions of uncleaved precursors have been shown to contain information for targeting to mitochondria (for review see Hurt and van Loon, 1986). In addition, it was recently demonstrated that other portions of precursor proteins can carry specific import information (Pfanner et al., 1987b,c). Nucleoside triphosphates are required for translocation-competent folding (“unfolding”) of the precursor proteins in the cytosol (for review see Eilers and Schatz, 1988; Pfanner and Neupert, 1988). The precursors interact with proteins of the mitochondrial outer membrane that are proposed to perform the function of import receptors. The precursors are then translocated into or across the mitochondrial membranes. In most cases, import occurs at contact sites between outer and inner membranes (Schleyer and Neupert, 1985; Hartl et al., 1986; Pfanner and Neupert, 1987b; Pfanner et al., 1987a,d; Schwaiger et al., 1987). Transfer into and across the inner membrane requires the electrical potential (Δψ) across the inner membrane (Pfanner and Neupert, 1985). The presequences are proteolytically cleaved by the processing peptidase of the mitochondrial matrix (Böhm et al., 1980, 1983; Conboy et al., 1982; McAda and Douglas, 1982; Miura et al., 1982; Zwizinski and Neupert, 1983; Schmidt et al., 1984; Hawlitschek et al., 1988). Several precursors destined for the intermembrane space or the outside of the inner membrane are retranslocated from the matrix back across the inner membrane (Hartl et al., 1986, 1987). The translocation of cytochrome c into the intermembrane space differs in several respects from the general import mechanism as it does not involve the action of the membrane potential or proteolytic cleavage (Zimmermann et al., 1981).

The following observations suggested that proteinaceous binding sites in the outer membrane are involved in protein import into mitochondria. (a) Pretreatment of isolated mitochondria with proteases inhibited subsequent import of precursor proteins (Gasser et al., 1982; Argan et al., 1983; Hennig et al., 1983; Riezman et al., 1983; Zwizinski et al., 1984; Schmidt et al., 1985; Hartl et al., 1986; Kleene et al., 1987; Ohba and Schatz, 1987a,b; Pfaller and Neupert, 1987; Pfanner and Neupert, 1987b; Pfanner et al., 1987b,c; Schwaiger et al., 1987). (b) Precursor proteins could be trapped at the level of binding to mitochondria by either lowering the temperature of the import reaction in the case of the outer membrane protein porin (Kleene et al., 1987; Pfanner and Neupert, 1987); dissipation of the membrane potential in the case of the inner membrane proteins ADP/ATP carrier (AAC), subunit 9 of the F0-ATPase (F09), and the intermembrane space protein cytochrome b1 (Zwizinski et al., 1983; Riezman et al., 1983; Pfanner and Neupert, 1985, 1987b; Schmidt et al., 1985; Pfanner et al., 1987b,c,d) or, in the case of the intermembrane space protein cytochrome c, inhibition of cytochrome c heme lyase.

Nikolaus Pfanner's present address is Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544.

1. **Abbreviations used in this paper:** AAC, ADP/ATP carrier; F1β, F1-ATPase subunit β; F09, F0-ATPase subunit 9; GIP, general insertion protein; TMPD, N,N,N',N'-tetramethylphenyldiamine; ws-porin, water-soluble porin.
(Hennig and Neupert, 1981). After relieving the import block, the precursor proteins were imported from their binding sites without prior release from the mitochondrial membrane. The binding sites for cytochrome c and porin were shown to be saturable (Hennig et al., 1983; Pfälzer and Neupert, 1987).

Recently, the binding reactions of AAC and porin were resolved into two sequential steps. The precursors first interact with protease-accessible receptor sites on the mitochondrial surface (stage 2 sites) and are then inserted into protease-resistant sites in the outer membrane (stage 3 sites; Pfänger and Neupert, 1987b; Pfanner et al., 1987c,d; Pfälzer and Neupert, 1987). We present evidence here that distinct stage 2 sites for AAC and porin are followed by a stage 3 site that is common for both precursors. Furthermore, stage 3 sites for AAC were saturable. The affinity and number of these binding sites for the AAC precursor, as determined by Scatchard analysis, were similar to the binding parameters found for the high-affinity binding of water-soluble (ws)-porin (Pfälzer and Neupert, 1987).

We have extended our studies of binding sites for several other precursor proteins destined for different mitochondrial compartments, including the Fe/S protein of the bc1-complex (intermembrane space), Fαβ (inner membrane), and subunit β of the F1-ATPase (Fαβ matrix). We report here that the precursors first interact with distinct protease-accessible binding sites on the mitochondrial surface (comparable with the stage 2 sites) and then the import pathways converge at a common insertion site (the stage 3 site) which is used by porin, the Fe/S protein, AAC, Fαβ, and Fαβ. We suggest that the protease-sensitive sites act as receptor sites for the specific recognition and binding of mitochondrial precursors, and that they subsequently direct the precursors to the general membrane insertion site.

**Materials and Methods**

**Materials**

L-[35S]Methionine (1,000 Ci/mmol) was purchased from Amersham Buchler GmbH (Braunschweig, FRG). BSA, ascorbic acid, and PMSF were from E. Merck (Darmstadt, FRG). ATP, NADH, proteinase K, and SP6 polymerase were from Boehringer-Mannheim GmbH (Mannheim, FRG). Apyrase (from potato), antimony A, oligomycin, N,N,N',N'-tetramethylphenylenediamine (TMPD), nucleotides, elastase (from porcine pancreas, EC 3.4.21.11), trypsin (from bovine pancreas, TPCK treated, EC 3.4.21.4), and soybean trypsin inhibitor were from Sigma Chemie GmbH (Deisenhofen, FRG).

**Synthesis of Precursor Proteins**

cDNA clones described previously were used in vitro transcription and translation of the AAC (Pfänger et al., 1987b), porin (Klee et al., 1987), Fαβ, and pFαβ, α5-dihydrofolate reductase (Pfänger et al., 1987d), and cytochrome c (Stuart et al., 1987).

Full-length cDNAs coding for Fαβ and the Fe/S-protein (Harnisch et al., 1985) were isolated from a Neurospora crassa library (Klee et al., 1987). For cloning into pGEN 3 vector (Promega Biotech, Madison, WI), the coding region of the cDNA of the Fe/S-protein was cut out with Hinf I. The cDNA of Fαβ was shortened at the 5' end by digestion with exonuclease III (Henikoff, 1984) leaving the start ATG codon intact. cDNA cloning and transformation into Escherichia coli strain DH 1 was carried out essentially as described before (Maniatis et al., 1982; Klee et al., 1987). cDNAs were transcribed using SP6 polymerase (Melton et al., 1984) and the transcripts were used in cell-free protein synthesis in rabbit reticulocyte lysate (Pelham and Jackson, 1976) in the presence of [35S]methionine (Pfänger and Neupert, 1985, 1986, 1987b; Hartl et al., 1986; Pfänger et al., 1987d).

**In Vitro Binding of Precursors and Import into Isolated Mitochondria**

Mitochondria were isolated from N. crassa as described (Pfänger and Neupert, 1985). In vitro binding and import studies were carried out in BSA-containing buffer consisting of 3% (wt/vol) BSA, 250 mM sucrose, 5 mM MgCl2, 80 mM KCl, 10 mM Mops/KOH, pH 7.2 (Pfänger and Neupert, 1985). Reticulocyte lysate containing 35S-labeled precursor proteins was added to import reactions in 10-20% (vol/vol) final concentration, except in experiments for Fig. 8 where binding of AAC to energized mitochondria was carried out in undiluted lysate.

**Pretreatment of Mitochondria with Trypsin**

Mitochondria were pretreated with trypsin essentially as described by Zwizinski et al. (1984) with the modification that mitochondria were incubated with trypsin for 20 min at 0°C. The protease treatment was stopped with soybean trypsin inhibitor (30-fold weight excess over trypsin) and 0.5 mM PMSF. Control mitochondria were treated in the same way except that trypsin was omitted.

**Miscellaneous**

Ws-porin was prepared as described previously (Pfänger et al., 1985; Pfänger and Neupert, 1987). SDS-PAGE (Laemmli, 1970) and fluorography (Chamberlain, 1979) were performed as described. Quantification of fluorographed bands was carried out by densitometry using a laser densitometer (Ultrascan XL; LKB Instruments, Inc., Gaithersburg, MD). In the case of titration experiments of binding sites for AAC, bands were excised from the gels, eluted in the presence of H2O2, and the 35S-radioactivity was determined by liquid scintillation counting (Nicholson et al., 1987). The values were corrected for the efficiencies of elution and counting (as determined by standard samples). The specific radioactivity of AAC was calculated by determination of the effective concentration of free methionine in the translation mixture and from the number of methionine residues in AAC.

**Results**

**Porin Competes for the Import of Precursor Proteins Destined for Different Mitochondrial Compartments**

Ws-porin, prepared from the purified membrane protein by denaturation/renaturation, has been found to have very similar binding and import properties compared to the biosynthetic porin precursor. This includes the requirement for a protease-sensitive site on the mitochondrial surface, competition with the biosynthetic porin precursor for binding, and two-step insertion into the outer membrane (Pfänger et al., 1985; Kleene et al., 1987; Pfänger and Neupert, 1987). Furthermore, ws-porin was able to form porin-specific membrane channels upon insertion into a lipid bilayer (Pfänger et al., 1985). Ws-porin was now used to investigate whether it would interact with binding sites for other precursor proteins (Fig. 1).

Isolated energized mitochondria and various 35S-labeled precursor proteins were incubated for 40 min at 0°C in the presence of unlabeled ws-porin at the concentrations indicated in Fig. 1. The mitochondria were reisolated and incubated for 15 min at 25°C in the absence of a membrane potential to allow for complete import of precursors which had only been partially imported at 0°C (Schleyer and Neupert, 1985; Pfänger and Neupert, 1987b). Ws-porin inhibited the import of the biosynthetic porin precursor and of AAC (Fig. 1; also see Pfänger et al., 1985; Pfänger and Neupert, 1987b). The import of Fe/S protein, Fαβ, and Fαβ was also inhibited (Fig. 1). The concentration of ws-porin required for half-maximal inhibition of import was in the range of 50-90 pmol/ml. This is similar to the concentration of ws-porin required for half-saturation of its binding sites on
Mitochondrial Protein Import

To demonstrate that the competition occurs at the level of binding of precursors to mitochondria and to exclude unspecific effects of ws-porin on precursors or mitochondria, we performed a series of control experiments (Figs. 2–4).

Repeated freezing and thawing of ws-porin renders it largely incompetent for binding and import into mitochondria (Pfaffler et al., 1985). Thus, it can be expected that ws-porin after freezing and thawing loses its ability to compete for the import of other precursors. Using this approach, we can exclude the possibility that the observed competition was due to an inactivation of precursor proteins by ws-porin. In the experiment described in Fig. 2, ws-porin was subjected to three cycles of freezing and thawing in the presence of a 35S-labeled precursor of F₁β (Fig. 2A), and in the presence or absence of a precursor of Fe/S-protein (Fig. 2B). In control samples, ws-porin was treated with reticulocyte lysate containing the radiolabeled precursor of either F₁β or Fe/S-protein; however, they were not subjected to freezing and thawing. The efficiency of competition was strongly decreased in samples where ws-porin was inactivated for binding and import, irrespective of whether the 35S-labeled precursors were present during the freezing and thawing or not (Fig. 2). We conclude, therefore, that competition of import by ws-porin requires the presence of import-competent porin.

Pretreatment of mitochondria with trypsin strongly reduces the import of porin but allows residual import of other precursor proteins to occur ("bypass import") (Pfaffler and Neupert, 1987; Pfaffler et al., 1988). We investigated the competition of import by ws-porin into trypsin-treated mitochondria in the case of F₁β (Fig. 3A) and Fe/S-protein (Fig. 3B). Competition of import by ws-porin was strong.

Mitochondria (Pfaffler and Neupert, 1987). The import of a chimeric protein consisting of the presequence of F₉ and the mouse cytosolic enzyme dihydrofolate reductase (Pfanner et al., 1987c,d) was competed for in a similar manner as the import of authentic F₉. The import of cytochrome c, however, was not significantly reduced by ws-porin under these conditions (Fig. 1). This is consistent with earlier studies that had shown the reciprocal case in which the precursor of cytochrome c did not compete for the import of AAC or F₉ (Zimmermann et al., 1981).
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Figure 3. Competition by ws-porin for the import of Fβ and Fe/S-protein does not occur with mitochondria pretreated with trypsin. (A and B) Mitochondria were either treated with 20 μg trypsin/mg mitochondria (+T) or without trypsin (-T). Competition assays were carried out as described in Fig. 1. (A) Competition for import of Fβ. (●) Untreated mitochondria (20 μg); (○) trypsin-treated mitochondria (60 μg). (B) Competition for import of Fe/S-protein. (●) Untreated mitochondria (15 μg); (○) trypsin-treated mitochondria (15 μg); (♂) trypsin-treated mitochondria (60 μg).

Figure 4. Import is not competed for by ws-porin at 25°C or after chase of prebound ws-porin. (A) Competition for import at 25°C. Competition assays were carried out essentially as described in Fig. 1. The incubation temperature, however, was 25°C instead of 0°C. Furthermore, the chase at 25°C was omitted. Import of Fe/S-protein, Fβ, and F09 was followed. (B) Competition after chase of prebound ws-porin. Binding of ws-porin to mitochondria was carried out as described in Fig. 1 except that reticulocyte lysate was omitted. After incubation for 30 min at 0°C mitochondria were reisolated, resuspended in 360 μl BSA-containing buffer, and incubated at 25°C for 15 min. Reticulocyte lysate (40 μl), containing 35S-labeled precursor proteins, was then added and import was performed at 25°C as described in the legend to Fig. 1. Import of Fe/S-protein, Fβ, and F09 was followed.

Import of Fβ Is Competed for by Porin at a Step Beyond the Interaction with Receptor Sites

In a previous report it has been shown that there is a different sensitivity of the import of porin and Fβ to pretreatment of mitochondria with elastase (Zwijinski et al., 1984). This suggested that different components of the import machinery were involved in the import of Fβ and porin. On the other hand, import of Fβ is efficiently competed for by ws-porin. To further localize the import step that is influenced by pretreatment of mitochondria with elastase, we compared the elastase sensitivity of the import of several precursor proteins (Fig. 5). Import of porin, AAC, F09, and the Fe/S-protein were sensitive to pretreatment with low amounts of elastase. Import of Fβ, in contrast, was not significantly affected by pretreatment of mitochondria with elastase (up to 10 μg/ml final concentration). It should be noted that the import of Fβ depended in a linear fashion on the amount of mitochondria and on the time of incubation (Pfanner et al., 1987c), so that the experiments were performed within the linear range for import.

Since import of all of these precursors was shown to be sensitive to pretreatment of mitochondria with trypsin (Zwijinski et al., 1984; Pfanner et al., 1988), it appears that the
trypsin-sensitive binding sites are different for porin and F1β. Therefore, ws-porin should not compete with F1β for import into elastase-pretreated mitochondria. Fig. 6 shows that this is indeed the case. Apparently, porin has to interact with its import sites on the mitochondrial surface in order to compete with the precursor of F1β for import. In addition to the control experiments shown in Figs. 2-4, this result indicates that the possibly nonspecific effects of porin (such as complex formation with the precursor of F1β or competition for the binding to cytosolic cofactors) can be excluded. Since the protease-accessible binding sites for F1β and porin are different, the competition of import appears to occur beyond these sites.

**Porin Does Not Compete for the Generation of the Stage 2 Intermediate of AAC**

The precursor of porin was shown to compete for the generation of the stage 3 intermediate of AAC, requiring a concentration of ws-porin of 60-90 pmol/ml for half-maximal inhibition. Translocation of AAC from the stage 3 sites into the inner membrane, on the other hand, was not competed for by ws-porin (Pfaller and Neupert, 1987). In the experiment described in Fig. 7A, we investigated whether the generation of the stage 2 intermediate of AAC was competed for by ws-porin. Fig. 7A, I, shows that the formation of the stage 2 intermediate of AAC was not competed for at any of the concentrations of porin tested (up to 250 pmol/ml). Binding and import of the precursor form of porin used is not affected by an ATP depletion of the import system (Pfaller et al., 1988). At least 85% of the AAC bound to mitochondria was a true stage 2 intermediate, since it was not found associated with mitochondria that had been pretreated with trypsin (Fig. 7A, II). Further translocation of AAC from the stage 2 site into mitochondria was competed for by porin (Fig. 7B). We conclude that the import pathways of AAC and of porin share a common component at the level of stage 3.

To assess whether the stage 3 site is a common component of the import pathways for porin and AAC, we titrated stage 3 sites for the AAC. For the experiment described in Fig. 8, isolated mitochondria were incubated with reticulocyte lysate containing increasing amounts of precursor of AAC for 30 min at 25°C in the absence of a membrane potential. Mitochondria were then reisolated and aliquots of the supernatant were saved to determine free AAC. Stage 3 intermediates were determined by trypsin treatment of the mitochondria containing bound AAC (Pfaller and Neupert, 1987b). Quan-
titative determination of free and bound AAC was performed by resolving the SDS and heat-denatured samples on SDS-polyacrylamide gels, excision of the bands corresponding to AAC, elution in the presence of H2O2, and counting of the 35S-radioactivity (see Materials and Methods).

Preincubation of mitochondria with reticulocyte lysate not containing AAC for varying periods of time did not lead to reduced binding of AAC in a subsequent incubation in the presence of labeled AAC precursor (not shown). Longer incubation times did not lead to higher amounts of bound AAC. We therefore conclude that the binding reactions were in or close to equilibrium. The number of binding sites and the apparent affinity constant were determined by Scatchard analysis. The number of stage 3 sites were determined to be 3.5 pmol/mg mitochondrial protein and had an apparent affinity constant (K_a) of 5 × 10^9 M^-1 (Fig. 8). Binding parameters determined in this way were in good agreement with those characterized for the high affinity binding of ws-porin to mitochondria (Pfaller and Neupert, 1987).

Discussion

Protein Import Pathways to Different Mitochondrial Compartments

The protein import pathways to the four different mitochondrial compartments appear to share common component(s). Our conclusions are summarized in the model shown in Fig. 9. The precursor proteins of porin, Fe/S protein, AAC, F_09, and F_β interact with proteins on the mitochondrial surface. Our working hypothesis suggests that these binding sites, which were described as stage 2 sites for AAC (Pfanner et al., 1987d), function as receptors which specifically recognize and bind mitochondrial precursor proteins. They transfer them to a common membrane insertion site, the general insertion protein (GIP), which corresponds to stage 3 sites for the other precursors. GIP and AAC use distinct protease-accessible receptor sites, distinct from those for the other precursors. Furthermore, porin and AAC use distinct protease-accessible receptor sites, since ws-porin does not compete for stage 2 binding of AAC. In summary, this suggests that at least three distinct receptor sites exist; i.e., for porin, for AAC, and for F_β. Our data allow the functional characterization of three distinct receptor sites. Without purification and functional reconstitution of the receptor sites, however, it cannot be determined whether these sites are completely separate entities or if they are structurally connected (i.e., different states of one or more proteins). It is also not excluded that receptors have overlapping specificity for the various precursor proteins. Furthermore, it is unknown if precursors first (specifically or unspecifically) interact with lipids of the outer membrane and are then, by binding to receptors, directed to their transport pathways into mitochondria.

The GIP

Beyond the receptor sites, the precursors interact with GIP which appears to be common for all these precursors. The precursor of porin competes for the import of the other five precursors that were investigated at a concentration of 50–90 pmol/ml for half-maximal inhibition. Competition by porin requires that native porin occupies GIP in the outer mitochondrial membrane. For AAC, the precursor of which can be trapped at the distinct stages of the import pathway (Pfanner and Neupert, 1987b; Pfanner et al., 1987d), we could directly demonstrate that the competition occurs for the interaction with GIP (also see Pfaller and Neupert, 1987). GIP participates in the insertion of precursor into the outer membrane (Pfanner and Neupert, 1987b; Pfanner et al., 1987d; Pfaller and Neupert, 1987; Söllner et al., 1988). This process is the most strongly ATP-dependent step of mitochondrial import, whereas the translocation of the AAC precursor from GIP into the inner membrane does not require ATP (Pfanner et al., 1987d). ATP was shown to be required for the cytosolic unfolding of precursors ("translocation-competent folding") (Chen and Douglas, 1987; Pfanner et al., 1987d; Pfanner and Neupert, 1987b).
Membrane common to the import pathways of the various precursor proteins takes place in a step that is strongly dependent on ATP. This step is mediated by GIP, a component of the outer membrane common to the import pathways of the various precursor proteins (presumably with the exception of cytochrome c). After the GIP-mediated insertion, outer membrane proteins are assembled while proteins destined to the other submitochondrial compartments are transferred to contact sites where the membrane potential–dependent translocation across the inner membrane takes place before further submitochondrial sorting events.

The specificity of mitochondrial protein import appears to be controlled at more than one site. The receptor proteins on the surface may have a major role in this function, but GIP and further components (in particular in contact sites) may also be important. This points to a “multiple check system” for mitochondrial protein import. Specificity at the level of precursor proteins relies to a large degree on the targeting sequences. We suggest that they interact with the receptors and thereby further import is enhanced. There may, however, be the chance for a targeting sequence to bypass the receptor (Pfaller et al., 1988). If so, it must have the ability to insert into the outer membrane, probably using GIP. Artificial presequences may therefore lack the ability to interact with receptors, but would require a certain membrane insertion activity for the bypass reaction (von Heijne, 1986; Roise et al., 1986). Furthermore, for entrance into the inner membrane, targeting sequences appear to require the presence of positively charged amino acid residues in order to respond to the membrane potential (Pfanner and Neupert, 1985; Horwich et al., 1985). Most interestingly, artificial sequences appear to fulfill the latter two requirements but probably not the first one, namely specific recognition by a receptor.

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References

Argan, V., C. J. Lusty, and G. C. Shore. 1983. Membrane and cytosolic components affecting transport of the precursor for ornithine carbamyltransferase into mitochondria. J. Biol. Chem. 258:6667-6670.

Böhni, P. C., G. Daum, and G. Schatz. 1983. Import of proteins into mitochondria. Partial purification of a matrix-localized protease involved in cleavage of mitochondrial precursor polypeptides. J. Biol. Chem. 258:4937-4943.

Böhni, P. C., S. Gasser, C. Leaver, and G. Schatz. 1980. A matrix-localized protease processing cytoplasmically made precursors to mitochondrial proteins. In The Organization and Expression of the Mitochondrial Genome. A. M. Kroon, and C. Saccone, editors. Elsevier Science Publishing Co., Amsterdam, The Netherlands. 423-433.

Chamberlain, J. P. 1979. Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor sodium salicylate. Anal. Biochem. 98:132-135.

Chen, W.-J., and M. G. Douglas. 1987. Phosphodiester bond cleavage outside mitochondria is required for the completion of protein import into the mitochondrial matrix. Cell. 49:651-658.

Cowboy, J. B., W. A. Fenton, and L. E. Rosenberg. 1982. Processing of the pre-ornithine transcarbamylase requires a zinc-dependent protease localized to the mitochondrial matrix. Biochem. Biophys. Res. Commun. 105:1-7.

Eilers, M., and G. Schatz. 1986. Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria. Nature (Lond.). 322:228-232.

Eilers, M., and G. Schatz. 1988. Protein unfolding and the energetics of protein translocation across biological membranes. Cell. 52:481-483.

Gasser, S. M., G. Daum, and G. Schatz. 1982. Import of proteins into mitochondria. Energy-dependent uptake of precursors by isolated mitochondria. J. Biol. Chem. 257:13034-13041.

Harnisch, U., H. Weiss, and W. Sebald. 1985. The primary structure of the iron-sulfur subunit of ubiquinol–cytochrome c reductase from Neurospora, determined by CDNA and gene sequencing. Eur. J. Biochem. 149:95-99.

Hartl, F.-U., J. Ostermann, B. Giard, and W. Neupert. 1987. Successive translocation into and out of the mitochondrial matrix: targeting of proteins to the intermembrane space by a bipartite signal peptide. Cell. 51:1027-1037.

Hartl, F.-U., B. Schmidt, E. Wachter, H. Weiss, and W. Neupert. 1986. Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol–cytochrome c reductase. Cell. 47:939-951.

Hawlitschek, G., H. Schneider, B. Schmidt, M. Tropschug, F.-U. Hartl, and W. Neupert. 1988. Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein. Cell. 53:795-806.

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Pfaller, R., H. Köhler, and W. Neupert. 1983. Receptor sites involved in post-translational transport of apocytochrome c into mitochondria: specificity, affinity and number of sites. Proc. Natl. Acad. Sci. USA. 80:4963–4967.

Hurt, E. C., and J. E. F. van Loon. 1986. How proteins find mitochondria and intramitochondrial compartments. Trends Biochem. Sci. 11:204–207.

Kleene, R., N. Pfanner, R. Pfaller, T. A. Link, W. Sebald, W. Neupert, and M. Tropschug. 1987. Mitochondrial porin of Neurospora crassa: cDNA cloning, in vitro expression and import into mitochondria. EMBO (Eur. Mol. Biol. Organ.) J. 6:2627–2633.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680–685.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

McAda, P., and M. G. Douglas. 1982. A neutral metallo endoprotease involved in the processing of an F1-ATPase subunit precursor in mitochondria. J. Biol. Chem. 257:3177–3182.

Melton, D. A., P. A. Krieg, M. R. Rebagliati, T. Maniatis, K. Zinn, and M. R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. 12:7035–7056.

Müira, S. M. Mori, Y. Amaya, and M. Tatibana. 1982. A mitochondrial protein that cleaves the precursor of ornithine transcarbamoyl transferase. Eur. J. Biochem. 122:641–647.

Nicholson, D. W., and W. Neupert. 1988. Synthesis and assembly of mitochondrial proteins. In Protein Transfer and Organelle Biogenesis, R. C. Das and P. W. Robbins, editors. Academic Press, New York. 677–746.

Nicholson, D. W., H. Köhler, and W. Neupert. 1987. Import of cytochrome c into mitochondria. Cytochrome c here lysase. Eur. J. Biochem. 164:147–157.

Oba, M., and G. Schatz. 1987a. Protein import into mitochondria is inhibited by antibodies raised against 45-kD proteins of the outer membrane. EMBO (Eur. Mol. Biol. Organ.) J. 6:2109–2115.

Oba, M., and G. Schatz. 1987b. Disruption of the outer membrane restores protein import to trypsin-treated yeast mitochondria. EMBO (Eur. Mol. Biol. Organ.) J. 6:551–556.

Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. 67:247–256.

Pfaller, R., and W. Neupert. 1987. High-affinity binding sites involved in the import of porin into mitochondria. EMBO (Eur. Mol. Biol. Organ.) J. 6:2635–2642.

Pfaller, R., H. Freitag, M. A. Harrey, R. Benz, and W. Neupert. 1985. A water-soluble form of porin from the mitochondrial outer membrane of Neurospora crassa: properties and relationship to the biosynthetic precursor form. J. Biol. Chem. 260:8188–8193.

Pfaller, R., N. Pfanner, and W. Neupert. 1988. Mitochondrial protein import. Bypass of proteaceous surface receptors can occur with low specificity and efficiency. J. Biol. Chem. In press.

Pfanner, N., and W. Neupert. 1985. Transport of proteins into mitochondria: a potassium diffusion potential is able to drive the import of ADP/ATP carrier. EMBO (Eur. Mol. Biol. Organ.) J. 4:2819–2825.

Pfanner, N., and W. Neupert. 1986. Transport of F-ATPase subunit β into mitochondria depends on both a membrane potential and nucleoside triphosphates. FEBS (Fed. Eur. Biochem. Soc.) Lett. 209:152–156.

Pfanner, N., and W. Neupert. 1987a. Biogenesis of mitochondrial energy transduction complexes. Curr. Top. Bioenerg. 15:177–219.

Pfanner, N., and W. Neupert. 1987b. Distinct steps in the import of ADP/ATP carrier into mitochondria. J. Biol. Chem. 262:7528–7536.

Pfanner, N., and W. Neupert. 1988. Bioenergetics of protein transport into mitochondria: role of ΔΨ and of nucleoside triphosphates. In The Roots of Modern Biochemistry. H. Kleinkauf, H. von Döhren, and L. Jaenicke, editors. Walter de Gruyter, Berlin, FRG. 707–712.

Pfanner, N., F.-U. Hartl, B. Guiard, and W. Neupert. 1987a. Mitochondrial precursor proteins are imported through a hydrophilic membrane environment. Eur. J. Biochem. 169:289–293.

Pfanner, N., P. Hoenen, M. Tropschug, and W. Neupert. 1987b. The Carboxyl-terminal two-thirds of the ADP/ATP carrier polypeptide contains sufficient information to direct translocation into mitochondria. J. Biol. Chem. 262:14851–14854.

Pfanner, N., H. K. Müller, A. Harrey, and W. Neupert. 1987c. Mitochondrial protein import: involvement of the mature part of a cleavable precursor protein in the binding to receptor sites. EMBO (Eur. Mol. Biol. Organ.) J. 6:3449–3545.

Pfanner, N., R. Pfaller, R. Kleene, M. Ito, M. Tropschug, and W. Neupert. 1988. Role of ATP in mitochondrial protein import. Conformational alteration of a precursor protein can substitute for ATP requirement. J. Biol. Chem. 263:4049–4051.

Pfanner, N., M. Tropschug, and W. Neupert. 1987d. Mitochondrial protein import: nucleoside triphosphates are involved in conferring import competence to precursors. Cell. 49:815–823.

Riesmann, H., R. Hay, C. Witte, N. Nelson, and G. Schatz. 1983. Yeast mitochondrial outer membrane specifically binds cytoplasmically synthesized precursors of mitochondrial proteins. EMBO (Eur. Mol. Biol. Organ.) J. 2:1113–1118.

Rise, D., J. J. Horvath, J. M. Tomich, J. H. Richards, and G. Schatz. 1986. A chemically synthesized pre-sequence of an imported mitochondrial protein can form an amphiphilic helix and perturb natural and artificial phospholipid bilayers. EMBO (Eur. Mol. Biol. Organ.) J. 5:1327–1334.

Schleyer, M., and W. Neupert. 1985. Transport of proteins into mitochondria: translocaional intermediates spanning contact sites between outer and inner membranes. Cell. 43:339–350.

Schmidt, B., R. Pfaller, N. Pfanner, M. Schleyer, and W. Neupert. 1985. Transport of proteins into mitochondria: receptors, recognition, and transmembrane movement of precursors. In Achievements and Perspectives of Mitochondrial Research, Vol. II. E. Quagliariello, E. C. Slater, F. Palmieri, C. Saccone, and A. M. Kroon, editors. Elsevier Science Publishers B. V., Amsterdam, The Netherlands. 389–396.

Schmidt, B., E. Wachter, W. Sebald, and W. Neupert. 1984. Processing peptidase of Neurospora crassa mitochondria. Two-step cleavage of imported ATPase subunit 9. Eur. J. Biochem. 144:561–588.

Schwaiger, M., V. Herzog, and W. Neupert. 1987. Characterization of translocation contact sites involved in the import of mitochondrial proteins. J. Cell Biol. 105:235–246.

Söllner, T., N. Pfanner, and W. Neupert. 1988. Mitochondrial protein import: differential recognition of various transport intermediates by antibodies. FEBS (Fed. Eur. Biochem. Soc.) Lett. 229:25–29.

Stuart, R. A., W. Neupert, and M. Tropschug. 1987. Deficiency in mRNA splicing in a cytochrome c mutant of Neurospora crassa: importance of carboxy terminus for import of apocytochrome c into mitochondria. EMBO (Eur. Mol. Biol. Organ.) J. 6:2131–2137.

Verner, K., and G. Schatz. 1987. Import of an incompletely folded precursor protein into isolated mitochondria requires an energized inner membrane, but no added ATP. EMBO (Eur. Mol. Biol. Organ.) J. 6:2449–2456.

von Heijne, G. 1986. Mitochondrial targeting sequences may form amphiphilic helices. EMBO (Eur. Mol. Biol. Organ.) J. 5:1335–1342.

Zimmermann, R. B. Hennig, and W. Neupert. 1981. Different transport pathways of individual precursor proteins into mitochondria. Eur. J. Biochem. 116:455–560.

Zwijinski, C., and W. Neupert. 1983. Precursoor proteins are transported into mitochondria in the absence of proteolytic cleavage of the additional sequences. J. Biol. Chem. 258:13340–13346.

Zwijinski, C., M. Schleyer, and W. Neupert. 1983. Transfer of proteins into mitochondria: precursor to the ADP/ATP carrier binds to receptor sites on isolated mitochondria. J. Biol. Chem. 259:4071–4074.

Zwijinski, C., M. Schleyer, and W. Neupert. 1984. Proteaceous receptors for the import of mitochondrial precursor proteins. J. Biol. Chem. 259:7850–7856.