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

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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 Neupert, 1988). 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 (∆V) 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; Pfaller and Neupert, 1987); dissipation of the membrane potential in the case of the inner membrane proteins ADP/ATP carrier (AAC),1 subunit 9 of the F0-ATPase (F9), 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.

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1. Abbreviations used in this paper: AAC, ADP/ATP carrier; F1β, F1-ATPase subunit β; F9, F0-ATPase subunit 9; GIP, general insertion protein; TMPD, N,N,N',N'-tetramethylphenylenediamine; 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 membranes. The binding sites for cytochrome c and porin were shown to be saturable (Hennig et al., 1983; Pfäffler 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; Pfannen and Neupert, 1987b; Pfannen et al., 1987b,d; Pfäffer 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 (Pfäffler 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), F1,9 (inner membrane), and subunit β of the F1-ATPase (F1β) (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, F1,9, and F1β. 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), antimycin A, oligomycin, N,N,N',N'-tetramethylphenylenediamine (TMPD), nucleotides, elastase (from porcine pancreas, EC 3.4.21.1), 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 in vitro transcription and translation of the AAC (Pfannen et al., 1987b), porin (Kleene et al., 1987), F1,9, and Pf1,9, dihydrofolate reductase (Pfannen et al., 1987d), and cytochrome c (Stuart et al., 1987).

Full-length cDNAs coding for F1β and the Fe/S-protein (Harnisch et al., 1985) were isolated from a Neurospora crassa library (Kleene et al., 1987). For cloning into pGEM 3 vector (Promega Biotech, Madison, WI), the coding region of the cDNA of the Fe/S-protein was cut out with Hind I. The cDNA of F1β 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 DH1 was carried out essentially as described before (Maniatis et al., 1982; Kleene 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 (Pfannen and Neupert, 1985, 1986, 1987b; Hartl et al., 1986; Pfannen et al., 1987d).

In Vitro Binding of Precursors and Import into Isolated Mitochondria

Mitochondria were isolated from N. crassa as described (Pfannen 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 (Pfannen 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 deenergized mitochondria was carried out in undiluted lysate.

Pretreatment of Mitochondria with Trypsin

Mitochondria were pretreated with trypsin essentially as described by Zwirinski 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 (Pfäffler et al., 1985; Pfäffer and Neupert, 1987). SDS-PAGE (Laemmli, 1970) and fluorography (Chamberlain, 1979) were performed as described. Quantitation 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 (Pfannen et al., 1985; Kleene et al., 1987; Pfäffer and Neupert, 1987). Furthermore, ws-porin was able to form porin-specific membrane channels upon insertion into a lipid bilayer (Pfäffler 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; Pfannen and Neupert, 1987b). Ws-porin inhibited the import of the biosynthetic porin precursor and of AAC (Fig. 1; also see Pfäffer et al., 1985; Pfäffer and Neupert, 1987). The import of Fe/S protein, F1,9, and F1β 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
mitochondria (Pflägger and Neupert, 1987). The import of a chimeric protein consisting of the presequence of F09 and the mouse cytosolic enzyme dihydrofolate reductase (Pfanner et al., 1987c,d) was competed for in a similar manner as the import of authentic F09. 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 F09 (Zimmermann et al., 1981).

To demonstrate that the competition occurs at the level of binding of precursors to mitochondria and to exclude unspecified 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 (Pfänner 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. 2 A), and in the presence or absence of a precursor of Fe/S-protein (Fig. 2 B). 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") (Pfaffer and Neupert, 1987; Pfanner et al., 1988). We investigated the competition of import by ws-porin into trypsin-treated mitochondria in the case of F1β (Fig. 3 A) and Fe/S-protein (Fig. 3 B). Competition of import by ws-porin was strong-
Competition assays were carried out essentially as described in Fig. 1. (A) Competition for import of Fe/S-protein. (●) 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).

As reported earlier, incubation of porin with isolated mitochondria at a temperature of 25°C led to almost complete import of porin into the outer membrane; only minor amounts were found remaining at the binding sites, thus these binding sites could only be saturated at low temperature (Pfaller and Neupert, 1987). When competition experiments with ws-porin were performed at 25°C instead of 0°C (see Fig. 1), the extent of competition was strongly reduced (Fig. 4 A). Under these conditions, significant inhibition of import was observed only if higher concentrations of ws-porin were applied (not shown).

In a further experiment, ws-porin was first bound to mitochondria at 0°C. The mitochondria were then reisolated, incubated at 25°C to allow import of the bound porin, and then 35S-labeled precursors were added. Competition of import was again strongly reduced (Fig. 4 B). Competition by ws-porin, thus, requires that it occupies its binding sites on mitochondria. In agreement with this, we have recently reported that at 0°C ws-porin enters binding sites at the outer membrane (Pfaller and Neupert, 1987). Our control experiments also exclude irreversible damaging of the mitochondrial protein import apparatus by ws-porin as a possible artifact.

In summary (Figs. 2-4; and Pfaller and Neupert, 1987), unspecific effects of ws-porin which might reduce protein import (such as complex formation with precursor proteins in the cytosol, disturbance of the membrane potential, or irreversible damage of mitochondria) can be excluded. The results rather suggest that competition occurs for a specific component of the mitochondrial import machinery.

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 (Zwizinski 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, F9, 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., 1987e), 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 (Zwizinski et al., 1984; Pfanner et al., 1988), it appears that the
trypsin-sensitive binding sites are different for porin and Fβ. Therefore, ws-porin should not compete with Fβ for import into elastase-pretreated mitochondria. Fig. 6 shows that this is indeed the case. Apparently, porin has to interact to compete with the precursor of Fβ 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 Fβ or competition for the binding to cytosolic cofactors) can be excluded. Since the protease-accessible binding sites for Fβ 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 (Pfanner 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. 7B). 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 (Pfanner and Neupert, 1987b). Quant...
The precursor proteins of porin, Fe/S protein, AAC, F09, insertion protein (GIP), which corresponds to stage 3 sites, 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.

**Receptor Sites for Mitochondrial Precursor Proteins**

Distinct receptors on the mitochondrial surface appear to be responsible for the specificity of mitochondrial protein uptake. The receptor for F\(\beta\) is not destroyed by elastase, in contrast to those for the other four precursors analyzed. This suggests the existence of a receptor site for F\(\beta\) which is 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\(\beta\). 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 protein 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).
the outer membrane 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 subcompartments are transferred to contact sites where the membrane potential-dependent translocation across the inner membrane takes place before further subcompartments insertion events.

et al., 1987d, 1988; Verner and Schatz, 1987; Pfanner and Neupert, 1988). On the other hand, it was demonstrated that mitochondrial precursor proteins were at least partially unfolded during import into mitochondria (Schleyer and Neupert, 1985; Eilers and Schatz, 1986; Pfanner et al., 1987d). Thus, the step which leads to interaction with GIP appears to need an unfolded precursor protein. GIP seems to be involved in the insertion of unfolded precursors into the outer membrane in such an arrangement that they become competent for translocation into or across the inner membrane (or for assembly in the outer membrane as in the case of porin). The proposed function of GIP in insertion and folding of precursors into the membrane fits well with the observation that many precursor proteins can interact with it. This confirms the general importance of GIP for protein import and may explain its relatively low specificity.

The molecular nature of GIP is unclear so far. It might represent a single protein or a protein complex. The following observations are relevant in this context. When yeast mitochondrial were pretreated with relatively high concentrations of trypsin and were then incubated with antibodies raised against 45-kD proteins of the outer membrane, import of precursor proteins was strongly inhibited (Ohba and Schatz, 1987a). These antibodies might bind to GIP or a component related to GIP which was made accessible by the pretreatment with trypsin. Thereby the antibodies would block import of precursor proteins. About 40% of the precursors of AAC trapped at GIP can be translocated via contact sites into the inner membrane at 0°C within a few seconds (Pfanner and Neupert, 1987b; Pfanner, N., and W. Neupert, unpublished observations). This might indicate that, upon binding, these precursors do not have to diffuse laterally in the lipid phase of the outer membrane before reaching contact sites. Thus, at least a certain fraction of GIP-bound precursor seems to be near to or already associated with translocation contact sites. Since separation of translocation contact sites from the bulk of the inner and outer membranes is possible (Schwaiger et al., 1987) this problem can be approached experimentally.

A Multiple Check System

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; Hirth 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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