Mitochondrial Protein Import

BYPASS OF PROTEINACEOUS SURFACE RECEPTORS CAN OCCUR WITH LOW SPECIFICITY AND EFFICIENCY*

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Rupert Pfaller, Nikolaus Pfanner,†, and Walter Neupert
From the Institut für Physiologische Chemie, Universität München, Goethestrasse 33, D-8000 München 2, Federal Republic of Germany

Proteolytic degradation of receptor sites on the mitochondrial surface strongly reduces the efficiency of mitochondrial protein import. The remaining residual import still involves basic mechanisms of protein import, including: insertion of precursors into the outer membrane, requirement for ATP and a membrane potential, and translocation through contact sites between both membranes. The import of a chloroplast protein into isolated mitochondria which occurs with a low rate is not inhibited by a protease-pretreatment of mitochondria, indicating that this precursor only follows the bypass pathway. The low efficiency of bypass import suggests that this unspecific import does not disturb the uniqueness of mitochondrial protein composition. We conclude that mitochondrial protein import involves a series of steps in which receptor sites appear to be responsible for the specificity of protein uptake.

A crucial step in mitochondrial protein import is the initial recognition of precursor proteins by mitochondria. This step precedes the subsequent reactions that direct precursors into the outer membrane and further on into the inner membrane and matrix (for review, see Pfanner et al., 1988b). The targeting information contained in the mitochondrial precursors must be initially decoded by structures on the mitochondrial surface. The nature of targeting information in mitochondrial precursors has been a subject of detailed studies. It has been shown convincingly that the amino-terminal cleavable presequences contained in most mitochondrial precursors carry sufficient and necessary targeting information (for review, see Hurt and van Loon, 1988). Targeting efficiency is influenced by certain hydrophobic sequences in the mature part of the precursors; however, the specificity appears to reside in the presequences (Pfanner et al., 1987c). In a number of cases, the specific targeting information is contained in the interior of the mature sequence, the best studied case being the ADP/ATP carrier (AAC).† The targeting information may be an abundance of positive charges. A frequent but not entirely general characteristic is a high content of hydroxylated amino acids (von Heijne, 1986). It has been repeatedly argued that the specific targeting information is hidden in a certain unknown secondary structure (e.g. amphipathic α-helix or β-sheet) (for review, see Roise and Schatz, 1988).

Little is known about the components on the mitochondrial surface which decode the targeting information of precursors. In the absence of translocation across the outer membrane, e.g. in the absence of a membrane potential and ATP, precursors can accumulate at the surface of mitochondria at specific binding sites (Zwizinski et al., 1983; Pfanner and Neupert, 1987; Pfanner et al., 1987a). From these sites they can be efficiently chased into the interior of mitochondria upon re-establishing a membrane potential. The surface bound precursors are apparently exposed on the outer face of the outer membrane, since they can be degraded by added proteases under conditions which do not compromise the outer membrane. Pretreatment of mitochondria with proteases strongly reduces their ability to bind or import precursors (Riezman et al., 1983; Zwizinski et al., 1984; Pfanner and Neupert, 1987; Pfaller and Neupert, 1987).

These observations have led to the concept of mitochondrial surface receptors which function in the recognition of precursors and in triggering their membrane insertion (Pfanner and Neupert, 1987; Pfaller and Neupert, 1987; Pfanner et al., in press). Here we ask whether these receptors are absolutely required for mitochondrial protein import. Our experiments show that import can still occur after destroying protease-sensitive receptors, although with low efficiency. This residual import seems to lack specificity; a chloroplast precursor can be imported into protease-pretreated mitochondria with the same efficiency as into untreated mitochondria. We conclude that import bypassing receptors is possible.

EXPERIMENTAL PROCEDURES

In Vitro Binding of Precursors and Import into Isolated Mitochondria—Neurospora crassa mitochondria were isolated in SEM medium (250 mM sucrose, 1 mM EDTA, 10 mM Mops/KOH, pH 7.2) as described previously (Pfanner and Neupert, 1985). For in vitro binding and import studies, BSA-containing buffer was used consisting of 3% (w/v) bovine serum albumin (BSA) (Merck, Darmstadt, Federal Republic of Germany), 250 mM sucrose, 5 mM MgCl₂, 80 mM KCl, 10 mM Mops/KOH, pH 7.2 (Pfanner and Neupert, 1985), except in experiments in which the effect of various concentrations of KCl or MgCl₂ on import was studied. Reticulocyte lysate containing 35S-
labeled precursor proteins was added to import reactions in 10 or 20% (v/v) final concentration.

Pretreatment of Mitochondria with Trypsin—Trypsin pretreatment of isolated mitochondria was carried out essentially as described by Zwizinski et al. (1984). Mitochondria, suspended in SEM buffer, were pretreated with trypsin (from bovine pancreas, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated, EC 3.4.21.4) for 20 min at 0 °C. After the incubation, trypsin was inhibited by adding soybean trypsin inhibitor (Sigma) (30-fold weight excess over trypsin) and 0.5 mM PMSF. Mitochondria were reisolated and washed in SEM medium containing 0.1 mg of trypsin inhibitor/ml and 0.5 mM PMSF. Control mitochondria were treated identically except trypsin was omitted.

Miscellaneous—cDNAs to the precursor proteins, cloned into pGEM vector (Promega) were employed in in vitro transcription using SP6 polymerase (Boehringer Mannheim) (Melton et al., 1984). The transcripts were translated by cell-free protein synthesis in rabbit reticulocyte lysates (Pelham and Jackson, 1976) in the presence of [35S]methionine (Pfanner and Neupert, 1985, 1986, 1987; Hartl et al., 1986; Pfanner et al., 1987a). After binding and import, the SDS- and heat-denatured samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and fluorography (Chamberlain, 1979). Quantification of fluorographed bands was carried out by densitometry on a LKB Ultrascan XL laser densitometer.

RESULTS

Import of Various Precursor Proteins Requires Trypsin-sensitive Components on the Mitochondrial Surface—35S-Labeled precursors of porin, Fe/S-protein of the bc1 complex, ADP/ATP carrier (AAC), F0-ATPase subunit 9 (F09), and F1-ATPase subunit β (F1β) were employed in in vitro import experiments. The mitochondria used were isolated from the fungus N. crassa. The amounts of precursor proteins in all experiments described here were such that their binding and import linearly depended on the amounts of mitochondria present (Zwijinski et al., 1984; Schwaiger et al., 1987; and data not shown); furthermore, in all parallel reactions equimolar amounts of the 35S-labeled precursors were employed (Pfanner et al., 1987c). The criteria for import of the precursor proteins were their proteolytic processing by the processing peptidase in the mitochondrial matrix (in the cases of Fe/S protein, F09, and F1β) and the protection of the proteins against protease K added to the isolated mitochondria. Precursors which were not imported were not proteolytically processed and were digested by the added protease K; furthermore, the import of Fe/S protein, AAC, F09, and F1β was dependent on the presence of the membrane potential (Schleyer et al., 1982; Zwizinski and Neupert, 1983; Zwizinski et al., 1983, 1984; Schleyer and Neupert, 1985).

Import of precursor proteins into control mitochondria and into trypsin-pretreated mitochondria was investigated under identical conditions. The import of the various precursors was inhibited by the trypsin-pretreatment to 5–25% of the control import (Fig. 1). The same reduction of import was achieved by pretreatment with protease K (10–30 μg/ml) (see below; Schwaiger et al., 1987).

Precursor Proteins Can Bypass the Protease-sensitive Components on the Mitochondrial Surface—The residual import into trypsin-pretreated mitochondria still showed several basic characteristics of mitochondrial protein import. Nucleoside triphosphates (Fig. 2A) were required as well as a membrane potential across the inner membrane for translocation of F1β into the matrix (Fig. 2B). Bypass import also occurred via contact sites between both mitochondrial membranes. When the precursor of F1β was incubated with trypsin-treated mitochondria at low temperature, the presequence was proteolytically removed by the processing peptidase in the mitochondrial matrix (Fig. 2C, lane 1). F1β was, however, still accessible to externally added protease K (Fig. 2C, lane 2). Raising the temperature in the absence of a mitochondrial membrane potential led to completion of import of already processed precursor (Fig. 2C, lane 4). The precursor trapped at low temperature thus fulfills the criteria of a translocation intermediate spanning both mitochondrial membranes at contact sites (Schleyer and Neupert, 1985).

Pretreatment of mitochondria with protease K or trypsin completely degraded the initial binding sites for AAC on the mitochondria ("stage 2 binding" Pfanner et al., 1987a; Pfaller et al., in press). In the experiment described in Fig. 2D, we investigated if the bypass import observed in protease-pretreated mitochondria still involved the "stage 3 sites" (also called "general insertion protein"). The stage 3 sites were shown to mediate insertion of AAC into the outer membrane in the absence of a membrane potential and, to release AAC precursor to the inner membrane after re-establishment of a
membrane potential (Pfanner and Neupert, 1987; Pfanner et al., 1987a). Two reactions were performed in parallel (Pfanner et al., 1987c): import of free precursor of AAC in the presence of a membrane potential ("import of free precursor") (Fig. 2D, lanes 1 and 2) and, binding of precursor in the absence of a membrane potential, resolation of mitochondria, and import of precursor from the bound state after re-establishing a membrane potential ("import of prebound precursor"). The ratio between import of prebound precursor and import of free precursor was taken as a measure for the efficiency of stage 3 binding (Fig. 2D, lane 3). Pretreatment of mitochondria with protease K did not change this ratio (Fig. 2D, lane 4). Washing of the reisolated mitochondria, which had precursor of AAC bound, also did not change this ratio indicating a relatively tight binding of AAC to mitochondria (Fig. 2D, lanes 5 and 6). We conclude that protease-pretreated mito-
chondria retain the functions which are ascribed to stage 3 sites and, that bypass import uses the protease-protected stage 3 sites.

The import kinetics, however, were slowed down considerably with protease-pretreated mitochondria (Fig. 3). With Fβ initial rates of import were 10-20-fold slower into protease- treated mitochondria as compared to untreated mitochondria. Thus, bypass of receptor sites results in a strongly reduced efficiency of import.

Receptor-mediated and Bypass Import Require Different Ionic Conditions—We asked the question whether import into mitochondria which were not trypsin-pretreated can be discriminated from bypass import on the basis of different import characteristics. In the first experiment, the influence of KCl was investigated. The usual import assay (see Fig. 1) was modified by omitting KCl from the BSA-containing buffer (which contained 80 mM KCl), such that KCl resulting from addition of reticulocyte lysate was present at a final concentration of about 15 mM. KCl was added to the import reactions to achieve additional concentrations of 0-500 mM. Import of Fβ and AAC into mitochondria, which had been either pretreated or not pretreated with trypsin was measured.

The salt dependence of import into intact mitochondria was very different with Fβ and AAC (Fig. 4A, −T). Import of Fβ was most efficient without any added KCl and was strongly reduced by increasing KCl. In contrast, with AAC the optimal concentration was between 150 and 250 mM of added KCl. At higher and lower concentrations of KCl, import of AAC was considerably decreased. The same behavior was observed for binding of AAC to de-energized mitochondria (not shown). This suggests that the import pathway of AAC is influenced by KCl at a step before the Δψ-requiring step.

Import of Fβ into trypsin-pretreated mitochondria also decreased with increasing KCl but more strongly than import into untreated mitochondria. In the case of AAC, bypass import was stimulated to a much lesser degree by KCl than import into untreated mitochondria (Fig. 4A, +T).

In the second experiment, the effect of various Mg2+ concentrations on import into trypsin-pretreated and non-pretreated mitochondria of AAC and Fβ was analyzed. The import reactions contained an endogenous Mg2+ concentration of about 1 mM; to this an additional 0-10 mM MgCl2 was added. AAC and Fβ were imported at 25 °C into mitochondria either pretreated or not pretreated with trypsin. Import of AAC into untreated mitochondria was strongly stimulated by added MgCl2; Fβ import was only slightly affected (Fig. 4B, −T). Bypass import of either AAC nor Fβ was influenced by MgCl2 (Fig. 4B, +T).

In summary, receptor-mediated import has different ionic requirements than bypass import. This again suggests that different components are involved in the two different initial import reactions. Variation of cofactors, ions, etc. may bias in vitro import to either the receptor-mediated or to the bypass pathway.

Import of a Chloroplast Precursor Protein into Mitochondria Does Not Require Protease-accessible Binding Sites—We investigated if mitochondrial protein import directed by a targeting signal which was not specific for mitochondria involved protease-accessible binding sites on the mitochondrial surface. Fig. 5 shows that the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (SSU; a nuclear-encoded chloroplast protein) from pea was imported into a protease-protected location in isolated Neurospora mitochondria. Dissipation of the mitochondrial membrane potential

**Fig. 3.** Rate of import of Fβ is reduced by trypsin pretreatment of mitochondria. Mitochondria were either not treated or treated with 20 μg of trypsin/mg of mitochondrial protein. Import of Fβ into these mitochondria was investigated as described in the legend to Fig. 1. Import was stopped at the times indicated by adding valinomycin (1 μM final concentration). Mitochondria were reisolated by centrifugation, washed in SEM buffer, and resolved by SDS-PAGE. Mature sized Fβ was analyzed by fluorography and densitometry.
stopped with 0.5 mM PMSF, mitochondria were reisolated, washed and

proteinase K was added in presence (+Asc/TMPD) or absence (1 μM valinomycin, +Val) of a membrane potential for further import. Protease treatment was stopped after 30 min at 0°C. Asc, ascorbate. B, kinetics of import of SSU into trypsin pretreated mitochondria. Mitochondria were treated either without (−Trypsin) or with 20 μg of trypsin/mg mitochondrial protein (+Trypsin). Import was carried out as described above. After the times indicated, samples were put on ice and proteinase K was added (200 μg/ml final concentration) to stop further import. Protease treatment was stopped after 30 min incubation at 0°C with 0.5 mM PMSF. C, dependence of SSU import on the amount of mitochondria in the import reaction. Import was performed as described above except the final concentrations of mitochondrial protein were varied as indicated.

strongly decreased import (Fig. 5A). The efficiency of import of SSU into isolated mitochondria was rather low (1–2% of total precursor added was imported). Hurt et al. (1996) reported that the transit sequence of SSU could direct “passenger” proteins to mitochondria. In that study the efficiency of import was also rather low. Pretreatment of mitochondria with trypsin did not inhibit the import of SSU (Fig. 5A). Furthermore, the kinetics of import of SSU were not affected by protease-pretreatment of mitochondria (Fig. 5B). Since the amount of import of SSU was linearly dependent on the amount of mitochondria in the import reaction (Fig. 5C), we conclude that the mitochondrial import of SSU does not involve protease-accessible sites on the mitochondrial surface.

In summary, precursor proteins enter the mitochondria even when the initial protease-accessible binding sites are destroyed. This bypass import seems to be the main import pathway used by a precursor protein not specific for mitochondria.

**DISCUSSION**

The ability of isolated mitochondria to import precursor proteins is not completely abolished upon removal of surface receptors by protease treatment. This observed import, however, occurs at a reduced rate. Under these conditions precursor proteins enter the existing import routes at a stage that normally follows the initial receptor-mediated step. We have shown here that in the absence of surface receptors the ADP/ATP carrier interacts with a component in the outer membrane, the stage 3 binding site, which corresponds to the general insertion protein, a component which is involved in the membrane insertion of all precursors studied (with the exception of cytochrome c) (Pfaller et al., in press). In the absence of specific receptors, import of precursors into inner membrane/matrix occurs through translocation contact sites and requires an electrical membrane potential. Thus the mechanism of protein import into protease-treated mitochondria still retains specific characteristics of the original, intact import pathway.

How can this residual bypass import be explained? We propose that signal sequences of mitochondrial precursors have at least a 3-fold function. First, they interact with mitochondrial surface receptors. The basis of this specific recognition is not clear. In view of the considerable heterogeneity of mitochondrial signal sequences one may speculate that this specific recognition is mediated by a certain structural element, such as an amphipathic α-helix or β-sheet structure. The second role of signal sequences is their ability to interact with the lipid phase of membranes and thereby to insert into membranes. Membrane activity of mitochondrial signals present in precursors as well as of the respective peptides has been demonstrated in a number of studies (reviewed by Roise and Schatz, 1988). Finally, mitochondrial signal sequences have the function to respond to the membrane potential across the inner membrane to trigger the initial translocation into the matrix space. Presumably for this reason mitochondrial signals without exception are positively charged while the mitochondrial membrane potential is negative inside.

The results presented here indicate that the interaction of signal sequences with receptors is not an obligatory step. Due to the membrane activity of their signals, at least in the in vitro system, precursors can insert into the outer membrane and thereby enter the translocation pathway. This can occur, however, only with reduced efficiency. We therefore suggest that surface receptors, by interacting with signal sequences, increase the efficiency of import. The question remains whether the bypass import would also occur in mitochondria with intact receptors. This appears to be quite possible, however, due to kinetic reasons, the import via receptors would be predominant in intact mitochondria.

It can be anticipated that artificial precursor sequences or non-mitochondrial signal sequences which conform to the second two of the three structural requirements discussed above may still be able to direct import into mitochondria although they cannot interact with receptors. Quite a number of artificial signals constructed by either genetic or chemical techniques have been investigated (for review, see Pfanner et al., 1988a). It appears that these precursors, provided they
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