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Mitochondrial Protein Import

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

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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, 1986). 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)[1] of the inner membrane (Adrian *et al.*, 1986; Pfanner *et al.*, 1987b). On the other hand, the secondary and tertiary structure of the targeting signals is not clearly understood.

The cleavable signals of the various precursor proteins differ considerably in their sequence. The only common feature is 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 mitochondria which decode the targeting information of precursors. In the absence of translocation across the inner 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; Pfaller *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 $MgCl_2$, 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 $MgCl_2$ on import was studied. Reticulocyte lysate containing ^{35}S-

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[1] The abbreviations used are: AAC, ADP/ATP carrier; SSU, a nuclear-encoded chloroplast protein; Mops, 4-morpholinepropanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; RBUp_2Case, ribulose-bisphosphate carboxylase/oxygenase; TMPD, *N,N,N,N'*-tetramethylphenylenediamine; Hsc, ascorbate.

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 incubated with trypsin (from bovine pancreas, L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated, EC 3.4.21.4) (Sigma) 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 [^{35}S]methionine (Pfanner and Neupert, 1985, 1986, 1987; Hartl *et al.*, 1986; Pfanner *et al.*, 1987a). After binding and import, the SDS- and heat-denaturated samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and fluorography (Chamberlain, 1979). Quantitation of fluorographed bands was carried out by densitometry on a LKB Ultroscan XL laser densitometer.

RESULTS

Import of Various Precursor Proteins Requires Trypsin-sensitive Components on the Mitochondrial Surface—^{35}S-Labeled precursors of porin, Fe/S-protein of the bc_1 complex, ADP/ATP carrier (AAC), F_O-ATPase subunit 9 (F_O9), and F_1-ATPase subunit β ($F_1\beta$) 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 linearily depended on the amounts of mitochondria present (Zwizinski *et al.*, 1984; Schwaiger *et al.*, 1987; and data not shown); furthermore, in all parallel reactions equimolar amounts of the ^{35}S-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, F_O9, and $F_1\beta$) and the protection of the proteins against proteinase K added to the isolated mitochondria. Precursors which were not imported were not proteolytically processed and were digested by the added proteinase K; furthermore, the import of Fe/S protein, AAC, F_O9, and $F_1\beta$ 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 proteinase 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 protease-pretreated mitochondria still showed several basic characteristics of mitochondrial protein import. Nucleoside triphosphates (Fig. 2*A*) were required as well as a membrane potential across the inner membrane for translocation of $F_1\beta$ into the matrix (Fig. 2*B*). Bypass import also occurred via contact sites between both mitochondrial membranes. When the precursor of $F_1\beta$ was incubated with trypsin-treated mitochondria at low temperature, the presequence was proteolytically removed by the processing peptidase in the mitochondrial matrix (Fig. 2*C, lane 1*). $F_1\beta$ was, however, still accessible to externally added proteinase K (Fig. 2*C, lane 2*). Raising the temperature in the absence of a mitochondrial

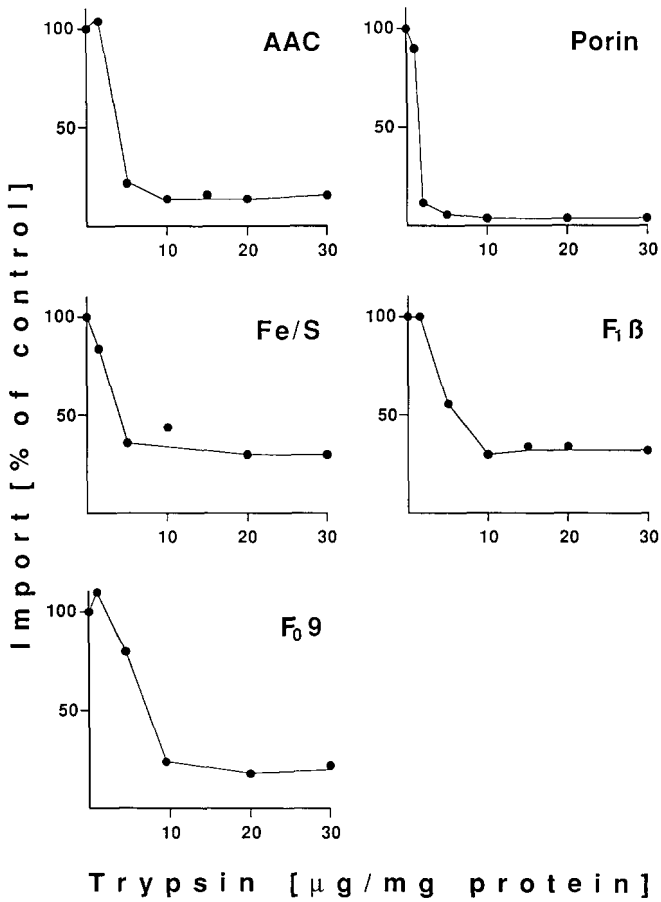

FIG. 1. **Pretreatment of mitochondria with trypsin inhibits protein import but a residual import remains.** Mitochondria were pretreated with various amounts of trypsin as described under "Experimental Procedures." Import reactions were performed in a final volume of 0.4 ml (adjusted with BSA-containing buffer) and contained 20 μg of mitochondrial protein and 40 μl of reticulocyte lysate with ^{35}S-labeled precursor proteins. The precursors of AAC, porin, F_O9, $F_1\beta$, and the Fe/S-protein were analyzed. In the case of AAC, F_O9, and $F_1\beta$, 16 mM ascorbate and 0.4 mM TMPD were present, in the case of the Fe/S-protein, 2 mM NADH and 1 mM ATP were added. No additions were made in the case of porin. After incubation for 20 min at 25 °C mitochondria were reisolated from import reactions by centrifugation. After import of F_O9, $F_1\beta$ and the Fe/S-protein mitochondrial pellets were washed and subjected to SDS-PAGE. Mitochondrial pellets from import reactions for porin and AAC were resuspended in BSA-containing buffer and treated with proteinsase K (100 μg/ml final concentration, 30 min at 0 °C) prior to SDS-PAGE. After electrophoresis the gels were dried and fluorographed. Mature sized F_O9, $F_1\beta$, and Fe/S-protein, and protease-protected AAC and porin were quantified by densitometry of the fluorographs.

membrane potential led to completion of import of already processed precursor (Fig. 2*C, 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 proteinase 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. 2*D*, 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

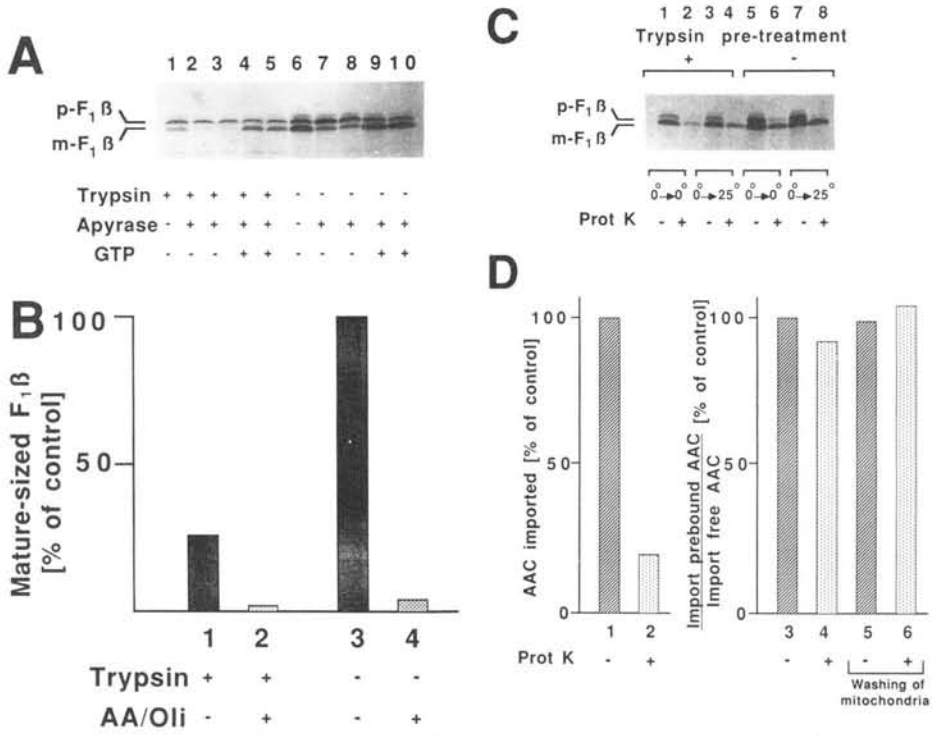

FIG. 2. **Bypass import shares characteristics of receptor-mediated import but the import efficiency is reduced.** *A*, bypass import of $F_1\beta$ requires nucleoside triphosphates. Mitochondria, either pretreated (*lanes 1–5*) or not pretreated (*lanes 6–10*) with trypsin (20 μg/mg mitochondrial protein), were subjected to apyrase treatment (5 units/ml) as described (Pfanner and Neupert, 1986). Reticulocyte lysate, containing ^{35}S-labeled precursor to $F_1\beta$ was also pretreated with apyrase (1 unit/ml in *lanes 2, 4, 7,* and *9* and 5 units/ml in *lanes 3, 5, 8,* and *10*) as described (Pfanner and Neupert, 1986). Heat-inactivated apyrase (corresponding to 5 units/ml) was applied in *lanes 1* and *6*. Import reactions for $F_1\beta$ were as described in the legend to Fig. 1, except that import reactions contained 50 μg of mitochondria and that antimycin A and oligomycin (8 and 20 μM final concentration, respectively) were included. Furthermore, *lanes 4, 5, 9,* and *10* received 6 mM GTP (added from a 0.2 M stock solution in 1 M Mops/KOH, pH 7.2), while only Mops buffer was added to the other samples. After incubation for 20 min at 25 °C, mitochondria were reisolated, washed in SEM buffer, subjected to SDS-PAGE, and analyzed by fluorography. A fluorograph of the dried gel is shown. *B*, bypass import of $F_1\beta$ requires a membrane potential. Pretreatment of mitochondria with trypsin was performed as in *A*. Conditions for import of $F_1\beta$ were the same as described in the legend to Fig. 1, except that 50 μg of either trypsin-treated (20 μg of trypsin/mg of mitochondrial protein, *lanes 1* and *2*) or untreated (*lanes 3* and *4*) mitochondria were present. The membrane potential was dissipated by adding antimycin A and oligomycin (8 and 20 μM final concentration, respectively, *lanes 2* and *4*) from a 100-fold concentrated stock solution in ethanol. Ascorbate and TMPD were omitted in these samples. Mature sized $F_1\beta$ were analyzed as described in the legend to Fig. 1. *C*, bypass import of $F_1\beta$ occurs via translocation contact sites. Contact site intermediates of $F_1\beta$ were accumulated by performing import at low temperature (Schleyer and Neupert, 1985) employing mitochondria either not pretreated or pretreated with 20 μg of trypsin/mg of mitochondria. After incubation for 20 min at 0 °C, valinomycin (1 μM final concentration, added from a 100-fold concentrated stock solution in ethanol) was added to each sample. Samples were further incubated for 20 min at 0 °C (*lanes 1, 2, 5,* and *6*) or for 20 min at 25 °C (*lanes 3, 4, 7,* and *8*). Finally, proteinase K was added (15 μg/ml final concentration), while control samples were left untreated. After incubation for 30 min at 0 °C all samples received 0.5 mM PMSF. Mitochondria were reisolated, washed in SEM buffer and analyzed by SDS-PAGE and fluorography. A fluorograph of the dried gel is shown. *D*, bypass import of AAC involves binding of precursor to the mitochondrial outer membrane. Mitochondria (1 mg/ml) were pretreated with proteinase K (20 μg/ml; *+Prot K*) as described (Pfanner and Neupert, 1987) or left untreated. Mitochondria and reticulocyte lysate containing ^{35}S-labeled precursor of AAC were incubated in the presence of ascorbate and TMPD (*lanes 1* and *2*) or antimycin A and oligomycin (*lanes 3–6*) for 15 min at 25 °C as described (Fig. 2*B*; Pfanner and Neupert, 1987). Mitochondria were reisolated and resuspended in BSA-containing buffer in the presence of antimycin A and oligomycin; for *lanes 5* and *6* this step was performed twice. *Lanes 3–6* received ascorbate and TMPD. All samples were incubated for 15 min at 25 °C, treated with proteinase K (200 μg/ml), and mitochondria were reisolated. Samples were analyzed by SDS-PAGE, fluorography, and densitometry. For *lanes 3–6*, the values obtained for *lanes 3–6* are expressed as percent of parallel control samples which had ascorbate and TMPD present in the first 25 °C incubation (as described for *lanes 1* and *2*).

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. 2*D*, *lanes 1* and *2*) and, binding of precursor in the absence of a membrane potential, reisolation 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. 2*D*, *lane 3*). Pretreatment of mitochondria with proteinase K did not change this ratio (Fig. 2*D*, *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. 2*D*, *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 $F_1\beta$ 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 $F_1\beta$ 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 $F_1\beta$ and AAC (Fig. 4*A*, $-T$). Import of $F_1\beta$ 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 $\Delta\Psi$-requiring step.

Import of $F_1\beta$ 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. 4*A*, $+T$).

In the second experiment, the effect of various Mg^{2+} concentrations on import into trypsin-pretreated and non-pretreated mitochondria of AAC and $F_1\beta$ was analyzed. The import reactions contained an endogenous Mg^{2+} concentration of about 1 mM; to this an additional 0–10 mM $MgCl_2$ was added. AAC and $F_1\beta$ were imported at 25 °C into mitochondria either pretreated or not pretreated with trypsin. Import of

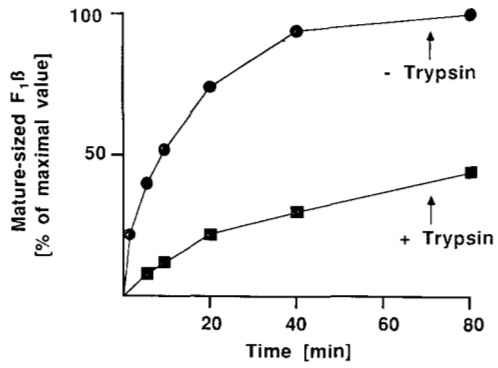

FIG. 3. **Rate of import of $F_1\beta$ is reduced by trypsin pretreatment of mitochondria.** Mitochondria were either not treated or treated with 20 μg of trypsin/mg of mitochondrial protein. Import of $F_1\beta$ into these mitochondria was investigated as described in the legend to Fig. 1. Import was 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 $F_1\beta$ was analyzed by fluorography and densitometry.

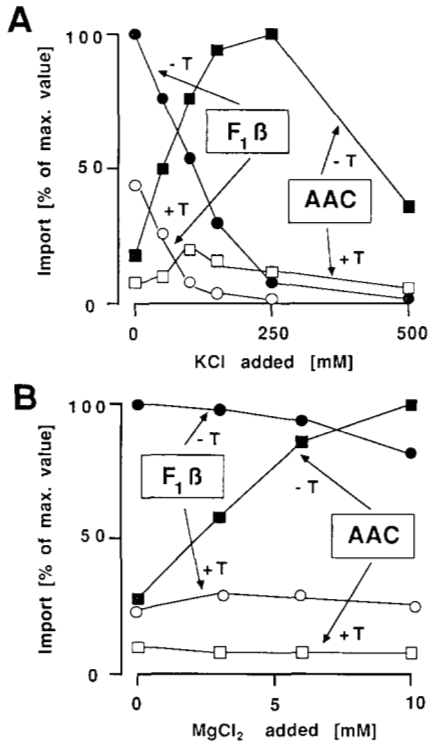

FIG. 4. **Import of $F_1\beta$ and AAC is dependent on the ionic composition of the *in vitro* system.** *A*, influence of KCl on the import of $F_1\beta$ and AAC. Mitochondria were either treated ($+T$) or not treated ($-T$) with trypsin (20 μg/mg protein) as described (see "Experimental Procedures"). Import of $F_1\beta$ and AAC was performed as described in the legend to Fig. 1 containing 10-fold diluted reticulocyte lysate. KCl was added to the import reactions at the concentrations indicated. After incubation for 40 min at 0 °C, mitochondria were reisolated, resuspended in BSA-containing buffer, and incubated for 15 min at 25 °C. Mitochondria were reisolated from samples in which import of $F_1\beta$ was analyzed; in the case of AAC, mitochondria were treated with proteinase K (200 μg/ml, 30 min at 0 °C) prior to reisolation. Mature sized $F_1\beta$ and proteinase K-resistant AAC were analyzed by SDS-PAGE, fluorography, and quantified by densitometry. *B*, influence of $MgCl_2$ on the import of $F_1\beta$ and AAC. Trypsin pretreatment of mitochondria and import of $F_1\beta$ and AAC were carried out as described in *panel A* except the import reactions were incubated for 20 min at 25 °C. A buffer containing 3% (w/v) BSA, 250 mM sucrose, and 10 mM Mops/KOH, pH 7.2, was used for dilution of reticulocyte lysate. $MgCl_2$ was added to the import reactions at the concentrations indicated.

AAC into untreated mitochondria was strongly stimulated by added $MgCl_2$; $F_1\beta$ import was only slightly affected (Fig. 4*B*, $-T$). Bypass import of neither AAC nor $F_1\beta$ was influenced by $MgCl_2$ (Fig. 4*B*, $+T$).

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

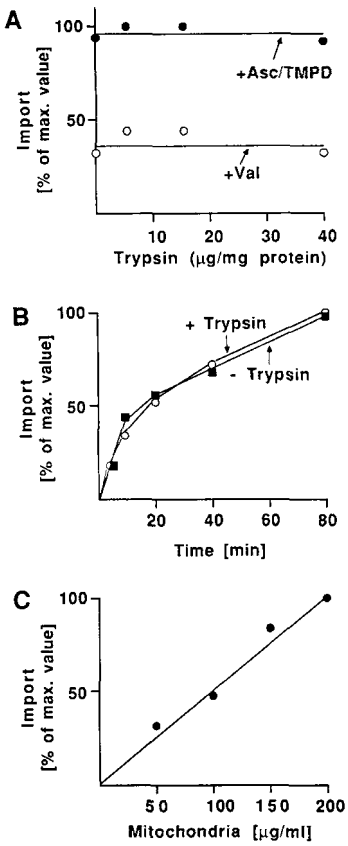

FIG. 5. **Import of the precursor to the small subunit of RUBp$_2$Case (SSU) in protease-pretreated mitochondria.** Import reactions contained 50 μg of mitochondrial protein (125 μg/ml), 40 μl of ^{35}S-labeled precursor of SSU, synthesized in reticulocyte lysate, 16 mM ascorbate, 0.4 mM TMPD. The final volume was adjusted to 0.4 ml with BSA-containing buffer (containing 2.7 mM $MgCl_2$). Processing peptidase was inhibited by adding 10 mM EDTA and 0.2 mM *o*-phenanthroline to the incubation mixtures. Imported SSU precursor was assessed by resistance to treatment with 250 μg of proteinase K/ml (30 min at 0 °C). Proteinase K treatment was stopped with 0.5 mM PMSF, mitochondria were reisolated, washed in SEM buffer, and analyzed by SDS-PAGE, fluorography, and densitometry of fluorographed bands. *A*, import into trypsin pretreated mitochondria and dependence of a membrane potential. Mitochondria were preincubated with the amounts of trypsin indicated. Import of SSU was carried out as described above either in the presence (+Asc/TMPD) or absence (1 μM valinomycin, +Val) of a membrane potential for 30 min at 25 °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 (250 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. 5*A*). The efficiency of import of SSU into isolated mitochondria was rather low (1–2% of total precursor added was imported). Hurt *et al.* (1986) 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. 5*A*). Furthermore, the kinetics of import of SSU were not affected by protease-pretreatment of mitochondria (Fig. 5*B*). Since the amount of import of SSU was linearly dependent on the amount of mitochondria in the import reaction (Fig. 5*C*), 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 presequences 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 presequences 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

have an amphipathic structure and a positive charge, are able to enter the translocation pathway after the step of mitochondrial surface receptor recognition. We show in this report that a non-mitochondrial signal sequence, the presequence of the small subunit of RBUp_2Case, can direct this chloroplast precursor into mitochondria. The chloroplast signal sequence is positively charged and may well have a membrane-active structure.

As a further possible explanation for residual import into proteased mitochondria one may consider that mitochondria possess two types of receptors, one being protease sensitive, the other being insensitive and that both types serve different precursor proteins. We consider this possibility rather unlikely as a general explanation since one would have to assume that by chance porin would have only a protease-sensitive receptor, whereas non-mitochondrial precursors (or artificial targeting sequences) would possess selectively protease-resistant receptors.

The observation that bypass of protease-sensitive receptors appears to be possible raises several important questions. For example, how then is the unique composition of the organelle maintained. As we have previously discussed, this may be due to the rather low efficiency of bypass import on the one hand and to a multiple-check system on the other hand, which controls import at several steps in a multistep pathway (Pfanner *et al.*, 1988b). In fact, the absence of mistargeting between mitochondria and chloroplasts has been described in plant cells (Boutry *et al.*, 1987). Our findings would also suggest that under certain conditions it would be quite conceivable that the receptors can be bypassed *in vivo*. Indeed a number of artificial signals can direct proteins into mitochondria *in vivo* (reviewed by Pfanner *et al.*, 1988a). If this is true, then it may be rather difficult to select for mutants deficient in receptor components, unless the screening system allows differentiation of the inefficient bypass import from the efficient receptor-mediated import. In this regard it is interesting that despite many efforts, yeast mutants deficient in import of precursor proteins have only been found to affect transport at a later stage, namely, the processing of precursors (Yaffe *et al.*, 1985; Hawlitschek *et al.*, 1988; Witte *et al.*, 1988). Furthermore, isolating mutations in receptor function might prove to be complicated if the receptors have overlapping specificity.

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