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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 bc_1-complex (intermembrane space), the ADP/ATP carrier (inner membrane), subunit 9 of the F_0-ATPase (inner membrane), and subunit β of the F_1-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, 1987*a*; 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., 1987*b*,*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, 1987*b*; Pfanner et al., 1987*a*,*d*; Schwaiger et al., 1987). Transfer into and across the inner membrane requires the electrical potential ($\Delta\Psi$) across the inner membrane (Pfanner and Neupert, 1985). The presequences are proteolytically cleaved by the processing peptidase of the mitochondrial matrix (Böhni 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, 1987*a*,*b*; Pfaller and Neupert, 1987; Pfanner and Neupert, 1987*b*; Pfanner et al., 1987*b*,*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 F_0-ATPase ($F_0$9), and the intermembrane space protein cytochrome b_2 (Zwizinski et al., 1983; Riezman et al., 1983; Pfanner and Neupert, 1985, 1987*b*; Schmidt et al., 1985; Pfanner et al., 1987*b*,*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; $F_1\beta$, F_1-ATPase subunit β; $F_0$9, F_0-ATPase subunit 9; GIP, general insertion protein; TMPD, *N*,*N*,*N*',*N*'-tetramethylphenylendiamine; ws-porin, water-soluble porin.

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(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; Pfaller 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; Pfanner and Neupert, 1987*b*; Pfanner et al., 1987*b*,*d*; Pfaller 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 (Pfaller 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 bc_1-complex (intermembrane space), $F_0$9 (inner membrane), and subunit β of the F_1-ATPase ($F_1\beta$) (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, $F_0$9, and $F_1\beta$. 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-[^{35}S]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 3.4.21.11), 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 in vitro transcription and translation of the AAC (Pfanner et al., 1987*b*), porin (Kleene et al., 1987), $F_0$9, and p$F_0$9$_{1\text{-}69}$-dihydrofolate reductase (Pfanner et al., 1987*d*), and cytochrome c (Stuart et al., 1987).

Full-length cDNA's coding for $F_1\beta$ 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 Biotec, Madison, WI), the coding region of the cDNA of the Fe/S-protein was cut out with Hinf I. The cDNA of $F_1\beta$ 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; Kleene et al., 1987).

cDNA's 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 [^{35}S]methionine (Pfanner and Neupert, 1985, 1986, 1987*b*; Hartl et al., 1986; Pfanner et al., 1987*d*).

In Vitro Binding of Precursors and Import into Isolated Mitochondria

Mitochondria were isolated from *N. crassa* as described (Pfanner 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 $MgCl_2$, 80 mM KCl, 10 mM Mops/KOH, pH 7.2 (Pfanner and Neupert, 1985). Reticulocyte lysate containing ^{35}S-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 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 (Pfaller et al., 1985; Pfaller 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 H_2O_2, and the ^{35}S-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 (Pfaller et al., 1985; Kleene et al., 1987; Pfaller and Neupert, 1987). Furthermore, ws-porin was able to form porin-specific membrane channels upon insertion into a lipid bilayer (Pfaller 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 ^{35}S-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; Pfanner and Neupert, 1987*b*). Ws-porin inhibited the import of the biosynthetic porin precursor and of AAC (Fig. 1; also see Pfaller et al., 1985; Pfaller and Neupert, 1987). The import of Fe/S protein, $F_0$9, and $F_1\beta$ 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

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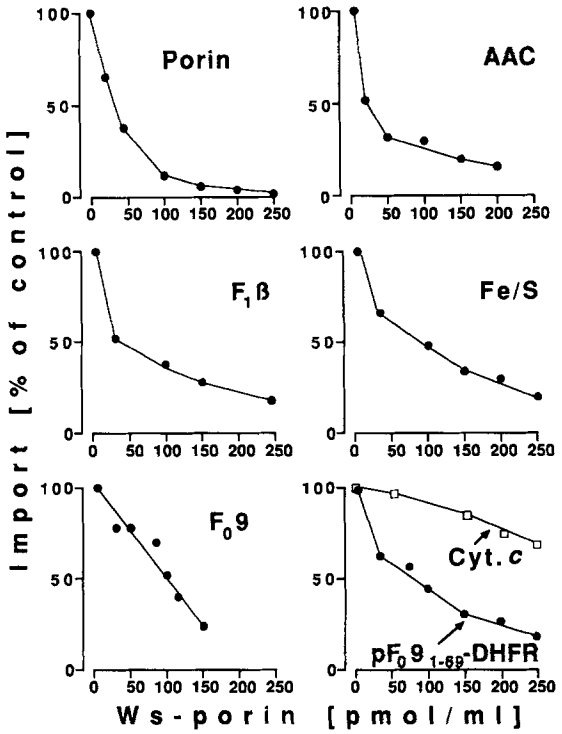

Figure 1. Import of various precursor proteins is competed for by ws-porin. Competition experiments were carried out by incubating 50 μg mitochondrial protein (suspended in a buffer containing 250 mM sucrose, 1 mM EDTA, 10 mM Mops/KOH, pH 7.2 [SEM buffer], in a concentration of 1 mg/ml) together with 40 μl reticulocyte lysate containing ^{35}S-labeled precursor proteins and 100 μl ws-porin (dissolved in 0.1 M NaP_i, pH 6.8, see Materials and Methods and Pfaller and Neupert, 1987) at the concentrations indicated. The final volume was adjusted to 0.4 ml with BSA-containing buffer. Import of AAC, $F_1\beta$, F_09, and pF_09_{1-69}-dihydrofolate reductase was carried out in the presence of ascorbate and TMPD. Import reactions for the Fe/S-protein received 2 mM NADH and 1 mM ATP. No additions were made in the case of porin or cytochrome c. After incubation for 40 min at 0°C mitochondria were reisolated, resuspended in 0.4 ml of BSA-containing buffer (including 8 μM antimycin A and 20 μM oligomycin), transferred to new tubes, and incubated for 15 min at 25°C. In the case of cytochrome c, 3 μM hemin and 1 mg $Na_2S_2O_4$/ml were added to allow formation of holocytochrome c (Nicholson et al., 1987). In the case of $F_1\beta$, F_09 and the Fe/S-protein mitochondria were reisolated and washed in SEM buffer. In the case of AAC and porin, mitochondria were treated with 200 μg proteinase K/ml before reisolation of the mitochondria. Imported proteins were analyzed by SDS-PAGE, fluorography, and densitometry. For cytochrome c, quantitation of holocytochrome c was performed by measuring radioactivity in the heme-containing tryptic peptide after HPLC separation (Nicholson et al., 1987).

mitochondria (Pfaller and Neupert, 1987). The import of a chimeric protein consisting of the presequence of F_09 and the mouse cytosolic enzyme dihydrofolate reductase (Pfanner et al., 1987*c*,*d*) was competed for in a similar manner as the import of authentic F_09. 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 F_09 (Zimmermann et al., 1981).

Competition Requires the Native Form of Porin Precursor Interacting with Binding Sites on the Outer Membrane

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 (Pfaller 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 ^{35}S-labeled precursor of $F_1\beta$ (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 $F_1\beta$ 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 ^{35}S-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") (Pfaller and Neupert, 1987; Pfaller et al., 1988). We investigated the competition of import by ws-porin into trypsin-treated mitochondria in the case of $F_1\beta$ (Fig. 3 *A*) and Fe/S-protein (Fig. 3 *B*). Competition of import by ws-porin was strong-

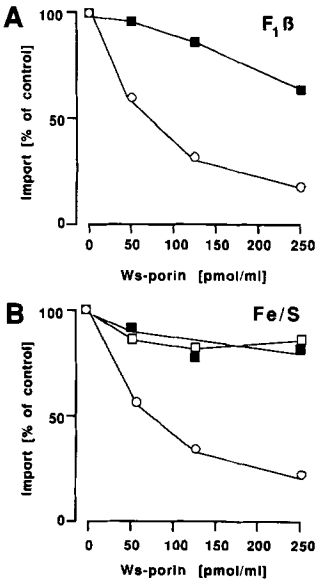

Figure 2. Ws-porin inactivated by freezing and thawing does not compete for the import of $F_1\beta$ or Fe/S-protein. (*A*) Competition for import of $F_1\beta$ was carried out as described in Fig. 1. Ws-porin was inactivated as follows. Samples containing 100 μl of ws-porin at the indicated concentrations together with 20 μl of reticulocyte lysate containing ^{35}S-labeled $F_1\beta$-precursor were incubated for 30 min at 0°C. The samples were subjected to three freeze-thaw cycles. The competition assay was carried out with freshly prepared mitochondria (■). In the control, freshly prepared ws-porin was incubated with $F_1\beta$-lysate for 30 min at 0°C and the competition assay was then performed (○). (*B*) Competition for import of Fe/S-protein was performed as described in Fig. 1. Inactivation of ws-porin was performed in the presence of reticulocyte lysate containing either ^{35}S-labeled Fe/S-precursor (■) or no precursor (cold lysate; □). Control competition was carried out with untreated ws-porin (○).

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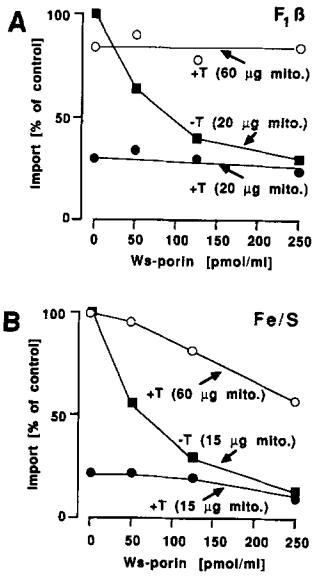

Figure 3. Competition by ws-porin for the import of $F_1\beta$ and Fe/S-protein does not occur with mitochondria pretreated with trypsin. (*A* and *B*) Mitochondria were either treated with 20 µg trypsin/mg mitochondria (+*T*) or without trypsin (−*T*). Competition assays were carried out as described in Fig. 1. (*A*) Competition for import of $F_1\beta$. (■) Untreated mitochondria (20 µg; (●) trypsin-treated 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).

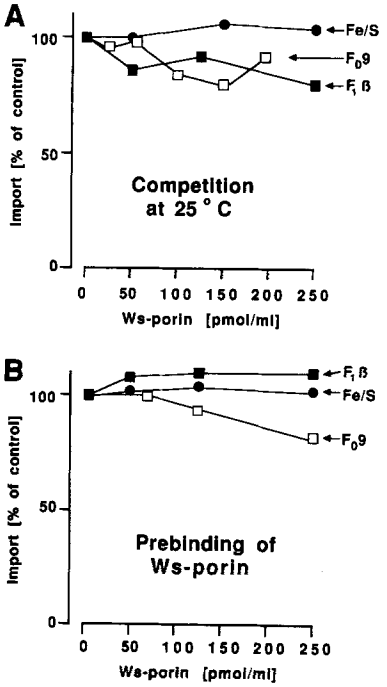

Figure 4. Import is not competed for by ws-porin at 25°C or after chase of prebound ws-porin. (*A*) Competition for import at 25°C. Competition assays were carried out essentially as described in Fig. 1. The incubation temperature, however, was 25°C instead of 0°C. Furthermore, the chase at 25°C was omitted. Import of Fe/S-protein, $F_1\beta$, and $F_0$9 was followed. (*B*) Competition of import 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 ^{35}S-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, $F_1\beta$, and $F_0$9 was followed.

ly reduced if protease-pretreated mitochondria were used in the import reactions (Fig. 3, *A* and *B*, +*T*). As indicated in Fig. 3, the amount of protease-pretreated mitochondria in the import assay was increased so that the bypass import reached the control value obtained with untreated mitochondria. The ability of ws-porin to compete for import, however, was still strongly reduced. We conclude that competition by ws-porin requires the specific interaction of ws-porin with mitochondria.

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 ^{35}S-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 $F_1\beta$ 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 $F_1\beta$ 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 $F_1\beta$ and porin. On the other hand, import of $F_1\beta$ 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, $F_0$9, and the Fe/S-protein were sensitive to pretreatment with low amounts of elastase. Import of $F_1\beta$, 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 $F_1\beta$ depended in a linear fashion on the amount of mitochondria and on the time of incubation (Pfanner et al., 1987*c*), 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; Pfaller et al., 1988), it appears that the

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trypsin-sensitive binding sites are different for porin and $F_1\beta$. Therefore, ws-porin should not compete with $F_1\beta$ 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 $F_1\beta$ 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 $F_1\beta$ or competition for the binding to cytosolic cofactors) can be excluded. Since the protease-accessible binding sites for $F_1\beta$ and porin are different, the competition of import appears to occur beyond these sites.

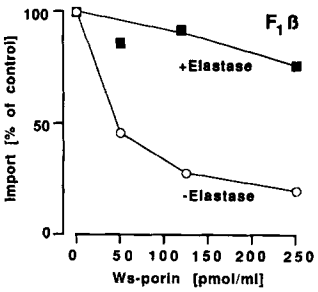

Figure 6. Import of $F_1\beta$ into mitochondria pretreated with elastase is not competed for by ws-porin. Mitochondria were pretreated or not pretreated with elastase (10 μg/ml) as described in the legend to Fig. 5. Import of $F_1\beta$ was performed as described above in the presence of varying concentrations of ws-porin as indicated.

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. 7 *A*, we investigated whether the generation of the stage 2 intermediate of AAC was competed for by ws-porin. Fig. 7 *A*, *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. 7 *A*, *II*). Further translocation of AAC from the stage 2 site into mitochondria was competed for by porin (Fig. 7 *B*). 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, 1987*b*). Quan-

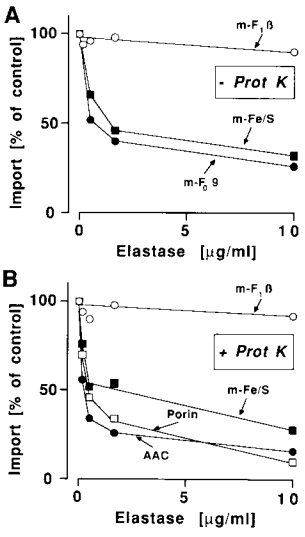

Figure 5. Pretreatment of mitochondria with elastase inhibits the import of several precursor proteins but not of $F_1\beta$. Isolated mitochondria (1 mg protein/ml) were treated with elastase essentially as described (Zwizinski et al., 1984), with the exception that after addition of PMSF the incubation was continued for 15 min at 25°C. Mitochondria were washed twice in SEM-buffer in the presence of PMSF. Mitochondria (10 μg protein) and reticulocyte lysate (70 μl), containing ^{35}S-labeled mitochondrial precursor proteins, were incubated together in the presence of 16 mM ascorbate, 0.4 mM TMPD in BSA-containing buffer as described previously (Pfanner and Neupert, 1987*b*) in a final volume of 200 μl. After incubation for 15 min at 25°C, the mitochondria were reisolated. They were then resuspended in BSA-containing buffer. Samples in *A* were directly analyzed for imported proteins. Samples in *B* were treated with proteinase K as described before (Pfanner and Neupert, 1987*b*). Mitochondria were reisolated and also analyzed for import by SDS-PAGE, fluorography, and densitometry. Note: controls showed that the total amount of precursor in the samples was not affected by the pretreatment with elastase.

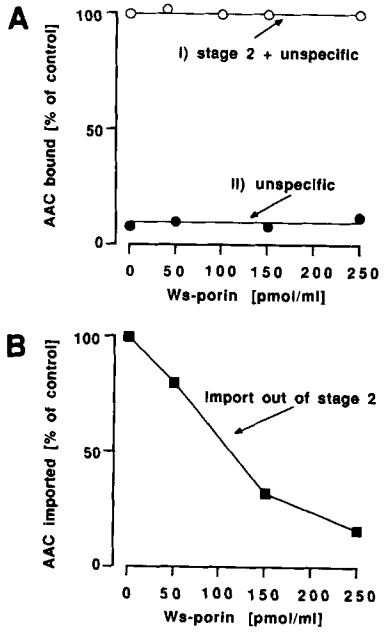

Figure 7. Ws-porin does not compete for the binding of AAC to stage 2 sites but does compete for the translocation to stage 3 sites. (*A*) Binding of AAC to mitochondria in the apyrase-treated system (Pfanner and Neupert, 1986; Pfanner et al., 1987*d*) was performed for 30 min at 0°C in the presence of the indicated concentrations of ws-porin as described in Fig. 1 (○). To determine the amount of unspecific binding, mitochondria that had been pretreated with trypsin (20 μg/ml) were used (●). (*B*) Competition was performed as described for *A*, except that the mitochondria were reisolated after the binding reaction, and then resuspended in BSA-containing buffer in the presence of antimycin A and oligomycin. After addition of ascorbate and TMPD, samples were incubated for 15 min at 25°C and treated with proteinase K (200 μg/ml).

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titative determination of free and bound AAC was performed by resolving the SDS and heat-denaturated samples on SDS-polyacrylamide gels, excision of the bands corresponding to AAC, elution in the presence of H_2O_2, and counting of the ^{35}S-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 5×10^8 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_0$9, and $F_1\beta$ 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., 1987*d*), 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 in the case of AAC (Pfanner and Neupert, 1987*b*) and high affinity binding sites in the case of porin (Pfaller and Neupert, 1987). After leaving GIP, the precursor of porin is transferred to its final destination in the outer membrane and assembled to the mature form. Other precursors are transported in a membrane potential–dependent step into the inner membrane via contact sites between both mitochondrial membranes, and are then finally sorted to their functional intramitochondrial location (matrix, inner membrane, intermembrane space) (Schleyer and Neupert, 1985; Hartl et al., 1986, 1987; Pfanner and Neupert, 1987*b*). The transport pathways of proteins into mitochondria thus start at distinct binding sites on the mitochondrial surface and converge at a common insertion site in the outer membrane. The pathways then diverge and the proteins are sorted within mitochondria by transport to either the outer membrane or to contact sites (Fig. 9). The import pathway of cytochrome c is clearly exceptional. It involves translocation across the outer membrane, probably directly into the intermembrane space, and apparently does not require GIP (Hennig et al., 1983; Nicholson et al., 1987).

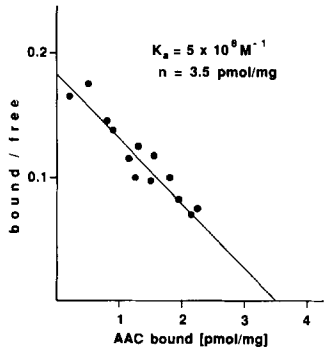

Figure 8. Scatchard analysis of binding of AAC to the stage 3 sites. Reticulocyte lysate containing varying concentrations of precursor of AAC were incubated with isolated *N. crassa* mitochondria (10 μg of mitochondrial protein) for 30 min at 25°C in the presence of antimycin A (8 μM) and oligomycin (20 μM). Mitochondria were reisolated and an aliquot of the supernatant was removed to determine free AAC (see Materials and Methods). The mitochondrial pellets were resuspended in BSA-containing buffer and treated with trypsin (10 μg/ml) as described (Pfanner and Neupert, 1987*b*). The amount of AAC bound to mitochondria was analyzed as described in Materials and Methods. Binding parameters were determined according to Scatchard.

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 $F_1\beta$ is not destroyed by elastase, in contrast to those for the other four precursors analyzed. This suggests the existence of a receptor site for $F_1\beta$ 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 $F_1\beta$. 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, 1987*b*; Pfanner et al., 1987*d*), 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, 1987*b*; Pfanner et al., 1987*d*; 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., 1987*d*). ATP was shown to be required for the cytosolic unfolding of precursors ("translocation-competent folding") (Chen and Douglas, 1987; Pfanner

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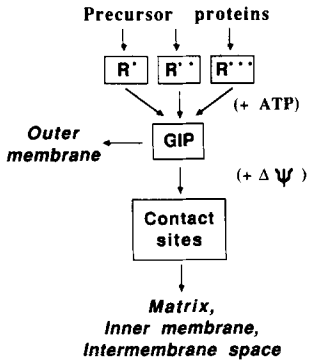

Figure 9. Working hypothesis on the initial recognition steps during the import of precursor proteins into mitochondria. After synthesis on cytoplasmically localized ribosomes, precursor proteins bind specifically to distinct receptor proteins on the mitochondrial surface (R'–R'''). At present, recognition sites can be distinguished for porin, AAC, and $F_1\beta$. After the specific initial binding to the mitochondrial surface, insertion into 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 submitochondrial compartments are transferred to contact sites where the membrane potential–dependent translocation across the inner membrane takes place before further submitochondrial sorting events.

et al., 1987*d*, 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., 1987*d*). 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 may be relevant in this context. When yeast mitochondria 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, 1987*a*). 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, 1987*b*; 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; 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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