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Mitochondrial protein import: involvement of the mature part of a cleavable precursor protein in the binding to receptor sites

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The precursor of F_0-ATPase subunit 9 was bound to mitochondria in the absence of a mitochondrial membrane potential ($\Delta\psi$). Binding was mediated by a protease-sensitive component on the mitochondrial surface. When $\Delta\psi$ was re-established, bound precursor was directly imported without prior release from the mitochondrial membranes. A chimaeric protein consisting of the complete subunit 9 precursor fused to cytosolic dihydrofolate reductase (DHFR) was also specifically bound to mitochondria in the absence of $\Delta\psi$. Two other fusion proteins, consisting either of the entire presequence of subunit 9 and DHFR or of part of the presequence and DHFR, were imported in the presence of $\Delta\psi$. In the absence of $\Delta\psi$, however, specific binding to mitochondria did not take place. We suggest that the hydrophobic mature part of subunit 9 is involved in the $\Delta\psi$-independent binding of the subunit 9 precursor to receptor sites on the mitochondrial surface.

Key words: mitochondria/protein import/import receptor/F_0F_1-ATPase subunit 9

Introduction

Mitochondrial protein import involves the specific recognition of precursor proteins by receptor sites on the mitochondrial surface (reviewed by Harmey and Neupert, 1985; Pfanner and Neupert, 1987a). Amino-terminal peptide extensions (presequences) appear to carry sufficient information for targeting of proteins to mitochondria and into the mitochondrial matrix (reviewed by Hurt and van Loon, 1986). Precursor proteins without cleavable presequences have been assumed to carry the targeting signal(s) in the extreme amino terminus of the mature protein (Hase *et al.*, 1984; Hurt *et al.*, 1985; Adrian *et al.*, 1986). Recent studies, however, suggested that at least in some cases carboxy-terminal precursor regions are specifically involved in the targeting (Pfanner *et al.*, 1987c; Stuart *et al.*, 1987).

Binding to specific receptor sites on the mitochondrial surface has been demonstrated for the precursors of the outer membrane protein porin (Zwizinski *et al.*, 1984; Pfaller *et al.*, 1985; Schmidt *et al.*, 1985; Kleene *et al.*, 1987; Pfaller and Neupert, 1987) and the intermembrane space protein cytochrome *c* (Hennig and Neupert, 1981; Hennig *et al.*, 1983), both precursors are imported independently of a membrane potential ($\Delta\psi$). In the case of precursor proteins that are $\Delta\psi$-dependently imported into or across the inner mitochondrial membrane, specific binding to the mitochondrial surface could only be clearly demonstrated for that of the ADP/ATP carrier (an inner membrane protein) (Zwizinski *et al.*, 1983, 1984; Pfanner and Neupert, 1985, 1987b; Schmidt *et al.*, 1985). Specific binding of the precursor of cytochrome b_2 to the mitochondrial surface in the absence of $\Delta\psi$ is low (Daum *et al.*, 1982; Gasser *et al.*, 1982; Riezman *et al.*, 1983). On the other hand, the precursor of F_1-ATPase subunit β does not bind at all to mitochondria in the absence of $\Delta\psi$ (Zwizinski *et al.*, 1984; Schleyer and Neupert, 1985), and the precursor of the Fe–S protein of the bc_1-complex appears only to bind in a non-specific manner in the absence of $\Delta\psi$ (Hartl *et al.*, 1986). High levels of specific binding in the absence of $\Delta\psi$ therefore appeared to be restricted to precursors without cleavable presequences (porin, apocytochrome *c*, and ADP/ATP carrier). Furthermore, the nature of the determinants of a precursor protein, which specified the specific binding in the absence of $\Delta\psi$, was unknown.

In this report, we show that the precursor of the inner membrane protein F_0-ATPase subunit 9, which carries a cleavable presequence, can specifically bind to the mitochondrial surface in the absence of $\Delta\psi$. Furthermore, we present evidence that the mature part of subunit 9 plays a role in this specific binding process.

Results

Import of F_0-ATPase subunit 9 and fusion proteins between subunit 9 and dihydrofolate reductase

The precursor proteins used in this study are shown in Figure 1: the precursor of F_0-ATPase subunit 9 ($pSu9_{1-147}$); a fusion

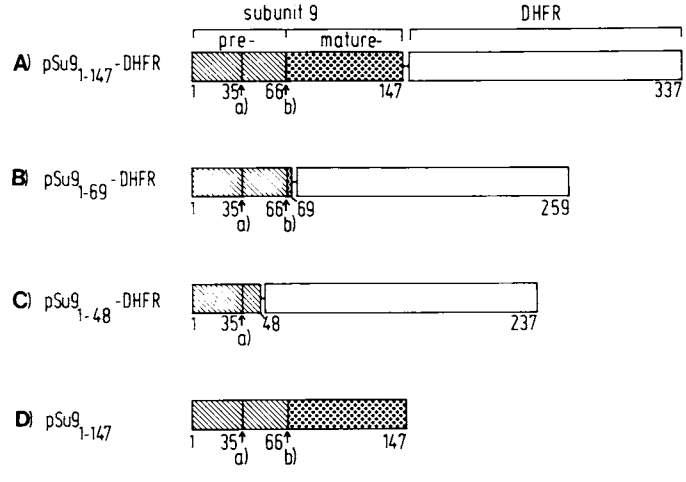

Fig. 1. Fusion proteins between F_0-ATPase subunit 9 and DHFR. The fusion proteins were constructed as described in Materials and methods. $pSu9_{1-147}$–DHFR, fusion protein between the complete subunit 9 precursor and DHFR; $pSu9_{1-69}$–DHFR, fusion protein between the presequence and three amino acid residues of the mature part of subunit 9 and DHFR; $pSu9_{1-48}$–DHFR, fusion protein between the 48 amino-terminal amino acid residues of the presequence of subunit 9 and DHFR; $pSu9_{1-147}$, authentic precursor of subunit 9. Numbers indicate the positions of amino acid residues (starting with the first amino acid residue of the subunit 9 presequence). (**a**) first cleavage site of the processing peptidase; (**b**) second cleavage site of the processing peptidase. Hatched boxes, presequence of subunit 9; pointed boxes, mature part of subunit 9; empty boxes, DHFR.

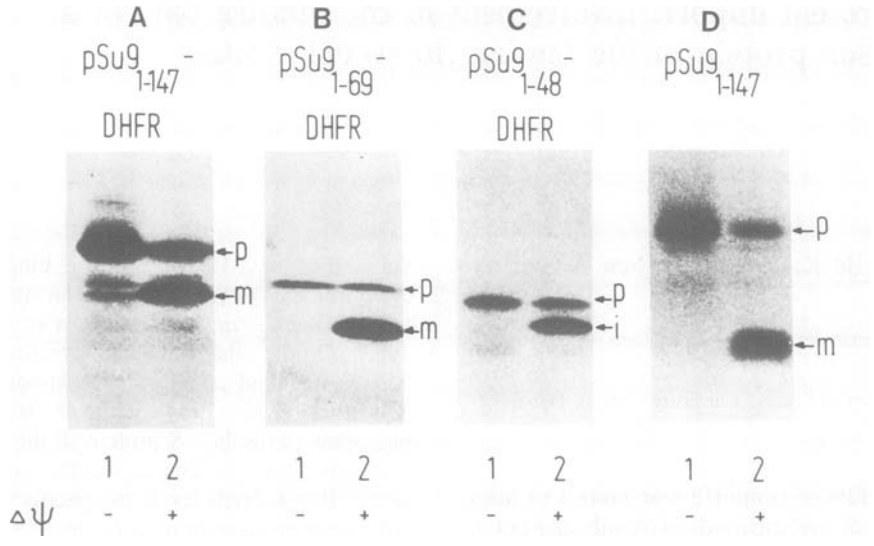

Fig. 2. Import of the fusion proteins requires the mitochondrial membrane potential. Reticulocyte lysate containing ^{35}S-labelled $pSu9_{1-147}$−DHFR (**panel A**), $pSu9_{1-69}$−DHFR (**panel B**), $pSu9_{1-48}$−DHFR (**panel C**) or $pSu9_{1-147}$ (**panel D**), and isolated *Neurospora* mitochondria were incubated in the presence of valinomycin, antimycin A, and oligomycin (**reactions 1**) or ascorbate plus TMPD (**reactions 2**). After 25 min at 25°C, mitochondria were re-isolated. Samples were resolved by SDS−polyacrylamide gel electrophoresis. Fluorographs of the dried gels are shown. **p**, precursor; **i**, intermediate-sized protein (corresponding to processing of $pSu9_{1-48}$−DHFR at the first cleavage site of the processing peptidase); **m**, mature-sized protein (corresponding to processing at the second cleavage site of the processing peptidase). The apparent mol. wts were: $Su9_{1-147}$−DHFR: p, 33.5 kd, m, 27 kd; $Su9_{1-69}$−DHFR: p, 28 kd, m, 22.5 kd; $Su9_{1-48}$−DHFR: p, 26 kd, i, 23 kd; $Su9_{1-147}$: p, 16 kd, m, 10 kd.

Table I. Import efficiency of ATPase subunit 9 and of derived fusion proteins

	$pSu9_{1-147}$−DHFR	$pSu9_{1-69}$−DHFR	$pSu9_{1-48}$−DHFR	$pSu9_{1-147}$
$\frac{\text{[Imported precursor]}}{\text{[Total precursor added]}} \times 100\,\%$	60.0 ± 2.9%	61.8 ± 4.2%	10.1 ± 2.1%	71.3 ± 1.5%

The experiments were carried out as described for reactions 2 of Figure 2, except that incubation at 25°C was for 35 min. Results were quantified by densitometry. The import efficiency is expressed as the percentage of total precursor added which is processed. The values given are the means of at least three experiments ± SEM). The values were corrected to allow for the different numbers of methionine residues in the precursors and processed proteins. Each assay contained equimolar amounts of precursor proteins based on the intensity of radiolabelling and on the number of methionine residues of the precursors. (The same condition applies to the experiments of Figures 3−5.)

protein between the entire subunit 9 precursor and mouse dihydrofolate reductase (DHFR) ($pSu9_{1-147}$−DHFR); a fusion protein between the presequence and three amino aicd residues of the mature part of subunit 9 and DHFR ($pSu9_{1-69}$−DHFR); and a fusion protein between the 48 amino-terminal amino acid residues of the subunit 9 presequence of DHFR ($pSu9_{1-48}$−DHFR).

Reticulocyte lysate containing the radiolabelled precursors and isolated *Neurospora* mitochondria were incubated at 25°C in the absence (Figure 2, lanes 1) or presence of $\Delta\psi$ (Figure 2, lanes 2). Only in the presence of $\Delta\psi$ were processed products generated. The subunit 9 precursor was processed to mature subunit 9 (Figure 2D) (Schmidt *et al.*, 1983, 1984). In the cases of $pSu9_{1-147}$−DHFR and $pSu9_{1-69}$−DHFR, the shift in the apparent mol. wt also corresponded to removal of the subunit 9 presequence (Figure 2A and B) (Pfanner *et al.*, 1987a). In the case of $pSu9_{1-48}$−DHFR the shift in the apparent mol. wt (Figure 2C) correlated well to cleavage at the first processing site identified for authentic subunit 9 by radiosequencing (Schmidt *et al.*, 1984). Since the processing depends on the presence of an energized inner membrane, it reflects transport of the precursors into mitochondria. Furthermore, the precursor proteins could be processed to the same mature-sized forms *in vitro* by the isolated processing peptidase (Hawlitschek and Neupert, unpublished). The two minor bands in the region of mature-sized $pSu9_{1-147}$−DHFR (Figure 2A, lane 1) were present in the reticulocyte lysate in the absence of mitochondria. They probably represent products of translational initiation at internal methionines. The precursor proteins were stable under the binding and import conditions used: unspecific degradation or decreased recovery of precursors after the incubation with mitochondria were not observed.

The efficiency of import (i.e. the percentage of total added precursor imported) after incubation at 25°C for 35 min was 60−70% for subunit 9 and the fusion proteins $pSu9_{1-147}$−DHFR and $pSu9_{1-69}$−DHFR (Table I). Thus these three precursors show a high import efficiency. The efficiency for import of $pSu9_{1-48}$−DHFR was considerably lower (~10%). It should be emphasized that this value represents a significant import and is higher than the import efficiency of many other fusion proteins studied so far. The decreased import efficiency of $pSu9_{1-48}$−DHFR may be caused by lack of the carboxy-

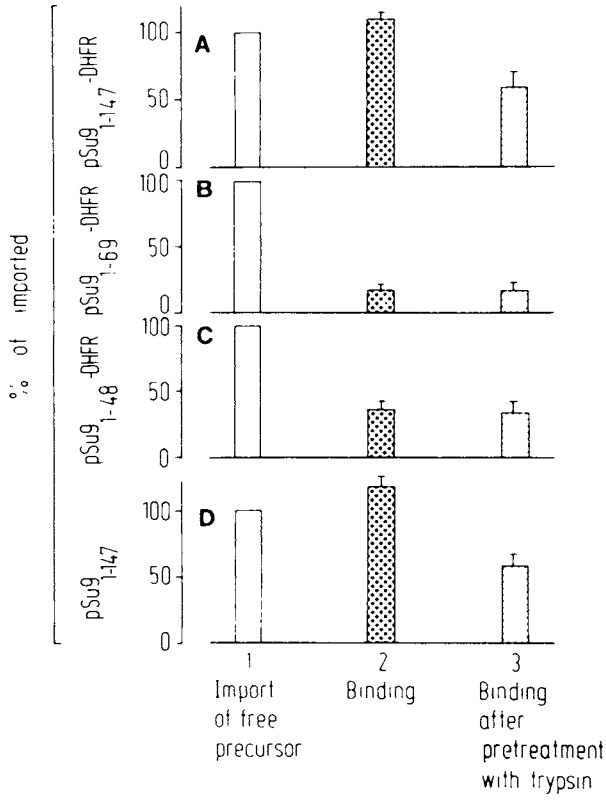

Fig. 3. Binding of ATPase subunit 9 and of the derived fusion proteins to mitochondria in the absence of $\Delta\psi$. **Reactions 1** and **2** were performed as described in the legend of Figure 2 except that reactions 1 contained ascorbate plus TMPD and reactions 2 contained valinomycin, antimycin A and oligomycin. **Reactions 3** were performed as described for reactions 2 except that the mitochondria were pretreated with trypsin (20 μg/ml for 25 min at 0°C). Results were quantified as described in the legend of Table I. The results represent the mean of at least three experiments; bar indicates SEM.

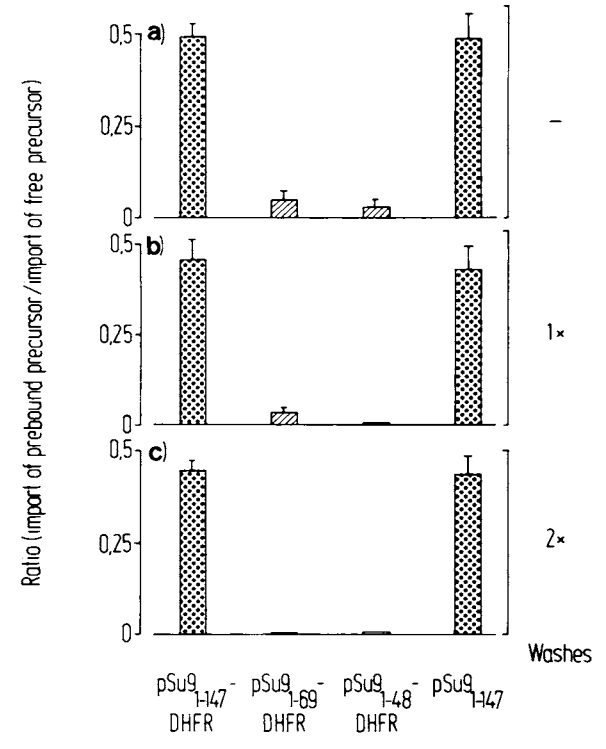

Fig. 4. Efficiency of import via the bound state. Reticulocyte lysate and isolated mitochondria were incubated in the presence of antimycin A and oligomycin for 20 min at 25°C. Mitochondria were re-isolated, resuspended in buffer containing 3% BSA (see Materials and methods), antimycin A, oligomycin and 20% (v/v) reticulocyte lysate (without labelled mitochondrial precursor proteins) (mix A), and transferred to new tubes. **Reactions a** were left on ice. Mitochondria of reactions **b** and **c** were re-isolated, washed once or twice in mix A, and finally resuspended in mix A. Ascorbate plus TMPD was added to all reactions, followed by incubation for 20 min at 25°C. Mitochondria were re-isolated. Gel electrophoresis and quantitation of processed proteins were performed as described in the legends of Figure 2 and Table I. Results are given as the ratio between the amount of processed proteins in the described reactions and the amount of processed proteins in parallel reactions, which were performed as described except that ascorbate plus TMPD was present in the first 25°C incubation. Results represent the average of at least three experiments; bar indicates SEM.

terminal portion of the subunit 9 presequence or by an unfavourable conformation of $pSu9_{1-48}$–DHFR compared to the other precursor proteins studied.

Binding of subunit 9 and of the fusion proteins to mitochondria in the absence of $\Delta\psi$

The precursor proteins were bound to mitochondria in the absence of $\Delta\psi$. The amount of bound precursor proteins (Figure 3, columns 2) was compared to the amount of precursors which were imported in the presence of $\Delta\psi$ (Figure 3, columns 1). The amount of binding of $pSu9_{1-147}$–DHFR and $pSu9_{1-147}$ was comparable to the amounts imported. The amount of bound $pSu9_{1-69}$–DHFR and $pSu9_{1-48}$–DHFR, however, was much lower than the amount imported.

The import of subunit 9 and of the three fusion proteins was strongly inhibited by mild protease-pretreatment of mitochondria (Zwizinski *et al.*, 1984; data not shown). This suggests that their import required a protease-sensitive component on the mitochondrial surface. We examined the effect of a protease pretreatment of mitochondria on the binding of precursor observed in the absence of $\Delta\psi$. This pretreatment of mitochondria with a low concentration of trypsin does not degrade the outer membrane barrier (Zwizinski *et al.*, 1984; Hartl *et al.*, 1986; Pfanner and Neupert, 1987b). The amount of binding of $pSu9_{1-147}$–DHFR and $pSu9_{1-147}$ to the trypsin-treated mitochondria (Figure 3, col-

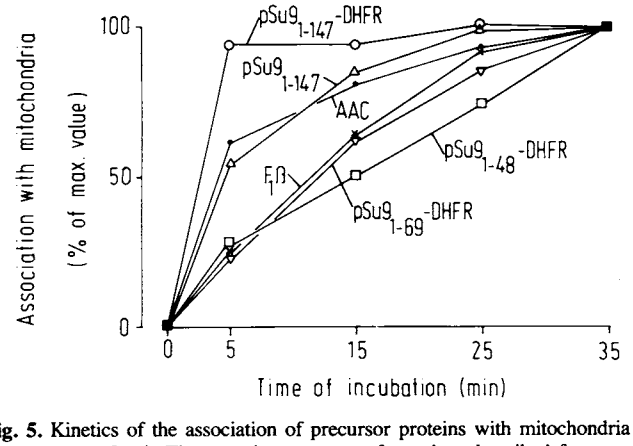

Fig. 5. Kinetics of the association of precursor proteins with mitochondria in the presence of $\Delta\psi$. The experiment was performed as described for reactions 2 of Figure 2 and in the legend of Table I. Incubations were performed at 25°C for the times indicated. Processed $pSu9_{1-147}$–DHFR, $pSu9_{1-69}$–DHFR, $pSu9_{1-48}$–DHFR and $pSu9_{1-147}$, and total mitochondria-associated ADP/ATP carrier (AAC) and $F_1\beta$ were quantified. Data represent the average of at least three experiments. The SEM was <6.5% in each case.

umns 3) was only half of the binding to control mitochondria (Figure 3, columns 2). This suggests that ~50% of the binding of $pSu9_{1-147}$-DHFR and $pSu9_{1-147}$ is due to a protease-sensitive component which is supposed to act as a receptor site (Zwizinski *et al.*, 1984; Schmidt *et al.* 1985; Pfanner and Neupert, 1987b). The low binding of $pSu9_{1-69}$-DHFR and $pSu9_{1-48}$-DHFR, however, was unaffected by the pretreatment with trypsin, suggesting that it was independent of the putative receptor sites.

In the experiment described in Figure 4, we examined which of the bound precursors were specifically located on the import pathway, i.e. could be imported from the binding sites. The precursor proteins were bound to mitochondria at 25°C in the presence of antimycin A and oligomycin which block the bc_1-complex and the F_0F_1-ATPase and thus inhibit the formation of a $\Delta\psi$ (Nicholls, 1982). After re-isolation, mitochondria were resuspended in buffer containing 3% bovine serum albumin (BSA) (Pfanner and Neupert, 1985), antimycin A, oligomycin and 20% reticulocyte lysate (without labelled mitochondrial precursor proteins) (mix A). The reaction mixtures were divided into three portions. Portions (a) were left on ice. Mitochondria of portions (b) and (c) were re-isolated, washed once or twice in mix A and finally resuspended in mix A. All samples received ascorbate plus *N,N,N',N'*-tetramethylphenylenediamine (TMPD), which supplied electrons to the respiratory chain at the level of cytochrome oxidase thereby generating a membrane potential. After incubation at 25°C, the amount of imported precursors was determined ('import of prebound precursor'). Control reactions were performed as described above except that ascorbate plus TMPD was added in the first 25°C incubation to allow import in this incubation ('import of free precursor'). The efficiency of import via the bound state was determined as the ratio between import of prebound precursor and import of free precursor. The import effciency of $pSu9_{1-147}$-DHFR and $pSu9_{1-147}$ via the bound state was ~50% when compared to the import of free precursor (Figure 4A and D). When the mitochondria (carrying the bound precursors) were washed in the same buffer which was used for import, the import efficiency via the bound state was virtually undiminished. This suggests that $pSu9_{1-147}$-DHFR and $pSu9_{1-147}$ were not released from the mitochondrial membranes. $pSu9_{1-69}$-DHFR and $pSu9_{1-48}$-DHFR, however, were not imported from the bound state. The small amounts of imported precursors (Figure 4a, B and C) were not found in the reactions where the mitochondria with the bound precursors were washed twice prior to import (Figure 4c, B and C). This indicates that the bound precursors $pSu9_{1-69}$-DHFR and $pSu9_{1-48}$-DHFR were released from the membranes and the import observed represented import of free precursor. We suggest that these two precursors were held on the mitochondrial surface by non-specific adhesion.

In summary, two different methods (binding to trypsin-treated mitochondria and import from the bound state) demonstrated that $pSu9_{1-147}$-DHFR and $pSu9_{1-147}$ could be specifically bound to mitochondria in the absence of $\Delta\psi$. Approximately 50% of the bound precursors was specifically associated with mitochondria. The other 50% appeared to be unspecifically bound. $pSu9_{1-69}$-DHFR and $pSu9_{1-48}$-DHFR were only unspecifically bound.

Kinetics of import into mitochondria in the presence of $\Delta\psi$

Do the precursors with different behaviour in the absence of $\Delta\psi$ also show different properties in the presence of $\Delta\psi$? We examined the kinetics of association with mitochondria and of import into mitochondria at 25°C in the presence of $\Delta\psi$.

The kinetics of association and of import (determined by specific processing) were similar. This may be explained by the earlier observations that the interaction of precursors with the binding sites on the mitochondrial surface are the rate-limiting step in import at 25°C (Pfanner and Neupert, 1985; Schmidt *et al.*, 1985). The transfer from the binding sites into mitochondria is rapid (<1 min at 25°C). Furthermore, the activity of the processing peptidase is not rate limiting at 25°C, as evidenced by the low levels of non-processed imported precursors (Figure 2; Schleyer and Neupert, 1985; and data not shown).

Import (and also association, see above) of $pSu9_{1-69}$-DHFR and $pSu9_{1-48}$-DHFR was significantly slower than the import of $pSu9_{1-147}$-DHFR and $pSu9_{1-147}$ (Figure 5). The import of $F_1\beta$, which also does not specifically bind in the absence of $\Delta\psi$, shows similar import kinetics to $pSu9_{1-69}$-DHFR and $pSu9_{1-48}$-DHFR. The import of ADP/ATP carrier, which can specifically bind in the absence of $\Delta\psi$, is as rapid as the import of $pSu9_{1-147}$. The most significant differences are seen in the initial import rates after 5 min at 25°C. The ratio between 5 min import and 35 min import ($ratio_{5/35}$) is <0.3 for $pSu9_{1-69}$-DHFR, $pSu9_{1-48}$-DHFR and $F_1\beta$, but >0.5 for $pSu9_{1-147}$ and ADP/ATP carrier. The import of $pSu9_{1-147}$-DHFR is even faster ($ratio_{5/35}$ >0.9). The reason for the very rapid import of $pSu9_{1-147}$-DHFR is not known. A possible explanation is that the release of precursors out of the cytosolic transport forms [e.g. ADP/ATP carrier and $pSu9_{1-147}$ have been shown to be transported in high mol. wt aggregates in the cytosol (Zimmermann and Neupert, 1980; Schmidt *et al.*, 1983)] might influence the kinetics of import and this step might be more rapid for $pSu9_{1-147}$-DHFR.

The proteins shown in Figure 5 can be divided into two different classes of precursor proteins. 'Class I' precursors specifically bind to mitochondria in the absence of $\Delta\psi$ and show rapid import in the presence of $\Delta\psi$. 'Class II' precursors do not specifically bind in the absence of $\Delta\psi$ and show slow import.

Discussion

We show here that a mitochondrial precursor protein with a cleavable presequence specifically binds to mitochondria in the absence of $\Delta\psi$. Binding requires a protease-sensitive component on the mitochondrial surface. In the presence of $\Delta\psi$, import from the bound state occurs without release of the precursor from the mitochondrial membranes. The ability to bind specifically in the absence of $\Delta\psi$ does not seem to be affected by the length of the precursor, since the authentic precursor of F_0-ATPase subunit 9 (147 amino acid residues) and a fusion protein between the precursor and DHFR (337 amino acid residues) show similar efficiency of specific binding.

Nine mitochondrial precursor proteins, which are imported in a $\Delta\psi$-dependent manner, have now been examined for their ability to bind specifically to mitochondria in the absence of $\Delta\psi$. Five of them (ADP/ATP carrier, a truncated form of ADP/ATP carrier, cytochrome b_2, F_0-ATPase subunit 9 and the fusion protein $pSu9_{1-147}$-DHFR) show this property (class I). The efficiency of import via the bound state (i.e. the ratio between import of precursor via the bound state and import of free precursor) is close to 1 in the case of the ADP/ATP carrier (Zwizinski *et al.*, 1983; Pfanner and Neupert, 1985) and the truncated ADP/ATP carrier (Pfanner *et al.*, 1987c), 0.5 in the case of subunit 9 and $pSu9_{1-147}$-DHFR, but low (≤0.1) in the case of cytochrome b_2 (Daum *et al.*, 1982; Gasser *et al.*, 1982; Riezman *et al.*, 1983). Four precursors ($F_1\beta$, Fe-S protein of the bc_1-complex, $pSu9_{1-69}$-DHFR, and $pSu9_{1-48}$-DHFR do not

specifically bind to mitochondria in the absence of $\Delta\psi$ (class II). On the other hand, these precursors are imported into mitochondria in the presence of $\Delta\psi$, and this import requires a protease-sensitive component on the mitochondrial surface. Obviously, the capacity for specific binding is very low with these precursors.

The question arises, do the class I and class II precursors also show different import properties in the presence of $\Delta\psi$? To provide an answer, the kinetics of association with mitochondria in the presence of $\Delta\psi$ was studied with three precursors of each class. The class II precursors showed signficantly slower import than the class I precursors.

Furthermore, each class I precursor contains one or more hydrophobic stretch(es) of amino acid residues in its sequence (Viebrock *et al.*, 1982; Arends and Sebald, 1984; Guiard, 1985). None of the class II precursors contains a long hydrophobic stretch (Harnisch *et al.*, 1985; Takeda *et al.*, 1985; T.Rassow, W.Neupert and M.Tropschug, unpublished).

It seems clear that positively charged hydrophilic sequences, which are usually contained in the presequences, can direct proteins to mitochondria and into the mitochondrial matrix ('matrix targeting signals') (Hurt and van Loon, 1986). We propose that one or more hydrophobic stretch(es) in a mitochondrial precursor protein support — in addition to the effect of the mentioned targeting signals — the specific interaction of the precursor with binding sites on the mitochondrial surface. This allows specific binding in the absence of $\Delta\psi$, and supports rapid import. In the case of subunit 9 and the derived fusion proteins, the presence of the (hydrophobic) mature protein part would suggest inclusion in class I, and its absence, inclusion in class II. We conclude that the hydrophobic mature part of subunit 9 is involved in the $\Delta\psi$-independent specific binding to the mitochondrial surface. The predicted hydrophobic stretch in the sequence of the cytochrome b_2 precursor contains a positive charge (Guiard, 1985). This might be the explanation for the low efficiency of specific binding of cytochrome b_2 in the absence of $\Delta\psi$. It should be emphasized that the putative function of the hydrophobic sequences is not related to the proposed role of 'stop transfer sequences'. These sequences were predicted to stop translocation of precursors across the outer or inner mitochondrial membranes by interaction with the lipid phases of the membranes (Hurt and van Loon, 1986). Furthermore, recent studies showed that the 'stop transfer signals' do not prevent translocation, but may rather act as second targeting signals, e.g. for the retranslocation of precursors from the matrix across the inner membrane (Pfanner and Neupert, 1987a; Pfanner *et al.*, 1987b; F.-U.Hartl, B.Guiard and W.Neupert, in preparation).

One possible explanation for the different binding properties of precursors is that class I precursors show a higher affinity than class II precursors for binding to the same receptor sites. This is supported by the observation that class I precursors associate with mitochondria more rapidly than class II precursors. As a further explanation, class I precursors may bind to other components of the outer membrane via their hydrophobic regions after having interacted with the protease-accessible receptor sites via the ('matrix')-targeting signals. Our studies of the import pathway of ADP/ATP carrier show that the binding of precursor occurs in two distinct steps: binding to a protease-accessible component, and subsequently insertion into a protease-protected site in the outer membrane. The second site also appears to be of proteinaceous nature. The import of ADP/ATP carrier into the inner membrane can occur from the second binding site, since it can take place when the initial (protease-sensitive) binding sites have been degraded by a protease treatment (for details see Pfanner and Neupert, 1987b; Pfanner *et al.*, 1987a). We suggest that both mechanisms (higher affinity to the initial receptor sites, and binding to a further component in the outer membrane) act in concert to increase the binding efficiency of class I precursors. Class II precursors also interact with the protease-sensitive receptor sites. In the presence of $\Delta\psi$ the precursors are then rapidly imported. Thus a sufficient (although slower) import may be guaranteed, despite the lower affinity and the inability to interact with the second binding sites.

Materials and methods

DNA manipulations

Isolation of a cDNA coding for subunit 9 of *Neurospora crassa* F_0-ATPase, construction and sequencing of the fusion proteins (shown in Figure 1), and transcription and translation were performed as described by Pfanner *et al.* (1987a). In the fusion protein $pSu9_{1-48}$-DHFR, the amino acids glycine and isoleucine were introduced between the subunit 9 and DHFR sequences. The cDNAs coding for the subunit 9 and the fusion proteins were ligated in the *Sma*I site of pGEM 3 (Promega).

Import of proteins into mitochondria

Isolation of *N. crassa* mitochondria via Percoll density gradient centrifugation, and binding and import of precursor proteins synthesized in rabbit reticulocyte lysates in the presence of [^{35}S]methionine were performed as described (Pfanner and Neupert, 1985, 1986, 1987b; Hartl *et al.* 1986; Pfanner *et al.*, 1987a). Binding and import was assayed in a buffer containing 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 3% (w/v) BSA and 10 mM 3-(*N*-morpholino)propanesulphonic acid, adjusted to pH 7.2 with KOH.

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