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Functional independence of the protein translocation machineries in mitochondrial outer and inner membranes: passage of preproteins through the intermembrane space

Bartolome Segui-Real, Gyula Kispal[1], Roland Lill and Walter Neupert

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie der Universität München, Goethestrasse 33, 8000 München 2, Germany
[1]Present address: Institute of Biochemistry, University Medical School, Szigeti ut 12, H-7624 Pécs, Hungary

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The protein translocation machineries of the outer and inner mitochondrial membranes usually act in concert during translocation of matrix and inner membrane proteins. We considered whether the two machineries can function independently of each other in a sequential reaction. Fusion proteins (pF-CCHL) were constructed which contained dual targeting information, one for the intermembrane space present in cytochrome *c* heme lyase (CCHL) and the other for the matrix space contained in the signal sequence of the precursor of F_1-ATPase β-subunit ($pF_1\beta$). In the absence of a membrane potential, $\Delta\Psi$, the fusion proteins moved into the intermembrane space using the CCHL pathway. In contrast, in the presence of $\Delta\Psi$ they followed the $pF_1\beta$ pathway and eventually were translocated into the matrix. The fusion protein pF51-CCHL containing 51 amino acids of $pF_1\beta$, once transported into the intermembrane space in the absence of a membrane potential, could be further chased into the matrix upon re-establishing $\Delta\Psi$. The sequential and independent movement of the fusion protein across the two membranes demonstrates that the translocation machineries act as distinct entities. Our results support a model in which the two translocation machineries can function independently of each other, but generally interact in a dynamic fashion to achieve simultaneous translocation across both membranes. In addition, the results provide information about the targeting sequences within CCHL. The protein does not contain a signal for retention in the intermembrane space; rather, it lacks matrix targeting information, and therefore is unable to undergo $\Delta\Psi$-dependent interaction with the protein translocation apparatus in the inner membrane.

Key words: cytochrome *c* heme lyase/F_1 ATPase/membrane potential/mitochondria/protein import

Introduction

The targeting of nuclear-encoded mitochondrial precursor proteins to the mitochondrial matrix is a complex, multi-step process involving their passage across two membranes, the outer and the inner membrane (Pfanner and Neupert, 1990; Glick and Schatz, 1991; Segui-Real *et al.*, 1992). The sub-organellar sorting of the preproteins requires that both membranes contain proteins that decipher the topogenic sorting signals on the preproteins. While many of the components of the outer membrane translocation apparatus have been characterized in some detail (Pfanner *et al.*, 1991), constituents of the inner membrane machinery have only recently started to become identified (Maarse *et al.*, 1992; Scherer *et al.*, 1992). With many preproteins it has been shown that translocation across both membranes is coupled and occurs at sites of close contact between the two membranes, the so-called contact sites (Schleyer and Neupert, 1985). Intermediates spanning both membranes can be accumulated using a variety of procedures (Wienhues and Neupert, 1992). The simultaneous passage through both membranes requires the active participation of the inner membrane; the electrochemical potential, $\Delta\Psi$, is needed for the insertion and/or translocation of the signal sequence into and across the inner membrane (Schleyer and Neupert, 1985). This, together with the binding of mitochondrial hsp70 to preproteins as they appear in the matrix, is believed to provide, at least in part, the driving force needed for the transfer across both membranes (Neupert *et al.*, 1990).

Despite the close cooperation of the two membranes, a preprotein *en route* to the matrix is accessible from the intermembrane space to externally added protease when the outer membrane is opened (Hwang *et al.*, 1991; Rassow and Pfanner, 1991). This has been used as an argument against a tightly sealed, continuous channel across the two membranes which directs the proteins into the matrix. Rather, it has been suggested that independent translocation machineries exist, one in the outer and another one in the inner membrane (Glick *et al.*, 1991; Pfanner *et al.*, 1992). Indeed, the inner membrane has been shown to contain an endogenous protein translocation activity which can transport preproteins independently of the outer membrane (Hwang *et al.*, 1989). Likewise, the outer membrane contains translocation activity (Mayer *et al.*, 1993) for proteins of the outer membrane and of the intermembrane space like cytochrome *c* heme lyase (CCHL) which is imported independently of a membrane potential (Lill *et al.*, 1992a,b). However, precursor proteins which are dependent on $\Delta\Psi$ for import are not translocated across the outer membrane without participation of the inner membrane. This has raised the question of whether proteins passing the inner membrane mechanistically require inner membrane translocation for passing also across the outer membrane in a simultaneous step, or whether passage across the two membranes can be dissected into two sequential and independent steps.

To address this question we have constructed fusion proteins between a matrix-targeted protein (F_1-ATPase β-subunit including its N-terminal signal sequence) and CCHL. The fusion proteins contain two targeting signals, one for the matrix and another for the intermembrane space. We show here that the fusion proteins are imported into isolated mitochondria from *Neurospora crassa*. The sub-mitochondrial localization in the intermembrane space or the matrix is determined by the absence or presence of an electrochemical potential showing that the utilization of the respective targeting signals may be adapted to the conditions

prevailing during import. Moreover, a protein first imported into the intermembrane space can be further transferred into the matrix if a membrane potential is re-established. This provides evidence for the existence in the two mitochondrial membranes of distinct protein translocation systems; these may be used sequentially by a precursor protein. Furthermore, the results show that the localization of CCHL to the intermembrane space is explained by the lack of a signal for targeting to the matrix rather than by a signal for retention in the intermembrane space.

Results

Construction of fusion proteins of the F_1-ATPase β-subunit and CCHL

Using the cDNAs encoding CCHL (Drygas *et al.*, 1989) and the precursor of the β-subunit of F_1-ATPase ($pF_1\beta$; Rassow *et al.*, 1990) from *N.crassa*, the N-terminal 51 or 425 amino acids of $pF_1\beta$ including the matrix targeting sequence were fused to the N-terminus of CCHL, producing pF51-CCHL and pF425-CCHL respectively (Figure 1 and Materials and methods). These fusion proteins contain two different targeting sequences, one for matrix localization contained in the signal sequence of $pF_1\beta$ (amino acids 1−40) and another for targeting to the intermembrane space located in the central part of CCHL (G.Kispal, unpublished). The first 21 amino acids of CCHL were deleted in these constructs. Previous results had shown that this part of CCHL does not contain targeting information, and that the truncated protein is transported to the intermembrane space with the same efficiency and rate as the full-length CCHL (G.Kispal, unpublished). Synthesis of the fusion proteins in an *in vitro* transcription/translation system in the presence of [^{35}S]methionine yielded translation products of the predicted molecular mass (Figure 1, inset) which could be immunoprecipitated with antisera against CCHL (data not shown). The import of these radioactively labeled proteins into isolated mitochondria from *N.crassa* was analyzed in some detail.

The sub-mitochondrial localization of imported fusion proteins is determined by the membrane potential

Upon incubation with isolated mitochondria a large fraction of pF51-CCHL was imported as measured by its resistance against externally added protease (Figure 2). In the presence of a membrane potential, the signal sequence was efficiently cleaved yielding the processed form, mF51-CCHL. The result indicates that the fusion protein or at least its N-terminal part had gained access to the matrix and import obviously occurred along the normal import pathway for $pF_1\beta$. In contrast, in the absence of a membrane potential the signal sequence was not removed, suggesting that under these conditions the protein remained in the intermembrane space. Apparently, the targeting information residing in CCHL is sufficient to direct the protein into mitochondria presumably by following the same pathway as CCHL which does not require a membrane potential for its import into the intermembrane space (Lill *et al.*, 1992a). In the fusion protein pF425-CCHL the two mitochondrial targeting signals are separated by almost the entire mature sequence of $pF_1\beta$. Upon incubation with energized mitochondria, pF425-CCHL was imported and the signal sequence was cleaved similarly to pF51-CCHL (Figure 2). However, in the absence of added ATP and a membrane potential, no significant import was observed. Thus, pF425-CCHL exhibits import characteristics similar to those of a typical matrix-targeted protein like $pF_1\beta$ (see below). When pF51-CCHL was treated with the purified components of the matrix processing

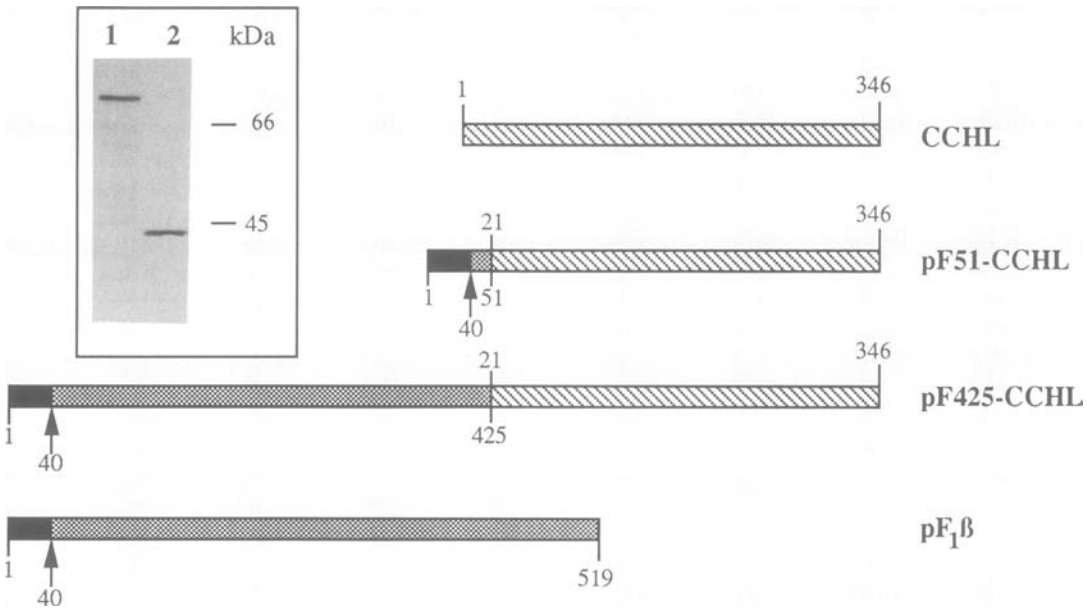

Fig. 1. Fusion proteins of N-terminal parts of the precursors of the β-subunit of F_1-ATPase and CCHL. The fusion proteins were constructed by using the cDNAs of the β-subunit of the F1-ATPase precursor ($pF_1\beta$, Rassow *et al.*, 1990) and of CCHL (Drygas *et al.*, 1989) from *N.crassa*. The first 51 or 425 amino acids of $pF_1\beta$ were joined to amino acids 21−346 of CCHL (producing constructs pF51-CCHL and pF425-CCHL respectively). The matrix targeting sequence of $pF_1\beta$ is shown in black, the arrow indicating the cleavage site for the matrix processing peptidase after residue 40. For *in vitro* transcription and translation the coding regions of the fusion proteins were cloned into pGEM3 vector under the control of SP6 promoter. The inset shows the respective translation products for pF425-CCHL (lane 1) and pF51-CCHL (lane 2) together with markers for molecular masses.

peptidase, α- and β-MPP (Hawlitschek *et al.*, 1988; M.Arretz *et al.*, unpublished) to cleave off the matrix targeting signal before the import reaction, the resulting mF51-CCHL was able to enter the mitochondria in both the absence and the presence of a membrane potential with similar efficiencies (data not shown). In contrast, with mF425-CCHL no or very inefficient import was observed. pF425-CCHL apparently can use the import pathway and the sorting signal of $pF_1\beta$, but is unable to follow the CCHL pathway. pF51-CCHL, on the other hand, shows a dual behaviour; it resembles authentic CCHL, when either the membrane potential is depleted or the signal sequence is removed, but in the presence of both it behaves like a matrix-targeted protein.

In order to determine the localization of the imported fusion proteins, the mitochondria were fractionated by employing the weak detergent digitonin. Digitonin preferentially opens the outer mitochondrial membrane, and only at higher concentrations is the inner membrane permeabilized giving access to the matrix components (Hartl *et al.*, 1986). The fractionation was performed in the presence of proteinase K to assay for the accessibility of the fusion proteins in the intermembrane space or in the matrix (Figure 3). When pF51-CCHL was imported in the presence of a membrane potential, the processed form of the fusion protein, mF51-CCHL, was found in the matrix as evident from its co-fractionation with mitochondrial hsp70 (Figure 3A). Endogenous CCHL, as a marker for the intermembrane space, became accessible to protease digestion at much lower concentrations of digitonin. An identical result was obtained for the import of pF425-CCHL in the presence of a membrane potential (Figure 3B). These results show that the entire fusion proteins and not just their N-terminal parts had entered the matrix space, i.e. the proteins had completed their translocation into the mitochondrial matrix.

In contrast, pF51-CCHL imported in the absence of a membrane potential was accessible to proteolytic attack in the same concentration range of digitonin as endogenous

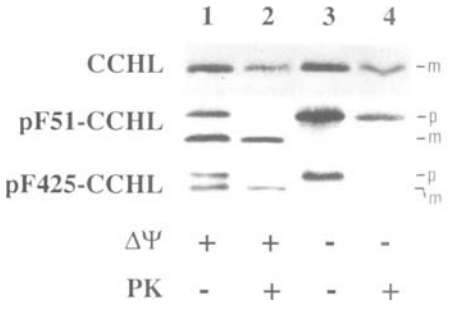

Fig. 2. Import of pF51-CCHL and of pF425-CCHL into mitochondria in the presence and absence of a membrane potential. ^{35}S-labeled precursors of pF51-CCHL, pF425-CCHL and CCHL were imported into freshly isolated mitochondria (100 μg protein per sample) at 25°C for 10 min. A membrane potential (ΔΨ) was generated where indicated by adding 8 mM potassium ascorbate and 0.2 mM *N,N,N',N'*-tetramethylphenylenediamine plus 2 mM ATP and 5% reticulocyte lysate, or depleted by a 3 min preincubation at 25°C in the presence of 30 μM oligomycin plus 12 μM antimycin A. After chilling on ice the samples were divided in half, and one aliquot was treated with proteinase K (PK, 50 μg/ml) while the other was kept on ice. Mitochondria were reisolated by centrifugation, and proteins were separated by SDS–PAGE, blotted onto nitrocellulose and analyzed by autoradiography. p, precursor form; m, mature form.

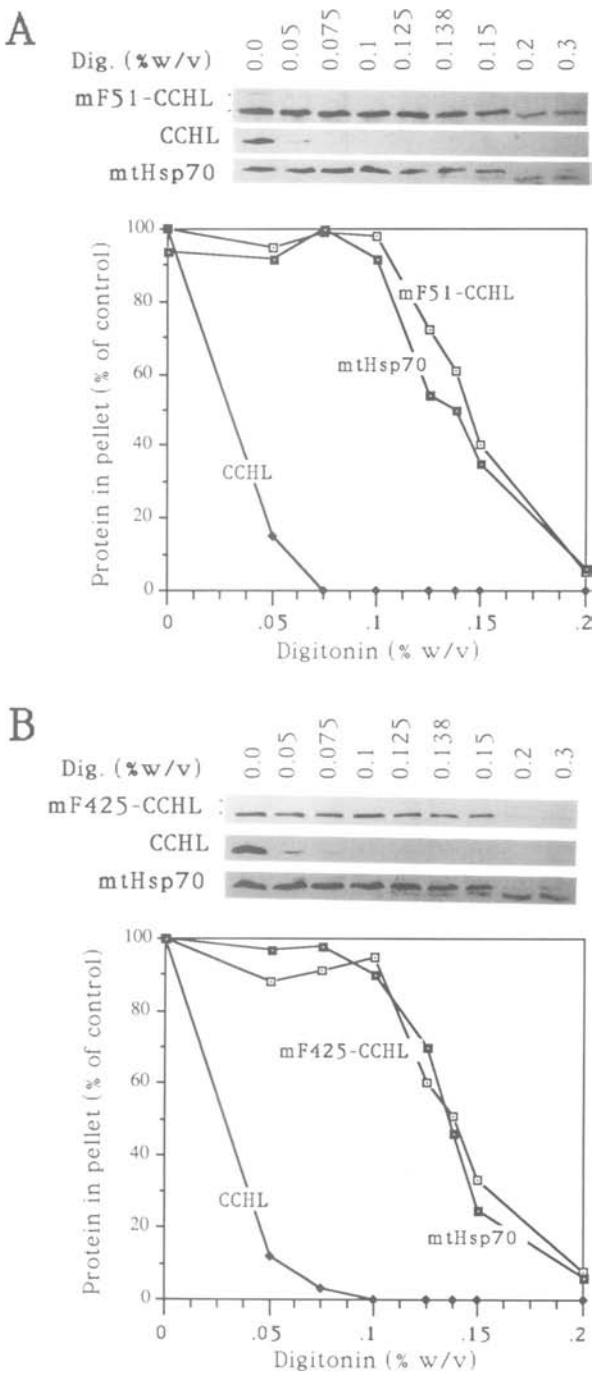

Fig. 3. Sub-mitochondrial localization of the fusion proteins imported in the presence of a membrane potential. Radioactively labeled precursors of (**A**) pF51-CCHL and (**B**) pF425-CCHL were imported into freshly isolated mitochondria for 15 min at 25°C in the presence of a membrane potential, 2 mM ATP and 5% reticulocyte lysate. Unimported material was degraded by trypsin treatment (20 min at 4°C with 40 μg/ml trypsin) followed by the addition of 1.4 mg/ml soybean trypsin inhibitor. Mitochondria were diluted with 1 ml SEMK buffer, reisolated by centrifugation, and resuspended in SEMK buffer at a final protein concentration of 11 mg/ml. Digitonin fractionation was performed as explained in Materials and methods. Samples were analyzed by SDS–PAGE, proteins blotted onto nitrocellulose and visualized by autoradiography (mF51-CCHL and mF425-CCHL) or immunodecoration (mtHsp70 and CCHL). Quantification of the bands (lower panels) was performed by laser densitometry, and the signal in the absence of digitonin was set to 100%.

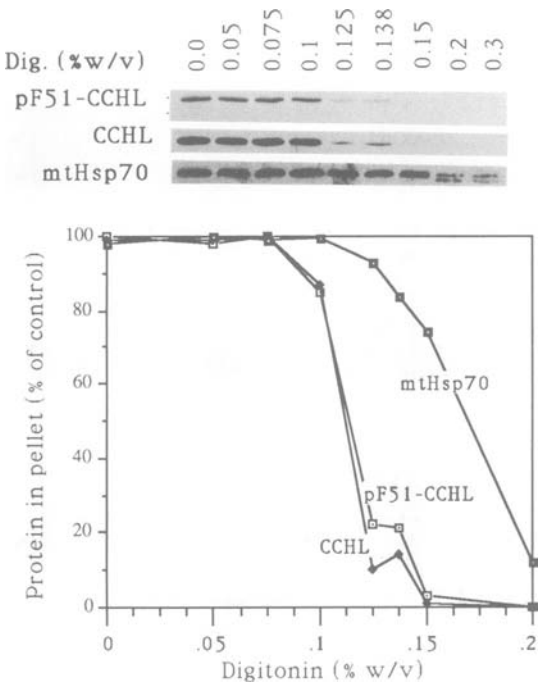

Fig. 4. Sub-mitochondrial localization of the pF51-CCHL imported in the absence of a membrane potential. The experiment was performed and evaluated as described in Figure 3. The import reaction was carried out for 20 min at 25°C.

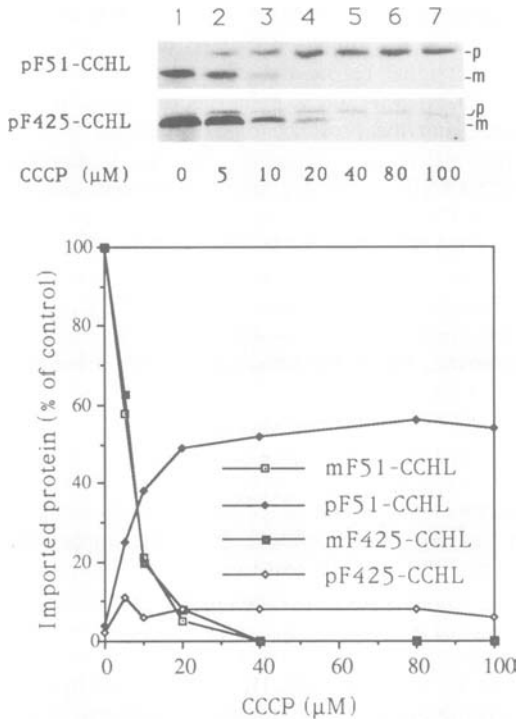

Fig. 5. Membrane potential-dependent targeting of the fusion proteins to the intermembrane space and the matrix. Freshly isolated mitochondria were energized by adding 8 mM potassium ascorbate and 0.2 mM *N,N,N′,N′*-tetramethylphenylenediamine, and the samples were supplemented with 2 mM ATP and 5% reticulocyte lysate. CCCP was added to the indicated concentrations by dilution from a 100-fold stock solution in ethanol followed by an incubation for 3 min at 25°C. Import of pF51-CCHL or pF425-CCHL was for 20 min at 25°C. Samples were treated with proteinase K and the mitochondria were reisolated by centrifugation. Further analysis of imported proteins and quantification of the data (lower panel) was as described in Figure 3. The respective values of mature proteins in the absence of CCCP was set to 100%. p, precursor form; m, mature form.

CCHL (Figure 4). This together with the lack of signal sequence cleavage suggests that translocation had terminated in the intermembrane space, presumably because a potential-dependent interaction with the translocation machinery of the inner membrane and subsequent transport could not occur. In the intermembrane space CCHL is tightly associated with the inner membrane (Nicholson *et al.*, 1988) behaving as a peripheral rather than an integral component of the inner membrane, since it is extracted under alkaline conditions (C.Hergersberg, unpublished; Dumont *et al.*, 1992). pF51-CCHL behaved similarly to the endogenous CCHL in that it strongly bound to membranes whether present in the intermembrane space or in the matrix (data not shown). Upon alkaline extraction of mitochondria both pF51-CCHL in the intermembrane space and mF51-CCHL in the matrix became solubilized. The fact that the fusion proteins can be solubilized in alkaline conditions shows that during import they were not integrated into the membrane, but rather peripherally associated with the membranes in a similar manner as seen for CCHL.

In order to study the $\Delta\Psi$-dependence of the import of pF51-CCHL into mitochondria in more detail, the membrane potential was gradually depleted by increasing amounts of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP; Figure 5). The decrease in the formation of mF51-CCHL was paralleled by an increase in the import of pF51-CCHL. The changes occur in the same concentration range of CCCP, which affects the import of $pF_1\beta$ into mitochondria (Lill *et al.*, 1992a). These results fit with the observation that the presequence is largely responsible for the degree of dependence on a membrane potential irrespective of the passenger protein imported (Martin *et al.*, 1991). However, in contrast to typical matrix-targeted proteins, which fail to become imported into mitochondria in the absence of $\Delta\Psi$, the fusion protein pF51-CCHL reaches the intermembrane space, when the membrane potential is insufficient to support the insertion and translocation of the presequence across the inner membrane. Thus, the import route taken by pF51-CCHL is modulated by the strength of the electrochemical potential across the inner membrane. A sufficiently strong membrane potential leads to the translocation of pF51-CCHL across both the outer and inner membranes, whereas in the case of a de-energized inner membrane, translocation is aborted after passage across the outer membrane.

With pF425-CCHL, import and the formation of mF425-CCHL were abolished by increasing CCCP concentrations in a similar fashion to that observed with pF51-CCHL (Figure 5). At higher concentrations of CCCP a small fraction of pF425-CCHL became protease-resistant indicating its import into the mitochondria along the CCHL pathway, even though this was inefficient. Obviously, upon using the CCHL pathway the short additional piece in pF51-CCHL can be transferred efficiently across the membrane, whereas the much longer segment in pF425-CCHL inhibits complete transfer. This inhibitory effect must be related to the folding of the $pF_1\beta$ part, since the import efficiency of pF425-CCHL was diminished by

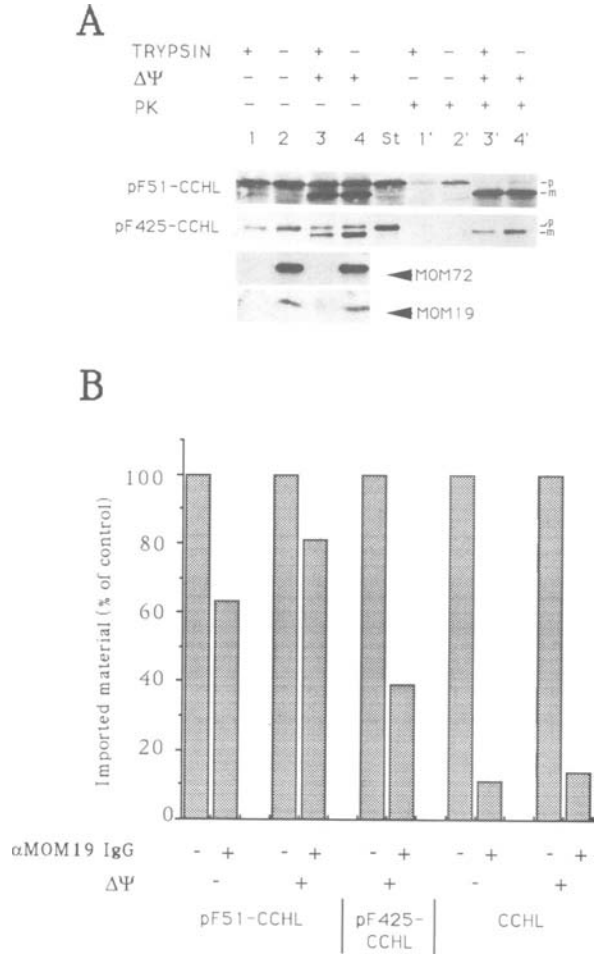

Fig. 6. Requirement of MOM19 receptor for import of pF51-CCHL and pF425-CCHL. (**A**) Mitochondria pretreated or not pretreated with trypsin (40 μg/ml for 15 min at 4°C) were incubated with fusion proteins pF51-CCHL and pF425-CCHL in the presence [addition of 5% (v/v) reticulocyte lysate, 2 mM ATP and 2 mM NADH] or absence (addition of 30 μM oligomycin and 12 μM antimycin A) of a membrane potential for 5 min at 25°C. The samples were divided in half and treated with proteinase K (PK) as indicated. Imported, radioactive proteins were analyzed as described in Figure 3. The lower part of the figure shows the efficiency of the trypsin pretreatment by immunoblotting of MOM19 and MOM72 receptors. st, standard for 30% of input; p, precursor form; m, mature form. (**B**) Mitochondria (20 μg/sample) in import buffer were preincubated with or without 100 μg IgG directed against MOM19 for 45 min at 0°C (Lill *et al.*, 1992a). Mitochondria were reisolated and pF51-CCHL, pF425-CCHL or CCHL were imported for 10 min at 20°C in the presence or absence of a membrane potential. After treatment with proteinase K the mitochondria were reisolated and further analysis was performed as described in Figure 3. Import in the absence of IgG was set to 100%.

removing ATP from the import reaction, and was considerably improved by denaturation of pF425-CCHL in 8 M urea prior to import (data not shown).

The fusion proteins use the outer membrane receptor complex for import

Even though the precursors of $F_1\beta$ and CCHL follow different import pathways, the two proteins share the initial translocation step across the outer membrane. For both proteins this step is dependent on the function of a protease-sensitive surface receptor, namely the MOM19 protein (Söllner *et al.*, 1989; Lill *et al.*, 1992a). To test whether pF51-CCHL also requires the participation of surface receptors, a fraction of the mitochondria was pretreated with trypsin to degrade MOM19 and MOM72 (Figure 6A, lower panel). In the absence of a membrane potential, import was reduced by a factor of three after the removal of the receptors (Figure 6A). However, translocation of pF51-CCHL into the intermembrane space was much less dependent on receptors than is CCHL or $pF_1\beta$ import, where a 10-fold reduction was observed (data not shown; see Lill *et al.*, 1992a). In the presence of a membrane potential, the import of pF51-CCHL was only slightly affected by the removal of the receptors. The import of pF425-CCHL was also dependent on the presence of protease-sensitive surface receptors, in fact to a somewhat higher degree than pF51-CCHL (Figure 6A). This shows that the import of these fusion proteins involves the function of surface receptors, although at a lesser extent than the proteins from which they are derived. The results are in keeping with a previous observation that multiple signal sequences of preproteins increase the efficiency of their translocation (Galanis *et al.*, 1991). In addition, the data are consistent with the notion that surface receptors serve to improve the efficiency of import, but can be bypassed under certain conditions (Pfaller *et al.*, 1988b; Miller and Cumsky, 1991).

To determine that specifically MOM19 function was involved in the import of the fusion proteins, mitochondria were preincubated with anti-MOM19 antibodies (Figure 6B). The import of the fusion proteins pF51-CCHL and pF425-CCHL was reduced in a fashion comparable to that observed for import into trypsin-pretreated mitochondria, both in the absence and in the presence of an electrochemical potential. Similarly, import of CCHL was affected more strongly than that of the fusion proteins. In contrast, antibodies against MOM72 did not result in a significant reduction of the import (data not shown). Taken together, the dependence on MOM19 function, although to a limited degree, demonstrates the functional participation of the outer membrane receptor complex in the import of the fusion proteins into the intermembrane space and the matrix of mitochondria. Thus, the fusion proteins use the same pathways and components as the proteins from which they are derived.

pF51-CCHL in the intermembrane space can be transferred to the matrix by re-establishing the membrane potential

We asked whether pF51-CCHL first imported into the intermembrane space in the absence of a membrane potential could engage into functional interaction with the inner membrane translocation machinery and, in a second, separate translocation step, reach the matrix. First, the fusion protein was imported into the intermembrane space in the absence of a membrane potential. When the membrane potential was re-established, a major fraction of the accumulated pF51-CCHL became processed, while no cleavage of the signal sequence was observed when the membrane potential was lacking also during the second step (Figure 7A). Digitonin fractionation of the mitochondria confirmed that the entire mature-sized mF51-CCHL was imported into the matrix, whereas pF51-CCHL remained in the intermembrane space as evident from its cofractionation with endogenous CCHL (Figure 7B). Obviously, the presequence of $pF_1\beta$, even when localized in the intermembrane space, is able to

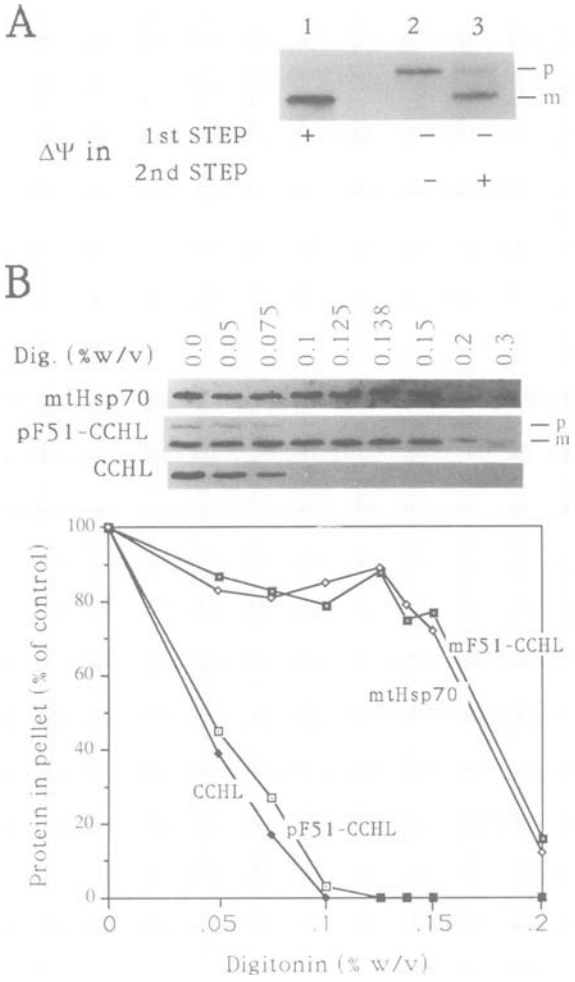

Fig. 7. Two-step import of pF51-CCHL into the matrix via the intermembrane space. (**A**) pF51-CCHL was imported into mitochondria in the presence (lane 1) or absence (lanes 2 and 3) of a membrane potential (see Figure 2). Both samples were treated with proteinase K. The mitochondria from the sample with a membrane potential were reisolated and precipitated using trichloroacetic acid. The sample without a membrane potential was divided in half, and the mitochondria were reisolated, resuspended in import buffer and supplemented with either 30 μM oligomycin and 12 μM antimycin A (lane 2) or 5% (v/v) reticulocyte lysate, 2 mM ATP, and 8 mM potassium ascorbate and 0.2 mM *N,N,N',N'*-tetramethylphenylenediamine (lane 3). After incubation of 25 min at 25°C mitochondria were reisolated and precipitated using trichloroacetic acid. Further analysis of imported proteins was as described in Figure 3. (**B**) pF51-CCHL was imported into de-energized mitochondria for 20 min at 25°C as described in Figure 4. Unimported material was degraded by trypsin treatment followed by addition of soybean trypsin inhibitor. Mitochondria were reisolated and resuspended in import buffer containing 5% (v/v) reticulocyte lysate, 2 mM ATP, and 8 mM potassium ascorbate and 0.2 mM *N,N,N',N'*-tetramethylphenylenediamine to generate a membrane potential. After incubation for 25 min at 25°C, mitochondria were reisolated and subjected to digitonin fractionation (see Figure 3). p, precursor form; m, mature form.

undergo $\Delta\Psi$-dependent interaction with the inner membrane translocation apparatus in order to initiate further movement of the entire protein to the matrix.

In summary, these experiments establish that pF51-CCHL can reach the matrix in a single step by simultaneous translocation across both membranes or by two independent steps with a transient pause in the intermembrane space (Figure 8). The pathway eventually taken is decided by the

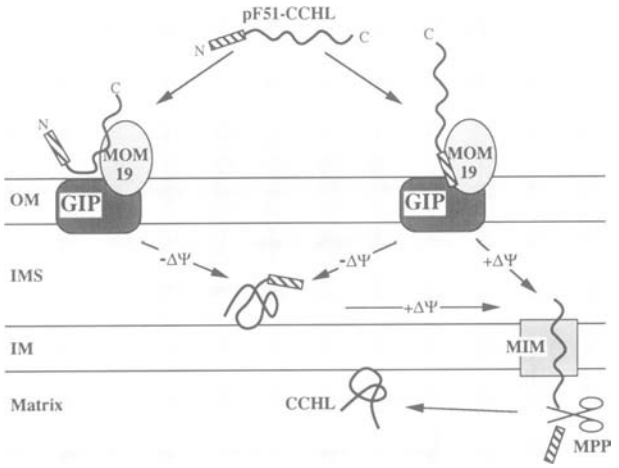

Fig. 8. Working model for the sequential and independent translocation of the fusion proteins across the outer and inner mitochondrial membranes. For details see text. GIP, general insertion pore (Pfaller *et al.*, 1988a); MIM, mitochondrial inner membrane translocation machinery; OM, outer membrane; IM, inner membrane; IMS, intermembrane space; MPP, matrix processing peptidase.

presence or absence of a membrane potential. For targeting to and crossing of the outer membrane either the signal sequence of $pF_1\beta$ or an internal signal in CCHL may be used. The two-step pathway involves the sequential utilization of the translocation machineries of the outer and the inner membrane and thereby demonstrates their functional independence.

Discussion

Translocation of proteins from the cytosol into the matrix space across both outer and inner membranes is an important reaction in mitochondrial biogenesis. As has recently become clear, this process is facilitated by two translocation machineries, one located in the outer membrane and another in the inner membrane (Glick *et al.*, 1991; Pfanner *et al.*, 1992). This raises several questions. Do precursor proteins use these two machineries in a sequential or in a concerted fashion? What are the respective signals for interacting with these two machineries? What are the driving forces that overcome the two membrane barriers? To date, only some of these problems have been addressed.

We recently proposed a model in which the two machineries interact in a dynamic manner (Pfanner *et al.*, 1992). According to this model, preproteins first interact with the outer membrane machinery which then makes contact with the translocation system of the inner membrane. This then leads to a simultaneous translocation of the preproteins across both membranes, i.e. a single precursor polypeptide engages both machineries at the same time. Thus, in this situation translocation across the outer membrane is coupled to translocation across the inner membrane. Moreover, in the absence of a membrane potential across the inner membrane, translocation across the outer membrane is also prevented. Transport across the outer membrane, however, does not require ongoing translocation of the precursor across the inner membrane. If the transfer into the matrix is stalled at early stages, e.g. by inhibiting the function of hsp70 in the matrix, the precursor can then slip into the intermembrane space, albeit much more slowly (Hwang *et al.*, 1991; Rassow and Pfanner, 1991; Jascur *et al.*, 1992). This

latter process is still dependent on the initiation of transfer across the inner membrane. On the other hand, transport of certain intermembrane space proteins from the cytosol across the outer membrane can take place without the apparent involvement of the inner membrane, indicating that the outer membrane by itself has translocation activity (Lill *et al.*, 1992b; Mayer *et al.*, 1993). It has yet to be explained why a matrix-targeted precursor protein is unable to enter the intermembrane space in the absence of functional inner membrane translocation.

In this paper we have investigated whether the two translocation machineries in intact mitochondria can, in principle, operate independently of each other in the translocation of proteins destined for the matrix space. For this purpose we have constructed precursor proteins containing dual targeting information, one for entering the matrix space (an N-terminal segment of the precursor of F_1-ATPase β-subunit) and another for entering the intermembrane space (present in the mature sequence of CCHL. According to our results, fusion proteins of this type can indeed use the translocation machineries in the outer and the inner membranes in two independent steps (Figure 8). The first step does not require a membrane potential and results in the protein being accumulated in the intermembrane space. The second step is triggered by the membrane potential and leads to a transfer of the protein from the intermembrane space to the matrix. When the membrane potential is present from the beginning of the import reaction, the fusion proteins are translocated directly into the matrix employing both machineries in a coupled fashion. Thus, the membrane potential acts as a switch to target proteins either to the intermembrane space or to the matrix. Apparently, coupled translocation across both membranes in this way is much more efficient in kinetic terms and shows a reduced dependence on the participation of surface receptors.

Our results also shed new light on the role of the matrix targeting signal in mitochondrial topogenesis. Proteins destined for the intermembrane space apparently remain there because they lack a targeting signal for further translocation across the inner membrane. This argues that a specific signal responsible for retention in the intermembrane space does not exist in proteins such as CCHL. The matrix targeting signal must become effective in a specific manner at the surface of the inner membrane, after it already has fulfilled its function by interacting with the receptors on the surface of the outer membrane. Most interestingly, intermembrane space proteins such as CCHL contain signals that are competent to interact with the receptors on the outer membrane and possibly with a transport channel in the outer membrane, but these signals are not able to interact with the corresponding machinery of the inner membrane.

The inability of matrix precursors to enter the intermembrane space in the absence of $\Delta\Psi$ would then be explained by the lack of a driving force for translocation (Neupert *et al.*, 1990; Simon *et al.*, 1992). These precursors probably interact with the outer membrane machinery but only limited segments may be able to become translocated. In contrast, a driving force must exist for sorting precursors such as CCHL into the intermembrane space. If CCHL is combined with a matrix precursor or a segment of it, the protein is transferred completely into the intermembrane space. By preferring a coupled translocation across both membranes under physiological conditions, not only may import be very rapid but also an accumulation of probably 'sticky' matrix precursors in the intermembrane space is avoided. Coupled translocation may also contribute to avoid partial refolding in the intermembrane space which most probably would have to be reversed upon further translocation across the inner membrane.

Materials and methods

Biochemical procedures

Published methods were used for the following: isolation of mitochondria from *N.crassa* wild-type strain 74A (Stuart *et al.*, 1990); protein determination with immunoglobulin G as a standard (Bradford, 1976); SDS–PAGE (Nicholson *et al.*, 1987); pretreatment of mitochondria with trypsin (Pfaller *et al.*, 1988a); raising antisera against various outer membrane proteins and the preparation of IgG from antisera (Söllner *et al.*, 1989); preincubation of mitochondria with IgG against various outer membrane proteins (Söllner *et al.*, 1989). The detection of proteins after blotting onto nitrocellulose was performed using the ECL detection system according to the instructions of the supplier (Amersham).

DNA manipulations and construction of the plasmids encoding the fusion proteins pF51-CCHL and pF425-CCHL

Isolation of DNA fragments and oligonucleotides, plasmid preparations, ligations, dephosphorylation of DNA, transformations and DNA sequencing were performed as previously described (Maniatis *et al.*, 1982). Enzymes for DNA modification were purchased from Boehringer Mannheim and New England Biolabs. For the construction of the plasmids encoding the fusion proteins (see Figure 1), the *Eco*RI–*Xho*II (blunted) fragment of the cDNA encoding *N.crassa* CCHL (Drygas *et al.*,1989) was ligated to the *Eco*RI and blunted *Bgl*II sites (yielding pF51-CCHL) or to the *Eco*RI and *Eco*RV sites (yielding pF425-CCHL) of the cDNA encoding the β-subunit of the F1-ATPase ($pF_1\beta$) from *N.crassa*. The $pF_1\beta$ gene inserted into the pGEM3 plasmid (Promega) was used (Rassow *et al.*, 1990). Correct construction of the plasmids was verified by DNA sequence analysis. The encoded fusion proteins were synthesized in the presence of [^{35}S]methionine in a coupled transcription/translation system in rabbit reticulocyte lysate (Söllner *et al.*, 1991). Production of correct translation products was confirmed by immunoprecipitation with anti-CCHL antibodies.

Import of precursor proteins into mitochondria

Import of radioactively labeled precursor proteins was performed in import buffer (250 mM sucrose, 10 mM MOPS–KOH pH 7.2, 80 mM KCl, 5 mM $MgCl_2$ and 1 mg/ml fatty acid free bovine serum albumin) for the time periods indicated. To generate a membrane potential the reactions were supplemented with 8 mM potassium ascorbate (adjusted to pH 7.2 before the addition), 0.2 mM *N,N,N',N'*-tetramethylphenylenediamine, and supplemented with 5% (v/v) reticulocyte lysate containing an ATP regenerating system (10 mM creatine phosphate and 22 units/ml creatine kinase plus 2 mM ATP; Rassow *et al.*, 1989). To dissipate the membrane potential reversibly, 12 μM antimycin A and 30 μM oligomycin were added and the samples were incubated for 3 min at 25°C before starting the import reactions. Import reactions were terminated by adding 40 μg/ml proteinase K followed by incubation for 15 min on ice. After the addition of 1 mM phenylmethanesulfonyl fluoride the samples were diluted with 0.5 ml SEM buffer (250 mM sucrose, 2 mM EDTA, 10 mM MOPS–KOH pH 7.2) and the mitochondria were pelleted by centrifugation. Imported proteins were analyzed by SDS gel electrophoresis, blotting onto nitrocellulose and autoradiography. The bands were quantified by laser densitometry (Pfaller *et al.*, 1988a).

Digitonin fractionation of mitochondria

After the import reactions mitochondria were collected by centrifugation (4 min in a benchtop centrifuge at 4°C) and gently resuspended in SEMK buffer (250 mM sucrose, 2 mM EDTA, 10 mM MOPS–KOH pH 7.2 and 100 mM KCl) at a protein concentration of 11 mg/ml. 13 μl aliquots were added to 7 μl of SEMK buffer containing proteinase K at a final concentration of 100 μg/ml (Glick, 1992) and varying concentrations of digitonin. Samples were incubated for 1.5 min at 4°C, diluted with 80 μl of SEMK and further incubated for 30 min at 4°C. Protease digestion was stopped with PMSF and samples were precipitated with trichloroacetic acid (10% w/v final concentration). Samples were analyzed by SDS–PAGE and subsequent autoradiography or immunostaining of nitrocellulose blots.

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