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Import of cytochrome *c* heme lyase into mitochondria: a novel pathway into the intermembrane space

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Cytochrome *c* heme lyase (CCHL) catalyses the covalent attachment of the heme group to apocytochrome *c* during its import into mitochondria. The enzyme is membrane-associated and is located within the intermembrane space. The precursor of CCHL synthesized *in vitro* was efficiently translocated into isolated mitochondria from *Neurospora crassa*. The imported CCHL, like the native protein, was correctly localized to the intermembrane space, where it was membrane-bound. As with the majority of mitochondrial precursor proteins, CCHL uses the MOM19–GIP receptor complex in the outer membrane for import. In contrast to proteins taking the general import route, CCHL was imported independently of both ATP-hydrolysis and an electrochemical potential as external energy sources. CCHL which lacks a cleavable signal sequence apparently does not traverse the inner membrane to reach the intermembrane space; rather, it translocates through the outer membrane only. Thus, CCHL represents an example of a novel, 'non-conservative' import pathway into the intermembrane space, thereby also showing that the import apparatus in the outer membrane acts separately from the import machinery in the inner membrane.

Key words: cytochrome *c* heme lyase/intracellular transport/mitochondria/*Neurospora crassa*/protein import

Introduction

Most mitochondrial proteins are encoded in the nucleus, translated on cytoplasmic ribosomes, and, after targeting to the mitochondrion, sorted to their final destination within the organelle (reviewed by Hartl *et al.*, 1989; Pfanner and Neupert, 1990; Baker and Schatz, 1991). The first specific interaction of precursor proteins with the mitochondrion is mediated through their binding to protease-sensitive receptors which are located on the cytoplasmic surface of the outer membrane (Riezman *et al.*, 1983; Zwizinski *et al.*, 1984; Pfaller and Neupert, 1987). MOM19 and MOM72 proteins from *Neurospora crassa* and MAS70 from yeast (Hines *et al.*, 1990) have recently been identified to participate in this initial step of protein import (for a review see Pfanner *et al.*, 1991). From the receptor-bound state the imported proteins are transferred to a site which can be blocked by excess import-competent porin (Pfaller *et al.*, 1988). This site has been termed general insertion protein (GIP), and has been shown to be used by most imported mitochondrial precursor proteins. A putative component of GIP, the *N.crassa* MOM38 or, in yeast, ISP42 (Vestweber *et al.*, 1989; Baker *et al.*, 1990), has been found in a stable complex with both MOM19 and precursor proteins (Kiebler *et al.*, 1990; Söllner *et al.*, 1991).

From the GIP site, proteins follow different pathways depending on their final destination. Outer membrane proteins which lack a cleavable signal sequence are assembled into the outer membrane after leaving GIP (Hase *et al.*, 1984; Kleene *et al.*, 1987). Matrix proteins are transferred through contact sites between outer and inner membranes (Schleyer and Neupert, 1985). Passage of the targeting sequence through the inner membrane is dependent on the presence of a membrane potential (Pfanner and Neupert, 1985). The N-terminal targeting sequence is cleaved off by the matrix processing activity (Hawlitschek *et al.*, 1988; Pollock *et al.*, 1988; Witte *et al.*, 1988). Interaction with the matrix heat shock protein hsp70 appears to be involved in the translocation reaction (Kang *et al.*, 1990). Folding and assembly of the imported polypeptides to oligomeric proteins requires the participation of hsp60 (Cheng *et al.*, 1989; Ostermann *et al.*, 1989).

Several proteins of the intermembrane space are not directly transferred to this compartment. They are first imported through contact sites to the matrix where the first part of a bipartite signal sequence is proteolytically removed by the matrix processing activity. During this transient path through the matrix at least some of the proteins interact with the heat shock proteins hsp60 and hsp70 (see above). Then they are targeted to and translocated through the inner membrane. Finally, the second part of the signal sequence is cleaved off by inner membrane protease I (Schneider *et al.*, 1991). This sorting mechanism was termed 'conservative sorting' (Hartl and Neupert, 1990) since the pathway resembles protein export through the bacterial plasma membrane, and thus provides support for the endosymbiont theory of mitochondrial origin.

For proteins of the inner membrane, alternative pathways have been described (Mahlke *et al.*, 1990). Proteins either follow the usual pathway to the matrix and, from there, are inserted into the inner membrane (conservative sorting). Alternatively, inner membrane proteins may dissociate from the GIP state and enter the inner membrane directly without transiently entering the matrix (termed non-conservative sorting). For proteins of the intermembrane space a non-conservative sorting pathway has not been described so far, with the exception of the unusual import mechanism for apocytochrome *c* (Stuart and Neupert, 1990) which is translocated independently of the receptor–GIP complex.

We have studied the import pathway of cytochrome *c* heme lyase (CCHL), a protein localized in the intermembrane space (Nicholson *et al.*, 1988), where it appears to be associated with the outer and inner membranes

(Hergersberg,C., Griffiths,G. and Neupert,W., in preparation). The enzyme catalyses the covalent attachment of the heme group to cytochrome *c* and is also involved in the import of cytochrome *c* into the intermembrane space (Nicholson *et al.*, 1988; Dumont *et al.*, 1988; Hergersberg *et al.*, in preparation). Here, we show that CCHL represents an example of a novel, 'non-conservative' sorting pathway to the intermembrane space. Unlike proteins imported along the standard 'conservative' pathway, CCHL lacks a cleavable signal sequence, and its import occurs independently of a membrane potential. Both are necessary for translocation through the inner mitochondrial membrane. The initial steps of import across the outer membrane, however, are shared with the majority of mitochondrial proteins, since CCHL uses the MOM19–GIP receptor complex of the outer membrane. CCHL thus selectively imports via the translocation machinery of the outer membrane that usually acts in conjunction with that in the inner membrane. This result, therefore, shows that translocation reactions across outer and inner membranes are not obligatorily coupled.

Results

CCHL imported in vitro is correctly localized to the intermembrane space

Radioactively labelled cytochrome *c* heme lyase (CCHL) was synthesized in an *in vitro* transcription–translation system. Upon the addition of mitochondria and incubation at 25°C for 15 min in a standard import buffer (Pfanner and Neupert, 1985), CCHL efficiently associated with the organelles as tested by reisolation of the mitochondria by centrifugation (Figure 1A and B, lanes 2). Since CCHL lacks a cleavable signal sequence, externally added protease was used as a means to discriminate between imported, protease-protected CCHL and CCHL which is only bound to the outside of the organelle. By this criterion, about half of the total CCHL was protease-resistant, i.e. was imported into the mitochondria (lanes 3–5). If the protease treatment was performed in the presence of detergent, CCHL was completely degraded even at the lowest concentration of protease used (lanes 6–8). The rate of CCHL import was dependent on the temperature (Figure 1C). At 25°C the import was completed with a half time of ~1 min. In contrast to other mitochondrial precursor proteins the import reaction was found to be very fast even at low temperatures. At 10°C the half time of import was 4 min, while at 0°C CCHL became protease-resistant with a half time of 10 min.

To test whether CCHL had reached its functional location within the intermembrane space or whether it was only bound to the outer membrane in a protease-resistant conformation, mitochondria containing imported CCHL were treated with the mild detergent digitonin, to open the outer and the inner membranes sequentially, thereby releasing soluble enzymes from the intermembrane space and the matrix. Mitochon-

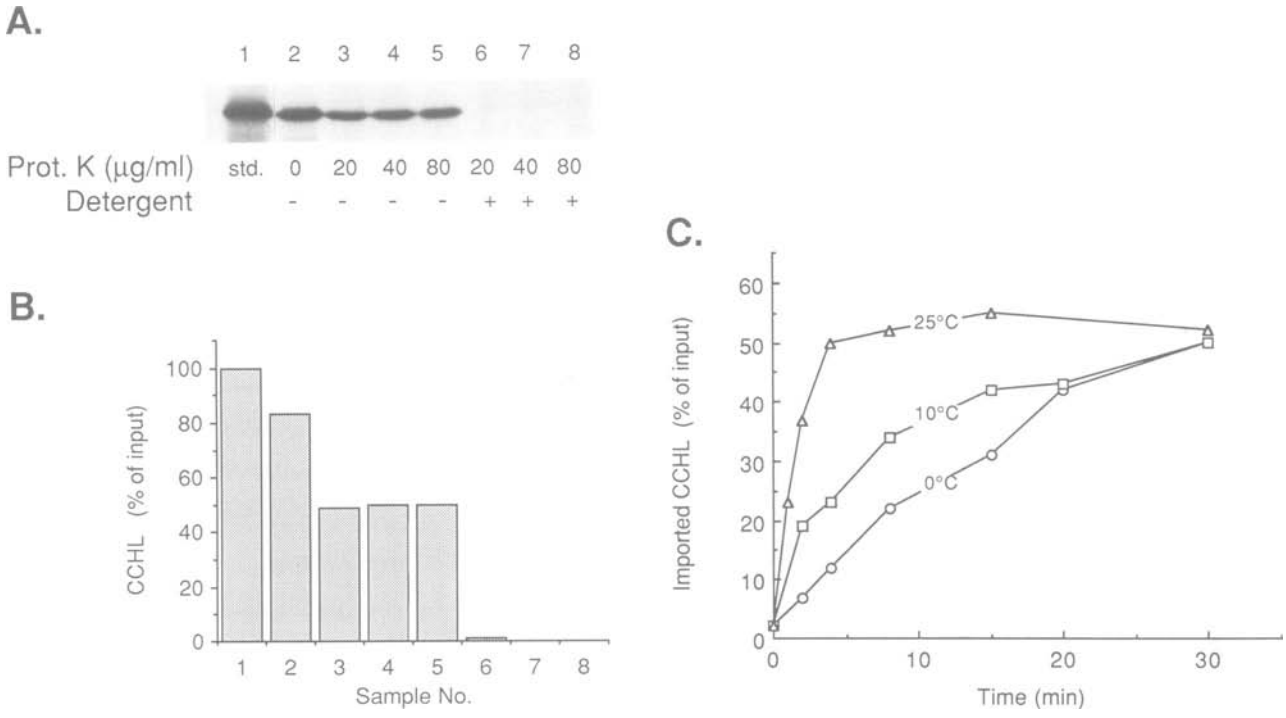

Fig. 1. Efficient *in vitro* import of CCHL into isolated mitochondria. (**A**) Freshly isolated mitochondria (30 μg protein) were incubated in BSA buffer for 15 min at 25°C with 6 μl reticulocyte lysate containing [^{35}S]methionine-labelled CCHL precursor in a total volume of 100 μl. Samples were transferred to 0°C and 1 ml of cold SME buffer was added. Mitochondria were reisolated by centrifugation for 12 min at 17 400 *g* in a Beckman JA-20 rotor and resuspended in 100 μl SME buffer at 0°C. Samples were treated with the indicated amounts of proteinase K for 15 min on ice in the absence or presence of 1% Triton X-100 detergent as indicated. Samples were precipitated with trichloroacetic acid (12.5% final concentration) and centrifuged in a microfuge. The pellets were washed once with cold acetone, dried, and subjected to SDS–PAGE. Radioactive CCHL was visualized by fluorography. Lane 1 represents the total input of CCHL used in the other import reactions. (**B**) Quantitation of the data from (A) by laser densitometry. The results are given relative to the total input radioactivity of CCHL (lane 1). (**C**) Temperature dependence of CCHL import. Mitochondria (30 μg protein) in 140 μl BSA buffer were mixed with 10 μl reticulocyte lysate containing [^{35}S]methionine-labelled CCHL precursor and incubated at 0°C (circles), 10°C (squares) or 25°C (triangles) for the indicated times. Samples were transferred to 0°C and treated with 30 μg/ml proteinase K for 15 min at 0°C. Then 1 ml of SMEP buffer was added, mitochondria were reisolated by centrifugation, and imported CCHL was analysed by SDS–PAGE, fluorography and laser densitometry.

dria were then isolated by centrifugation and analysed for radioactively labelled CCHL by gel electrophoresis and fluorography (Figure 2A). In addition, the enzyme activity of soluble markers for the intermembrane space (adenylate kinase) and the matrix (fumarase) was followed. Imported CCHL appeared to be tightly associated with the mitochondrial membranes (Figure 2A), i.e. it behaved indistinguishably from the enzyme activity of the endogenous protein (see Nicholson *et al.*, 1988). When digitonin-treated mitochondria were incubated with low amounts of protease, both the imported CCHL (Figure 2B) and the endogenous CCHL enzyme activity (Nicholson *et al.*, 1988) were degraded concomitantly with the opening of the outer membrane. These results indicate that CCHL imported *in vitro* is transferred to its functional environment, namely the intermembrane space where it is bound to mitochondrial membranes in a similar manner to that observed for the endogenous protein (Nicholson *et al.*, 1988; Hergersberg *et al.*, in preparation).

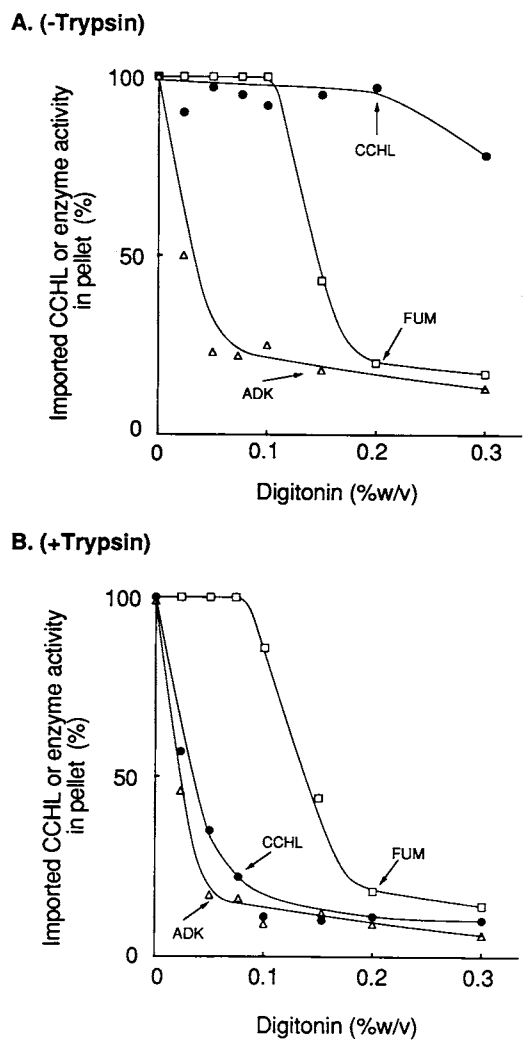

Fig. 2. Imported CCHL is membrane-bound and located within the intermembrane space. The [^{35}S]methionine-labelled CCHL precursor was imported into mitochondria (1.5 mg protein in 2 ml BSA buffer) at 25°C for 15 min. Following proteinase K treatment (20 μg/ml, 30 min at 0°C), the import reaction was diluted 2-fold with SMEP buffer. Mitochondria were reisolated by centrifugation, washed and resuspended in SME buffer containing 100 mM KCl (SMEK) and divided into eight aliquots. Digitonin treatment at the indicated concentrations was for 2 min at 0°C in a final volume of 40 μl at a protein concentration of 5 mg/ml. Samples were diluted 20-fold with SMEK and divided in half. One half was allowed to stand on ice (**A**), while the other half was treated with 25 μg/ml trypsin for 30 min at 0°C (**B**). After the addition of 1 vol of SMEP buffer containing 2 mg/ml soybean trypsin inhibitor, samples were again divided in half and separated into pellet and supernatant fractions by centrifugation for 10 min at 27 000 *g* (JA-20 rotor, Beckman). The pellets from one set of samples were subjected to SDS–PAGE, and analysed for imported CCHL by fluorography and laser densitometry (●). The remaining set of pellets was resuspended in SMEK buffer plus 1% (w/v) Genapol and used for determination of marker enzyme activities, adenylate kinase (ADK) and fumarase (FUM). The activities recovered in the samples with no digitonin were set as 100%.

Import of CCHL into mitochondria is independent of ATP and an electrochemical potential as energy sources

Most mitochondrial proteins like the β-subunit of F_1-ATPase need ATP for efficient import (Pfanner *et al.*, 1987). On the one hand, ATP has been shown to have a role in maintaining the import competence of the precursor form of imported proteins in the cytosol. This, at least in part, is achieved by the reversible binding of the precursor to the cytosolic heat shock proteins of the hsp70 class (Chirico *et al.*, 1988; Deshaies *et al.*, 1988). On the other hand, ATP is required for folding of imported polypeptides by and release from the chaperones hsp60 and hsp70 in the mitochondrial matrix (Ostermann *et al.*, 1989; Kang *et al.*, 1990). To test whether ATP is also required for the import of CCHL, the reticulocyte lysate containing the translated CCHL was first treated with increasing amounts of apyrase to deplete endogenous ATP and then used for import of

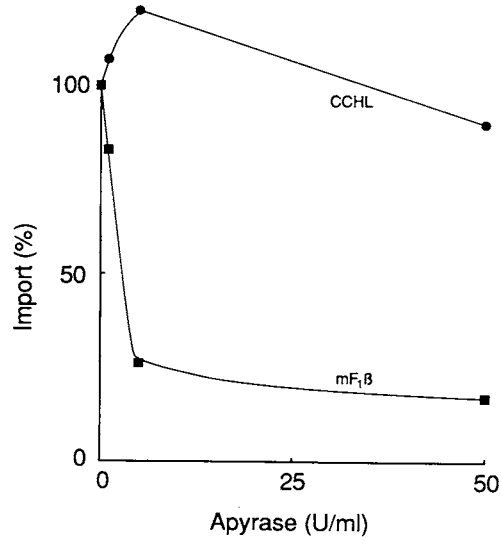

Fig. 3. Import of CCHL *in vitro* does not require ATP as an energy source. Reticulocyte lysate containing [^{35}S]methionine-labelled CCHL and the precursor of the β-subunit of F_1-ATPase were treated with the indicated concentrations of apyrase (Sigma grade VIII, ~250 U/mg protein) for 15 min at 30°C and 15 min at 25°C. Aliquots (10 μl) were incubated with isolated mitochondria (10 μg protein) for 10 min at 25°C in 100 μl BSA buffer containing 20 μM oligomycin, 0.2 μM carboxyatractyloside, 8 mM potassium ascorbate (adjusted to pH 7.2 before addition), and 0.2 mM *N,N,N',N'*-tetramethylphenylenediamine. The samples were treated for 30 min at 0°C with 10 μg/ml proteinase K and diluted with 1 ml SMEP buffer. Mitochondria were reisolated by centrifugation, washed once with SMEP buffer, and radioactive proteins analysed by SDS–PAGE, fluorography and laser densitometry. Import was assessed by the amount of protease-resistant CCHL or of protease-resistant, processed $F_1\beta$ ATPase ($mF_1\beta$). Results are given relative to the import observed without apyrase treatment.

CCHL into mitochondria. No influence of the ATP concentration on the import of CCHL was observed even at apyrase concentrations well above that completely inhibiting the import of the β-subunit of F_1-ATPase (Figure 3). Consistent with this result, the addition of up to 10 mM γS-ATP to the import reaction did not influence the efficiency of translocation of CCHL into mitochondria either in the presence or in the absence of ATP (data not shown). As determined by fractionation of mitochondria with digitonin, CCHL imported in the absence of ATP behaved like the endogenous protein and like CCHL imported with ATP (cf. Figure 2B), i.e. CCHL became degraded by externally added protease upon the opening of the intermembrane space (data not shown). Thus, import of CCHL is independent of free ATP as an energy source which suggests that CCHL either does not interact with cytosolic hsp70 proteins prior to the import reaction or, as a less likely interpretation, does not need ATP hydrolysis for dissociation from hsp70 proteins.

A membrane potential across the mitochondrial inner membrane is required for proteins to be translocated through the inner mitochondrial membrane (Pfanner and Neupert, 1985; Martin *et al.*, 1991). This is the case for proteins of the mitochondrial matrix and for proteins of the intermembrane space following the 'conservative sorting' pathway involving a transient passage through the mitochondrial matrix (Hartl *et al.*, 1987). Unlike the proteins traversing the inner membrane, outer membrane proteins like porin do not require a membrane potential (Freitag *et al.*, 1982). If during the *in vitro* import of CCHL the electrochemical potential was depleted by the addition of the uncoupler CCCP, no influence was detectable on the import efficiency of CCHL (Figure 4A). In comparison, import of the β-subunit of F_1-ATPase was strongly inhibited. Similar results were obtained when the import was performed in the presence of the ionophore valinomycin and of inhibitors of both the electron transport chain and the F_1F_0-ATPase, antimycin A and oligomycin (Figure 4B). The slight reduction of CCHL import by ~20% was caused by a partial opening of the intermembrane space upon the addition of valinomycin. The reduction correlated well with the accessibility for digestion by proteinase K of a marker enzyme for the intermembrane space, adenylate kinase (data not shown). These data indicate that imported CCHL reached its correct location within the intermembrane space without requiring an electrochemical potential. The results suggest that CCHL does not pass through the inner membrane during import into the intermembrane space. In addition, CCHL does not possess cleavable signal sequences (Dumont *et al.*, 1987; Drygas *et al.*, 1989) which could direct import into and export out of the mitochondrial matrix.

CCHL uses the MOM19 – GIP complex for import

Protein import into mitochondria is generally mediated by interaction of precursor proteins with surface receptors on the outer membrane. The receptors are degraded by treating mitochondria with low amounts of proteinase thereby leading to a block of import (Zwizinski *et al.*, 1984; Pfaller and Neupert, 1987; Pfaller *et al.*, 1988). If mitochondria were pretreated with low amounts of proteinase K (Figure 5), trypsin, or elastase (data not shown), import of CCHL was greatly diminished. Similarly, import of the β-subunit of F_1-ATPase was affected in the same concentration range of the protease (Figure 5).

To establish whether CCHL uses one of the so far characterized receptor proteins in the outer membrane, namely MOM19 and MOM72 (Söllner *et al.*, 1989, 1990; Steger *et al.*, 1990), these receptors were functionally

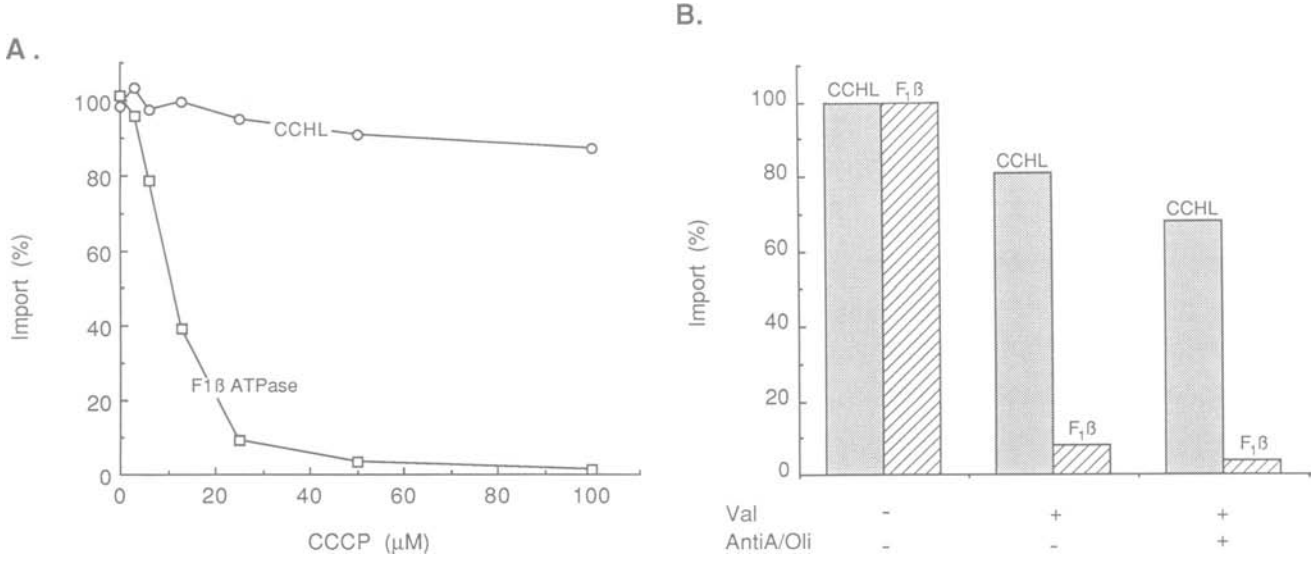

Fig. 4. Import of CCHL does not require an electrochemical potential across the inner mitochondrial membrane. (**A**) Freshly isolated mitochondria (0.3 mg/ml) were energized by the addition of 8 mM potassium ascorbate and 0.2 mM *N,N,N',N'*-tetramethylphenylenediamine. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added at the indicated concentrations by dilution from a 50-fold concentrated stock solution in ethanol. 5 μl reticulocyte lysate containing the [^{35}S]methionine-labelled precursors of either CCHL or the β-subunit of F_1-ATPase were added to a final volume of 100 μl of BSA buffer. Samples were incubated for 10 min at 25°C and treated with 30 μg/ml proteinase K for 15 min at 0°C. After the addition of 1 ml SMEP buffer, mitochondria were reisolated by centrifugation and washed once with SMEP. Imported, protease-protected proteins were resolved by SDS–PAGE and quantified by laser densitometry of the fluorographs. Results are shown relative to the import without CCCP. (**B**) The import reactions were performed and analysed as in (A) in the presence or absence of 0.5 μM valinomycin (Val) and 8 μM antimycin A plus 20 μM oligomycin (AntiA/Oli) as indicated. Import results are given for protease-resistant CCHL and for protease-resistant, processed $F_1\beta$ ATPase relative to the respective import without inhibitors.

blocked by specific antibodies. Subsequent import of CCHL (and, as a control, of the β-subunit of F_1-ATPase) was affected specifically by antibodies against MOM19 (Figure 6), the receptor which has been shown to be required for the import of a large number of mitochondrial proteins. Antibodies against MOM72, the import receptor used by the ADP/ATP carrier, had only a minor effect on CCHL import, and did not interfere with the import of the β-subunit of F_1-ATPase. Neither antibodies derived from pre-immune serum nor antibodies directed against porin influenced the amount of imported CCHL or of the β-subunit of F_1-ATPase. It appears that CCHL during import into mitochondria uses the entry site common to most mitochondrial proteins, namely MOM19.

Import of the majority of mitochondrial precursor proteins can be inhibited by blocking the import sites in the outer membrane with chemical amounts of water-soluble (i.e. import-competent) porin. Based on the extractability behaviour of porin in this state it was concluded that porin is bound to a hydrophilic, proteinaceous site. This site was termed the general insertion protein (GIP), since it is apparently used by almost all precursor proteins analysed so far (Pfaller *et al.*, 1988). Most likely, the GIP function resides in a stable complex consisting of a set of outer membrane proteins and the MOM19 receptor (Kiebler *et al.*, 1990; Söllner *et al.*, 1991). To substantiate further our observation that CCHL uses this MOM19–GIP complex for translocation across the outer membrane, import of CCHL was performed in the presence of inhibiting amounts of water-soluble porin in order to block the import sites. Reticulocyte lysate containing radioactively labelled CCHL and, as a control, porin, were incubated with mitochondria together with increasing concentrations of water-soluble porin. The import of CCHL and that of porin was strongly inhibited in a similar concentration range of water-soluble porin (Figure 7). This result further establishes that CCHL, like porin and other precursor proteins, uses the MOM19–GIP complex for its translocation into the mitochondrial intermembrane space.

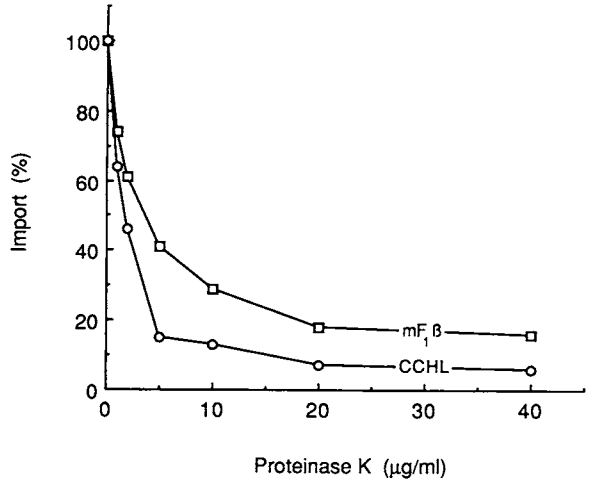

Fig. 5. Import of CCHL requires a protease-sensitive receptor in the outer membrane. Freshly isolated mitochondria (0.4 mg protein/ml SME) were treated with the indicated concentrations of proteinase K for 15 min at 0°C. Protease activity was halted by the addition of 1 mM PMSF. 1 ml SMEP was added and mitochondria were reisolated by centrifugation, and resuspended in BSA buffer containing 8 mM potassium ascorbate (adjusted to pH 7.2 before addition) and 0.2 mM *N,N,N',N'*-tetramethylphenylenediamine at a protein concentration of 0.4 mg/ml. The protease-treated mitochondria (40 μg protein) were mixed with the [^{35}S]methionine-labelled precursors of the β-subunit of F_1-ATPase and of CCHL (in 10 μl reticulocyte lysate). Samples were incubated for 15 min at 25°C and treated with 40 μg/ml proteinase K for 15 min at 0°C. After dilution with 1 ml SMEP buffer, mitochondria were collected by centrifugation and washed once with SMEP buffer. The resulting pellets were subjected to SDS–PAGE. The gels were fluorographed and radioactive bands corresponding to protease-protected, matured $F_1\beta$-ATPase ($mF_1\beta$) or CCHL were quantified by laser densitometry. The data are given relative to the import without protease pretreatment.

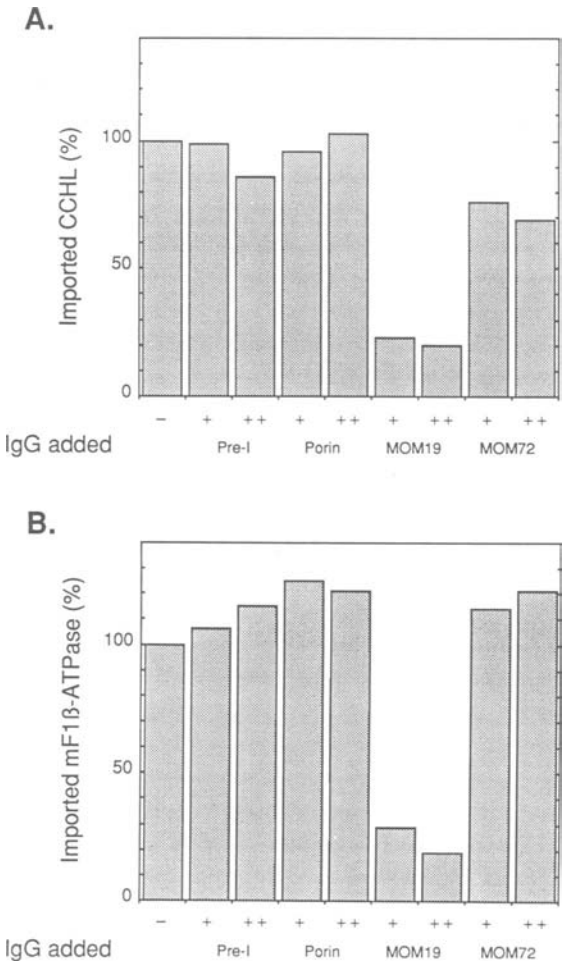

Fig. 6. Import of CCHL requires functional MOM19. Mitochondria (0.1 mg/ml) were preincubated with immunoglobulin G (IgG) isolated from preimmune serum (Pre-I) or IgG raised against the outer membrane proteins porin, MOM19 and MOM72. The binding of IgG was performed in 200 μl BSA buffer containing 12 μg *N.crassa* proteinase inhibitor fraction (Schmidt *et al.*, 1984) and 2 μg α_2-macroglobulin. Final concentrations of IgG were 40 μg/ml (+) and 100 μg/ml (++). After 40 min at 0°C mitochondria were reisolated by centrifugation, resuspended at 0.1 mg/ml protein concentration, and divided in half. To one half 5 μl of reticulocyte lysate containing [^{35}S]methionine-labelled CCHL precursor was added. The other half received 5 μl of [^{35}S]methionine-labelled β-subunit of F_1-ATPase together with 8 mM potassium ascorbate and 0.2 mM *N,N,N',N'*-tetramethylphenylenediamine in order to form a membrane potential. Samples were incubated for 15 min at 25°C and then treated with 15 μg/ml proteinase K for 15 min at 0°C. After the addition of 1 ml SMEP, mitochondria were reisolated by centrifugation and washed once with SMEP buffer. Imported, protease-protected proteins were resolved by SDS–PAGE and quantified by laser densitometry of the fluorographs. Results are given for (**A**) protease-resistant CCHL and (**B**) protease-resistant, processed $F_1\beta$-ATPase ($mF_1\beta$) relative to the import observed without preincubation with IgG (–).

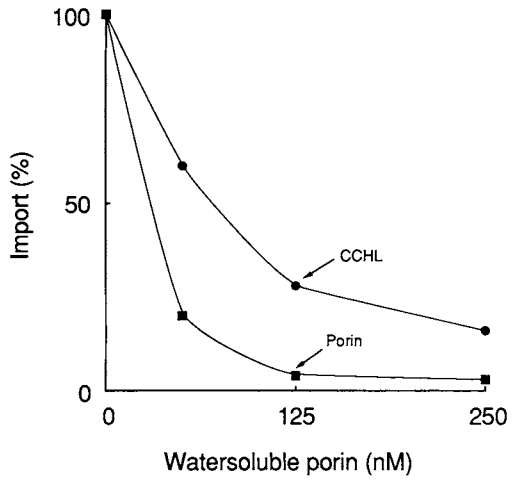

Fig. 7. Import of CCHL is inhibited by water-soluble porin. The precursors of porin and CCHL were synthesized in reticulocyte lysate in the presence of [^{35}S]methionine. 20 μl of the lysates were incubated with mitochondria (10 μg protein) in the presence of water-soluble porin dissolved in 50 μl of 0.1 M sodium phosphate, pH 6.8 (Pfaller and Neupert, 1987) at the concentrations indicated. The final volume was adjusted to 200 μl with BSA buffer and the samples were incubated for 40 min at 0°C. Mitochondria were then recovered by centrifugation, resuspended in 100 μl of BSA buffer, and incubated for 15 min at 25°C. Samples of the porin and the CCHL import reactions were treated with 200 μg/ml and 20 μg/ml of proteinase K, respectively, at 0°C for 30 min. Protease activity was halted by the addition of 1 ml SMEP buffer. Mitochondria were reisolated by centrifugation and washed once with SMEP buffer. Imported porin and CCHL were quantified by SDS–PAGE and laser densitometry of the resulting fluorographs. Results are given relative to the import observed in the absence of water-soluble porin.

Discussion

Upon import into isolated mitochondria cytochrome *c* heme lyase (CCHL) reaches its functional location, the mitochondrial intermembrane space, without undergoing a proteolytic cleavage. Such a result is expected from the protein sequence which suggests the lack of a cleavable signal sequence (Dumont *et al.*, 1987; Drygas *et al.*, 1989). CCHL uses the MOM19–GIP receptor complex for its initial interaction with the mitochondrial outer membrane, and then moves into the intermembrane space directly without transient passage across the inner membrane and the matrix according to the 'conservative sorting' pathway (Hartl and Neupert, 1990). Thus, import of CCHL defines a novel, 'non-conservative' import pathway into the intermembrane space.

The conclusion that CCHL obviously does not traverse the inner membrane but enters the intermembrane space directly is supported by the following arguments. First, CCHL lacks a typical mitochondrial signal sequence which is thought to mediate the transfer across the inner membrane. Second, CCHL lacks a sorting sequence which could redirect it from the matrix side back through the inner membrane into the intermembrane space (Dumont *et al.*, 1987; Drygas *et al.*, 1989) Third, transport of CCHL does not require an electrochemical potential across the inner membrane. An energized membrane seems to be a necessary requirement for all proteins transported through the inner membrane. It is believed that positive charges within the signal sequence mediate the movement of the precursor proteins through the inner membrane (Martin *et al.*, 1991). According to this model one would not predict an interaction of CCHL with and translocation through the inner membrane. Taken together, the data presented provide evidence that CCHL translocates into the mitochondrial intermembrane space by using a direct pathway through the outer membrane rather than the more commonly used pathway through the inner membrane and the matrix (Hartl and Neupert, 1990).

The import pathway of CCHL is unusual in yet another aspect. Practically all precursor proteins require ATP in the cytosol for import which is believed to be necessary for the release of bound protein factors, in particular of cytosolic heat shock protein hsp70. Release of hsp70 is assumed to be the rate-limiting step of the import reaction, since treatment of precursor proteins with 8 M urea followed by rapid dilution leads to release of hsp70 and unfolding of the precursor. At the same time, urea-treated precursors are imported quite rapidly and efficiently even at low temperatures. Therefore, in contrast to most mitochondrial precursor proteins, import of CCHL does not require the presence of ATP, occurs at a high rate, and is affected to only a minor degree by lowering the temperature to 0–10°C. All these findings support the notion that the CCHL precursor does not interact strongly with hsp70 or related factors. The reason for this behaviour may be related to the unusually high content of proline residues (11.6%) in the *N.crassa* CCHL.

The fact that CCHL requires neither ATP hydrolysis nor a membrane potential for import, raises the interesting question of what energetically drives targeting to and import into mitochondria. The targeting signal most likely does not reside in the extreme N-terminus of CCHL, since nine or 36 N-terminal amino acids can be deleted without any effect on the import efficiency (unpublished results). In addition, no amphipathic helix typical of mitochondrial presequences (von Heijne, 1986) can be drawn for the N-terminus of CCHL. Thus, the import signal must reside somewhere internal as found in the case of proteins which also lack a cleavable N-terminal signal sequence like porin (Kleene *et al.*, 1987), the ADP/ATP carrier or MOM72 (Steger *et al.*, 1990). The driving force for import may come from folding of CCHL within the intermembrane space. It will be interesting to see whether such folding reaction is assisted by other, yet to be discovered factors. Alternatively, CCHL may be pulled into the intermembrane space by specific interaction with a binding partner. Since CCHL avidly binds to liposomes, such an interaction may include binding to lipid molecules in the outer and inner membranes. These questions can now be addressed by attempting import of CCHL as a model protein into vesicles of isolated outer membranes.

At a first glimpse, the import of CCHL into the intermembrane space bears some resemblance to that of apocytochrome *c*. The latter also only crosses the outer membrane (Stuart and Neupert, 1990), and its import occurs independently of an electrochemical potential (Zimmermann *et al.*, 1981). A closer look, however, shows that these two proteins use distinct mechanisms for their passage through the outer membrane. Apocytochrome *c*, in contrast to CCHL, does not use the MOM19–GIP receptor complex in the outer membrane for its import (Nicholson *et al.*, 1988). Rather, the protein spontaneously inserts with low affinity into the outer membrane by interacting with the acidic phospholipids of the outer membrane (Rietveld *et al.*, 1985). Subsequent interaction with the CCHL enzyme which serves as a high-affinity binding site (Nicholson *et al.*, 1988) leads

to a stable intermediate if the covalent attachment of the heme group is precluded (Hergersberg *et al.*, in preparation). The covalent modification with heme is thought to induce a conformational change in the protein chain thereby triggering its membrane transit (Hennig and Neupert, 1983). Thus, the molecular import mechanisms for apocytochrome *c* and CCHL differ in a number of aspects, and thus can be regarded as distinct, independent processes.

Aside from describing a novel import pathway into the intermembrane space, our studies bear relevance to the general protein import pathway into mitochondria. Import of proteins across the outer membrane is not necessarily coupled to translocation across the inner membrane, i.e. the two translocation machineries in the two membranes do not necessarily form a continuous channel. Indication for this has come already from studies showing that segments of polypeptide chains in transit can be exposed to the intermembrane space, either before they engage in translocation across the inner membrane or when translocation across the outer membrane is completed but not that across the inner membrane (Rassow and Pfanner, 1991).

The identification of this novel, non-conservative sorting pathway of CCHL into the intermembrane space opens a number of interesting questions. What is the mitochondrial targeting signal within CCHL? How does CCHL bind to the membranes in the intermembrane space? How does CCHL leave the general import route to enter the intermembrane space directly? Is it simply the lack of interaction with the inner membrane which allows it to escape the default pathway? Is CCHL imported on the whole surface of the outer membrane or only at contact sites? How does the import pathway of cytochrome c_1 heme lyase which shares homology with CCHL (A.Haid, personal communication) compare with that of CCHL? These questions are now accessible to a direct experimental examination, as an *in vitro* import system for CCHL has been developed.

Materials and methods

Biochemical procedures

The following published procedures were used: isolation of mitochondria from *N.crassa* (wild type 74A) (Stuart *et al.*, 1990); protein determination with immunoglobulin G (IgG) as a standard (Bradford, 1976); fractionation of mitochondria with digitonin (Schwaiger *et al.*, 1987); measurement of the enzyme activities of adenylate kinase (Schmidt *et al.*, 1984) and fumarase (Kanarek and Hill, 1964); separation of proteins by electrophoresis on 15% SDS-polyacrylamide gels and the preparation of the gels for fluorography (Nicholson *et al.*, 1987); quantification of the fluorographs by laser scanning densitometry (Pfaller *et al.*, 1988); treatment of the reticulocyte lysates with apyrase (Pfanner *et al.*, 1987); preparation and competition studies with water-soluble porin (Pfaller and Neupert, 1987; Pfaller *et al.*, 1988); 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).

Import of precursor proteins into mitochondria

Precursor proteins of CCHL and the β-subunit of F_1-ATPase were synthesized in the presence of [^{35}S]methionine in a coupled transcription-translation system (Pelham and Jackson, 1976; Stueber *et al.*, 1984; Rassow *et al.*, 1989). Post-ribosomal supernatants were prepared and supplemented as described (Zimmermann and Neupert, 1980). A full-length cDNA encoding cytochrome *c* heme lyase (CCHL) cloned into bluescript vector (Stratagene) was used. The cDNA for the β-subunit of F_1-ATPase was inserted into pGEM vector (Promega). If not otherwise stated, import of the precursors (in 5-10 μl lysate) into 20-40 μg (protein) freshly isolated *N.crassa* mitochondria was performed in 100 μl BSA buffer (250 mM sucrose, 3% (w/v) fatty acid free BSA, 80 mM KCl, 5 mM $MgCl_2$ and 10 mM MOPS/KOH, pH 7.2). Import times and temperatures are given in the individual experiments. The additions of oligomycin, antimycin A, valinomycin, potassium ascorbate and of *N,N,N',N'*-tetramethylphenylenediamine were according to Pfanner and Neupert (1986). Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was freshly dissolved in ethanol as a 2 mM stock solution and further diluted to give 50-fold concentrated stock solutions. To assess import of precursor proteins into mitochondria samples were treated with 30 μg/ml proteinase K for 15 min on ice, unless stated otherwise. 2 mM phenylmethylsulphonylfluoride (PMSF) was added from a freshly prepared 200 mM stock solution in ethanol, and samples were diluted with 1 ml SMEP buffer (250 mM sucrose, 10 mM MOPS/KOH, pH 7.2, 2 mM EDTA and 1 mM PMSF). Mitochondria were reisolated by centrifugation for 12 min at 17 400 *g* in a Beckman JA-20 rotor. The pellets were washed once with 1 ml SME buffer (250 mM sucrose, 10 mM MOPS/KOH, pH 7.2, 2 mM EDTA) and analysed for imported radioactive protein by gel electrophoresis, fluorography and laser densitometry.

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