Biogenesis of Mitochondrial Heme Lyases in Yeast

IMPORT AND FOLDING IN THE INTERMEMBRANE SPACE*

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Heme lyases are components of the mitochondrial intermembrane space facilitating the covalent attachment of heme to the apoforms of c-type cytochromes. The precursors of heme lyases are synthesized in the cytosol without the typical N-terminal mitochondrial targeting signal. Here, we have analyzed the mode of import and folding of the two heme lyases of the yeast Saccharomyces cerevisiae, namely of cytochrome c heme lyase and of cytochrome c₁ heme lyase. For transport into mitochondria, both proteins use the general protein import machinery of the outer membrane. Import occurred independently of a membrane potential, ΔΨ, across the inner membrane and ATP in the matrix space, suggesting that the inner membrane is not required for transport along this direct sorting pathway. The presence of a large folded domain in heme lyases was utilized to study their folding in the intermembrane space. Formation of this domain occurred at the same rate as import, indicating that heme lyases fold either during or immediately after their transfer across the membrane. Folding was not affected by depletion of ATP and ΔΨ or by inhibitors of peptidylprolyl cis-trans isomerases, i.e. it does not involve homologs of known folding factors (like Hsp60 and Hsp70). The energy derived from folding cannot be regarded as a major driving force for import, since the folded domain could be imported into mitochondria with the same efficiency as the intact protein. We conclude that protein folding in the intermembrane space obeys principles different from those established for other subcellular compartments.

Heme lyases are involved in the biogenesis of c-type cytochromes and are thought to catalyze the covalent attachment of heme to the apoforms of the cytochromes (1–3). At present, the genes of three enzymes have been identified, the cytochrome c heme lyases (CCHL)1 of Neurospora crassa (4) and the yeast Saccharomyces cerevisiae (5) as well as cytochrome c₁ heme lyase (CC₁HL) (6) of yeast. These proteins are homologous and share about 35% amino acid sequence identity (50% similarity). They are located in the mitochondrial intermembrane space, where they are peripherally associated with the inner membrane (7, 8). The functional role of heme lyases is only partially understood. CCHL appears to have a dual function during the biogenesis of cytochrome c. First, it has been demonstrated to serve as a high affinity binding site for apocytochrome c (7, 9). By forming a stable complex, CCHL renders the reversible passage of apocytochrome c across the mitochondrial outer membrane unidirectional (10). Second, CCHL is required for the covalent attachment of heme to apocytochrome c (5, 7). In this reaction, the two vinyl groups of heme are linked with cysteines 14 and 17 of apocytochrome c to form thioether bonds. Apart from the fact that the reduced form of heme is required for the conversion to holocytochrome c (11), the molecular mechanisms underlying this complicated reaction are still enigmatic. Even less is known about the involvement of CC₁HL in heme attachment to cytochrome c₁ in the intermembrane space. A direct function for CC₁HL has been inferred from studies showing heme addition as a prerequisite for the maturation of the intermediate form of cytochrome c₁ (6, 12–14).

Unlike most other mitochondrial precursor proteins, heme lyases do not contain typical mitochondrial targeting sequences (presequences) at their N termini. Thus, their biogenesis is expected to be distinct from that of the bulk of mitochondrial preproteins. The import pathway has been investigated in some detail for CCHL from N. crassa (15). Its precursor is transported to the functional location, the outer face of the inner membrane, by direct passage across the mitochondrial outer membrane without a requirement for the inner membrane (see also Ref. 16). Import occurs independently of the inner membrane potential, ΔΨ, and does not require external energy sources like ATP. Thus, this pathway is markedly different from that of other constituents of the intermembrane space, e.g. the cytochromes c₁ and b₂, which require an energized inner membrane for import (12).

Many important aspects of the biogenesis of heme lyases are not understood. For example, it is unknown whether the pathway defined by N. crassa CCHL is also shared by the homologous CC₁HL and possibly by other preproteins. The independence of the import on external energy sources like ATP poses the problem of the driving force of this reaction. The absence of an N-terminal presequence raises the important question of the location and nature of the targeting signal in heme lyases. Finally, it is not known how heme lyases become folded in the intermembrane space and whether the free energy change of folding can be regarded as a driving force for import. At present, no information on the mechanism of protein folding in this compartment is available.

To address some of these questions, we decided to investigate the biogenesis of the two heme lyases from the yeast S. cerevisiae. First, we have elucidated the import pathway of these

1 The abbreviations used are: CCHL, cytochrome c heme lyase; CC₁HL, cytochrome c₁ heme lyase; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; MPP, matrix processing peptidase; FCCP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone.

2 I. van der Klei and R. Lill, unpublished data.
proteins into their functional location. According to our results, yeast heme lyases are imported directly across the outer membrane with no apparent requirement for the inner membrane. This makes it likely that other proteins of this compartment use a similar sorting pathway. Second, we examined the folding of these enzymes into their native structure by following the generation of a protease-resistant domain upon import into the intermembrane space. Folding was found to occur during or immediately after import. It did not involve folding factors acting similarly to those known from other cellular compartments, e.g. Hsp60 and Hsp70 chaperone proteins (17) or peptidyl-prolyl cis-trans isomerases. Thus, folding in the intermembrane space either occurs in a spontaneous fashion without the assistance of chaperone proteins or folding is mediated by so far unknown factors and mechanisms. Finally, our studies render folding of the heme lyases as a driving force for their import unlikely.

**MATERIALS AND METHODS**

**DNA Manipulations**—Genomic DNA coding for either CCHL (5) or CC1HL (6) was cloned into the Smal site of the transcription vector pGEM4 (Promega) as either blunt-ended Dral-DralI or AflII-EcoRV fragments using standard molecular biology techniques (18). Antibodies Against Yeast Heme Lyases—For generation of antisera against yeast CCHL, a blunt-ended BanI-Sall fragment was cloned into the blunt BamHI site of plasmid pGEX4T3 (Pharmacia Biotech Inc.). This created a glutathione S-transferase-CCHL fusion protein lacking the 25 amino-terminal amino acid residues of CCHL. The fusion protein was expressed in E. coli cells strain BL21(DE3), isolated from inclusion bodies, and used as an antigen to immunize rabbits. Antibodies against yeast CC1HL were raised against a TrpE-CC1HL fusion protein as previously described (6).

Biochemical Procedures—The following published methods were used: isolation of mitochondria from S. cerevisiae wild type strain D273-10B (19), pretreatment of mitochondria with trypsin (20), osmotic shock (21) to generate mitoplasts (21), use of protease-sensitive markers of either the intermembrane space (cytochrome b$_{559}$) (21) or the matrix space (MIM44) (22) and precipitation of proteins with trichloroacetic acid (23), alkaline extraction (24), urea denaturation of radiolabeled precursor proteins (25), and SDS-PAGE and fluorography of resulting gels and quantitation of fluorographs by densitometry (16). Immunoblotting and detection by chemiluminescence using the ECL kit (Amersham International, Amersham, United Kingdom) and protein determination using bovine serum albumin as a standard (Bio-Rad) was performed according to the instructions of the suppliers. The inhibitors of peptidyl prolyl cis-trans isomerases, cyclosporin A and FK506, were obtained from Sandoz and Fujisawa Co., respectively.

Import of Precursor Proteins into Mitochondria—Precursor proteins of CCHL, CC1HL, cytochrome C$_{b}$, the $b$-subunit of the F$_{1}$-ATPase, and the $\alpha$-subunit of matrix processing peptidase were synthesized by in vitro transcription and translation in reticulocyte lysate using $^{35}$S-methionine as a label (26). Import of radiolabeled precursor proteins was performed in 100 µl of import buffer (0.6 M sorbitol, 50 mM HEPES-KOH (pH 7.0), 50 mM KCl, 10 mM MgCl$_{2}$, 2 mM KH$_{2}$PO$_{4}$, 2.5 mM EDTA, and 1 mg/ml fatty acid-free bovine serum albumin) containing 2 mM NADH and 50 µg of mitochondrial protein. After incubation for 3 min at 25°C, radiolabeled precursor proteins in reticulocyte lysate (1-5 µl) or 2 µl of urea-denatured precursor proteins (final urea concentration, 160 mM) were added. Unless stated otherwise, incubation was continued for 30 min at 25°C. Samples were chilled on ice and treated with 200 µg/ml proteinase K for 30 min at 0°C. Proteinase digestion was halted by the addition of 1 mM PMSF from a freshly prepared 100 mM stock solution in ethanol. After 5 min on ice, samples were diluted with SoH buffer (0.6 M sorbitol, 20 mM HEPES-KOH, pH 7.4) containing 1 mM PMSF to a final volume of 1 ml. Mitochondria were isolated by centrifugation for 12 min at 10,000 x g in a Beckman J18.1 rotor at 2°C. Further manipulations were to be done, centrifugation was for 7 min at 6,000 x g. Mitochondria were resuspended in 100 µl of SoH buffer, and proteins were precipitated with trichloroacetic acid. Following SDS-PAGE and fluorography, radioactive imported proteins were quantitated by densitometry.

**Manipulation of ATP Levels**—Inside and Outside of Mitochondria—Extracellular ATP outside the mitochondrial inner membrane was selectively depleted by incubating mitochondria in import buffer (containing 2 mM NADH and 20 mM glucose) in the presence of 20 µM carboxyatractylonide for 5 min at 0°C. A mixture of hexokinase/myokinase (50–100 µg/ml each, Boehringer Mannheim) was added, and incubation was continued for 10 min at 25°C. In parallel, reticulocyte lysates containing radiolabeled precursor proteins were depleted of ATP by treatment with hexokinase/myokinase in the presence of 20 mM glucose as above. Depletion of internal matrix ATP was as described (27). For the nonselective depletion of total ATP, mitochondria (samples con-
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Heme Lyases Are Transported into the Intermembrane Space via a Direct Sorting Pathway—The import pathway of yeast heme lyases was investigated using radioactively labeled preproteins that had been synthesized by in vitro translation in reticulocyte lysates. After incubating the precursors of CCHL and CC1HL with isolated mitochondria, a fraction of both proteins became resistant to added protease (Fig. 1A). Upon lysis of the mitochondria by detergent, the heme lyases became completely degraded, indicating that the protease resistance was a result of import and was not caused by other reasons, e.g., aggregation of the precursors outside the mitochondria. A strong temperature dependence of the import reaction was observed (Fig. 1B). While CC1HL was imported at 0°C, albeit at a very slow rate, no import was observed with CCHL at 10°C and below. With the latter protein, the import efficiency was considerably lower, even if the import was performed at 25°C. The amounts of imported material could not be significantly increased by denaturing CCHL in urea before the import reaction (data not shown). This makes it unlikely that the low import competence of CCHL was the result of the tight folding of this preprotein.

Does the transport of heme lyases require the protein import complex of the mitochondrial outer membrane? To examine this question, the protease-sensitive receptors at the mitochondrial surface were inactivated by pretreating the mitochondria with trypsin. Import of both heme lyases was strongly reduced, applying trypsin concentrations known to degrade the surface receptors (Fig. 1C, higher than 20 μg/ml) (cf. Refs. 28 and 29). The behavior of a matrix-targeted protein, the precursor of the α-subunit of matrix processing peptidase (preMPP), was identical. Thus, import of heme lyases into mitochondria depends on protease-sensitive components of the outer membrane, suggesting that these proteins use the general protein import machinery of the mitochondrial outer membrane.

The submitochondrial localization of imported heme lyases was determined by subfractionation of the mitochondria. After import, the organelles were treated in hypotonic buffer to effect swelling of the mitochondria and rupture of the outer membrane (21). As a result, proteins of the intermembrane space and the inner membrane become accessible to digestion by added protease, while components beyond the inner membrane remain resistant. Both imported heme lyases behaved identical to the endogenous enzymes and were degraded after opening of the outer membrane (Fig. 2A). Thus, they had reached their functional location, the intermembrane space. Unlike cytochrome b2, a soluble constituent of the intermembrane space, imported and endogenous heme lyases sedimented with the mitoplasts (i.e. mitochondria with a disrupted outer membrane) (21). As a result, proteins of the intermembrane space and the inner membrane become accessible to digestion by added protease, while components beyond the inner membrane remain resistant. Both imported heme lyases behaved identical to the endogenous enzymes and were degraded after opening of the outer membrane (Fig. 2A). Thus, they had reached their functional location, the intermembrane space. Unlike cytochrome b2, a soluble constituent of the intermembrane space, imported and endogenous heme lyases sedimented with the mitoplasts (i.e. mitochondria with a disrupted outer membrane), indicating that they were membrane bound. The heme lyases remained stably associated with the membranes even at higher ionic strength (Fig. 2B), in contrast to soluble constituents of the intermembrane space like cytochrome c. To further analyze the sub-mitochondrial localization of the imported proteins, an extraction with alkaline buffers was performed (24). About half of both endogenous and imported CCHL was recovered in the supernatant, whereas CC1HL was fully resistant to space. MIM44 and Hsp70 (heat shock protein of 70 kDa) are located in the matrix; the ADP/ATP carrier (AAC) is an integral protein of the inner membrane.

**RESULTS**

**A**

![Diagram showing import and protease-sensitive components of the outer membrane.](http://www.jbc.org/)

**B**

![Diagram showing import and protease-sensitive components of the outer membrane.](http://www.jbc.org/)

**C**

![Diagram showing import and protease-sensitive components of the outer membrane.](http://www.jbc.org/)
supernatant (cytochrome solubilization (Fig. 2C)). Control proteins were recovered in the forces anchor heme lyases to the membrane. Interaction is unexpected. It remains to be determined which heme lyases are hydrophilic proteins and do not contain any suggests the correct localization of the imported material. Since efficient import can occur in the absence of external energy sources like a membrane potential, Δψ, or ATP in the matrix, the inner membrane translocation machinery is apparently not involved in import.

CC1HL becomes rapidly folded upon import—To date, nothing is known about the components and mechanisms involved in folding of proteins in the intermembrane space. In particular, the question of how heme lyases adopt their functional conformation has not been addressed. To examine the folding of heme lyases in the intermembrane space, we sought to identify a domain in these proteins that is resistant to the attack by added proteases. Digestion of endogenous CC1HL and CCHL in mitoplasts with trypsin resulted in the generation of stable fragments of 25 and 21 kDa, respectively (Fig. 4A; the apparent molecular masses of the intact proteins are 31 and 34 kDa, respectively). As observed for the intact proteins (cf. Fig. 2), these large fragments were tightly associated with nitrocellulose, and analyzed for CCHL, CC1HL, and their folded fragments by immunostaining. B, CC1HL was imported into mitochondria (30 min at 25°C) by diluting the precursor 50-fold from reticulocyte lysate (RL) or from 8M urea into the import mixture. Following proteinase K treatment, mitochondria were reisolated by centrifugation and resuspended in SoH buffer, and aliquots were subjected to hypotonic treatment in the presence of the indicated amounts of trypsin for 20 min at 0°C. Protease digestion was stopped by addition of 1 mM PMSF, and samples were precipitated with trichloroacetic acid. Proteinase K treatment, mitochondria were reisolated by centrifugation and resuspended in SoH buffer, and aliquots were subjected to hypotonic treatment in the presence of the indicated amounts of trypsin for 20 min at 0°C. Protease digestion was stopped by addition of 1 mM PMSF, and samples were precipitated with trichloroacetic acid. Proteins were separated by SDS-PAGE, and imported CC1HL and its 25-kDa fragment were analyzed by fluorography. As a control, CC1HL precursor was diluted into SoH buffer lacking mitochondria (Free). The standard lane (St) contains 10% of input precursor protein.

Previous studies (27, 31, 32). Taken together, these import studies show that yeast heme lyases become imported into the mitochondrial intermembrane space via direct transfer across the outer membrane by utilizing the general protein import machinery. Since efficient import can occur in the absence of external energy sources like a membrane potential, Δψ, or ATP in the matrix, the inner membrane translocation machinery is apparently not involved in import.

We investigated the energy requirements for the import of yeast heme lyases. First, the membrane potential, Δψ, was depleted by increasing concentrations of the uncoupler FCCP. No reduction of import was observed for the two heme lyases, even at 10-fold higher concentrations of FCCP than those needed to completely inhibit the import of a matrix-targeted preprotein, preMPP (Fig. 3A). Second, ATP was depleted selectively inside or outside the mitochondrial inner membrane (27, 31). In both cases, the removal of ATP did not cause a significant change of the import efficiency of the heme lyases (Fig. 3B). Control proteins such as the β-subunit of F1-ATPase and precytochrome c1 were affected in their import as reported in previous studies (27, 31). Taken together, these import studies show that yeast heme lyases become imported into the mitochondrial intermembrane space via direct transfer across the outer membrane by utilizing the general protein import machinery. Since efficient import can occur in the absence of external energy sources like a membrane potential, Δψ, or ATP in the matrix, the inner membrane translocation machinery is apparently not involved in import.
shown, see below). For the subsequent studies of the folding process in the intermembrane space, we analyzed the 25-kDa fragment of CC1HL.

We tested whether this fragment was generated in the course of in vitro import of CC1HL. When CC1HL precursor synthesized in reticulocyte lysate was imported into mitochondria, the 25-kDa fragment was formed with high efficiency (more than 50% of intact CC1HL; Fig. 4B). In comparison, only a small amount of fragment (5-10%) was generated by trypsin treatment of CC1HL precursor in the absence of mitochondria. When urea-denatured CC1HL precursor was used in these experiments, trypsin treatment generated the 25-kDa fragment only following import into mitochondria. No such fragment was formed upon dilution of the denatured precursor into buffer lacking mitochondria. In summary, upon import into mitochondria, CC1HL folds efficiently into a conformation that is indistinguishable from its native form. The presence of the 25-kDa domain in reticulocyte lysate, yet in low amounts, indicates that folding is not strictly dependent on the entry into the intermembrane space. However, the 5-10-fold increase in folding efficiency in the latter case may indicate the participation of specific factors during the folding reaction.

The time courses of import and folding were compared. The formation of the 25-kDa domain proceeded at the same rate as the import of CC1HL, even when the experiments were performed at lower temperatures to ensure that import occurred in the linear range (Fig. 5). Apparently, folding of CC1HL in the intermembrane space is at least as rapid as the import reaction, i.e. folding must take place either during or immediately after the entry of the protein into the intermembrane space.

Protein folding in the mitochondrial matrix or in the cytosol has been reported to be assisted by heat shock proteins (Hsp60 and Hsp70; e.g. Refs. 25, 33, 34). As these chaperone proteins require ATP for their function, we asked whether the folding of CC1HL would display a similar dependence on ATP. The folding of the 25-kDa fragment was analyzed after import of CC1HL in the absence of ATP. No influence of the presence or absence of the nucleotide on the amount of fragment formation was observed (Fig. 6). Likewise, depletion of the membrane potential, $\Delta \Psi$, by the ionophore valinomycin during import of CC1HL did not alter the amount of the 25-kDa fragment generated by tryptic digestion (Fig. 6). Therefore, external energy sources do not appear to be important cofactors in the folding of CC1HL in the intermembrane space.

Heme lyases contain an unusually high number of proline residues (8-12%). Therefore, the potential role of mitochondrial peptidylprolyl cis-trans isomerases, namely of cyclophilin and FK506 binding protein (35, 36), for the folding of CC1HL was analyzed by using specific inhibitors of these enzymes (37). The latter protein has been found to be localized in the intermembrane space.$^3$ CC1HL was imported in the presence of 10 $\mu$M of either cyclosporin A or FK506, i.e. at concentrations known to completely inactivate the peptidylprolyl cis-trans isomerase activities (36). After the import reactions, the generation of the 25-kDa fragment was tested. No significant influence of the drugs on the yield of the 25-kDa fragment was observed (Fig. 6). Even the combined presence of the inhibitors did not cause any reduction of folding (not shown). Thus, according to these criteria, neither chaperone proteins, which require ATP for their function, nor the known mitochondrial peptidylprolyl cis-trans isomerase appear to be involved in the folding of CC1HL.

The Folded 25-kDa Fragment Can Be Efficiently Imported into Mitochondria—As shown above, the 25-kDa domain of CC1HL can be generated in soluble form by trypsin treatment of CC1HL in reticulocyte lysate, albeit in low amounts (cf. Fig. 4B). This allowed us to address a central question of heme lyase biogenesis, namely whether the energy derived from folding in the intermembrane space could drive the import reaction. No net energy would be gained during the translocation if the 25-kDa fragment of CC1HL was already folded before its membrane passage. Reticulocyte lysate containing CC1HL precursor was treated with increasing amounts of trypsin to gen-

\[ r^A \text{S. A. Stuart and A. Mayer, unpublished results.} \]
Therefore, the energy derived from folding of the polypeptide chain cannot be regarded as a major contribution to the driving force for mitochondrial import of CC1HL.

**DISCUSSION**

According to our in vitro import studies, yeast heme lyases reach the intermembrane space by direct transfer across the outer membrane. The use of the general protein import complex of this membrane is suggested by the dependence of the import on protease-sensitive receptors in the same way as found for matrix-targeted preproteins. The independence of transport from the inner membrane follows from the energy requirements of the import reaction. Neither a membrane potential, \( \Delta \psi \), across the inner membrane nor ATP in the matrix space is required for import. Both conditions are known to be essential for protein transport into and across the inner membrane (12, 27, 32, 38). Thus, the yeast heme lyases studied here use a direct, “non-conservative” import pathway. A similar route has been deduced recently to be taken by CCHL from N. crassa (15).

The import route taken by the three known heme lyases differs markedly from that of other constituents of the intermembrane space. In the case of cytochromes \( c_1 \) and \( b_2 \), the involvement of a membrane potential during import into mitochondria is well established (12). At least parts of these preproteins are transiently exposed to the matrix (39, 40, 41), showing that participation of the inner membrane is a characteristic feature of their sub mitochondrial sorting. The pathway defined by apocytochrome \( c \), on the other hand, is similar to that of heme lyases in that import only requires the outer membrane (for reviews, see Refs. 1 and 3). However, other features of the import of this preprotein are unique. Transport of apocytochrome \( c \) across the outer membrane occurs independently of protease-sensitive components, in particular of the general protein import complex (7, 42). Nonetheless, specific interaction with this membrane seems to involve a hitherto uncharacterized component, which also might facilitate the passage of the apoprotein across the membrane (10). Import of apocytochrome \( c \) is driven by the specific interaction with CCHL in the intermembrane space, which serves as a high affinity “trans side receptor” before attaching heme to the apoprotein (7–10). Thus, there exist at least three fundamentally different pathways of protein sorting into the mitochondrial intermembrane space, one of which is taken by the heme lyases. These pathways may have been developed as a result of the evolutionary different origins of the various intermembrane space proteins. For instance, cytochrome \( b_2 \), a constituent derived from the bacterial endosymbiont ancestor, apparently follows a pathway reminiscent of protein export from bacteria (see Refs. 40 and 43). Heme lyases, on the other hand, do not appear to have structural counterparts in bacteria (44), and therefore may have evolved a novel way of entering the mitochondrial intermembrane space.

Practically nothing is known about the mechanism of protein folding in the mitochondrial intermembrane space. To study this process, we have taken advantage of a large fragment of CC1HL that folds into a trypsin-resistant domain during import. The folded fragment formed rapidly during or immediately after the import of the protein. To a low degree, this domain was also generated during translation in reticulocyte lysate. Therefore, folding does not require the participation of specific factors from the intermembrane space. However, the 5–10-fold increase in the efficiency of folding upon import may indicate assistance by constituents of the intermembrane space. Even though our study does not identify any factor involved in the folding reaction, a few components may be excluded. The independence of folding on the presence of ATP.
renders it unlikely that chaperone proteins like those involved in protein folding in, e.g. the cytosol or the mitochondrial matrix, may be involved. The central components of these folding processes, namely Hsp70 and Hsp60 proteins, need ATP for their function (17). Furthermore, our study rules out an essential function of known mitochondrial peptidyl-prolyl cis-trans isomerases (35, 36), which in part are localized in the mitochondrial intermembrane space. Since all known heme lyases have an exceptionally high content of proline residues (8–12%), catalysis of their isomerization might have been expected to accelerate the rate of folding. Clearly, our studies show that protein folding in the intermembrane space obeys different principles than those established for other subcellular compartments.

The mechanism of folding of CC1HL may be a paradigm for other components of the intermembrane space. A recent study of the folding of other proteins located in the intermembrane space and of reporter proteins elucidated remarkable parallels to the folding of heme lyases. Folding occurred rapidly and could not be kinetically resolved from translocation. There was no apparent requirement for the presence of external energy sources like ATP. Thus, one might expect from these studies that for this mitochondrial subcompartment, the folding process may be assisted by so far unknown chaperone activities. It will be interesting to identify these components and to further elucidate the mechanisms underlying the folding reactions.

An open question concerning the biogenesis of heme lyases has been the nature of the driving force for their transfer into the intermembrane space. No external energy sources like cytosolic or mitochondrial ATP were found to be required for import of all three known heme lyases. As a possibility for driving the import reaction, the specific folding of heme lyases in the intermembrane space seems possible (45). However, our data do not support this idea; they rather demonstrate that folding cannot be regarded as the energetic basis for the overall import process. A domain of CC1HL comprising 80% of the total protein could be efficiently transported into mitochondria despite its folded character. Therefore, no net energy could be gained from folding in this case. With the apparent lack of a driving force, how might the import process be viewed in energetic terms? We would like to suggest a scenario in which the import is driven by a series of specific interactions heme lyases undergo during and after import. The energy derived from these interactions may substitute for the well studied action of Hsp70 under expense of ATP. A first interaction may be the recognition of the internal targeting signal of heme lyases by receptors at the mitochondrial surface. Similar to the case found for N-terminal presequences, this interaction may be very labile, thereby facilitating rapid insertion of the polypeptide chain into the translocation pore (46). Further movement across the membrane may then lead to an interaction of the targeting signal with a specific binding site on the trans side of the outer membrane. If this interaction is comparatively stable, as has been found with N-terminal presequences (46), such a situation would inevitably lead to net translocation of the targeting signal and regions surrounding the signal. This would explain why CCHL can be imported into isolated outer membrane vesicles (16). Refolding of the translocated polypeptide chain could then lead to dissociation from the “trans site” and thus be prerequisite for interaction of the heme lyases with the inner membrane. Most likely, this involves a specific binding partner. The stable interaction with this putative heme lyase receptor may render the translocation process irreversible and in addition will ensure the exclusive localization to the outer face of the inner membrane. These ideas are now open for direct experimental testing.

It is remarkable that a protein which is folded before the transport reaction can traverse the outer membrane with the same efficiency as the unfolded protein. It is well known that preproteins pass the mitochondrial membranes in an unfolded, extended conformation (47). Thus, it seems plausible that also the soluble, folded 25-kDa domain of CC1HL has to undergo an unfolding step to be able to translocate across the outer membrane. In general, mitochondrial Hsp70 is believed to participate in such unfolding reactions (48), either by actively pulling on the incoming polypeptide chain (49) or by binding to segments spontaneously entering the matrix space (50). In the latter case, Hsp70 would act as a “molecular ratchet” which is driven by the expense of ATP. Since in the case of CC1HL precursor Hsp70 molecules are not involved in the import process, the outer membrane itself may facilitate unfolding. Recently, an example of such an unfolding reaction has been demonstrated to accompany initiation of translocation of sequence-containing preproteins across the isolated outer membrane (46).

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