Sorting of d-Lactate Dehydrogenase to the Inner Membrane of Mitochondria

ANALYSIS OF TOPOGENIC SIGNAL AND ENERGETIC REQUIREMENTS*

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D-Lactate dehydrogenase (D-LD) is located in the inner membrane of mitochondria. It spans the membrane once in an N-in-C-out orientation with the bulk of the protein residing as a folded domain in the intermembrane space. D-LD is synthesized as a precursor with an N-terminal cleavable presequence and is imported into the mitochondria in a Δψ-dependent, but mt-Hsp70-independent manner. Upon import in vitro D-LD folds in the intermembrane space to attain a conformation indistinguishable from endogenous D-LD. Sorting of D-LD to the inner membrane is directed by a composite topogenic signal consisting of the hydrophobic transmembrane segment and a cluster of charged amino acids C-terminal to it. We propose a model for the mode of operation of the sorting signal of D-LD. This model also accounts for the driving force of translocation across the outer membrane, in the apparent absence of mt-Hsp70-dependent assisted import and involves the folding of the D-LD in the intermembrane space.

Nuclear encoded proteins of the inner membrane of mitochondria contain topogenic signals which function to sort them to the membrane following their import. These topogenic signals comprise hydrophobic cores of varying length and are flanked usually by charged amino acids. In many instances these signals form integral parts, the transmembrane anchors, of the sorted protein. In a small number of cases they are proteolytically cleaved following the sorting event. These topogenic signals are distinct from mitochondrial targeting sequences that serve to target the precursor initially to the mitochondria and to initiate membrane potential (Δψ)-dependent translocation across the inner membrane. In some cases these topogenic signals are located after N-terminal mitochondrial targeting signals (presequences) and operate in conjunction with them. This is not the case for all topogenic signals as many proteins of the inner membrane are synthesized without presequences but rather contain internal mitochondrial targeting signals.

Bearing these differences in mind, together with the wide variety of orientations displayed by inner membrane proteins, it would appear unlikely that the topogenic signals operate in an uniform manner. How do they act to ensure the sorting of proteins to the inner membrane? Recently we have described that the topogenic signals of a subset of inner membrane proteins serve as export signals directing the export of domains of the protein from the matrix to the intermembrane space following the complete import of the preprotein into the matrix (1–3). On the other hand, it has been suggested that some topogenic signals function as translocation arrest signals at the level of the inner membrane, thereby preventing further import into the matrix (4–8).

To further our understanding of the mechanisms of sorting of inner membrane proteins, we have addressed the biogenesis of the (d+)-lactate dehydrogenase (D-LD)† protein. D-LD together with cytochrome b2, an (l+)-lactate dehydrogenase, are involved in lactate utilization in the yeast Saccharomyces cerevisiae and catalyze the oxidation of d- and l-lactate, respectively, to pyruvate. Cytochrome c acts as an electron acceptor in the oxidation reaction, connecting the reaction to the mitochondrial respiratory chain. Recently the gene for D-LD was cloned and appears to contain an N-terminal mitochondrial targeting signal (9).

The data presented here demonstrate that D-LD is a mitochondrial protein anchored to the inner membrane with an N-in-C-out orientation. We present here information on the topogenic signal sequence and energetic requirements necessary for D-LD to achieve this membrane orientation.

EXPERIMENTAL PROCEDURES

Isolation of Yeast Mitochondria—Saccharomyces cerevisiae wild-type strain (D273-10B) was grown in lactate medium (10) at 30 °C, while the temperature-sensitive mutant of mt-Hsp70, ssc1-3 (PK83) and its corresponding wild type (PK82) (11) were grown at 24 °C. Cells were harvested at an A578 of ~1, and mitochondria were isolated, as described previously (10), with the exception that the zymolyase treatment of PK82 and PK83 strains was performed at 24 °C. Isolated mitochondria were resuspended in 0.6 M sorbitol, 20 mM HEPES, pH 7.4, 1 mM EDTA (SEH-buffer) at a protein concentration of 10 mg/ml.

Recombinant DNA Techniques and Plasmid Constructions—The recombinant DNA techniques applied were as described by Sambrook et al. (12). The D-LD gene was obtained by amplification of yeast genomic DNA of strain D273-10B by a polymerase chain reaction. The resulting DNA fragment was cloned into pGEM4 (Promega, Madison, WI) yielding the plasmid pLDL. To construct the plasmids for the expression of pLDLD(1–42)-dihydrofolate reductase (DHFR), pLDLD(1–72)-DHFR, and pLDLD(1–66)-DHFR, the relevant D-LD DNA fragments were synthesized by polymerase chain reaction and subcloned intermediate to a BamHI site in front of the DNA encoding mouse DHFR. Standard polymerase chain reaction and site-directed mutagenesis techniques

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1 The abbreviations used are: LD, lactate dehydrogenase; DHFR, dihydrofolate reductase; PAGE, polyacrylamide gel electrophoresis; MPP, mitochondrial processing peptidase; TIM, translocase of the inner membrane.

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were used to create the other pDLD(1–72)-DHFR derivatives used in this study.

Precursor Proteins—DNA encoding precursor proteins were cloned in pGEM4 vectors and were transcribed with SP6 RNA polymerase. All precursor proteins were then synthesized in rabbit reticulocyte lysate (Promega) in the presence of [35S]methionine (13).

Import into Mitochondria—Import was performed essentially as described previously in S1 buffer (0.6 M sorbitol, 80 mM KCl, 50 mM HEPES pH 7.2, 3% (w/v) bovine serum albumin, 10 mM MgCl2, 2 mM potassium phosphate, 2.5 mM EDTA, 2.5 mM MnCl2) (14). Import mixtures contained 2 mM NADH, 2 mM ATP, 0.25 mg/ml mitochondrial protein/ml, and 1% (v/v) reticulocyte lysate containing the radiolabeled precursor proteins and were incubated at 25 °C, unless otherwise indicated. Following import for the time indicated, samples were either mock-treated or treated with proteinase K for 15 min at 0 °C either under nonswelling (0.6 M sorbitol, 20 mM HEPES, pH 7.4) or hypotonic swelling (20 mM HEPES, pH 7.4) conditions (14). Mitochondria and mitoplasts were reisolated by centrifugation, and samples were analyzed by SDS-PAGE and immunoblotting onto nitrocellulose. The efficiency of swelling of the mitochondria was assessed following immunodecoration of the blot with antibodies against endogenous cytochrome c peroxidase (soluble intermembrane space protein) and Mge1p (matrix protein).

Depletion of Matrix ATP—Isolated mitochondria were depleted of their matrix ATP, as described previously (15). Briefly, following dilution in import buffer, mitochondria were incubated at 25 °C for 3 min, and then oligomycin (20 μM), carbacholactactylase (5 μM), and NADH (2 mM) were successively added at 3-min intervals.

Assessment of the Folding State of d-LD—Assessment of the folding state of imported and endogenous d-LD was performed as follows. Mitochondria were lysed with 0.1% (w/v) Triton X-100, 150 mM NaCl, and 10 mM Tris/Cl, pH 7.4, for 10 min on ice in the absence or in the presence of 7.5 μg/ml proteinase K or of 25 μg/ml trypsin. Proteins were then trichloroacetic acid-precipitated and analyzed by SDS-PAGE and Western blotting with an antibody directed against the C-terminal region of d-LD.

Antiserum Generation—A BglII-HindIII fragment encoding for the C-terminal residues (amino acids 373–576) of d-LD was cloned into the Escherichia coli expression vector pQE9 (digested BsiHII-HindIII), thus generating a (His6)-tagged d-LD C-terminal fragment upon induction with isopropyl-l-thio-β-D-galactopyranoside, which was purified by Ni-nitrilotriacetic acid chromatography and used to immunize rabbits to raise a polyclonal serum.

Miscellaneous—Protein determination, SDS-PAGE, and Western blotting were performed according to the published methods of Bradford (16), Laemmli (17), and Towbin et al. (18), respectively. The detection of proteins after blotting onto nitrocellulose was performed using the ECL detection system (Amersham Corp.).

RESULTS

Topology of the d-LD—The amino acid sequence deduced from the cDNA sequence of the d-LD protein indicated the presence of an N-terminal targeting signal and a single transmembrane domain (residues 46–61, see Fig. 1A) (9). The predicted mitochondrial targeting signals comprises amino acids 1–25, thus leaving 20 residues prior to the transmembrane segment. The C-terminal hydrophilic segment comprises residues 62–576. To determine the submitochondrial localization of the d-LD, isolated mitochondria were subjected to protease treatment and subfractionation by hypotonic swelling, which disrupts the integrity of the outer membrane while leaving the inner membrane intact (Fig. 1A). d-LD was not accessible to externally added proteinase K in intact mitochondria. Upon hypotonic swelling, d-LD remained associated with the mitoplasts but was accessible to the added protease.

We further analyzed the topology of d-LD by digitonin fractionation (Fig. 1B). Mitochondria were incubated in the presence of proteinase K and in increasing concentrations of the detergent digitonin to sequentially disrupt the outer and the inner mitochondrial membranes. Immunoblotting of the resulting Western blots showed that d-LD was inaccessible to the added protease when both outer and inner membrane were intact. Upon opening of the outer membrane (as indicated by the loss of cytochrome c peroxidase) d-LD became accessible to added protease. Analysis of the supernatants indicated that a C-terminal fragment of d-LD of ~50 kDa had been released by this protease treatment.

In conclusion, the d-LD spans the inner membrane once whereby its N terminus protrudes into the matrix, while the bulk of the protein, C-terminal to the transmembrane stretch, is in the intermembrane space; this latter segment forms a tightly folded domain.

Imported Radiolabeled Pre-d-LD Attains the Correct Orientation in Vitro—The open reading frame of d-LD was cloned into a pGEM4 vector and transcribed with SP6 RNA polymer-
Fig. 2. Importance of the transmembrane segment for the sorting of d-LD to the intermembrane space. A, hydrophathy plot of d-LD, indicating amino acid residues 46–61 form the transmembrane domain. B, d-LD-DHFR fusion constructs. Zig-zag line, N-terminal presequence; the arrow indicates the putative site for MPP cleavage; black box, transmembrane domain. DHFR, mouse cytosolic dihydrofolate reductase, used as passenger protein for the fusion constructs. Positioning of amino acid residues is indicated. pre-DLD(1–72)/ΔTM)-DHFR lacks the transmembrane domain (amino acids 46–61). C, radiolabeled pre-DLD(1–42)-DHFR, pre-DLD(1–72)-DHFR, and pre-DLD(1–72/ΔTM)-DHFR were imported into isolated mitochondria at 25 °C for 30 min. Samples were further processed as described in Fig. 1C.

Translation of the resulting mRNA in rabbit reticulocyte lysate in the presence of [35S]methionine gave rise to a radiolabeled product of approximately 63 kDa, termed pre-d-LD (Fig. 1C). Upon incubation with isolated mitochondria, pre-d-LD was imported into a protease-resistant location where it was processed to its mature size form of 61 kDa, termed m-d-LD. The processing is apparently catalyzed by the divalent metalloendopeptidase MPP, which was applied as reagent at 25 °C for 30 min. Samples were analyzed by SDS-PAGE, fluorography, and quantified densitometry. The percentage of total imported mature species protease protected in mitoplasts is indicated.

The necessary information to ensure correct sorting of the pre-protein to an Nin-Cout topology across the inner membrane, a series of fusion proteins comprised of N-terminal regions of d-LD and fused to the mouse cytosolic DHFR were created (Fig. 2B).

A fusion protein pre-d-LD(1–42)-DHFR, consisting of the initial 42 amino acids of pre-d-LD, was targeted to the mitochondrial matrix where it was processed by MPP (Fig. 2C). The fusion protein pre-d-LD(1–72)-DHFR, which encompasses the transmembrane segment of d-LD fused to DHFR, was accessible to exogenously added proteases when the outer membrane was perturbed following its import into mitochondria, thus indicating correct sorting to an Nin-Cout orientation. Specific deletion of the transmembrane domain from this d-LD-DHFR derivative, pre-d-LD(1–72/ΔTM)-DHFR (Fig. 2B), however, resulted in its missorting to the mitochondrial matrix (Fig. 2C). This emphasizes the importance of the transmembrane segment for the sorting process.

Fig. 3. Role of the charges for the sorting of d-LD to the intermembrane space. A, site-directed mutagenesis analysis of the topogenic signal. The amino acid residues (numbers) preceding the transmembrane segment (black box) and those C-terminal to it (62–72) are indicated together with the fusion site to DHFR. In pre-d-LD(1–66 4)-DHFR the cluster of charges (RETKEK) is replaced by the sequence NLYS. Lysines K45 and K62 were mutated in the context of pre-d-LD(1–72)-DHFR to generate pre-d-LD(1–72)K45E-DHFR, pre-d-LD(1–72)K45N-DHFR and pre-d-LD(1–72)K62N-DHFR, respectively. B, radiolabeled preproteins were imported into isolated mitochondria for 30 min at 25 °C. Mitochondria were either mock-treated or converted to mitoplasts in the presence of proteinase K (100 μg/ml). Samples were analyzed by SDS-PAGE, fluorography, and quantified densitometry. The percentage of total imported mature species protease protected in mitoplasts is indicated.

Signal Requirements for Sorting of d-LD—To test whether the N-terminal region including the transmembrane region (amino acid residues 46–61) of pre-d-LD (Fig. 2A) contains all the necessary information to ensure correct sorting of the pre-protein to an Nin-Cout topology across the inner membrane, a series of fusion proteins comprised of N-terminal regions of d-LD and fused to the mouse cytosolic DHFR were created (Fig. 2B).
destabilizing for the sorting process. Replacing lysine 62, C-terminal to the transmembrane segment, by an uncharged amino acid, asparagine (K62N), did not affect the sorting to the N\textsubscript{int}\textsubscript{out} orientation across the inner membrane.

In addition, the possible role of a cluster of charged amino acids (RETKEKD, amino acid residues 67–72) C-terminal to the transmembrane domain in the sorting event was addressed (Fig. 3B). Substitution of this cluster of charges by a repetition of the four uncharged amino acids preceding it (NLYS) (pre-D-LD(1–66+4)-DHFR) caused missorting to the mitochondrial matrix (Fig. 3B). Point mutation of the five charged amino acids of this cluster separately to neutral ones did not affect the sorting of D-LD to the intermembrane space (results not shown). This suggests that the individual charges of the cluster do not have a specific role, but rather act together as a topogenic entity.

Thus the sorting signal of pre-D-LD is a composite one, comprising a number of essential elements, the hydrophobic membrane anchor and the charged amino acids N-terminal and C-terminal to it.

Role of Hydrophobicity for the Sorting of D-LD to the Inner Membrane—To analyze the role of hydrophobicity in the targeting signal of D-LD, a series of proteins were created in which the hydrophobic core of the transmembrane segment was lengthened at its C-terminal end by the introduction of either 4 or 8 leucine additional residues. These modifications were performed either in pre-D-LD-(1–72)-DHFR and thus in the context of the cluster of charges or in the pre-D-LD-(1–66+4)-DHFR, i.e. in the absence of the charged cluster (Fig. 4A).

Extension of the transmembrane segment core by the addition of 4 leucine residues had no adverse effect on sorting. Radiolabeled pre-D-LD-(1–74+4L)-DHFR was imported into isolated mitochondria, where it was processed by MPP and correctly sorted to the N\textsubscript{int}\textsubscript{out} orientation with the same efficiency as pre-D-LD(1–72)-DHFR (Fig. 4B). The cluster of charges remained important for this sorting as its deletion, pre-D-LD(1–62+4L)-DHFR, resulted in missorting into the mitochondrial matrix in a similar fashion as pre-D-LD(1–66+4)-DHFR (Fig. 4B).

The addition of a total of 8 leucines to the hydrophobic core dramatically affected the efficiency of both import into mitochondria and processing by MPP (Fig. 4C). The precursors of both species pre-D-LD-(1–74+8L)-DHFR and pre-D-LD(1–62+8L)-DHFR accumulated as their precursor forms at the outer membrane where they were accessible to added proteinase K (Fig. 4C). If, however, the outer membrane was disrupted by the formation of mitoplasts prior to import thus allowing precursors to bypass the outer membrane and gain direct access to the inner membrane, efficient processing and import was observed. Under these conditions sorting of pre-D-LD-(1–74+8L)-DHFR to the inner membrane and pre-D-LD(1–62+8L)-DHFR to the matrix was achieved.

In conclusion, the degree of hydrophobicity of the hydrophobic core plays a crucial role in sorting of D-LD to the inner membrane. Extension of this core by an additional 8 leucine residues results in the early partitioning of the protein into the outer membrane. This missorting to the outer membrane can be relieved by disruption of the outer membrane by hypotonic swelling prior to import. Under these conditions the corresponding proteins access to the inner membrane directly and are processed and sorted like wild-type hydrophobic core containing proteins. The cluster of charges remains necessary for correct sorting irrespective of the length of the transmembrane segment, as its removal resulted in missorting to the matrix.

Matrix ATP and mt-Hsp70 Requirements for Import and Sorting of D-LD—To address whether the import of pre-D-LD is supported by the ATP-dependent mt-Hsp70 action in a similar manner as matrix-targeted proteins, we tested if depletion of matrix ATP levels had an adverse effect on the import and sorting of the process. Treatment of mitochondria prior to import with oligomycin and carboxyatractyloside serves to reduce the matrix levels so they become limiting for mt-Hsp70 action (15, 19). The efficiency of import of the pre-D-LD was only slightly reduced when imported into ATP-depleted mitochondria, as compared with matrix ATP-containing mitochondria (Fig. 5A). This was in marked contrast to the import of a hydrophilic matrix targeted protein, preSu9(1–69)-DHFR, whose processing by MPP and import were significantly reduced by the depletion of the matrix ATP levels. Likewise the import and the sorting of pre-D-LD(1–72)-DHFR was not affected by prior matrix-ATP depletion. In contrast, the import, but not the processing by MPP, of the matrix mistargeted pre-D-LD(1–66+4)-DHFR, was inhibited (Fig. 5B).

In addition, pre-D-LD was efficiently imported into mitochondria from the mutant ssc1-3 harboring a temperature sensitive mt-Hsp70, following their prior exposure to their nonpermissive temperature (Fig. 5C). Thus the import and attainment of the correct topology of the D-LD across the inner membrane apparently do not require ATP-dependent action of mt-Hsp70 in the matrix.
Imported v-LD Folds in the Intermembrane Space to Its Native Conformation—As shown in Fig. 1, endogenous v-LD contains a large C-terminal folded domain in the intermembrane space. When mitochondria were solubilized with detergent and subjected to a proteinase K treatment, a C-terminal fragment was recovered (Fig. 6A). When mitochondria were solubilized with detergent and treated with proteinase K to assess the folding state of the imported species. Imported radiolabeled v-LD became folded, as proteinase K treatment of detergent extracts of mitochondria generated the 50 kDa fragment of v-LD (Fig. 6B). An increase in the levels of protease-resistant fragments formed was not observed if the initial trypsin treatment was omitted. This observation suggested that the pre-v-LD bound outside the mitochondria was not folded in the same manner as that of the imported species. Furthermore, if import was performed in the absence of Δψ, protease treatment of the precursor species accumulated outside the mitochondria did not give rise to the characteristic protease-resistant fragments upon lysis (Fig. 6B). When trypsin only was used during lysis, a slightly larger protease-protected fragment of 56 kDa was observed. Two smaller fragments of 22 and 24 kDa, respectively, were generated by this trypsin treatment, but they were also present in the −Δψ sample; thus they were not specific for import. Quantitation of these results showed that 50% of imported v-LD (Fig. 6B) and 25% of the processed species were folded (Fig. 6B).

When imported into isolated mitoplasts in the presence of a Δψ, the MPP-processed v-LD also became correctly folded as indicated by lysis and trypsin treatment. As was observed with import into the mitochondria, folding of v-LD in mitoplasts was not observed in the absence of Δψ. The efficiency of folding in mitoplasts was, however, low; only 4% of MPP-processed material was folded as opposed to 27% for the mitochondrial import control (Fig. 6C, lane 2 versus 6). This indicates that soluble components of the intermembrane space may be necessary for this folding process. Attempts to improve the folding efficiency in mitoplasts by addition of cytosol, cold lysate, ATP, FADH2, or Zn2+ (v-LD is a flavin protein that also binds Zn2+) (9) were unsuccessful.

The requirement for a Δψ to observe folding of radiolabeled v-LD suggested that the N terminus of the pre-v-LD must be imported across the inner membrane before the C-terminal region of the protein may become folded to its native conformation in the intermembrane space. We then addressed whether MPP processing, import, and folding of v-LD were concomitant events or whether they could be dissected into distinct processes. Radiolabeled pre-v-LD was imported into isolated mitochondria, and after various time points samples were removed and MPP processing, import, and folding of v-LD were assessed. Processing of pre-v-LD to its mature form was a very rapid process and kinetically preceded completion of import across the outer membrane (Fig. 6D, a). Furthermore, folding of v-LD occurred with similar kinetics as those of import (Fig. 6D, b), suggesting they may be coupled events.

In a separate analysis the temperature dependence of import and folding of v-LD in mitochondria was analyzed. Translocation of the N-terminal region of v-LD across the inner membrane into the matrix, as monitored by MPP maturation of the presequence, occurred efficiently even at low temperatures (Fig. 7A). Thus this initial step in the import of v-LD is not a temperature-sensitive one. In contrast, completion of translocation of C-terminal regions of v-LD across the outer membrane, as measured by inaccessibility of radiolabeled v-LD to exogenously added protease, was inhibited at low temperatures and occurred only at 25 °C (Fig. 7B). On the basis of this observation, the temperature sensitivity of the folding process was assessed following import of v-LD into isolated mitoplasts (Fig. 7C). Folding of v-LD, as judged by protease resistance of v-LD in detergent extracts, was observed only following import at 25 °C (Fig. 7C).

Thus in contrast to the initial step of presequence entry into the matrix, the later steps of v-LD import, namely, completion of translocation across the outer membrane followed by folding in the intermembrane space, are strictly temperature-dependent events.

DISCUSSION

The sequence information responsible for targeting v-LD to the mitochondria and for sorting into the inner membrane resides entirely in the N-terminal 72-amino acid residues of v-LD. As shown here this short region contains both a cleavable...
mitochondrial targeting signal, which is followed by the inner membrane topogenic signal. The topogenic signal apparently serves to arrest the D-LD in the inner membrane during import.

Dissection of the topogenic signal revealed it to be a composite one, comprising essential hydrophobic and hydrophilic features. The hydrophobic transmembrane region is essential for sorting D-LD to the inner membrane. Furthermore, our results suggest that the degree of hydrophobicity of this segment is crucial for sorting of D-LD to the inner membrane. If the length (and thereby the hydrophobicity) is increased, D-LD preferentially partitions into the outer membrane in such a manner that further translocation to the inner membrane is prevented. The hydrophobic transmembrane segment is flanked directly on both sides by a positively charged amino acid. These charges appear to play a minor role in the sorting process of D-LD. Deletion of the cluster of charged amino acids located C-terminally of the hydrophobic domain, however, led to a massive missorting of the protein to the matrix. The importance of this cluster indicates a degree of protein:protein interactions that may actually occur at the level of the translocase of the inner membrane (TIM machinery), where the transmembrane segments of Tim17 or Tim23, components of the TIM complex, may display an affinity for hydrophobic segments of incoming preproteins, and thus serve to retard further translocation across the inner membrane (20). Alternatively, it is feasible that a channel of the translocase may open out to the lipid bilayer and thus the hydrophobic domain of incoming D-LD may preferentially partition into the membrane from the TIM machinery, thus aborting further translocation.

Not every hydrophobic topogenic signal, however, functions to arrest preproteins in the inner membrane. Indeed many are imported into the matrix where they serve as re-export signals targeting insertion into the inner membrane (1–3). Hence there must be a second control mechanism to distinguish whether a topogenic signal should be arrested or further translocated.

Hydrophobic interactions may actually occur at the level of the translocase of the inner membrane (TIM machinery), where the transmembrane segments of Tim17 or Tim23, components of the TIM complex, may display an affinity for hydrophobic segments of incoming preproteins, and thus serve to retard further translocation across the inner membrane (20).

Sorting of D-LD to Inner Membrane of Mitochondria

FIG. 6. Imported D-LD folds to its native conformation in the intermembrane space. A, isolated mitochondria were lysed with 0.1% (w/v) Triton X-100, 150 mM NaCl, and 10 mM Tris/Cl, pH 7.4, for 10 min on ice in the absence or in the presence of 7.5 µg/ml proteinase K or 25 µg/ml trypsin, as indicated. Proteins were then trichloroacetic acid-precipitated and analyzed by SDS-PAGE and Western blotting with an antibody directed against the C-terminal part of D-LD. Molecular weight markers are indicated. B, radiolabeled pre-D-LD was imported into isolated mitochondria in the presence (+Δψ) or absence (−Δψ) of membrane potential. After import, samples were treated with trypsin (20 µg/ml) where indicated. Mitochondria were resolated and lysed as in A, in the presence of proteinase K (PK, 7.5 µg/ml) or trypsin (25 µg/ml), as indicated. Samples were analyzed by SDS-PAGE and blotting onto nitrocellulose. The resulting blot was decorated with an antibody directed against the C terminus of D-LD (endogenous D-LD). C, radiolabeled pre-D-LD was imported into isolated mitoplasts in the presence (+Δψ) or absence (−Δψ) of membrane potential. After import, samples were lysed as in A in the presence of trypsin (25 µg/ml). D, radiolabeled pre-D-LD was imported into isolated mitochondria at 25 °C for the times indicated. Import was stopped by addition of valinomycin. Samples were divided and were either mock-treated (to assess MPP processing (depicted in a)), treated with 20 µg/ml trypsin (to assess import (depicted in b)), or recovered by centrifugation and lysed, and folding was assessed as described in A (depicted in b). Samples were analyzed by SDS-PAGE and fluorography and quantified with a PhosphorImager.
interaction may also be involved in the mode of operation of the sorting signal. How this cluster of charges functions for the sorting of D-LD remains a matter of speculation. Due to their positioning with respect the transmembrane anchor, these charged residues are probably recognized by (a) proteinaceous factor(s) exposed to the intermembrane space; hydrophilic domains of Tim17 or Tim23, which are exposed to the intermembrane space could conceivably be involved. Alternatively, one could imagine that the cluster of charged amino acids might function to slow down the translocation across the inner membrane and thus serve to enhance the partitioning of the hydrophobic domain into the lipid bilayer of the inner membrane.

A similar cluster of charged amino acids are found at the C-terminal side of the transmembrane domain of other inner membrane proteins with an N_in-C_out orientation, such as Sco1p, Sco2p, and CoxVa. The mechanism which D-LD uses to achieve its membrane orientation may therefore be common to these other membrane proteins. The transmembrane domains of all these proteins are relatively short (16–18 amino acids) and are flanked on each side by a positive charge, with the exception of CoxVa which has a negative charged residue at the C-terminal position. The importance of both the short transmembrane segment and of the charged cluster in sorting of these other inner membrane proteins remains to be studied. A similar charged cluster is not found in the sorting signals of other proteins, e.g. cytochrome b_2 and cytochrome c_1, where a stop-transfer mechanism of sorting is currently being debated, thus supporting the case for an alternative sorting mechanism of these proteins (4, 5, 21–23).

What is the driving force for the import of D-LD across the outer membrane? Translocation of the N terminus of D-LD across the inner membrane into the matrix occurs in response to the Δψ and does not require mt-Hsp70 and matrix ATP. Import of the N terminus to the matrix is a rapid process even at lower temperature. The topogenic signal of D-LD appears to function in halting import in the inner membrane. No evidence for the transient passage of part or all of D-LD through the matrix during its sorting to the inner membrane was obtained. As we have demonstrated previously, the presence of a hydrophobic sorting signal in proximity to the N-terminal matrix-targeting signal of a preprotein can secure the translocating polypeptide chain in the import channel in a manner that does not require mt-Hsp70 activity (20). Completion of translocation of C-terminal elements across the outer membrane thus occurs independently of mt-Hsp70, and in the case of D-LD, appears to be the rate-limiting step for import. Upon import across the outer membrane, D-LD folds to attain a protease-resistant conformation. The kinetics and temperature sensitivity of this folding step paralleled those of import. The folding of translocated domains on the trans side of the outer membrane may be the driving force for the completion of translocation across the membrane.

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