Minireview

Protein translocation pathways of the mitochondrion

Carla M. Koehler*

Department of Chemistry and Biochemistry, Box 951569, University of California, Los Angeles, CA 90095-1569, USA

Received 5 May 2000

Edited by Gunnar von Heijne

Abstract The biogenesis of mitochondria depends on the coordinated import of precursor proteins from the cytosol coupled with the export of mitochondrially coded proteins from the matrix to the inner membrane. The mitochondria contain an elaborate network of protein translocases in the outer and inner membrane along with a battery of chaperones and processing enzymes in the matrix and intermembrane space to mediate protein translocation. A mitochondrial protein, often with an amino-terminal targeting sequence, is escorted through the cytosol by chaperones to the TOM complex (translocase of the outer membrane). After crossing the outer membrane, the import pathway diverges; however, one of two TIM complexes (translocase of inner membrane) is generally utilized. This review is focused on the later stages of protein import after the outer membrane has been crossed. An accompanying paper by Lithgow reviews the early stages of protein translocation. © 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Mitochondrion; Protein targeting; Membrane; Protein translocation; Translocase

1. Introduction

The mitochondrion is a structurally complex organelle in the eukaryotic cell, containing an outer and inner membrane, which separate the matrix from the intermembrane space. This organelle contains its own small genome that encodes a handful of inner membrane proteins of the mitochondrial energy producing system. As in bacteria, these proteins are exported from the matrix to the inner membrane, although the export components generally are quite different between bacteria and the mitochondrial inner membrane [1]. Even though mitochondrial protein import has been studied intensively for the past two decades, new protein translocation systems have recently been identified in the mitochondrial inner membrane that mediate the import (and export) of inner membrane proteins (Fig. 1).

Most mitochondrial precursors contain an amino-terminal targeting presequence, but many proteins, particularly those of the outer and inner membrane, contain targeting and sorting information within the mature part of the protein. Based on studies focused on the import and sorting of model mitochondrial proteins or synthetic fusion proteins between a mitochondrial targeting sequence and a passenger protein, a translocation system is present in both the outer and inner membrane (reviewed by [2–6]). The translocase of the outer membrane (TOM) consists of protein import receptors and the import channel. The receptors (Tom20, 22, 37 and 70, with the number indicating molecular weight) on the mitochondrial surface recognize targeting information on mitochondrial precursors, while components Tom40 and the small Tom proteins 5, 6 and 7 form the channel through which the translocating precursor passes [6]. After passage through the TOM complex, proteins are sorted via a number of mechanisms either directly to the outer membrane, the intermembrane space, or the translocase of the inner membrane (TIM). Generally, the TIM23 machinery mediates protein translocation into the matrix and the TIM22 machinery mediates insertion into the inner membrane.

2. The TIM23 complex is the translocase of the general import pathway

Precursors with an amino-terminal targeting presequence follow the general import pathway (Fig. 2; [7–9]); their import is mediated by the Tim17/Tim23 complex (designated TIM23) and the translocation motor consisting of Tim44, mitochondrial heat shock protein hsp70 and the nucleotide exchange factor mGrpE. This translocation is dependent upon the presence of a membrane potential (ΔΨ) and gener-

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*Fax: (1)-310-206 4038.
E-mail: koehlerc@chem.ucla.edu

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PHI: S0014-5793(00)01664-1
To date, additional proteins in this TIM machinery have been identified, but their specific role in protein import has not been determined. Tim11 was identified because of its intimate association with the Tim channel [32]. Studies with a cytochrome b2 arrested translocation intermediate and a cross-linker with a short spacer arm cross-linked Tim11 with very high specificity. Further studies revealed it is also the $\gamma$-subunit of the mitochondrial ATPase and is an ATPase assembly factor [33]. Studies by Endo and colleagues, based on the presence of site-specific cross-links with a mitochondrial precursor with a classical targeting sequence, have revealed other proteins that also might play a role in import [34]. Of these, a 50 kDa protein is identified as a potential new import component [34].

### 3. TIM22 protein import pathway mediates insertion of inner membrane proteins

Many inner membrane proteins lack a cleavable targeting sequence, carrying instead their targeting and sorting information within the ‘mature’ part of the polypeptide chain. This category of proteins includes at least 34 members of the yeast mitochondrial carrier family [35], which span the inner membrane six times, as well as the TIM components. The mechanism by which these inner membrane proteins cross the hydrophilic intermembrane space and then insert correctly into the inner membrane has been uncertain until recently; a new protein import pathway (designated TIM22) that acts specifically on inner membrane proteins has been identified (Fig. 3) [36–41]. Components in this pathway are located in the mitochondrial inner membrane and intermembrane space.

#### 3.1. Inner membrane components of TIM22 import pathway

TIM22, an essential inner membrane protein, was the first component identified based on homology to Tim17 and...
Tim23 [42]. Surprisingly, depletion of Tim22 did not affect the general import pathway but inhibited the insertion of inner membrane proteins, particularly those of the carrier family. Although the new protein seemed to participate in mitochondrial import, it was not part of the well-characterized Tim17/Tim23 complex. Rather, Tim22 was recovered from detergent-solubilized mitochondria in a separate high molecular weight complex [42]. A second component, Tim54, was identified through a two hybrid interaction with the mitochondrial outer membrane protein Mmm1 [39]. Subsequent analysis revealed that Tim54 is an integral inner membrane protein and partners with Tim22. Inactivation of Tim54 in a temperature-sensitive strain inhibited import of AAC into isolated mitochondria [39].

Tim18 was recently identified because it interacted genetically with a temperature-sensitive strain mutant [43] and co-immunoprecipitated with Tim54 [44]. Tim18 is an integral inner membrane protein that is 40% identical to Sdh4, the membrane anchor of succinate dehydrogenase [45]. Tim18, Tim22 and Tim54 with the tiny Tim proteins of the intermembrane space form a 300 kDa complex. While a direct role in protein import has not been established, Tim18 may regulate assembly of the 300 kDa complex because depletion of Tim18 yielded a functional complex of 250 kDa [43,44].

3.2. ‘Tiny Tims’ of the intermembrane space

A family of small proteins in the mitochondrial intermembrane space mediates import of inner membrane proteins across the intermembrane space [36-38,40,41]. Five proteins, Tim8, Tim9, Tim10, Tim12 and Tim13, have been identified in the yeast intermembrane space, while similar complements are present in other metazoans (Fig. 3). The amino acid sequences of the small Tim proteins are 25% identical and 50% similar to each other. They also share a ‘twin CX3C’ motif, in which two cysteine residues are separated by three amino acids and each cysteine block is separated from the other by 11-16 amino acids [38]. This motif is reminiscent of a canonical zinc finger, but with a longer spacer [46]. Recombinants Tim10 and Tim12 fusion proteins bind zinc, and interaction between Tim10 and AAC is inhibited by zinc chelators [40], suggesting that the small Tim proteins bind zinc and that zinc binding is required for their function in vivo.

Tim10 and Tim12 were the first two identified components of the intermembrane space to mediate protein import [36,40]. Fractionation of yeast mitochondria showed that most of Tim10 was located in the soluble intermembrane space whereas Tim12 was peripherally bound to the outer surface of the inner membrane. Both proteins could be cross-linked chemically to a partly imported AAC precursor, indicating that they interact directly with the imported protein. However, the different intramitochondrial locations of Tim10 and Tim12 reflect their different functions in the import pathway. Inactivation or deletion of Tim12 did not interfere with import of AAC into the intermembrane space, but prevented insertion of AAC into the inner membrane. In contrast, inactivation or deletion of Tim10 blocked import of AAC, PiC and Tim22 across the outer membrane. Thus, Tim10 functions before Tim12, probably by binding the incoming precursor as it emerges from the TOM complex.

Tim9 was identified as a partner protein with Tim10 through genetic and biochemical approaches [37,41]. Most of Tim9 is located in the mitochondrial intermembrane space as a soluble 70 kDa complex containing approximately equimolar amounts of the Tim9 and Tim10 [37,41]; the rest is present in the 300 kDa insertion complex. A single serine-cysteine mutation in Tim9 allowed the protein to suppress the temperature-sensitive mutation in Tim10 [37].

The other two yeast proteins related to Tim10 and Tim12, Tim8 and Tim13 [38,47], were found in the intermembrane space as a distinct 70 kDa complex that could be separated from the Tim9/Tim10 complex by ion exchange chromatography [38]. Deletion of Tim8 or Tim13, alone or in combination, had no notable effect on cell growth and did not significantly affect import of AAC or PiC into isolated mitochondria. However, deletion of Tim8 in combination with a temperature-sensitive Tim10 mutation was lethal [38]. Studies with a broader spectrum of precursors in strains lacking Tim8 or Tim13 revealed that Tim8/Tim13 mediated import of Tim23 [48]. Thus the Tim8/Tim13 complex most likely works in parallel with the Tim9/Tim10 complex by mediating the import of a subset of integral inner membrane proteins.

The specific route taken by the substrate to reach the inner membrane is still uncertain. One possibility is that the small Tim complexes act as chaperone-like molecules to guide the precursor across the aqueous intermembrane space, yielding a soluble intermediate in which the precursor is bound to the 70 kDa complexes in the intermembrane space (Fig. 3). This model is supported by import studies with temperature-sensitive Tim10 and tim12 mutants, and by the fact that an AAC translocation intermediate bound to Tim10 in intact mitochondria is protected from added protease [36,37]. It predicts a transient complex in which Tim9/Tim10 or Tim8/Tim13 are bound directly to the precursor. Equally plausible is a model in which the 70 kDa complexes form a link between the TOM and the TIM complexes. In this model, the precursor is not released into the intermembrane space, but binds to the small Tim proteins as it emerges from the TOM complex. Further transfer to the Tim22/Tim54 complex could then occur without release into the intermembrane space. This model is supported by the recent finding that an AAC translocation intermediate is partially degraded by added protease [49]. It predicts a transient complex in which the TOM complex as well as the small Tim proteins are bound to the precursor.

3.3. Defective protein import: a novel type of mitochondrial disease

Humans contain at least six homologs of the small Tim proteins found in the yeast mitochondrial intermembrane space. One of these homologs had already been termed deafness-dystonia peptide (DDP1) because its loss results in the severe X-linked Mohr–Tranebjaerg syndrome, characterized by deafness, dystonia, muscle weakness, dementia and blindness [50,51].

DDP1 is most similar to yeast Tim8 and, when expressed in monkey or yeast cells, is located in mitochondria [38]. Mohr–Tranebjaerg syndrome is thus almost certainly a new type of mitochondrial disease caused by a defective protein import system of mitochondria. Loss of DDP1 function probably lowers the mitochondrial abundance of some inner membrane proteins that are critical for the function, development or maintenance of the sensorineural and muscular systems in mammals. The findings in yeast suggest that DDP1 functions as a complex with related partner proteins, perhaps with
hTim13. As mutations in DDP1 partner proteins may also be deleterious, and as all potential partner proteins are autoso-
mally encoded, non-X-linked diseases with symptoms resemble-
ing those of Mohr–Tranebjaerg syndrome may well have a
related etiology. Further, the link between a mitochondrial
import defect and a neurodegenerative disease may provide
insights into the molecular basis of other more frequent neu-
rological diseases such as Parkinsonism that have been corre-
lated with mitochondrial dysfunction.

4. Mitochondrial protein export pathways

As with protein import pathways, recent studies in protein
export pathways for mitochondrially coded proteins have re-
vealed new membrane components. While the topology of
mitochondrial export resembles that of bacterial secretion,
the yeast genome does not encode detectable homologs of the
bacterial Sec translocase [1]. However, at least two path-
ways have been identified for protein export from the matrix
to the inner membrane (Fig. 1). Oxa1 is a nuclear-coded inner
membrane protein that mediates export of N- and C-tails of
the mitochondrially coded precursor cytochrome c oxidase
subunit II (Cox2) and also plays a role in ATP synthase for-
mation [52–54]. Oxa1 interacts directly with nascent mito-
ochondrially synthesized polypeptides [54]. However, its precise
role in membrane insertion is not clear because oxa1 mutants
can be suppressed by mutations in the nuclear gene coding the
cytochrome c1 subunit of the bc1 complex [53]. This suppres-
sion suggests that the conserved Oxa1 function can be by-
passed in the membrane insertion process. Interestingly,
Oxa1p has a homolog in the chloroplast, termed ALB3 in
Arabidopsis thaliana, which is an essential protein involved in
chlorophyll biosynthetic pathways [55].

A second export component, Pnt1, has been identified in an
elegant genetic screen to identify yeast mutants defective for
the export of mitochondrially coded proteins [56]. Pnt1 is an
integral inner membrane protein facing into the matrix that
mediates export of the C-terminus of Cox2. However, its pre-
cise role in export has not been determined because deletion of
pnt1 in S. cerevisiae did not impair Cox2 processing. Dele-
tion of the PNT1 ortholog from Kluyveromyces lactis,
KIPNT1, resulted in a non-respiratory phenotype, absence of
cytochrome oxidase activity, and a defect in the assembly of
KICox2 that appears to be due to a block of C-tail export.
Thus, it may be possible that Oxa1 and Pnt1 have overlapping
functions in S. cerevisiae. PNT1 was previously identified as a
gene that caused resistance to the antimicrobial drug pentam-
idine [57]. Given the coordination that must be required to
assemble the large respiratory complexes of the inner mem-
brane, one might expect that additional components will be
identified.

5. Concluding remarks

Biogenesis of the various import components itself is com-
plicated, with individual subunits using different pathways
[47]. Tim54 is imported via Tim9/Tim10 [48] and inserted
into the inner membrane through the TIM23 machinery
[47], whereas Tim22 is imported via the TIM22 complex
[36,41,47]. Import of the small Tim proteins bypasses the
Tim machinery altogether, requiring Tom5, but no membrane
potential [47]. The complex interplay between the different

machineries may ensure coordinated regulation of the assem-
bly of the mitochondrial protein import systems.

The recent discoveries of new import components and new
import pathways imply how little we know about mitochon-
drial biogenesis, particularly the inner membrane, but also
suggest that the answers to these questions will reveal exciting
insights into a complicated biological process. Because new
protein import components are still being identified, we are
only at the tip of the iceberg when it comes to understanding
the mechanisms of protein import.

Acknowledgements: C.M. Koehler is the recipient of the Damon Run-
yon Scholar Award from the Cancer Research Fund of the Damon
Runyon-Walter Winchell Foundation (DRS18). This author’s re-
search is also supported in part by the Burroughs Wellcome Fund
New Investigator Award in the toxicological sciences, American Heart
Association #0030147N, Muscular Dystrophy Association #022398,
Deafness Research Foundation, and Research Corporation #R10459.

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