Molecular & Biochemical Parasitology 244 (2021) 111393

Contents lists available at ScienceDirect

Molecular & Biochemical Parasitology

journal homepage: www.elsevier.com/locate/molbiopara

Minimal peptide length required to span the mitochondrial protein translocases in *Trypanosoma brucei*

Christoph Wenger, André Schneider *

Department of Chemistry, Biochemistry and Pharmaceutical Sciences, University of Bern, Freiestr. 3, CH-3012, Bern, Switzerland

ARTICLE INFO

Keywords:
Trypanosoma
Mitochondrial protein import
ATOM complex
TIM complex

ABSTRACT

Mitochondrial protein import depends on heterooligomeric translocases in the outer and inner membranes. Using import substrates consisting of various lengths of the N-terminal part of mitochondrial dihydrolipoamide dehydrogenase (LDH) fused to dihydrofolate reductase we present an *in vivo* analysis showing that in *Trypanosoma brucei* at least 96 aa of mature LDH are required to efficiently produce an import intermediate that spans both translocases. This is different to yeast, where around 50 aa are sufficient to achieve the same task and likely reflects the different arrangement and architecture of the trypanosomal mitochondrial translocases. Furthermore, we show that formation of the stuck import intermediate leads to a strong growth inhibition suggesting that, depending on the length of the LDH, the import channels in the translocases are quantitatively blocked.

Mitochondria import more than 95 % of their proteins from the cytosol. The process is mediated by protein translocases termed TOM and TIM complexes in the outer and the inner membrane (OM and IM) of the organelle, respectively. The TOM and TIM complexes have been studied in great detail in yeast and to a lesser extent in mammals [1,2]. However, yeast and mammals belong to the same eukaryotic supergroup of the Opisthokonts and experimental studies of mitochondrial protein import in other eukaryotic supergroups have been scarce. One exception is the parasitic protozoan *Trypanosoma brucei*, a member of the Discoba of the paraphyletic Excavate supergroup [3], which was shown to have the most highly diverged TOM and TIM complexes known to date [4–7]. As the TOM complex in yeast, the trypanosomal atypical TOM complex (ATOM) consists of seven subunits [7,8]. However, except for ATOM40 and ATOM14, which are highly diverged orthologues of yeast Tom40 and Tom22 respectively, the remaining 5 subunits are restricted to the Kinetoplastids. The trypanosomal TIM complex is also very unusual. While Opisthokonts have two TIM complexes, the TIM23 complex specialized for the presequence pathway and the TIM22 complex specialized for the insertion of IM proteins with multiple transmembrane domains, *T. brucei* has a single TIM complex that deals with both types of substrates [9]. The only trypanosomal protein that is homologous to integral membrane TIM subunits of Opisthokonts is TbTim17 [10], a remote Tim22 orthologue. All other five TIM subunits are restricted to the Kinetoplastids.

Here, we have determined the minimal length of a peptide that is sufficient to span both the unique ATOM and TIM complexes of *T. brucei* using an approach that has been pioneered in yeast many years ago [11, 12]. We produced tetracycline-inducible transgenic trypanosomes expressing mouse dihydrofolate reductase (DHFR) that is C-terminally HA-tagged and N-terminally fused to different lengths (78–154 aa) of the N-terminal part of trypanosomal dihydrolipoamide dehydrogenase (LDH) including its 14 aa presequence (Fig. 1A). The folate analogue aminopterin (AMT) strongly binds to DHFR and stabilizes its 3D conformation preventing its unfolding. Since mitochondrial protein import requires unfolding of the translocated substrates, AMT prevents import of the DHFR moiety of the fusion proteins. Depending on the length of the LDH part, this allows the formation of import intermediates that are stuck in the import channels of the OM and IM. As would expected based on data from yeast and mammals, the formation of the import intermediate depends on mHsp70 of the presequence-associated import motor (PAM) module, which pulls the unfolded protein across the two mitochondrial membranes [13].

In order to see which of the LDH-DHFR import substrates (Fig. 1A) accumulated in an ATOM-containing high molecular weight complex, crude mitochondrial extracts of all 9 transgenic cell lines were analyzed by blue native (BN) PAGE (Fig. 1B), and the resulting immunoblots were probed for the HA-tagged DHFR substrate (Fig. 1B, top panel) as well as the ATOM subunit ATOM40 (Fig. 1B, bottom panel), respectively. Moreover, the same fractions were also subjected to immunoprecipitations (IPs) using anti-HA antibodies to pull down the LDH-DHFR

* Corresponding author.
E-mail address: andre.schneider@dcb.unibe.ch (A. Schneider).

https://doi.org/10.1016/j.molbiopara.2021.111393
Received 28 May 2021; Received in revised form 23 June 2021; Accepted 24 June 2021
Available online 29 June 2021

substrates (Fig. 1C). The IP fractions were analyzed by SDS PAGE and the resulting immunoblots were probed for the ATOM subunits ATOM40 and the import receptor ATOM69 as well as for the TIM core subunit TbTim17. VDAC served as control for the specificity of the IPs.

The previously analyzed LDH-DHFR fusion with 154 of the LDH N-terminus (LDH-154-DHFR) served as a positive control [9]. It was efficiently imported into mitochondria in the absence of AMT but accumulated in a high molecular weight complex in its presence (Fig. 1B). Pull down analysis shows that this complex corresponds to an ATOM-TIM supercomplex as it contains the ATOM40 and ATOM69 as well as TbTim17 (Fig. 1C). The remaining LDH-DHFR substrates can be divided in three groups regarding their behavior in the presence of AMT.

The LDH-78 and LDH-83-DHFR fusion proteins do not accumulate in the ATOM-TIM supercomplex. In agreement with this observation, the corresponding ATOM40-containing complex shows the range of molecular weights expected for the ATOM complex only (Fig. 1B). Pull down experiments show that LDH-78-DHFR does not interact with ATOM subunits, whereas LDH-83-DHFR pulls down a small amount of ATOM69 and ATOM40 but not TbTim17 (Fig. 1C). Thus, LDH-78-DHFR does not accumulate at either translocase, but a very small amount of LDH-83-DHFR seems to accumulate at the ATOM complex. For the three fusion proteins LDH-88/93/100-DHFR a small fraction that increases proportionally with the length of the LDH moiety, accumulates in a high molecular weight complex (Fig. 1B, upper panel). In line with this observation, a small portion of ATOM40 gets shifted to a higher molecular weight (Fig. 1B, lower panel). Moreover, pull down experiments with LDH-88/100-DHFR recovered some TbTim17 confirming the formation of a small amount of ATOM-TIM supercomplexes (Fig. 1C). The LDH-110/120/130/140-DHFR fusion proteins, finally, behaved like the positive control LDH-154-DHFR, producing large amounts of ATOM-TIM supercomplexes containing ATOM40, ATOM69 as well as TbTim17 (Fig. 1B and C).

Fig. 2 shows that the fitness of cell lines expressing selected LDH-DHFR fusion proteins in the presence of AMT is in line with the results of Fig. 1B. Expression of the shortest fusion protein LDH-78-DHFR did not inhibit growth. However, this changed for LDH-88-DHFR and LDH-110-DHFR, which both caused slow growth phenotypes. Expression of LDH-154-DHFR finally completely abolished growth (Fig. 2). The phenotypes are tightly linked to the expression of the various LDH-DHFR fusion proteins since they were only seen in the presence of tetracycline. Both uninduced and induced cell lines were treated with 8 μM of AMT, which only marginally inhibited growth of the uninduced *T. brucei* cell lines (Fig. 2). Thus, LDH-78-DHFR cannot form ATOM-TIM supercomplexes and, therefore, does not inhibit growth. The modest growth reduction caused by LDH-88-DHFR is likely caused by a partial blockage of the ATOM complex only since the fusion protein seems to efficiently

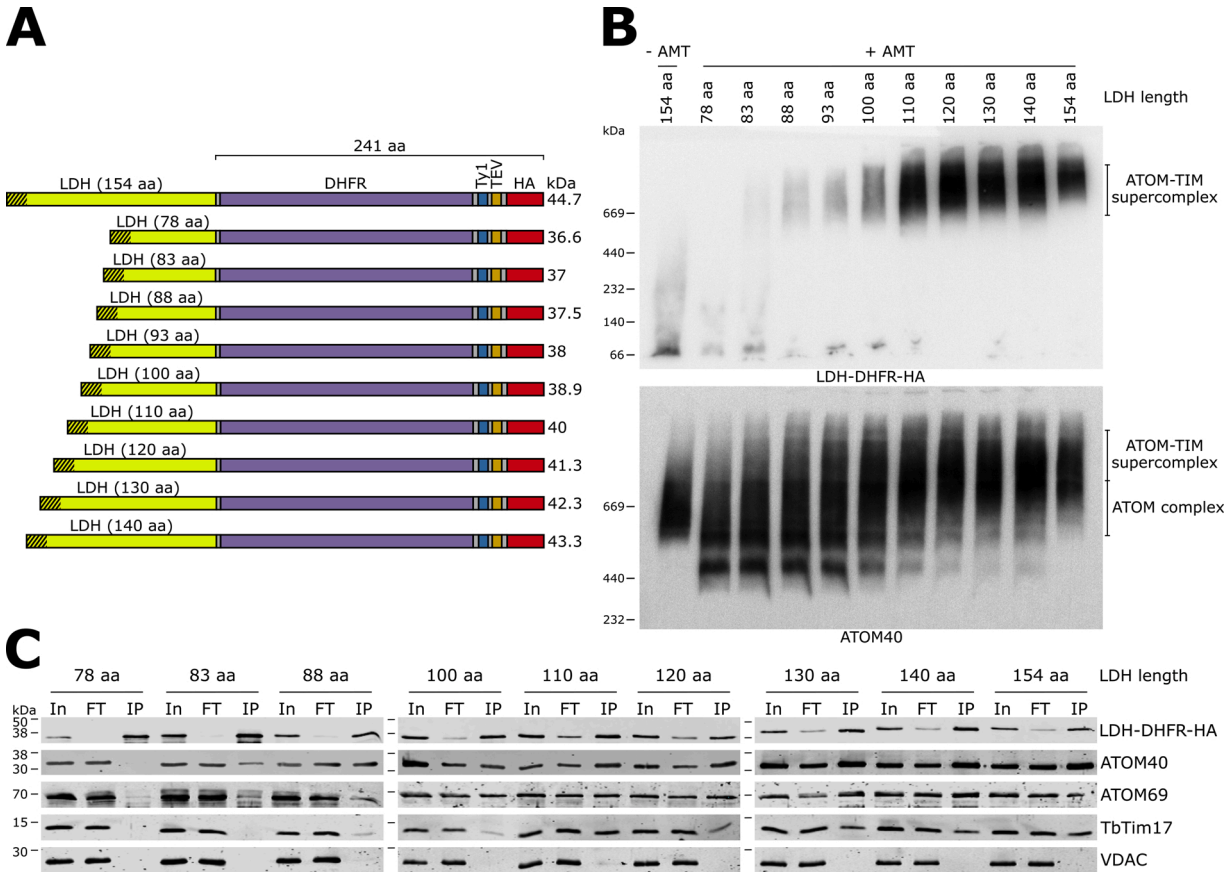

Fig. 1. Formation of import intermediates using LDH-DHFR substrate of different lengths. A. Constructs encoding fusion proteins consisting of the first 154, 78, 83, 88, 93, 100, 110, 120, 130 and 140 aa of LDH (yellow) including its 14 aa presequence (yellow, shaded) and mouse DHFR (purple), followed by a Ty1-tag (blue), a TEV protease site (orange) and a triple HA-tag (red). Length of the DHFR moiety is indicated at the top and the predicted molecular weight of the fusion protein at the right. **B.** BN-PAGE immunoblot analysis of solubilized mitochondrial fractions of the transgenic cell lines expressing the indicated LDH-DHFR fusion proteins grown in the absence (-AMT) or presence of AMT (+AMT). Top panel was probed for the LDH-DHFR substrates using anti-HA antibodies. Lower panel was probed for the ATOM complex using anti-ATOM40 antibodies. **C.** IPs using anti-HA antibody containing beads of solubilized mitochondrial fractions from transgenic cell lines expressing the indicated HA-tagged LDH-DHFR fusion proteins grown in the presence of AMT. The input (In) (10 %), unbound flowthrough (FT) (10 %) and eluate (IP) (100 %) fractions were analysed by SDS-PAGE (14 %) and processed for immunoblotting. The resulting blots were probed for the HA-tagged LDH-DHFR fusion proteins, ATOM40, ATOM69 and TbTim17. The voltage dependent anion channel (VDAC) served as a negative control. Preparation of constructs, transgenic cell lines are (based on procyclic *T. brucei* 29-13), mitochondrial fractions, BN-PAGE analysis and IPs were done as previously described [9].

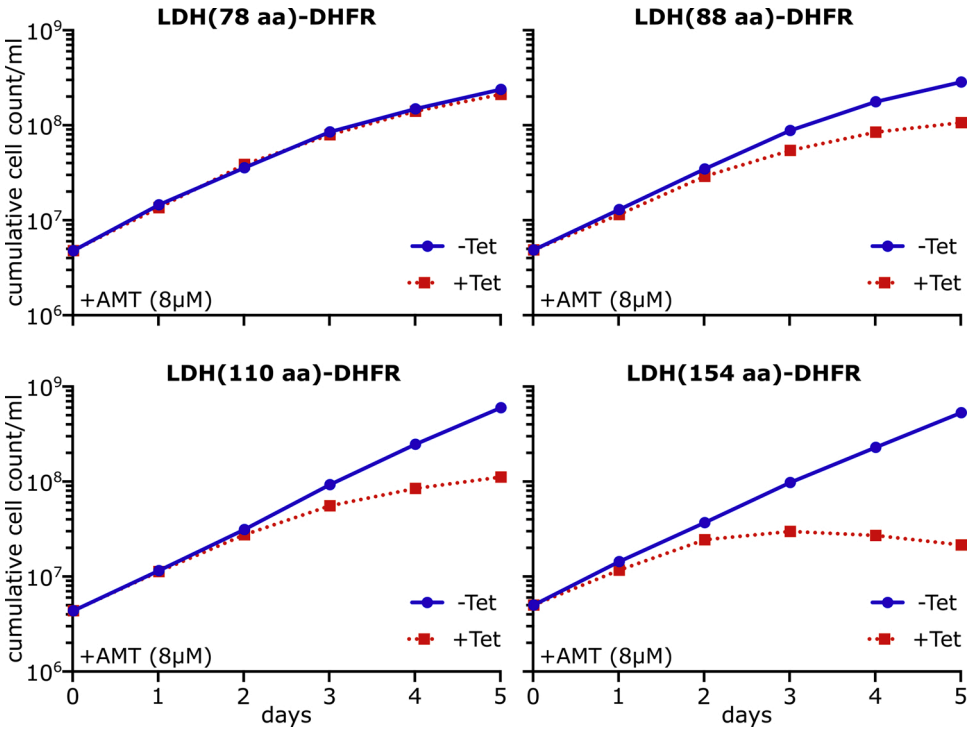

Fig. 2. Expression of LDH-DHFR fusion proteins can affect growth. A. Growth curves of transgenic *T. brucei* cell lines grown in SDM-79 medium in the presence of 8 μM of AMT not expressing (-Tet) or expressing (+Tet) the indicated LDH-DHFR fusion proteins.

interact with the ATOM40 and ATOM69. LDH-110-DHFR which appears to cause a slightly stronger growth phenotype than LDH-88-DHFR in addition also interacts with TbTim17. The strong growth inhibition seen by LDH-154-DHFR, finally, likely is caused by efficient formation of ATOM-TIM supercomplexes and blockage of the import channels (Fig. 1B).

In summary, we show that a 78 aa long peptide sequence fused to tightly folded AMT-stabilized DHFR can neither efficiently engage with the ATOM nor with the TIM complex. Increasing the LDH length to 83, 88, 93 or 100 aa, allows efficient binding to the ATOM complex but only residual binding to the TIM complex. Efficient interaction with both ATOM and TIM complexes, finally, requires at least around 110 aa of the LDH sequence.

It is interesting to compare these results to similar experiments that have been performed in *Saccharomyces cerevisiae* [14]. Using a cytochrome b2-DHFR fusion protein in *in vitro* import assays, it was shown that a sequence of around 50 aa, excluding the processed mitochondrial presequence, is enough to span both the yeast TOM and TIM23 complexes. In *T. brucei*, however, even 83 aa are too short to achieve that same task. If we subtract the 14 aa presequence of LDH [15], which should allow direct comparison to the yeast experiment, this suggests that 69 aa are not enough to span ATOM and TIM in trypanosomes. It should be noted that we were not able to determine whether the presequence of LDH gets processed in the various fusion proteins, although we would expect this to be the case at least for the long LDH-DHFR fusion proteins which efficiently form ATOM-TIM supercomplexes. In fact, even when the length of the LDH moiety is increased to 100 aa (86 aa without presequence) only small amounts of ATOM-TIM supercomplexes are formed and most of the TIM complex does not engage with the substrate. These results strongly suggest that the minimal peptide length to span the ATOM and TIM complexes in *T. brucei* is at least 86 aa, 40 % longer, than the one required to span the TOM and TIM23 complexes in yeast.

The architecture of the ATOM-TIM supercomplex is very different from the TOM-TIM23 complex in yeast:

- The single trypanosomal TIM complex contains five essential integral membrane subunits whose specific roles in the import process are unknown, these includes two inactive rhomboid-like proteins that are specifically associated with the presequence pathway [9]. The yeast presequence translocase TIM23, however, consists of only three essential subunits [16] and thus is much smaller than its counterpart in trypanosomes
- The trypanosomal TIM is permanently associated, on the intermembrane space side, with heterooligomeric complexes consisting of five different small TIM chaperones [17], even when it is engaged in the import of presequence-containing proteins [9]. This is in contrast to the yeast presequence translocase TIM23 which is not associated with small TIM chaperones, since the small TIM complexes of yeast are specifically bound to the TIM22 complex.
- Efficient formation of supercomplexes requires an active PAM module. It was recently shown that the trypanosomal PAM is quite different from the one in yeast. It lacks a Tim44 orthologue and the TbPam27, which serves as a functional analogue of yeast Pam18, is much larger than its yeast counterpart [13]. Furthermore, as yet unknown PAM subunits may exist in *T. brucei*. It can, therefore, be speculated that the trypanosomal PAM is larger than the PAM module of yeast.

In summary, all these unique features of the trypanosomal protein translocases may increase the distance that a precursor protein is required to bridge between the ATOM and TIM complexes, compared to the situation in yeast. Thus, we show that adapting an old classic experiment from yeast [12] allows to probe the *in vivo* organization and architecture of the unique trypanosomal protein translocases.

Author statement

Christoph Wenger: Conceptualization, Investigation, Visualization, Writing – Review & Editing. André Schneider: Writing Original draft, Supervision, Funding acquisition.

Acknowledgments

This study was supported by grant 175563 and in part by the NCCR "RNA & Disease" both funded by the Swiss National Science Foundation.

References

[1] N. Pfanner, B. Warscheid, N. Wiedemann, Mitochondrial proteins: from biogenesis to functional networks, Nat. Rev. Mol. Cell Biol. 20 (2019) 267–284.
[2] K.G. Hansen, J.M. Herrmann, Transport of proteins into mitochondria, Protein J. 38 (2019) 330–342.
[3] F. Burki, A.J. Roger, M.W. Brown, A.G.B. Simpson, The new tree of eukaryotes, Trends Ecol. Evol. 35 (1) (2020) 43–55.
[4] A. Harsman, A. Schneider, Mitochondrial protein import in trypanosomes: expect the unexpected, Traffic 18 (2017) 96–109.
[5] M. Chaudhuri, C. Darden, F.S. Gonzalez, U.K. Singha, L. Quinones, A. Tripathi, Tim17 updates: a comprehensive review of an ancient mitochondrial protein translocator, Biomolecules 10 (2020).
[6] A. Schneider, Evolution of mitochondrial protein import - lessons from trypanosomes, Biol. Chem. 401 (2020) 663–676.
[7] A. Schneider, Mitochondrial protein import in trypanosomatids: variations on a theme or fundamentally different? PLoS Pathog. 14 (2018), e1007351.
[8] J. Mani, S. Desy, M. Niemann, A. Chanfon, S. Oeljeklaus, M. Pusnik, O. Schmidt, C. Gerbeth, C. Meisinger, B. Warscheid, et al., Mitochondrial protein import receptors in Kinetoplastids reveal convergent evolution over large phylogenetic distances, Nat. Commun. 6 (2015) 6646.
[9] A. Harsman, S. Oeljeklaus, C. Wenger, J.L. Huot, B. Warscheid, A. Schneider, The non-canonical mitochondrial inner membrane presequence translocase of trypanosomatids contains two essential rhomboid-like proteins, Nat. Commun. 7 (2016) 13707.
[10] U.K. Singha, E. Peprah, S. Williams, R. Walker, L. Saha, M. Chaudhuri, Characterization of the mitochondrial inner membrane protein translocator Tim17 from Trypanosoma brucei, Mol. Biochem. Parasitol. 159 (2008) 30–43.
[11] M. Eilers, G. Schatz, Binding of a specific ligand inhibits import of a purified precursor protein into mitochondria, Nature 322 (1986) 228–232.
[12] A. Schneider, Dihydrofolate reductase and membrane translocation: evolution of a classic experiment, EMBO Rep. 19 (2018).
[13] C. von Känel, S.A. Muñoz-Gómez, S. Oeljeklaus, C. Wenger, B. Warscheid, J. G. Wideman, A. Harsman, A. Schneider, Homologue replacement in the import motor of the mitochondrial inner membrane of trypanosomes, eLIFE 9 (2020) e52560.
[14] J. Rassow, F.U. Hartl, B. Guiard, N. Pfanner, W. Neupert, Polypeptides traverse the mitochondrial envelope in an extended state, FEBS Lett. 275 (1990) 190–194.
[15] A.J. Else, D.W. Hough, M.J. Danson, Cloning, sequencing, and expression of *Trypanosoma brucei* dihydrolipoamide dehydrogenase, Eur. J. Biochem. 212 (1993) 423–429.
[16] C. Schulz, A. Schendzielorz, P. Rehling, Unlocking the presequence import pathway, Trends Cell Biol. 25 (2015) 265–275.
[17] C. Wenger, S. Oeljeklaus, B. Warscheid, A. Schneider, A. Harsman, A trypanosomal orthologue of an intermembrane space chaperone has a non-canonical function in biogenesis of the single mitochondrial inner membrane protein translocase, PLoS Pathog. 13 (2017), e1006550.