Powering mitochondrial protein import

Nikolaus Pfanner and Kaye N. Truscott

Proteins imported into mitochondria must be unfolded in order to pass through translocation pores present in the mitochondrial membranes. An article in this issue suggests that not only the heat shock protein 70 in the matrix, but also the electrical membrane potential across the inner membrane can actively unfold preproteins *via* a pulling mechanism.

The vast majority of mitochondrial proteins are synthesized as precursors in the cytosol and must be imported into the organelle[1–5]. Many of these precursors carry N-terminal targeting sequences, termed presequences, that direct the proteins to receptors on the mitochondrial surface and subsequently across the membranes into the inner compartment, the matrix (Fig. 1). Two major energy sources drive translocation of preproteins into mitochondria: (i) a membrane potential ($\Delta\psi$) across the inner membrane and (ii) ATP that powers an import motor, the matrix heat shock protein 70 (mtHsp70) in association with the translocase of the inner membrane (TIM). The membrane potential is thought to promote the initial insertion of presequences into the inner membrane, while mtHsp70 drives the completion of translocation and supports unfolding of precursor domains.

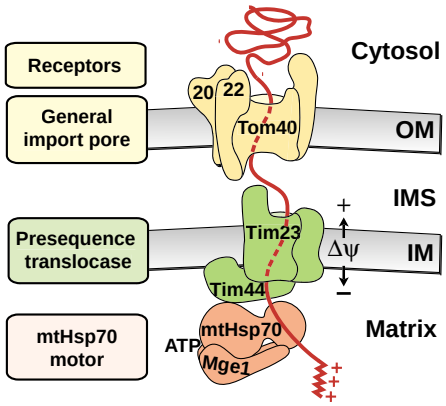

Fig. 1 Protein translocation into the mitochondrial matrix. The precursor protein (shown in red) is recognized by the receptors Tom20 and Tom22 of the mitochondrial outer membrane (OM). It passes through hydrophilic cation-selective channels formed by Tom40 and Tim23 of the outer membrane and inner membrane (IM), respectively. The membrane potential ($\Delta\psi$) across the IM is required for translocation of the positively charged presequence of the precursor protein. The mitochondrial heat shock protein 70 (mtHsp70) forms an ATP-driven import motor in cooperation with Tim44 and the nucleotide exchange factor Mge1. IMS, intermembrane space.

Although the requirement of these energy sources is well known, the molecular mechanisms of how they are converted into import-driving activities are the subject of an ongoing debate. Does the import machinery actively pull preproteins into the mitochondria, or does it merely trap spontaneous diffusive movements into the organelle? Does mtHsp70 catalyze the unfolding of precursor domains during import by an active or a passive mechanism? On page 301 of this issue of *Nature Structural Biology*, Huang *et al.*[6] provide a new twist: they show that not only mtHsp70, but also the $\Delta\psi$ can actively promote the unfolding of precursor domains. This study indicates that the electrophoretic effect produced by $\Delta\psi$ on presequences could lead to catalyzed unfolding of precursor domains, strongly supporting the view that an active pulling mechanism occurs within mitochondria.

Protein transport into the matrix

Mitochondrial presequences sequentially interact with a series of import components and thus direct the attached protein into mitochondria[7–9] (Fig. 1). The presequence first binds the receptors Tom20 and Tom22 of the translocase of the outer membrane (TOM)[1,2,4]. After passage through the general import pore formed by Tom40, the presequence interacts with the intermembrane space domain of Tom22 and is then transferred to Tim23, which forms a channel across the inner membrane. The affinities of preproteins for each individual binding partner are relatively low[7,8], and it is thought that matrix-destined preproteins can in principle move back and forth at distinct binding sites within the TOM machinery. Unidirectionality of the transport reaction is achieved by the strong import forces localized at the inner membrane, $\Delta\psi$ and mtHsp70. The membrane potential is essential for insertion of presequences into the Tim23 channel[10–12]. Upon exit from the channel, the presequence and mature portion of precursors are bound by the chaperone mtHsp70, which, together with Tim44 and the nucleotide exchange factor Mge1, drives the completion of transport into the matrix in an ATP-dependent manner[3,13–16].

The pore diameters of the TOM and TIM channels are so small that preproteins cannot be transported into mitochondria in a folded conformation. The inner diameter of the Tom40 channel is ~20 Å, whereas that of Tim23 is even smaller (~13 Å), probably reflecting the need to maintain an electrochemical proton gradient across the inner membrane[12,17–19]. The Tim23 channel is just wide enough to allow the passage of one polypeptide chain in an α-helical conformation[12], while Tom40 can accommodate two polypeptide segments simultaneously (for example, for transport of precursor proteins in a loop formation)[4]. Since most mitochondrial proteins are imported after completion of their synthesis, the preproteins are not necessarily in an unfolded conformation — indeed, several preproteins have been found to contain stably folded domains in the cytosol *in vivo*[20,21]. The N-terminal presequences are loosely folded and can enter the mitochondrial import machinery, while the compact domains are excluded and thus remain trapped at the outer side of the

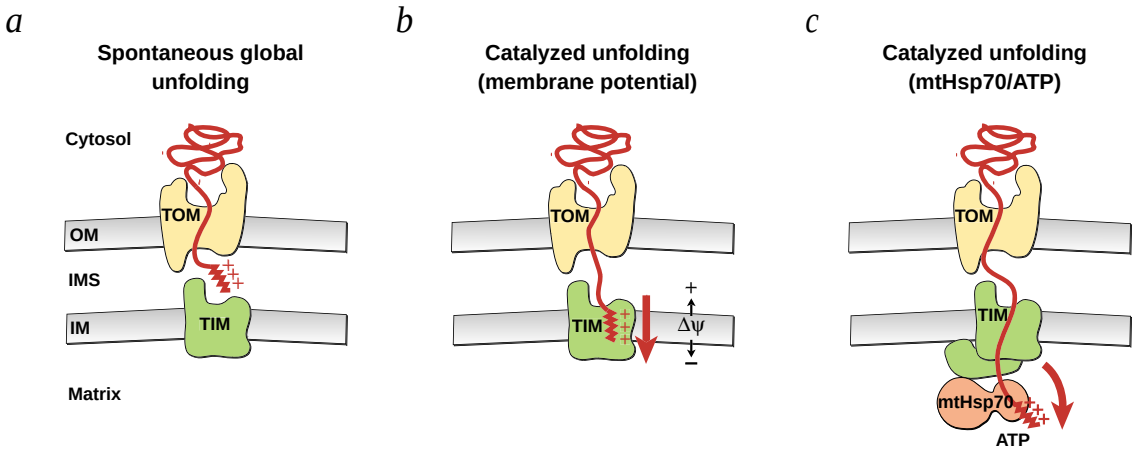

Fig. 2 Three mechanisms for protein unfolding during mitochondrial import. ***a***, Spontaneous global unfolding. The targeting sequence of a precursor protein (red) is so short that the import-driving activities of the inner membrane (Δψ) and matrix (mtHsp70) cannot act on it. Thus global spontaneous unfolding of folded domains is required before import can take place. ***b***, Unfolding of protein domains catalyzed by Δψ as shown by Huang *et al.*[6] The positively charged presequence is positioned in the electrical field of the inner membrane. Δψ can trap small unfolding fluctuations occurring at the N-terminus of the folded domain (biased diffusion of preprotein segments into mitochondria) and can pull the presequence in. Δψ thus unravels the precursor protein from its N-terminus. ***c***, Unfolding of protein domains catalyzed by the ATP-driven import motor mtHsp70. The N-terminal segment of the precursor protein is so long that it can reach the mtHsp70 system in the matrix. By a combination of trapping and pulling, mtHsp70 unravels the precursor protein from its N-terminus. TOM, translocase of outer membrane; TIM, translocase of inner membrane.

TOM channel (Fig. 1). So far it has been assumed that the unfolding of these domains occurs either spontaneously (when the N-terminal segment is too short to reach mtHsp70; Fig. 2*a*) or is promoted by mtHsp70 (for longer preproteins; Fig. 2*c*).

Role of the membrane potential

The membrane potential has two known functions in protein import: (i) it is required for the insertion of every presequence into the inner membrane channel. This requires only the electrical component (Δψ) of the electrochemical proton gradient across the inner membrane, supporting the view that Δψ produces an electrophoretic effect on the positively charged presequences[11]; and (ii) Δψ activates the Tim23 channel[12,22].

Huang *et al.*[6] discovered a novel role of Δψ by using a model protein, a construct consisting of the N-terminal portion of a mitochondrial preprotein fused to a passenger protein, barnase. The folding and unfolding of barnase have been studied in detail; hence distinction between spontaneous and catalyzed unfolding of the protein is possible. Huang *et al.*[23] previously showed that mitochondria can actively unfold barnase by first unraveling it at the N-terminus. In contrast, spontaneous unfolding of barnase is a global process, originating within the middle portion of the protein. A high affinity protein ligand, barstar, inhibits this spontaneous unfolding process, but not the active unfolding by mitochondria, providing an elegant assay for studying the mechanism of protein unfolding during import into isolated mitochondria.

Huang *et al.*[6] used three preproteins that differ in the lengths of the loosely folded N-terminal segments — a short (35 amino acids), an intermediate (65 amino acids) or a long (95 amino acids) polypeptide — fused to barnase. Import of the short preprotein depends on the spontaneous unfolding of barnase since binding of the ligand barstar strongly inhibits this process (Fig. 2*a*). Import of the other two preproteins, however, is not inhibited by binding of barstar, demonstrating that their unfolding pathway has changed; it is actively catalyzed by the mitochondrial import machinery (Fig. 2*b*,*c*). Unfolding of the long preprotein depends on the activity of mtHsp70 as expected (Fig. 2*c*), but, surprisingly, the unfolding of the intermediate-sized preprotein depends on the magnitude of Δψ (Fig. 2*b*). When Δψ is partially reduced, import of the intermediate-sized preprotein is strongly impaired, whereas import of the long preprotein is only slightly affected. The effect of reduced Δψ can be rescued by first denaturing the intermediate-sized preprotein before import. Interestingly, barstar binding inhibits import of the intermediate-sized preprotein (with folded barnase) at low Δψ, demonstrating that spontaneous unfolding of barnase is needed under this condition. Thus, unfolding of this preprotein proceeds along different pathways at high and low Δψ.

The sophisticated analysis of Huang *et al.*[6] directly shows that the mitochondrial membrane potential promotes active unfolding of certain passenger proteins. This function requires a high Δψ created by the intact proton gradient across the inner membrane. Furthermore, the results show that the action of Δψ depends on the length of the loosely folded N-terminal segment of the preprotein . This dependence is explained by the accessibility of the presequence to the import-driving forces of mitochondria. The presequence of the long preprotein can reach all the way into the matrix while barnase is still folded, and the mtHsp70 chaperone is used to drive the unfolding reaction (Fig. 2*c*). The intermediate-sized preprotein is just long enough such that the presequence is in the electrical field of the inner membrane, and the electrophoretic effect drives the unfolding reaction (Fig. 2*b*). When the presequence is too short, the preprotein does not respond to either of the catalyzed unfolding processes and thus spontaneous unfolding is necessary (Fig. 2*a*).

Pulling preproteins in

The molecular mechanism of protein unfolding by mitochondria is a contentious issue, with two hotly debated views on the role of mtHsp70 in the process. In one view, mitochondria play a passive role: preprotein segments diffuse into the organelle by Brownian motion; the inward movements are simply trapped by the import machinery[24]. In this model, folded domains located on the cytosolic

side must undergo spontaneous global unfolding to allow insertion of further precursor segments into mitochondria and subsequent trapping by mtHsp70. In the second view, mtHsp70 actively contributes to the unfolding process[3,16,25]: mtHsp70 bound to the inner membrane import channel undergoes ATP-dependent conformational changes to capture local unfolding fluctuations in the preproteins and generate a pulling force on the preprotein. However, the two views do not need to be mutually exclusive. In fact, recent evidence suggests a dual role of mtHsp70. The passive trapping of preproteins by mtHsp70 without major conformational changes of the chaperone is sufficient for import of loosely folded preproteins[16,26]; whereas an active pulling mechanism involving conformational changes of membrane-bound mtHsp70 is needed for preproteins with tightly folded domains. Thus, both trapping and pulling by mtHsp70 contribute to preprotein import into mitochondria[3,16,26].

Does $\Delta\psi$ promote protein unfolding by pulling at the preprotein? This would most likely be the case if the $\Delta\psi$-catalyzed unfolding is mediated by an electrophoretic effect of $\Delta\psi$ on the presequence. To test this hypothesis, Huang *et al.*[6] increased the positive charge density at the beginning of the presequence of the short preprotein; they found that import of this modified preprotein indeed becomes catalyzed by $\Delta\psi$. The result thus supports the proposed role of $\Delta\psi$. As with mtHsp70, $\Delta\psi$ may operate by a double mechanism, trapping spontaneous unfolding fluctuations at the N-terminus of barnase by biased diffusion and exerting a pulling force that facilitates labilization of folded domains[6].

For future studies, it will be interesting to use different structural folds to compare the unfolding power of $\Delta\psi$ and mtHsp70. It also remains open whether the pulling force of $\Delta\psi$ is strictly limited to the presequence or whether $\Delta\psi$ can also facilitate the translocation of positively charged segments located in the mature portion of preproteins (for example, when the activity of mtHsp70 is limiting). While previous models for mitochondrial protein import assign individual and single functions to the import driving forces, it is becoming more and more evident that multiple mechanisms operate. The membrane potential has at least three functions: activation of the channel of the inner membrane (Tim23), insertion of presequences into the channel and active unfolding of some preproteins. MtHsp70 promotes protein import by trapping and pulling of preproteins and, in addition, supports proper protein folding in the matrix. Finally, depending on the preprotein and the distance between presequence and folded domain, there are at least three mechanisms for protein unfolding during mitochondrial import: spontaneous unfolding and catalyzed unfolding mediated by either mtHsp70 or the membrane potential.

Nikolaus Pfanner and Kaye N. Truscott are in the Institut für Biochemie und Molekularbiologie, Universität Freiburg, Hermann-Herder-Straβe 7, D-79104 Freiburg, Germany. Correspondence should be addressed to N.P. email: Nikolaus.Pfanner@biochemie.uni-freiburg.de

1. Koehler, C.M., Merchant, S. & Schatz, G. *Trends Biochem. Sci.* **24**, 428–432 (1999).
2. Bauer, M.F., Hofmann, S., Neupert, W. & Brunner, M. *Trends Cell Biol.* **10**, 25–31 (2000).
3. Matouschek, A., Pfanner, N. & Voos, W. *EMBO Rep.***1**, 404–410 (2000).
4. Pfanner, N. & Geissler, A. *Nature Rev. Mol. Cell Biol.* **2**, 339–349 (2001).
5. Jensen, R.E. & Johnson, A.E. *Nature Struct. Biol.* **8**, 1008–1010 (2001).
6. Huang, S., Ratliff, K.S. & Matouschek, A. *Nature Struct. Biol.* **9**, 301–307 (2002)
7. Brix, J., Dietmeier, K. & Pfanner, N. *J. Biol. Chem.* **272**, 20730–20735 (1997).
8. Komiya, T. *et al. EMBO J.* **17**, 3886–3898 (1998).
9. Meisinger, C. *et al. Mol. Cell. Biol.* **21**, 2337–2348 (2001).
10. Schleyer, M. & Neupert, W. *Cell* **43**, 339–350 (1985).
11. Martin, J., Mahlke, K. & Pfanner, N. *J. Biol. Chem.* **266**, 18051–18057 (1991).
12. Truscott, K.N. *et al. Nature Struct. Biol.* **8**, 1074–1082 (2001).
13. Kang, P.J. *et al. Nature* **348**, 137–143 (1990).
14. Schneider, H.-C. *et al. Nature* **371**, 768–774 (1994)
15. Horst, M. *et al. EMBO J.* **16**, 1842–1849 (1997).
16. Voisine, C. *et al. Cell* **97**, 565–574 (1999).
17. Künkele, K.-P. *et al. Cell* **93**, 1009–1019 (1998).
18. Hill, K. *et al. Nature* **395**, 516–521 (1998).
19. Schwartz, M.P. & Matouschek, A. *Proc. Natl. Acad. Sci. USA* **96**, 13086–13090 (1999).
20. Wienhues, U. *et al. J. Cell Biol.* **115**, 1601–1609 (1991).
21. Bömer, U. *et al. J. Biol. Chem.* **272**, 30439–30446 (1997).
22. Bauer, M.F., Sirrenberg, C., Neupert, W. & Brunner, M. *Cell* **87**, 33–41 (1996).
23. Huang, S., Ratliff, K.S., Schwartz, M.P., Spenner, J.M. & Matouschek, A. *Nature Struct. Biol.* **6**, 1132–1138 (1999).
24. Gaume, B. *et al. EMBO J.* **17**, 6497–6507 (1998).
25. Matouschek, A. *et al. EMBO J.* **16**, 6727–6736 (1997).
26. Geissler, A., Rassow, J., Pfanner, N. & Voos, W. *Mol. Cell. Biol.* **21**, 7097–7104 (2001).

Effector regulation in a monomeric enzyme

Martha L. Ludwig and Rowena G. Matthews

The monomeric B_{12}-dependent ribonucleotide reductase from *L. leichmannii* has the central 10-stranded α/β-barrel found in all ribonucleotide reductases but incorporates two distinctive structural features, a novel cobalamin-binding fold and an insert forming part of a specificity control site that mimics the allosteric site found in the oligomeric di-iron dependent reductases.

"It is usually the comparison of structures... that is most enlightening about function."
Gregory A. Petsko

Ribonucleotide reductases (RNRs) catalyze the reduction of ribonucleotides to deoxyribonucleotides. Since no pathway exists for the *de novo* biosynthesis of deoxyribonucleotides, which can only be formed by reduction of ribonucleotides, these enzymes play essential roles in all living organisms. All known reductases employ a radical mechanism for ribonucleotide reduction, in which catalysis is initiated by hydrogen atom transfer from the substrate to a thiyl radical[1,2]. Quite surprisingly for essential enzymes, ribonucleotide reductases employ three different chemical strategies to generate the essential thiyl radical. The structure of the B_{12}-dependent ribonucleotide reductase from *Lactoba-*