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# Protein unfolding: mitochondria offer a helping hand

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**Post-translocational protein import into mitochondria requires that the protein be unfolded prior to import. Mapping the mitochondrial-assisted unfolding pathway of a cargo protein and demonstrating that it deviates from the spontaneous unfolding pathway have provided valuable insight into the energetics of the translocation process.**

Since Anfinsen's seminal studies, there has been an enormous effort vested in understanding how proteins are folded and unfolded in solution. In recent years, we have also seen explosive progress in the study of protein folding in the cell. The connections between folding of proteins *in vitro* and *in vivo*, however, have been lacking. On page 1132 of this issue of *Nature Structural Biology*, Huang *et al.*[1] demonstrate such a rare connection and in doing so provide critical insight into how proteins are translocated across the mitochondrial membranes.

To elucidate the complete folding pathways of a protein, researchers for many years have studied the unfolding of proteins after the addition of denaturant since unfolding is the reverse of folding. Protein unfolding is essential to many cellular processes, including the degradation of proteins by ATP-dependent proteases and the translocation of proteins across membranes. The vast majority of proteins are synthesized by cytosolic ribosomes; therefore the translocation of proteins across membranes is a process that is critical to the operation of all organelles. Mitochondria face the most daunting of translocation tasks — the post-translational insertion of, in some cases, a folded protein through a double set of membranes (for reviews see refs 2, 3). For a folded protein to be threaded through proteinaceous channels that reside in mitochondrial membranes, it first has to be unfolded on the cytosolic side of the membrane. Therefore, how a protein is unfolded prior to its import into mitochondria is an important biological question.

Matouschek and colleagues were uniquely positioned to address this question, having previously characterized in detail the *in vitro* unfolding pathway of a prototypic protein, barnase[4]. The unfolding pathway was mapped by analyzing how the addition or removal of domain specific stabilizing factors affected the kinetics of barnase unfolding. By fusing barnase with a mitochondrial presequence that targets the protein to mitochondria, they were able to dissect the unfolding pathway during mitochondrial import, utilizing mutations of barnase that affect its unfolding, and crosslinking or ligand binding that stabilizes specific folding domains[1]. Interestingly, the spontaneous unfolding pathway in free-solution differs from the unfolding pathway during import. Instead of unfolding starting in the core of the protein and progressing outward to the termini as occurs in free-solution, the fusion protein unravels vectorially from the N- toward the C-terminus in the presence of mitochondrial membranes (Fig. 1). Evidently the import machinery of the mitochondria is able to act as an enzyme, catalyzing and altering the unfolding pathway.

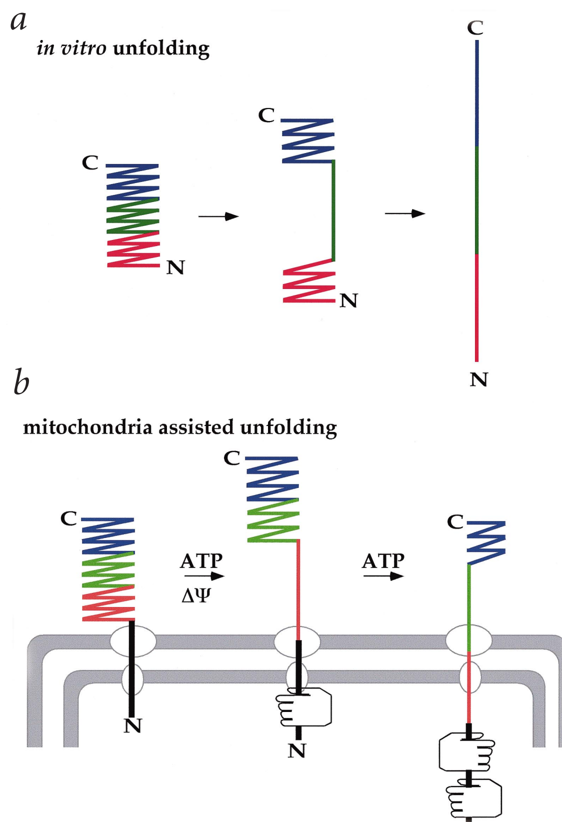


**Fig. 1** Alteration of the protein unfolding pathway by mitochondria. ***a***, A cartoon diagram illustrates the spontaneous unfolding pathway of barnase in free-solution. ***b***, A cartoon diagram indicates the unfolding pathway of barnase that has been targeted to mitochondrial membranes by the addition of a mitochondrial presequence (solid black bar). Unfolding and import requires an electrical membrane potential ($\Delta\Psi$) across the inner membrane and the ATP-dependent assistance of mtHsp70 (hands) in the mitochondrial matrix.

By combining this biophysical approach of studying protein unfolding with the cell-free mitochondrial import system, Huang *et al.*[1] also provide valuable information that impacts an area of protein import that is currently being vigorously debated. This controversy focuses on understanding how metabolic energy is used to translocate a protein through a membrane[5]. For protein import into mitochondria, translocation is driven by the combined assistance of an electrical membrane potential ($\Delta\Psi$) across the inner membrane and ATP[6–8]. Although the electrochemical potential of ~100 mV helps to force the positively charged presequences through the translocon, the proteinacious channel containing the translocation machinery, the membrane potential alone is not sufficient to drive import.

Two models have been proposed to explain the requirement for ATP and both involve the mechanism of action of the mitochondrial matrix protein, mtHsp70. The first model describes the actions of hsp70 as a Brownian ratchet[9,10], while the second model depicts hsp70 as a molecular motor fueled by ATP[11,12]. These two models are not mutually exclusive. Both models agree that

 

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mtHsp70 binds the presequence of the cargo protein and ATP regulates hsp70's binding cycle. The peptide binding pocket is open in the ATP-bound state and closed in the ADP-bound state. The replacement of ADP with ATP initiates the release of the bound peptide. Rebinding takes place upon restoration of the ADP-bound state after ATP hydrolysis, which is stimulated by a partner J domain protein (for a review see ref. 13). In mitochondria, the J domain protein (Tim44) resides in the inner membrane and positions mtHsp70 at the entrance to the matrix.

The root of the discrepancy between the two models is in the magnitude of force that is generated by hsp70 on the translocating polypeptide. The Brownian ratchet model proposes that the binding of hsp70 to the matrix side of the membrane prevents the backward sliding of the protein in the channel[9,10]. Therefore passive diffusion would only permit forward motion, biasing the movement of the protein into the organelle. Translocation-competent substrates are generated by spontaneous unfolding or by cytosolic chaperones that delay folding. In contrast, the motor model proposes that hsp70 provides a power-stroke that unfolds the polypeptide chain on the *cis* side of the outer membrane and is involved in pulling the protein across the membranes into the matrix[11,12]. Here, ATP-driven conformational changes in hsp70 are capable of creating a force that actively pulls the protein inward.

Recent studies by Matlack *et al.*[14] have convincingly demonstrated that a protein ratchet mechanism is sufficient for the import of an unfolded protein through a purified endoplasmic reticulum (ER) translocon. As predicted by the model, the polypeptide was able to slide backwards in the channel in the absence of hsp70 binding. However, transport also occurred when antibodies, which do not bind and hydrolyze ATP, instead of hsp70 were used to bind to the translocating peptide on the *trans* side of the membrane. They concluded that trapping alone permitted the import of a polypeptide chain and that a ratchet mechanism was capable of translocating an unfolded protein across the ER translocon.

The post-translational import of mitochondrial proteins, however, encounters a higher degree of complexity since the protein first has to be unfolded to an extended structure on the *cis* face of the mitochondrial membranes prior to being threaded through membrane pores with an internal diameter of ~20 Å (refs 15, 16). The co-translational transport of proteins into the eukaryotic ER alleviates this requirement for unfolding by placing the translating ribosome directly on the translocon. If the mitochondrial import system relied on spontaneous unfolding to generate transport-competent substrates then the transport rate would be dependent upon the spontaneous unfolding rate, thereby precluding the import of stably folded molecules such as barnase.

Several studies indicate that mitochondria play an active role in unfolding proteins prior to their import. Mutations in mtHsp70 inhibit protein unfolding, import and refolding in the matrix[17–19]. Recently, Voisine *et al.*[20] provided additional support for the direct involvement of a pulling action by mtHsp70 in mitochondrial import. By using mutants of mtHsp70 that permitted the separation of the trapping and unfolding functions of mtHsp70, they demonstrated that trapping or ratcheting alone did not permit the unfolding and subsequent import of a folded preprotein; rather, an additional ATP-dependent pulling force was required to import a stably folded preprotein. The key to the power stroke of mtHsp70 was found to depend on its interaction with Tim44 in the inner membrane to correctly position the hsp70 so that the directionality of the force could be controlled and harnessed.

The efforts to understand the energetics of protein import into the mitochondria have focused on the mechanism of the import machinery directly. Matouschek and coworkers address this concern from a different perspective[1]. While the ratchet model relies either on cytosolic factors to maintain the preprotein in a loosely folded state or on the spontaneous unfolding of the polypeptide chain, the motor model postulates that a power-stroke can be actively involved in the unfolding of the preprotein. Therefore monitoring the unfolding pathway of the preprotein during import could provide an approach to discriminate between the two current models.

The perturbation of the spontaneous unfolding pathway by the mitochondria import machinery conflicts with the current version of the ratchet model. These results are, however, consistent with the motor model with mtHsp70 providing a force to unravel the preprotein on the *cis* side of the membrane. The results by Huang *et al.*[1] clearly indicate that the import machinery can influence the unfolding pathway and are supportive of a motor model. However, a caveat is that the unfolding pathway could also be affected simply by the loss of freedom at the N-terminus when it is tethered or trapped at the mitochondrial surface. One key experiment to test this would be to determine whether the unfolding pathway is affected by immobilization of its N-terminus.

The determination of how the mitochondrial machinery unfolds proteins will impact our understanding of how proteins are unfolded by the ATPases of the degradative pathway where vectorial unfolding is also critical. Huang *et al.*[1] have provided a new perspective to begin to address these problems. In the future, further biochemical and biophysical experiments will have to be performed to fully answer this important cell biological question of how proteins are unfolded and translocated across membranes in the cell.

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