

# Gazing at Translocation in the Mitochondrion

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Most mitochondrial proteins are synthesized in the cytosol and imported into the mitochondrion via molecular machines called translocons on the outer and inner mitochondrial membranes. Alder et al. (2008b) examine protein translocation into intact mitochondria by adapting fluorescent techniques first used to study translocation in the endoplasmic reticulum.

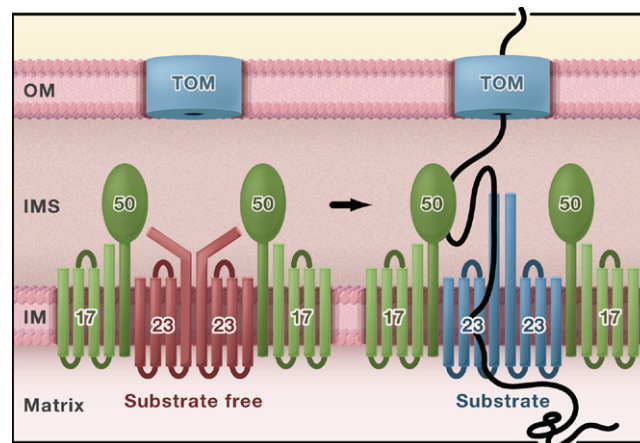
The mitochondrion is a complex organelle to assemble because it contains four compartments due to the presence of an outer membrane and an inner membrane that separate the matrix from the intermembrane space. Although the mitochondrion contains a small genome that encodes a handful of proteins for electron transport, the majority of mitochondrial proteins are encoded in the nuclear genome and must be imported from the cytosol. The precursor proteins contain targeting sequences for guidance to the mitochondrion, and the outer and inner membranes contain translocon machines that mediate import. Historically, studies of mitochondrial protein translocation have relied on the use of mutants and conventional biochemical techniques including chemical crosslinking (Kutik et al., 2007; Neupert and Herrmann, 2007). These approaches have established a basic picture of the translocation machinery—its components, the residues in the precursor proteins necessary for targeting, and the basic mechanisms of translocon function. By comparison, studies of protein translocation in the endoplasmic reticulum (ER) are more advanced, in part due to the successful application of protein crystallography, which has provided a detailed picture of how these translocons are assembled (Rapoport, 2007). In addition, Johnson and colleagues have developed a fluorescent-based experimental system that allows direct examination

of the microenvironment during translocation into the ER (Alder et al., 2005; Johnson, 2005). Protein translocation studies in the mitochondrion are just beginning to utilize this technology, as demonstrated by the new work of Alder et al. (2008b) presented in this issue. The authors have adapted the fluorescent approaches first used to study ER protein translocation to examine how a translocon operates during protein import into intact, functioning mitochondria.

Alder et al. focus on the protein Tim23, a component of the TIM23 (translocase of the inner mitochondrial membrane) translocon, which mediates the import of proteins into the matrix.

The TIM23 translocon consists of two membrane proteins, Tim23 and Tim17, that form a channel (Figure 1). Tim50 is an additional subunit that functions as a receptor to guide precursor proteins from the translocase of the outer mitochondrial membrane (TOM) complex to the TIM23 translocon (Yamamoto et al., 2002). All of these components are essential for viability, indicating that these core components of the TIM23 complex are critical for mitochondrial biogenesis. Precursors are threaded in an unfolded conformation from the TOM complex to the TIM23 complex, and the initial stages of protein translocation depend on the presence of a membrane potential ( $\Delta\Psi$ ) that exerts an electrophoretic effect on the positively charged presequence and promotes insertion into the translocon. The TIM23 translocon also associates with the protein-associated motor (PAM) complex that uses ATP hydrolysis to pull precursors into the mitochondrial matrix, completing import.

Tim23 is a multifunctional protein in the translocon. It contains four membrane-spanning domains in the C-terminal half that form part of the translocation channel along with Tim17. The N-terminal 100 amino acid domain is hydrophilic and has several activities. These include acting as a gate to close the translocation channel (along with Tim50) when a substrate



**Figure 1. The Translocation Machinery in Mitochondria**

The mitochondrial outer membrane (OM) contains the translocase of the outer membrane (TOM) that precursor proteins pass through to reach the inner membrane (IM). The translocase of the inner membrane (TIM)-23 complex contains Tim23, Tim17, and Tim50 and functions in the import of precursors to the mitochondrial matrix. In the resting state (left), when a precursor is not engaged in the translocon, Tim50 and the intermembrane space (IMS) domain of Tim23 shield the translocation channel. In a study using fluorescent probes (Alder et al., 2008b), fluorescence of the labeled resting (substrate-free) Tim23 protein is red-shifted (left). However, in the presence of a substrate that is being imported (right), the fluorescently labeled Tim23 shifts to a lower wavelength, indicating interactions with substrate and the exclusion of water molecules.

is not engaged, functioning in a sorting/ guiding capacity to transfer precursors from the TOM complex to the TIM23 complex, and serving as a tether in the outer membrane (Donzeau et al., 2000). Thus, it is a central component of the TIM23 translocon.

To date, biochemical studies have shown that the TIM23 translocation channel has an opening of approximately 13–24 Å that can accommodate an unfolded, extended polypeptide chain (Truscott et al., 2001). From electrophysiology studies with recombinant TIM23 and isolated TIM23 complexes, the channel is voltage gated, and precursors or peptides derived from an N-terminal targeting sequence inhibit channel activity. However, it is not obvious how the permeability barrier across the inner membrane is maintained during protein import, and essentially nothing is known about how a TIM23 translocon in an intact mitochondrion imports substrates. The study by Alder et al. is the first glimpse into how the TIM23 translocon functions in intact mitochondria.

In prior work, Johnson and colleagues developed a methodology to incorporate fluorescent probes into specific sites of a translated ER substrate, revealing the translocation process based on the spectral changes of the probe during movement into ER-derived vesicles.

Alder et al. (2008b) have now adapted this technique to probe translocation in the mitochondrion. Site-specific fluorescent probes have been sequentially incorporated into Tim23 to investigate the microenvironment of the translocation channel in intact mitochondria at rest and during translocation of a substrate (Alder et al., 2008a, 2008b). The new work focuses primarily on transmembrane domain 2 because it interacts with a translocating substrate and lines the translocation channel (Alder et al., 2008a). After fluorescent labeling, Tim23 was imported into isolated mitochondria and subsequently integrated into mature TIM23 translocons. Using comprehensive and sophisticated spectral analysis, the probes in trans-

membrane 2 of Tim23 demonstrated that this region occupies an amphipathic environment in the TIM23 complex; notably, a subset of residues face a polar (aqueous) environment and a subset of residues face a hydrophobic environment (Figure 1). Moreover, when a substrate was arrested in the translocon, the probes in the hydrophobic environment remained unchanged, but probes in the polar environment shifted fluorescence parameters, consistent with an interaction with the translocating substrate or the exclusion of water molecules (Figure 1). Therefore, one face of Tim23's transmembrane 2 seemingly contacts the aqueous environment of the channel and also is near the translocating substrate, whereas the other face remains in a hydrophobic environment, potentially interacting with lipids or other hydrophobic sections of neighboring proteins. These results were surprising because transmembrane domain 2 of Tim23 was not obviously amphipathic and orientation of the transmembrane domain in a channel could not have been predicted from the primary sequence alone.

Given that the translocation pore is an aqueous channel, it might be continuous with the intermembrane space. Alder et al. addressed this by adding a quenching agent to the mitochondria, which enters the intermembrane space through the porous outer membrane. If the quenching agent reaches the aqueous TIM23 pore, quenching of fluorescence should be observed. Surprisingly, the quenching agent did not strongly alter fluorescent properties of probes in transmembrane 2, either in the presence or in the absence of a translocating substrate. In contrast, the quenching agent abrogated fluorescence in probes that were placed in the intermembrane space domain of Tim23, indicating that the N-terminal region of Tim23 is present in the intermembrane space. This evidence demonstrates that the inner membrane permeability barrier is maintained during protein translocation by the strong interactions between the channel and the translocating substrate. This is an important

result because translocation of proteins across the inner membrane must not be accompanied by a proton leak if the proton motive force across the inner membrane is to be maintained. Thus, the translocating substrate appears to occupy the entire translocation channel, and intimate associations between the substrate and the helices lining the pore prevent leaking of ions during import.

This fluorescence approach therefore provides high-resolution structural information on the microenvironment of the transmembrane domain 2 of Tim23 during translocation. Continued studies to characterize the microenvironment of other regions of Tim23 will be insightful given that a crystal structure of the TIM23 translocon is currently lacking. Moreover, new challenges include structural determination of the mitochondrial translocons and additional mechanistic studies using this fluorescent approach to decipher how precursors are sorted, how transmembrane domains are accommodated in the translocons, and how the translocons are assembled.

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