Chapter 2

In Vitro Analysis of the Mitochondrial Preprotein Import Machinery Using Recombinant Precursor Polypeptides

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Abstract

The import of proteins into mitochondria represents an essential process for the survival of eukaryotic cells. Most mitochondrial proteins are synthesized as cytosolic precursor proteins. A complex chain of reactions needs to be followed to achieve a successful transport of these precursors from the cytosol through the double membrane system to their final destination inside the mitochondria. In order to elucidate the details of the translocation process, in vitro import assays have been developed that are based on the incubation of isolated active mitochondria with natural or artificial precursor proteins containing the appropriate targeting information. Using this basic system, most of the protein components of the import machinery have been identified and functionally characterized. However, a detailed definition of the molecular mechanisms requires more specialized assay techniques. Here we describe modifications of the standard in vitro import assay technique that are based on the utilization of large amounts of recombinant preprotein constructs. The application of saturating amounts of substrate preproteins is a prerequisite for the determination of translocation kinetics and energy requirements of the import process. Accumulation of preproteins as membrane-spanning translocation intermediates further provides a basis for the functional and structural characterization of the active translocation machinery.

Key words Mitochondria, *Saccharomyces cerevisiae*, Protein import, Precursor proteins, Import kinetics, Translocation intermediates

1 Introduction

According to proteomic studies, yeast mitochondria contain roughly 1,000 different proteins [1]. Only about 1 % of these proteins are encoded by the mitochondrial genome. Consequently, the vast majority of proteins has to be imported into the organelle after their synthesis on cytosolic ribosomes (*see* Fig. 1a). To ensure their proper destination, mitochondrial proteins are generated as precursor polypeptides carrying specific targeting signals. Most matrix-destined precursors, as well as monotopic inner membrane proteins possess N-terminal, typically cleavable presequences. Polytopic outer and inner membrane proteins, by contrast, carry internal signals that are usually distributed throughout the mature protein.
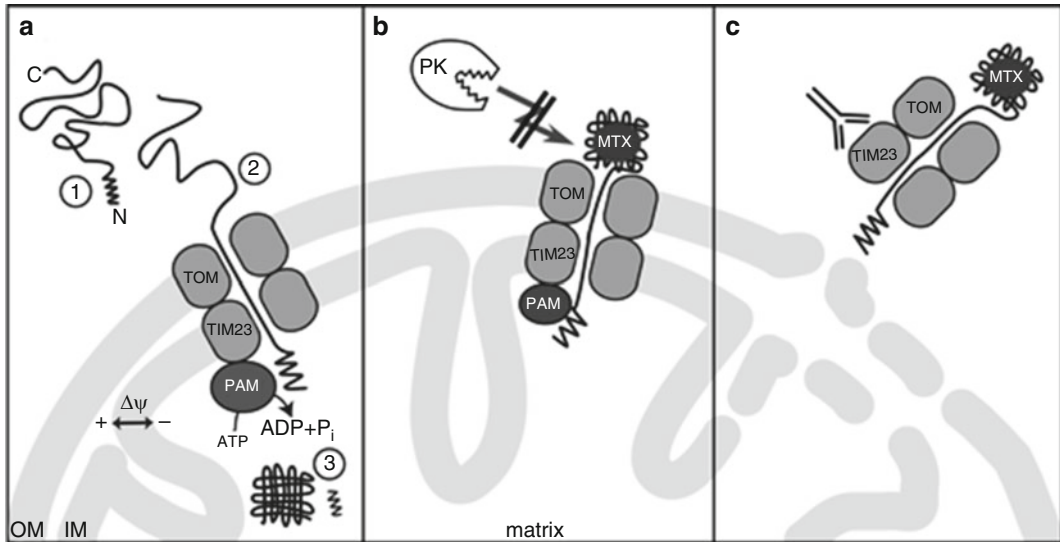
Fig. 1 Principles of the analysis of mitochondrial preprotein import. (**a**) Import of a presequence-containing protein into the mitochondrial matrix. Matrix-targeted preproteins contain N-terminal signal sequences (1) that are recognized by receptors on the outer face of the outer mitochondrial membrane. The preprotein is inserted into the outer membrane by the translocase of the outer membrane (TOM complex), and subsequently handed over to the translocase of the inner membrane (TIM23 complex), where the presequence is inserted in a reaction dependent on the electric potential $\Delta\psi$ (2). Completion of matrix-import requires the mtHsp70-containing import motor (PAM) that couples protein import with ATP hydrolysis. Cleavage of the presequence and folding of the protein occurs in the matrix, resulting in the mature size protein in its native state (3). (**b**) Generation of protease-resistant translocation intermediates using preproteins with a C-terminal dihydrofolate reductase (DHFR) domain. Addition of the specific ligand methotrexate (MTX) stabilizes the folding state of the DHFR-domain, thereby preventing its translocation. The N-terminal b_2-part crosses both membranes and contacts the import motor. Stable folding of the DHFR domain inhibits proteolysis by externally added Proteinase K (PK) when the translocation machinery is active. (**c**) Isolation of the MTX-generated TOM-TIM supercomplex by use of detergent. Addition of digitonin solubilizes the mitochondrial membranes, whereas the protein complex remains stable and can be extracted intact. Detection of the complex can be done by use of anti-Tim23 antibodies. OM: outer membrane; IM: inner membrane

To date, at least five distinct import pathways into the different mitochondrial subcompartments have been identified, all comprising specialized protein components organized as "translocase" complexes [2]. So far, recombinant preproteins have been employed to analyze the targeting pathway into the mitochondrial matrix compartment, also termed "the presequence translocase pathway." The first step of the preprotein import process into the matrix is the specific recognition of the targeting information by receptor proteins at the mitochondrial surface. This reaction also governs the insertion of preproteins into the *T*ranslocase of the *O*uter mitochondrial *M*embrane (TOM complex) which represents the general entry pore for essentially all nuclear-encoded proteins. As a second step, translocation across the inner membrane is mediated by the *T*ranslocases of the *I*nner *M*embrane, the TIM23 complex, typically

also called presequence translocase [2]. Proteins destined for the matrix require a membrane potential across the inner membrane as an initial energy source for insertion of the N-terminal presequence into the inner membrane pore provided by the TIM23 complex. Completion of matrix import furthermore requires chemical energy in the form of ATP-hydrolysis, catalyzed by the matrix heat shock protein of 70 kDa (mtHsp70, Ssc1 in yeast), which provides the driving force for the unfolding of preprotein domains and the translocation of the bulk polypeptide chain across the membrane [3]. MtHsp70 represents the core component of the *P*resequence-*T*ranslocase-*A*ssociated *M*otor (PAM). The motor complex is recruited to the inner face of the inner membrane import complex by an interaction with the peripheral membrane protein Tim44. Further essential PAM-components are the nucleotide exchange factor Mge1 (*M*itochondrial *G*rp*E* homolog), as well as the ATPase-stimulating J-domain protein Pam18, and its partner Pam16. In the matrix, the presequence is cleaved off (often by the *m*atrix-*p*rocessing *p*eptidase, MPP), resulting in the mature-sized protein. Folding of imported proteins is initially promoted by mtHsp70, which stabilizes, and thereby protects unfolded proteins until they acquire their native conformation (assisted by the Hsp60 chaperonin system) or in the case of multimeric proteins, assemble into the respective protein complexes [4].

Due to its convenience, especially with regards to genetic manipulation, the yeast *Saccharomyces cerevisiae* represents an ideal model organism for the study of the mitochondrial biogenesis processes typical for eukaryotic cells. Although research in the recent years has revealed significant additional functions of mitochondria in mammalian cells compared to yeast, the preprotein import machineries are remarkably conserved. An experimental analysis of the mitochondrial protein import first requires the isolation of functionally intact mitochondria, as well as an assessment of their structural integrity. The isolation of yeast mitochondria includes an enzymatic digestion of the cell wall, followed by a mechanical disruption of the resulting spheroblast. Enrichment of the mitochondrial fraction is achieved by subsequent differential centrifugation of the crude cell lysate. The structural integrity of the mitochondrial preparation is assessed by checking the membrane potential across the inner mitochondrial membrane, which is of particular importance for the comparative analysis of mitochondria derived from different yeast strains. The assay described here is based on the reversible interaction of a fluorescent dye with membranes in a membrane potential-sensitive manner [5, 6]. Secondly, an appropriate precursor protein, consisting of a mitochondrial signal sequence and a reporter component has to be generated. The precursor can be either synthesized as a radiolabeled polypeptide by translation in a cell-free system, or isolated from *E. coli* cells as a recombinant protein. The import reaction in vitro is finally performed by combining intact mitochondria with precursor polypeptides

under appropriate buffer conditions. For the import reaction, mitochondria are additionally energized by ATP, as well as NADH, which represents the main substrate of the respiratory chain in yeast. A successful translocation reaction is typically monitored by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyzing the shift in relative molecular mass generated by the removal of the targeting sequence by the matrix processing peptidase (MPP) [7]. Information about the completion of poly-peptide translocation can be gained by assessing the resistance of the processed preproteins against externally added proteases, which cannot reach the fully imported protein molecules in intact mitochondria.

Precursor synthesized by in vitro translation or generated by heterologous expression are functionally equivalent but their differ-ent properties have major implications for the import reaction. Generally speaking, the in vitro synthesis of a radiolabelled precur-sor in a cell-free system is more convenient and less time-consuming. It is ideally performed in commercially available rabbit reticulocyte lysate, supplemented with [35S]methionine and/or [35S]cysteine, after in vitro transcription from appropriate template DNA using the SP6 or T7 RNA polymerase system (*see* Chapter 27). Template DNA for in vitro transcription can be conveniently generated by PCR reactions directly from yeast genomic DNA since most yeast genes contains no intron sequences. Using this method almost any polypeptide or open reading frame can be tested for its mito-chondrial import properties [7]. However, experiments using radioactive precursor proteins, which are usually produced in sub-stoichiometric amounts, only permit a qualitative analysis of the import reaction. In order to apply more stringent conditions, or to obtain reliable kinetic data, saturating amounts of precursor poly-peptides have to be used. Recombinant preproteins obtained by expression and purification from *E. coli* cells have been shown to be import competent even in the absence of cytosolic cofactors and are virtually unlimited in their amounts [8]. Using saturating amounts of precursor proteins, conditions can be achieved that represent the maximal rate of the mitochondrial import reaction (V_{max}). These conditions are a prerequisite for the quantitative characterization of the preprotein translocation kinetics [8]. Besides, applying excess preprotein may accentuate potential import defects in mutant forms of individual components of the import machinery.

This chapter describes the expression and isolation, as well as in vitro import of a fusion protein, consisting of a modified mito-chondrial presequence derived from the yeast protein cytochrome (b_2), and a heterologous passenger protein (mouse dihydrofolate reductase, DHFR). These so called b_2-DHFR fusion proteins and their variants have been routinely used in in vitro import anal-yses with yeast mitochondria [9, 10]. The use of a heterologous protein moiety allows one to take advantage of the highly specific antigen–antibody recognition for analysis of the import reaction,

as interference from endogenous proteins is bypassed. Instead of DHFR other heterologous protein domains or peptide tags can be utilized at the C-terminal end of the respective fusion proteins, provided an appropriated mitochondrial targeting signal is inserted at the N-terminal end. In our lab, we have also successfully employed Strep- and SNAP-tag fusions proteins. An important issue is the availability of a specific and sensitive antiserum for the immunodetection of the respective tag by Western blot. Previous experiments indicated a maximal import rate of about 25 pmol protein per min per mg mitochondrial protein [8], at least under the conditions described here. The absolute amount of imported polypeptides is therefore rather small and has to be reliably detected over the background of the complete mitochondrial proteome.

Based on the import of precursor proteins carrying a DHFR-domain, the mitochondrial protein import can be analyzed in greater detail by the generation of specific translocation intermediates (*see* Fig. 1b). To that end, the DHFR-ligand Methotrexate (MTX) is employed, which strongly stabilizes the native folding state of the DHFR domain. The full translocation of preproteins containing a C-terminal DHFR moiety is thereby prevented while the insertion of N-terminal segments into the translocation channel is not affected [11, 12]. Thus, the precursor accumulates in an intermediate state in which the N-terminal b_2-part contacts the TIM23 complex (also termed Presequence Translocase), whereas the C-terminal DHFR-domain remains in the cytosol [9, 13]. Since these intermediates represent preproteins engaged in active translocation, they can be used for an assessment of the inward-directed translocation force exerted by the import motor. Provided that the mtHsp70-containing PAM is functional and active, the preprotein is stably held in the intermediate complex, whereas it is lost in cases of a motor defect [14]. Since the preprotein spans both translocation complexes (TOM and TIM23), a physical connection between the machinery of the outer and inner membrane is generated that allows the extraction and analysis of the whole translocation machinery in its active state [13], termed the TOM-TIM supercomplex (*see* Fig. 1c). Formation of this supercomplex is typically analyzed by Blue Native PAGE (BN-PAGE) that allows for the electrophoretic separation of multimeric proteins in their native state [15].

2 Materials

Prepare all buffers in distilled water.

2.1 Preparation of Functional Mitochondria from Yeast Cells

1. DTT softening buffer: 100 mM Tris/H_2SO_4 pH 9.4; 10 mM DTT.

2. Zymolyase buffer: 20 mM KPi pH 7.4; 1.2 M Sorbitol.

3. Homogenizing buffer (prepare fresh, 4 °C): 10 mM Tris/HCl pH 7.4; 0.6 M Sorbitol; 0.5 % BSA; 1 mM PMSF (0.5 M (500×) Stock: 87 g/ml in EtOH).

4. SEM buffer: 250 mM sucrose; 10 mM MOPS/KOH pH 7.2; 1 mM EDTA; add 1 mM phenylmethanesulfonyl fluoride (PMSF) (from freshly prepared 0.5 M or 500× stock solution) where indicated.

2.2 Measurement of the Mitochondrial Membrane Potential by Fluorescence Spectrometry

1. Isolated mitochondria in SEM buffer (*see* Subheading 2.1): 10 mg/ml protein, freshly prepared or frozen and thawed *on ice*.

2. 1 M KP_i, pH 7.2: Prepare 50 ml each of 1 M KH_2PO_4 and of K_2HPO_4, mix solutions by adding the appropriate volume of KH_2PO_4 (high pH) to 50 ml of K_2HPO_4 (low pH), until the required pH 7.2 is reached.

3. Potential buffer: 0.6 M sorbitol, 0.1 % (w/V) bovine serum albumin (BSA, for molecular biology, e.g., *Roche*), 10 mM $MgCl_2$, 20 mM KP_i, pH 7.2.

4. 3,3′-dipropylthiadicarbocyanine iodide ($diSC_3(5)$, *Molecular Probes, Life Technologies*), prepare fresh as 1 mM stock in EtOH, wrap tube in aluminum foil as the dye is light sensitive.

5. 100× inhibitor mix: 800 μM antimycin A (blocks electron transfer within complex III of the respiratory chain), store as 8 mM stock in EtOH p.a. at 20 °C, 50 μM valinomycin (dissipates membrane potential by acting as K^+-ionophore), store as 1 mM stock in EtOH p.a. at –20 °C, 2 mM oligomycin (inhibits F_1 subunit of F_1F_o-ATP synthase), store as 10 mM stock in EtOH p.a. at –20 °C; for preparation of 1 ml 100× working solution, mix 100 μl of antimycin A stock with 50 μl of valinomycin stock and 200 μl of oligomycin stock in 650 μl EtOH p.a., store at –20 °C, stable for years.

6. 100× valinomycin: dilute 50 μl of valinomycin stock in 950 μl EtOH p.a., store at –20 °C, stable for years.

7. Substrates: 1 M succinate in H_2O, pH 8.5 (L-succinic acid, di-sodium salt), 1 M malate in H_2O, pH 8.0 (L-malic acid, di-sodium salt), prepare 1 ml each, store at –20 °C, stable for years.

8. Fluorescence spectrometer (e.g., *AMINCO-Bowman*), Cuvette (1-cm-pathlength) for fluorescence measurements (e.g., *Hellma 101-OS*).

2.3 Preparation of Cytochrome b_2-DHFR Precursor Proteins from E. coli

1. LB-medium: 1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl.

2. Isopropyl-β-D-thiogalactopyranoside (IPTG): prepare a 1 M stock and store at –20 °C, stable for years.

3. Pre-lysis buffer: 30 % sucrose, 20 mM KP_i, pH 8.0, 1 mM EDTA, 10 mM DTT (*see* **Note 1**), 1 mM PMSF (dissolve in

EtOH p.a., keep on ice once dissolved; *see* **Note 2**); precool to 4 °C before use.

4. Buffer A: 20 mM Tris–HCl, pH 7.0, 1 mM EDTA, 10 mM DTT (*see* **Note 1**), 1 mM PMSF (*see* **Note 2**), protease-inhibitors (1.25 μg/ml leupeptin, 2 μg/ml antipain, 0.25 μg/ml chymostatin, 0.25 μg/ml elastinal, 5 μg/ml pepstatin, e.g., "Complete EDTA-free," *Roche*); add 1 mg/ml lysozyme, 0.1 % Triton X-100 (keep as 10 % (V/V) solution at room temperature), and 0.1 mg/ml DNAse, as well as 50 % glycerol, as indicated; precool to 4 °C before use.

5. Buffer B: Buffer A plus 1 M NaCl.

6. Sonifier (e.g., *Branson*).

7. Cellulose acetate membranes, 0.22 μm pore size (e.g., *Millipore*).

8. MonoS HR 10/10 cation-exchange column for FPLC, 6 ml (e.g., *GE Healthcare*).

9. Low Molecular Weight-SDS Calibration Kit for electrophoresis (e.g., *GE Healthcare*) consisting of marker proteins for determination of absolute protein amounts.

2.4 In Vitro Import of Recombinant Precursor Proteins into Isolated Mitochondria

1. Isolated mitochondria in SEM buffer: 10 mg/ml protein content, freshly prepared or thawed *on ice*.

2. Preprotein to be imported, freshly prepared, or thawed on ice.

3. Import buffer: 3 % (w/V) fatty-acid free bovine serum albumine (BSA), 250 mM sucrose, 80 mM KCl (*see* **Note 3**), 5 mM $MgCl_2$, 2 mM KH_2PO_4, 10 mM MOPS-KOH, pH 7.2, store in 10–20-ml aliquots at –20 °C.

4. 0.2 M NADH, prepare fresh, keep solution on ice.

5. 0.2 M ATP, store as stock solution after pH adjustment to 7.0 with 5 M KOH in working aliquots at –20 °C, stable for years, do not refreeze.

6. 1 M KP_i (*see* Subheading 2.2).

7. 100× inhibitor mix (*see* Subheading 2.2).

8. 100× valinomycin (*see* Subheading 2.2).

9. 0.1 M PMSF (*see* **Note 2**).

10. SEM buffer (*see* Subheading 2.2).

11. 2.5 mg/ml Proteinase K (PK, specificity: hydrophobic and aromatic residues), dissolved in SEM buffer, store in working aliquots at –20 °C, stable for years.

12. Import samples need to be incubated on ice for various experimental purposes; we use metal blocks with holes corresponding to the diameter of 1.5 ml reaction tubes, placed on ice.

2.4.1 Import of Urea-Denatured Precursor Proteins

1. Cold-saturated ammonium sulfate, pH 7.2; store at 4 °C, stable for years.
2. Urea buffer: 7 M urea, 30 mM MOPS-KOH, pH 7.2, 1 mM DTT (*see* **Note 1**), prepare fresh.

2.5 Analysis of the Import Reaction

1. Standard materials required to perform SDS-PAGE and Western blot.
2. Highly specific antiserum against DHFR (or against other utilized protein tags).
3. Quantification of Western blot signals by image analysis software (i. e. ImageJ).

2.6 Dissection of a Functional Import Intermediate on Blue Native PAGE

1. Gel preparation and running settings for Blue Native PAGE.
   (a) ATP-regenerating system: creatine kinase, 10 mg/ml, prepare fresh; creatine phosphate, store as 1 M stock at −20 °C, stable for years.
   (b) 5 % digitonin in water, prepare a 5 % stock solution in water (*see* **Note 4**), keep at 4 °C, stable for months.
   (c) Solubilization buffer: 1 % digitonin, 20 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl, 10 % glycerol, 1 mM PMSF (*see* **Note 4**). Prepare a 2× solution w/o digitonin, and add detergent prior to use.
   (d) Acrylamide solution: 49.5 % Acrylamide (e.g., *Roth*), 1.5 % bis-acrylamide, store at room temperature in the dark, stable for months.
   (e) 3× gel buffer: 200 mM ε-amino-*n*-caproic acid, 150 mM bis-tris/HCl, pH 7.0, store at 4 °C, stable for months.
   (f) 10× Loading Dye: 5 % Coomassie blue G250 (water soluble, whereas Coomassie R is not), 500 mM ε-amino-n-caproic acid, 100 mM bis-tris/HCl, pH 7.0. Prepare 10 ml and store at 4 °C, stable for months.
   (g) 10× anode buffer: 500 mM bis-tris/HCl, pH 7.0, store at 4 °C, stable for months; 1× solution can be used for up to three times.
   (h) 10× cathode buffer: 500 mM tricine, 150 mM bis-tris/HCl, pH 7.0, prepare 1 L, divide into halves, and dissolve 0.2 % Coomassie blue G250 in 500 ml.
   (i) Marker protein set (e.g., *Sigma-Aldrich*).
   (j) Gradient mixer, peristaltic pump, cooled vertical gel electrophoresis system (e.g., SE 600 Series, *GE Healthcare*).

2. Generation of the TOM-TIM-Supercomplex.
   Materials are the same as listed in Subheading 2.4 with the following variations:

   (a) Import buffer as described in Subheading 2.4 without BSA.

   (b) Methotrexate (MTX): keep as 10 mM solution in 0.1 M MOPS-KOH, pH 7.2, store at –20 °C, stable for years; dilute in import buffer w/o BSA as indicated.

2.7 Determination of Matrix-Directed Import Forces Exerted by Hsp70

1. Isolated mitochondria in SEM buffer: 10 mg/ml in protein content, freshly prepared or thawed *on ice*.

2. Import buffer: *see* Subheading 2.4.

3. 0.1 mM Methotrexate in import buffer w/o BSA, store at –20 °C, stable for years.

4. 0.2 M NADH, prepare fresh, keep on ice.

5. 0.2 M ATP (*see* Subheading 2.4).

6. 1 U/μl apyrase, store at –20 °C, stable for years.

7. 10 mM oligomycin (*see* Subheading 2.4).

8. ATP-regenerating system (*see* Subheading 2.6).

9. Buffer R1 (–ATP): 20 μM oligomycin, 5 μM MTX, in P80 w/o BSA, prepare fresh.

10. Buffer R2 (+ATP): 2 mM ATP, 20 mM creatine phosphate, 200 μg/ml creatine kinase, 5 μM MTX, prepare fresh.

11. 100× inhibitor mix (*see* Subheading 2.4).

12. 100× valinomycin (*see* Subheading 2.4).

13. 0.1 M PMSF (*see* **Note 2**).

14. 2.5 mg/ml Proteinase K (PK, *see* Subheading 2.4).

Miscellaneous: Buffers, solutions, and equipment for SDS-PAGE, Western Blot, and Immunodecoration.

3   Methods

All centrifugation steps are to be carried out at 4 °C, unless stated otherwise.

3.1 Preparation of Functional Mitochondria from Yeast Cells

1. Measure OD 660 nm of yeast culture: harvest ideally at 1.5–2.5 OD/ml.

2. Determine and note (!) weight of centrifugation bottles (i.e., 500 ml bottles for Beckman JA-10 rotor). Pellet cells for 5 min at $1,600 \times g$ and RT. Discard medium and resuspend cell pellet in monodest. water. Fill up to 3/4 of total volume.

3. Centrifuge cell suspension again for 5 min at $1,600 \times g$ and RT. Remove supernatant completely and etermine wet weight of pellet.

4. Resuspend pellet in prewarmed DTT buffer, 2 ml/g pellet. Shake gently (about 100 rpm) for 20 min at 30 °C in Erlenmeyer flask.

5. Pellet cells for 5 min at $2,800 \times g$. Discard supernatant and resuspend pellet in approx. 100 ml zymolyase buffer (without enzyme).

6. Pellet cells 5 min at $2,800 \times g$. Resuspend pellet in prewarmed zymolyase buffer (7 ml/g pellet). Add 3 mg zymolyase per gram wet weight and incubate for 30 min at 30 °C.

7. Mix 50 μl spheroblast suspension with 2 ml water for zymolyase test (shake vigorously: a clear suspension indicates complete digestion of the cell wall and formation of spheroblasts)—compare lysis efficiency with sample before zymolyase step.

8. Pellet spheroblasts for 5 min at $2,800 \times g$. Wash pellet carfully with about 100 ml zymolyase buffer (without enzyme). Centrifuge for 5 min at $1,600 \times g$.

9. Perform the following steps on ice.

10. Resuspend in homogenizing buffer 6.5 ml/g wet weight and homogenize 15–20× (up and down = 1×). Add additional homogenizing buffer (less than 100 ml total volume) and transfer to cenrtifuge tubes (i.e., 50 ml tubes for Beckman JA-25.50 rotor).

11. Centrifuge for 2 min at $300 \times g$, save supernatant and directly spin again for 4 min at $1,100 \times g$. Save supernatant again and centrifuge for 5 min at $2,000 \times g$.

12. Save supernatant and centrifuge 15 min at $17,500 \times g$, resuspend pellet carefully in 1 ml SEM buffer with blue tips. Rinse centrifugation tube with 1 ml SEM buffer.

13. Fill centrifugation tube (i.e., 50 ml tubes for Beckman JA-25.50 rotor) with resuspended mitochondria to 3/4 of total volume. Centrifuge for 5 min at $2,000 \times g$. Save supernatant and centrifuge for 10 min at $17,500 \times g$ to obtain a pellet containing the purified mitochondria.

14. Resuspend pellet in a small volume (1–2 ml) of SEM buffer. Determine total protein concentration and adjust mitochondria solution to 10 mg/ml protein (if possible).

15. Prepare aliquots of 200 or 400 μl and snap-freeze in liquid N_2. Store at –80 °C

3.2 Measurement of the Mitochondrial Membrane Potential by Fluorescence Spectrometry

The assessment of the membrane potential ($\Delta\psi$) by use of a cyanine dye is based on the changes in fluorescence emission in response to a membrane potential [5, 6]. Upon addition of mitochondria to the $diSC_3(5)$-containing buffer, the dye is potential-dependently partitioned between mitochondria and surrounding medium.
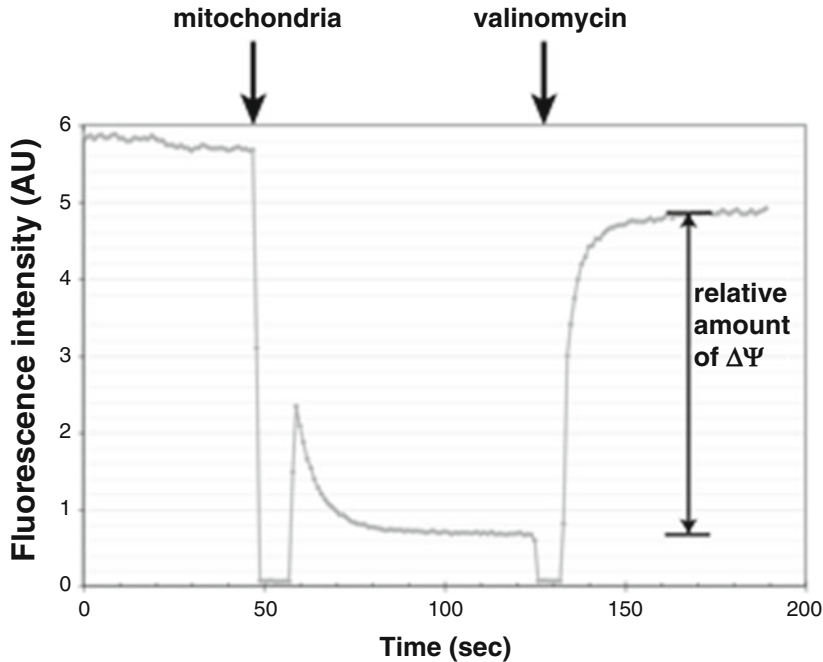
Fig. 2 Assessment of the membrane potential $\Delta\psi$ by use of the fluorescent dye $diSC_3(5)$. Mitochondria are added to $diSC_3(5)$-containing potential buffer where indicated. A decrease of the fluorescent signal is observed due to uptake of the dye by the mitochondria and subsequent quenching of the fluorescence. After the signal has stabilized, uncoupling of the respiratory chain and destruction of the membrane potential is achieved by addition of the K^+-ionophore valinomycin. The relative amount of the membrane potential ($\Delta\psi$) is reflected by the restoration of $diSC_3(5)$-fluorescence after addition of valinomycin

The fluorescence emission from mitochondria-associated $diSC_3(5)$ is decreased (quenched) with increasing $\Delta\psi$. This fluorescence quenching can be reversed by depolarization of the mitochondrial membranes. $\Delta\psi$ is reflected by the restoration in $diSC_3(5)$ fluorescence emission at 670 nm, in response to the addition of depolarizing (uncoupling) agents (*see* Fig. 2).

1. Add 3 ml of potential buffer to the cuvette.

2. At this step, you may need to add substrates to a final concentration of 5 mM each (*see* **Note 5**).

3. Add 3 μl $diSC_3(5)$, mix by covering the cuvette with a piece of laboratory film (e.g., *Parafilm*) and inverting once. Start your measurement using the following settings:

   - Excitation wavelength 622 nm.
   - Emission wavelength 670 nm.
   - Measuring period of 200–250 s.
   - Response interval 1 s.

4. When $diSC_3(5)$ fluorescence has reached a stable value (~40–50 s) add 3–5 μl mitochondria, mix, and continue measurement until the decrease in fluorescence finishes.

5. Add 3 μl of 100× inhibitor mix to uncouple respiratory chain complexes, and trace fluorescent changes until the increase has reached its maximum again (*see* **Note 6**).

3.3 Preparation of Precursor Proteins from E. coli

Authentic cytochrome b_2 reaches the intermembrane space (IMS) of mitochondria via a "stop-transfer" mechanism that comprises the insertion of N-terminal segments into the matrix followed by a retranslocation and processing event in the IMS. The fusion proteins described here consist of the N-terminal 167 residues of the cytochrome b_2 precursor fused to the complete mouse DHFR [9, 10]. The b_2-sequence encompasses the complete mitochondrial targeting signal but lacks residues 47–65 of the intermembrane spacing sorting signal (*see* Fig. 3a). Thus, the resulting preprotein, named $b_2(167)_\Delta$-DHFR, is targeted to the mitochondrial matrix instead of being exported to the intermembrane space.

For isolation of the preprotein in high amounts, *E. coli* cells expressing the construct are lysed and the protein is purified using cation-exchange chromatography. The protein is eluted by application of a salt gradient (0–0.5 M NaCl).

1. Grow *E. coli* cells expressing the fusion protein $b_2(167)_\Delta$-DHFR at 37 °C in LB medium to an OD_{600} of 0.8.

2. Add IPTG to a final concentration of 1 mM, and continue growth at 30 °C for additional 2–3 h.

3. Harvest cells by centrifugation at $3,500 \times g$ for 8 min.

4. Wash cell pellet by resuspension in pre-lysis buffer and centrifugation as in **step 3**.

5. Resuspend cells in buffer A with 0.1 g cell pellet per ml lysis buffer. Destabilize cell walls by freezing the suspension in liquid N_2 and subsequent thawing at room temperature for two times. Add lysozyme to a final concentration of 1 mg/ml, and 0.1 mg/ml DNAse, and incubate on ice for 10 min.

6. Add 0.1 % (V/V) Triton X-100 and solubilize cells on ice for 10 min.

7. Disrupt cells by sonication on ice: 3×20 pulses (chill cells on ice briefly between pulse intervals), 40 % duty cycle, micro tip setting 7.

8. Pellet cell debris by centrifugation at $15,000 \times g$ for 20 min. Take samples of both pellet and supernatant (lysate) to analyze lysis efficiency via SDS-PAGE.

9. Pour crude cell lysate over a cellulose acetate filter membrane. Take sample of filtered lysate for SDS-PAGE analysis.
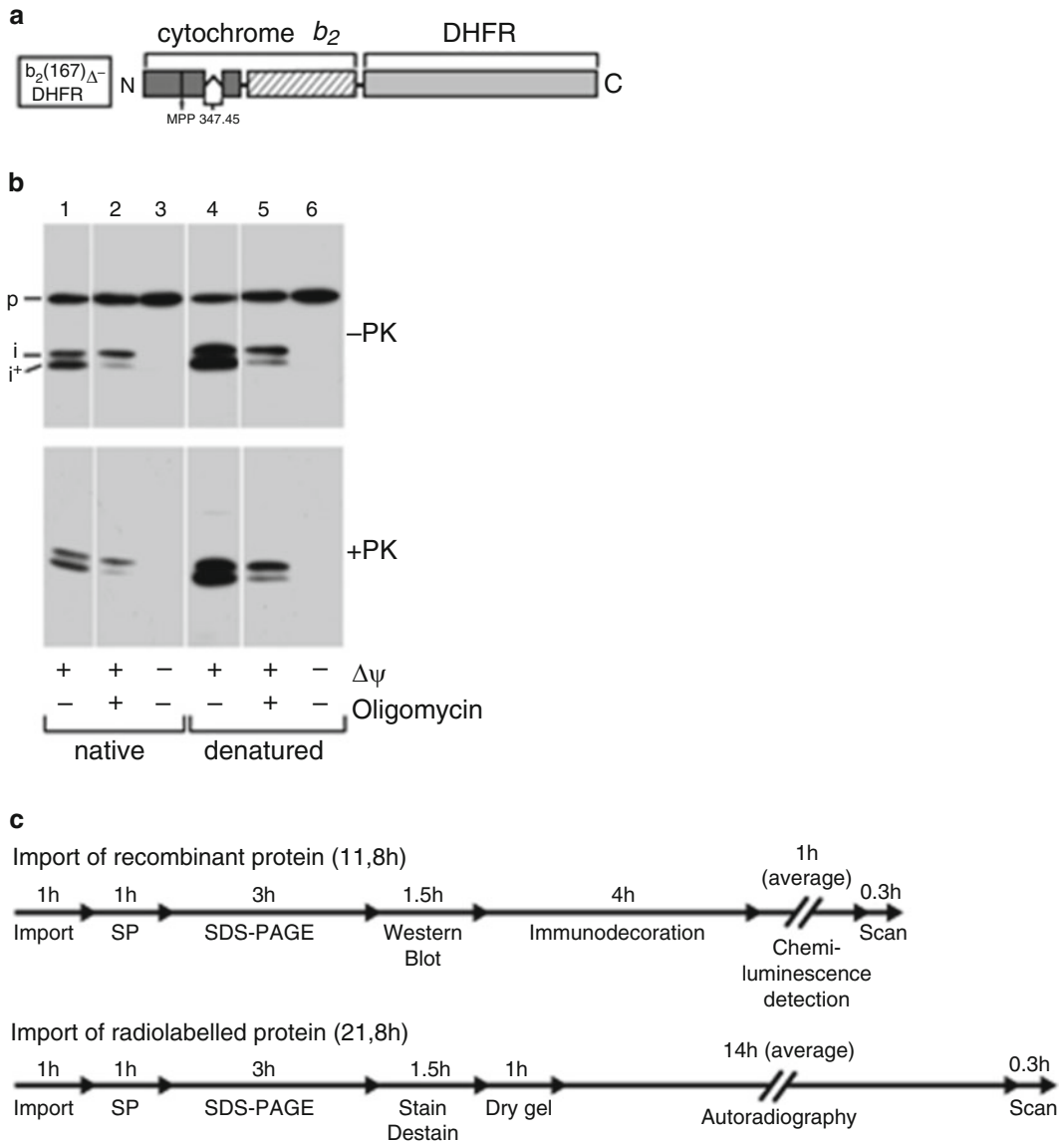
Fig. 3 Import of recombinant presequence-containing DHFR-fusion proteins. (**a**) Schematic drawing of reporter proteins $b_2(167)_\Delta$-DHFR and $b_2(167)_\Delta$-DHFR-SNAP representing fusion constructs of the N-terminal 167 residues of the cytochrome b_2 precursor and the complete mouse dihydrofolate reductase (DHFR). The b_2 precursor lacks residues 47–65 of the intermembrane space targeting signal. The cleavage site by the matrix processing peptidase (MPP) is indicated. The lower construct additionally contains the C-terminal SNAP-tag. The site for fluorescence labelling is indicated by an *asterisk*. (**b**) In vitro import of native (*lanes 1–3*) and urea-denatured (*lanes 4–6*) pre-$b_2(167)_\Delta$-DHFR into the mitochondrial matrix in dependency of $\Delta\psi$. *Lanes 1* and *4* represent a standard import reaction showing the appearance of processed, protease protected forms of the precursor protein. *Lanes 2* and *5* show decreased protein import after inhibition of the F_1F_0-ATP-synthase by oligomycin, which reduces the ATP concentration in the matrix compartment. In *lanes 3* and *6*, the membrane potential was dissipated by addition of the inhibitors antimycin A, valinomycin, and oligomycin, resulting in a complete block of the import reaction. Proteins were separated by SDS-PAGE, transferred to a PVDF-membrane, and immunodecorated with anti-mouse DHFR. The precursor (p) was processed twice (i, i*). In the lower panel, nonspecifically associated preproteins at the outer surface of mitochondria were degraded by treatment with Proteinase K (PK). (**c**) Timelines for import and analysis of recombinant and radiolabelled precursors. Given are the time periods for each individual step, as well as the overall time of the assays presented. SP: sample processing for SDS-PAGE

10. Load filtered lysate on a MonoS HR 10/10 cation exchange chromatography column. Take a sample of the flowthrough for SDS-PAGE.

11. Wash column with three column volumes of buffer A.

12. Elute column by addition of ten column volumes applying a NaCl gradient from 0–50 % (0.5 M NaCl). Collect eluate in 1-ml-fractions, and take a sample each for SDS-PAGE.

13. Check the efficiency of your purification by SDS-PAGE and Western Blot analysis using anti-mouse DHFR.

14. To the fractions containing the $b_2(\Delta)$-DHFR fusion protein, add glycerol to a final concentration of 50 %, and snap-freeze them in small aliquots in liquid N_2 for storage at –80 °C.

3.4 In Vitro Import of Recombinant Precursor Proteins into Isolated Mitochondria

A standard import reaction into yeast mitochondria contains 2 mM ATP, 4 mM NADH, as well as 20 mM KP_i, and 25 μg of mitochondria in a total volume of 100 μl import buffer. Substrate-saturating conditions are reached by an addition of at least tenfold excess of recombinant preprotein over available import sites. Based on the assumption of 0.25 pmol import sites per μg of yeast mitochondria [9], this corresponds to 6.25 pmol preprotein for a standard reaction (in the case of the described preprotein $b_2(167)_\Delta$-DHFR, about 240 ng per reaction). After terminating the import reaction by dissipation of the membrane potential, nonspecifically bound and non-imported precursors are degraded by the addition of Proteinase K. Further analysis requires Western transfer and immunodecoration, using anti-mouse DHFR antiserum. Figure 3b shows the results from in vitro import of $b_2(167)_\Delta$-DHFR, gained by immunodecoration, under different import conditions. Figure 3c shows a comparison of the typical timelines of an import reaction utilizing radiolabeled versus recombinant preproteins.

1. Thaw 17 μl (170 μg) mitochondria and 7 μl ATP on ice.

2. Prepare an import master mix for 6.6 time points on ice (*see* **Note 7**; total volume: 654 μl, minus 6.6× the amount of precursor added, 6.6 μl ATP, 13.2 μl NADH, 13.2 μl KP_i, pH 7.2, ad 660 μl import buffer, minus the amount of precursor added. Add 16.5 μl (165 μg) mitochondria, mix carefully by inverting the tube and spin down at low speed for 2–3 s (*see* **Note 8**).

3. Divide your master mix into six portions of 100 μl, minus the amount of precursor added.

4. Dissipate the membrane potential in mitochondria of sample #6 by addition of 1× inhibitor mix from the 100× stock; add 1 μl EtOH p.a. to samples #1–5 (mock). Mix carefully by flicking the tubes with your index finger.

5. Pre-incubate samples at 25 °C for 2 min, add 240 ng precursor (corresponding to 6.25 pmol of $b_2(167)_\Delta$-DHFR) to sample #6, mix, and leave at 25 °C for the entire import incubation period.

6. Initiate import reaction by addition of precursor to sample #5 (longest incubation period) and subsequent precursor addition to samples #4–1 (with #1 = shortest incubation period). Mix after addition by flicking the tubes.

7. Stop import reactions according to time scheme by addition of 1× valinomycin from 100× stock and moving samples on ice.

8. Prepare six fresh tubes (samples #7–12) with 1 μl PK (f.c.: 50 μg/ml) in 50 μl SEM buffer.

9. Add 50 μl each of samples #1–6 to PK-containing samples #7–12 and incubate for 15 min on ice; add 50 μl SEM buffer to samples #1–6 and likewise keep on ice.

10. Stop PK treatment by addition of 1 mM PMSF from 0.1 M stock (*see* **Note 5**); for equal treatment of all samples, add PMSF to non-PK-treated samples as well.

11. Keep samples on ice for other 10 min, before pelleting mitochondria by centrifugation at $12,000 \times g$ for 10 min.

12. Remove supernatants carefully, wash mitochondrial pellet by addition of 200 μl SEM buffer containing 1 mM PMSF, and centrifugation at $12,000 \times g$ for 8 min.

13. After removal of supernatants denature mitochondria in 1× Laemmli buffer.

3.4.1 Import of Urea-Denatured Precursor Proteins

The import of precursor proteins in a denatured state can be useful for the analysis of mitochondrial import defects in more detail. Denaturation of the preprotein to be imported facilitates its import, as the import reaction is no longer dependent on the functionality of the mtHsp70 system for unfolding. ATP-driven precursor unfolding is of particular importance for the import of tightly folded proteins or protein domains. Thus, comparison of the import efficiencies of both natively folded and denatured preproteins in mitochondrial preparations from different strains can have implications both for the tertiary structure of the preprotein imported, and for the mtHsp70 protein (un)folding capacity.

1. Thaw the preprotein to be imported on ice.

2. Add three volumes of cold-saturated $NH_4(SO_4)_2$ and mix by vortexing at low speed.

3. Incubate on ice for 30 min with occasional vortexing.

4. Centrifuge precipitates down at $20,000 \times g$ for 15 min.

5. Aspirate supernatant carefully, and resuspend protein pellet in the required volume of urea buffer by shaking at room temperature on a thermomixer at maximum speed for 15 min.

6. Pellet non-denatured protein material by centrifugation at $20,000 \times g$ for 5 min.

7. Transfer supernatant to a fresh tube and keep on ice until use.

8. Perform import reaction essentially as described in Subheading 3.4.

3.5 Analysis of the Import Reaction

1. Run samples on a sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel with a resolving gel acrylamide concentration of 12 %. Always run three control lanes with 1, 2 and 5 % of the preprotein amount used in the assay for quantification.

2. Transfer proteins to a Western Blot membrane (e.g., *Millipore*).

3. Immunodecorate with antibodies directed against mouse DHFR.

4. Detect signals using peroxidase-coupled secondary antibodies and enhanced chemiluminescence.

5. Evaluate raw data with image analysis software (*see* Subheading 3.4.1).

Evaluation of import kinetics can be done by quantification of protein bands using standard image analysis software (i.e., ImageJ) provided that the signals have not saturated. Band intensities in samples not treated with protease provide information on processing efficiencies, while determination of the efficiency of complete import is done from the protease-treated samples. Use the control lanes as references for protein amounts.

3.6 Dissection of a Functional Import Intermediate on Blue Native PAGE

The ligand methotrexate (MTX) stabilizes the tertiary structure of the DHFR domain and thereby prevents translocation of fusion proteins containing a C-terminal DHFR domain across the mitochondrial membranes [11, 12]. When $b_2(\Delta)$-DHFR is imported in the presence of MTX, it accumulates in a translocation intermediate that spans both mitochondrial membranes. Determination of the constituents of this complex by Blue Native gel electrophoresis (BN-PAGE) revealed the presence of TOM-components, as well as the core components of the TIM23 complex of the inner membrane. The translocase complexes are connected to each other by the preprotein in transit resulting in the formation of a large multiprotein complex comprising both outer and inner membrane components [9, 13]. Formation of the TOM-TIM-supercomplex is dependent on the membrane potential across the inner membrane, and results in an almost quantitative shift of the TIM23 core subunits from a size of ~90 kDa to a size of ~600 kDa, on BN-PAGE (*see* Fig. 4a).

The accumulation of $b_2(167)_\Delta$-DHFR in import sites is achieved by pre-incubation of the preprotein in the presence of 5 μM MTX, and addition of MTX in the import reaction, which results in the stable arrest of the preprotein inside the import channels.

Formation of the supercomplex can be monitored by Western blot using anti-Tim23 antiserum. BN-PAGE is specifically suited
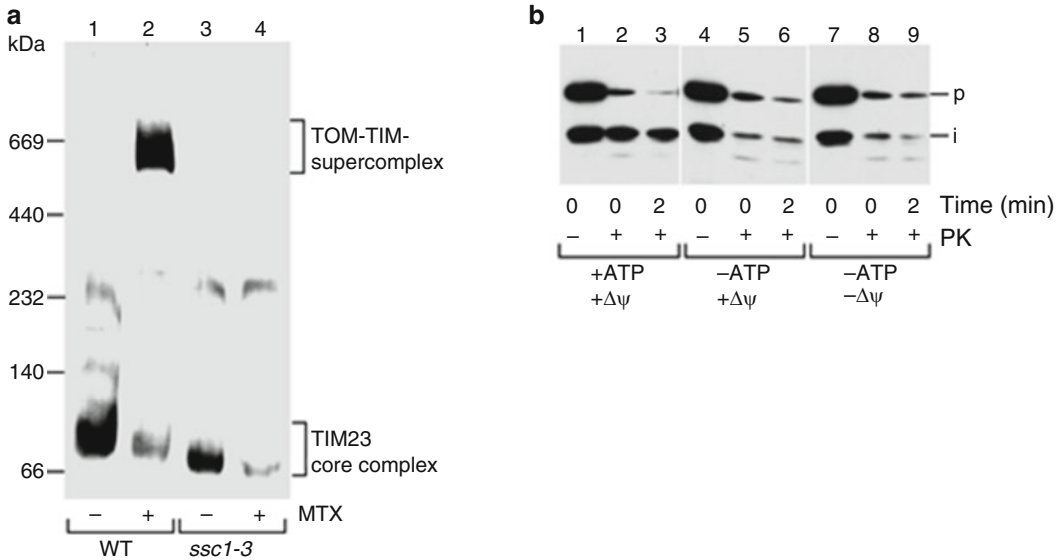
Fig. 4 Generation and analysis of import intermediates. (**a**) Dissection of the TOM-TIM supercomplex on Blue Native gel electrophoresis (BN-PAGE). Wild type (WT; *lanes 1* and *2*) and mtHsp70 mutant (*ssc1-3*; *lanes 3* and *4*) mitochondria were incubated with pre-b$_2$(167)$_\Delta$-DHFR in the presence (*lanes 2* and *4*) or absence (*lanes 1* and *3*) of 5 µM Methotrexate (MTX). Addition of MTX resulted in the accumulation of the preprotein in a location spanning both mitochondrial membranes. Mitochondria were extracted with the mild detergent digitonin and protein complexes resolved by BN-PAGE under native conditions. Analysis by Western blot using anti-Tim23 antibodies indicates the formation of the TOM-TIM23 supercomplex at a size of ~600 kDa only in the presence of MTX (compare *lanes 1* and *2*). The temperature-sensitive mtHsp70-mutant *ssc1-3* is not able to support the completion of the translocation at nonpermissive conditions. Hence, the supercomplex is not observed in *ssc1-3* mutant mitochondria (*see lanes 3* and *4*) (**b**) Assessment of the inward-directed import force exerted by mtHsp70. Mitochondria were incubated in the presence of pre-b$_2$(167)$_\Delta$-DHFR and 5 µM MTX for 10 min. A sample was taken directly after the incubation (*lanes 1*, *4*, and *7*). Another sample was taken directly after Proteinase K (PK) treatment (*lanes 2*, *5*, and *8*). A third sample was taken after a 2 min incubation following PK-treatment (*lanes 3*, *6*, and *9*). In the presence of a membrane potential (+$\Delta\psi$) and high ATP levels in the matrix (+ATP), an active import motor rendered 20–30 % of the accumulated intermediates resistant to protease treatment (*lanes 2* and *3*). Depletion of the membrane potential (−$\Delta\psi$) or removal of ATP (−ATP) inactivated the translocation machinery resulting in a rapid and complete loss of protease resistance

for the analysis of membrane bound protein complexes [15]. The BN technique includes the solubilization of mitochondria by the non-ionic detergent digitonine (*see* **Note 9**), and subsequent substitution of the detergent for water-soluble Coomassie blue. Thereby, proteins become negatively charged, and are thus separated mainly by molecular size in the electrophoresis.

1. Dilute MTX stock 200-fold (f.c.: 50 mM) in import buffer w/o BSA.

2. Incubate the appropriate amount of preprotein with 5 µM MTX for 10 min at 25 °C.

3. Set up an import reaction (*see* Subheading 3.4) for the accumulation; add creatine kinase, and creatine phosphate to a concentration of 200 µM, and 20 mM, respectively. Add 5 µM MTX.

4. Initiate accumulation by addition of a tenfold excess of MTX-coupled precursor (240 ng, corresponding to 6.25 pmol in the case of $b_2(167)_\Delta$-DHFR), and incubate for 15 min at 25 °C.

5. Stop reaction by addition of 1× inhibitor mix, and move samples on ice.

6. Pellet mitochondria by centrifugation at $12,000 \times g$ for 10 min.

7. Wash mitochondria by addition of 200 μl SEM buffer and centrifugation at $12,000 \times g$ for 8 min.

8. Solubilize mitochondrial pellet in 55 μl ice-cold digitonin buffer by pipetting up and down carefully with a 20–200-μl-pipet-tip for 15–20 times without causing digitonin foam. Keep on ice for 15 min.

9. Spin down unsolubilized material by centrifugation at $20,000 \times g$ for 15 min.

10. Prepare fresh tubes with 5 μl 10× Loading Dye, to which 45 μl of solubilized material (supernatants) is added after centrifugation. Take 5 μl of remaining supernatants each, and analyze on SDS-PAGE in parallel.

11. Load samples on a 6–16.5 % acrylamide gradient BN gel (*see* Table 1 and **Note 10**). Cover samples with 1× cathode buffer containing 0.2 % coomassie blue G250. Gel run is performed in a cooled chamber (4 °C). Keep at 100 V for passage through the stack gel, afterwards the voltage can be raised to 600 V. Always keep the current below 15 mA.

12. After the proteins have migrated into the separation gel, the Coomassie blue-containing cathode buffer is replaced by the one without dye (excess dye might interfere with subsequent Western transfer of the proteins).

13. Before Western transfer, the gel is incubated in 1x SDS-PAGE running buffer for 5 min (facilitation of protein transfer).

Table 1
Pipetting scheme for blue native gel preparation

% Acrylamide	6	8	10	13	16.5	Stack gel
3× gel buffer (ml)	3	3	3	3	3	2.5
Acrylamide (ml)	1.07	1.46	1.82	2.35	3.05	0.6
Glycerol (ml)	–	–	1.8	1.8	1.8	–
Water (ml)	4.888	4.507	2.347	1.817	1.117	4.367
10 % APS (μl)	38	38	38	38	38	30
TEMED (μl)	3.8	3.8	3.8	3.8	3.8	3

14. Decorate BN-membrane for Tim23 using TBS(T), supplemented with 5 % skim milk powder (*see* **Note 11**). SDS Western membrane is decorated against anti-mouse DHFR.

3.7 Assessment of the Inward-Directed Translocation Force

The interaction of the precursor protein inserted into the import channels with the functional import motor is required to generate the matrix-directed import force driving polypeptide movement and unfolding [16]. In case of methotrexate-stabilized $b_2(\Delta)$-DHFR translocation intermediates the protease-resistant DHFR domain is pulled tightly against the outer mitochondrial membrane by the activity of the import motor. The close apposition of stable DHFR and the outer membrane prevents the access of external proteases to the preprotein translocation intermediates. However, resistance against proteases is achieved only in the case of an active ongoing translocation reaction. Therefore the quantification of the protease resistance of translocation intermediates is an indirect measure for the import driving activity of the translocation machinery. In combination with the protease treatment, the membrane potential and the ATP-conditions can be manipulated to assess the role of the mitochondrial energy sources and the activity of the mtHsp70 import motor in the import process (*see* Fig. 4b).

1. Thaw 60 μl (600 μg) mitochondria and 12 μl ATP on ice.

2. Prepare an import master mix on ice (*see* **Note 7**; total volume: 2,080 μl): 2 mM ATP (23 μl), 4 mM NADH (46 μl), 1,978 μl import buffer, 57.5 μl mitochondria, and 5 μM MTX. Mix carefully by inverting the tube and spinning at low speed for 2–3 s.

3. Divide the master mix into two portions of 1,000 μl.

4. Preparation of precursor: Dilute precursor in import buffer w/o BSA to a concentration of 0.7 pmol/μl in 230 μl. Add 5 μM MTX and incubate for 5 min on ice.

5. Start accumulation of translocation intermediates: add 110 μl of precursor to 1,000 μl import master mix, and incubate for 10 min at 25 °C. Place on ice afterwards.

6. Depletion of ATP: add 11 μl apyrase (f.c.: 0.01 U/μl) and 2 μl oligomycin (f.c.: 20 μM) to one of the 1,000 μl import reactions. The other is treated as mock with 2 μl ethanol. Incubate for 10 min on ice.

7. Re-isolate mitochondria by centrifugation at $12,000 \times g$ for 10 min.

8. Resuspend mitochondria carefully in 1,100 μl buffer R1 (w/o ATP) or 1,100 μl buffer R2 (w/ ATP).

9. Take 2×500 μl of each buffer R to achieve the following incubation conditions:

   A: $+\Delta\psi/-$ATP.
   B: $-\Delta\psi/-$ATP.

C: $+\Delta\psi/+$ATP.

D: $-\Delta\psi/+$ATP.

10. Start incubation of conditions A–D (500 µl) at 25 °C, take sample 1 and 2 (100 µl each, corresponding to 25 µg mitochondria) immediately and put on ice.

11. Dissipate the membrane potential in mitochondria for condition B and D by addition of 3 µl inhibitor mix, and mix carefully by flicking the tube.

12. Add 3 µl EtOH p.a. to condition A and C (*mock*) and mix carefully flicking the tube.

13. Take sample 3 after 2 min, and sample 4 after 15 min (100 µl each) and put on ice.

14. PK-treatment: add 2 µl PK (f.c. 50 µg/ml) to samples 2, 3 and 4 (condition A–D) and incubate for 15 min on ice.

15. Stop PK treatment by addition of 1 mM PMSF (*see* **Note 2**); for equal treatment of all samples add PMSF to non-PK-treated samples as well. Keep samples on ice for further 5 min, pellet mitochondria by centrifugation at $12,000 \times g$ for 10 min.

16. Remove supernatants and wash mitochondrial pellet by addition of 150 µl SEM buffer, containing 1 mM PMSF, and centrifugation at $12,000 \times g$ for 8 min.

17. Remove supernatants and denature mitochondria in 1× Laemmli buffer.

18. Run samples on a Polyacrylamide gel with a resolving gel concentration of 12 % polyacrylamide.

19. Transfer your samples to a PVDF Western Blot membrane, and immuno-decorate with antibodies directed against mouse-DHFR.

4   Notes

1. Always prepare a fresh 0.5 M stock solution, and add DTT just prior to use.

2. PMSF addition should always be followed by immediate vortexing, otherwise it precipitates. In aqueous solution, PMSF has a half-life of about 30 min. Thus, always add it just prior to use. Alternatively, isopropanol stocks which are stable for up to 6 months can be used.

3. Depending on the preprotein, and the mitochondria used, rising of K^+ concentration up to 200 mM might help to increase import fidelity.

4. It is more convenient to use already recrystallized digitonin that can be directly dissolved in water (e.g., Calbiochem).

Heating up the solution significantly increases the solubility of digitonin.

5. Alternatively, 2 mM NADH plus 2 mM ATP might be added. Wild type mitochondria usually contain sufficient internal substrates, i.e., the addition of substrates will not further increase the fluorescence quenching.

6. Fluorescence intensity will not reach starting (maximal) value again, as you will always have unspecific fluorescence quenching to a certain extent.

7. For preparation of a master mix, it is useful to always use 5–10 % in excess of the volumes needed.

8. To maintain the full import-competence, do not vortex mitochondria at all before performing the import reaction as this might result in damages of the membranes.

9. Other detergents such as Dodecylmaltoside or Triton X-100 may also be suitable.

10. Prepare the BN gel always in advance and keep it at 4 °C until it is loaded.

11. Addition of sodium azide to a final concentration of 0.02 % (from a 100× stock) and storage at –20 °C is useful to preserve the antibody solution for subsequent experiments. Handle sodium azide with care as it is highly toxic.

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