Isolation and characterisation of the intermembrane space components of the mitochondrial TIM22 protein import machinery of *Neurospora crassa*

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To my grandmothers, Jelena and Marija

Table of Contents

1. Introduction

1.1. Mitochondrial protein translocation machineries

1.1.1. Mitochondrial structure and function

Mitochondria are semi-autonomous intracellular organelles of eukaryotic organisms. They have essential roles in the iron-sulfur cluster biogenesis (Mühlenhoff and Lill, 2000), and in the production of ATP (the main cellular energy-transducing molecule) by the means of oxidative phosphorylation (Mitchell, 1979; Schägger, 2002; Kadenbach, 2003). Furthermore, they perform functions related to the cell stress response and programmed cell-death (Hengartner, 2000; Zamzami and Kroemer, 2001; Newmeyer and Ferguson-Miller, 2003), as well as aging (Finkel and Holbrook, 2000; de Souza-Pinto and Bohr, 2002). They are also important for the maintenance of cellular Ca^{2+} homeostasis (Rizzuto *et al.*, 1992; Pozzan and Rizzuto, 2000; Orrenius *et al.*, 2003; Parekh, 2003). Moreover, oxidative decarboxylation of pyruvate, reactions of the citric/tricarboxylic acid cycle, certain steps of the urea cycle and the biosynthesis of haem and metabolites such as amino acids and lipids take place in mitochondria (Voet and Voet, 1995).

Mitochondrial functions are affected in various genetically inherited diseases (Ohta, 2003; Zeviani and Carelli, 2003). Mitochondrial morphology and abundance in the cell depend on the type of organism, type of cell and the metabolic/physiological state of the cell. Mitochondria differ in size, which ranges from less than 1 μm, to more than 10 μm. They can be ovoid, bean-shaped or spherical, thread-like, elongated tubules, or highly branched nets (Frey and Mannella, 2000). Their morphology is maintained through balanced fusion and fission events which take place throughout the cell cycle (Nunnari *et al.*, 1997). Even their position in the cell can vary depending on the metabolic, energetic and various other cellular requirements and environmental conditions. They manoeuvre around through the association with cytoskeletal elements and linger in the vicinity of high energy consumption sites.

It is important to note that no *de novo* synthesis of the organelle occurs. Instead, these organelles continuously grow throughout the cell cycle, and the daughter cells inherit a portion of them upon cell division (Yoon and McNiven, 2001). Mitochondria probably arose monophyletically from a single α-proteobacterial ancestor that underwent symbiotic fusion

with a nucleus-containing eukaryotic host resembling extant amitochondriate protists (Gray *et al.*, 1999; Emelyanov, 2003). This event took place approximately 1.5-2.0 billion years ago. During evolution the ancestral endosymbiotic genome was significantly reduced, with most of the genes being lost or transferred to the nucleus of the host organism (Herrmann, 2003). Nowadays mitochondria contain rather small genomes (mtDNA nucleoids), that code for a handful of proteins and some of its RNA species, while most of the genes required for supporting its activity are located in the nucleus.

Mitochondria contain two membranes: the outer membrane which is the physical barrier separating the mitochondrion from the cytoplasm, and the convoluted inner membrane, physically dividing the intermembrane space from the dense matrix (Figure 1), adapted from Frey and Manella, 2000).

Due to its highly convoluted character, the inner mitochondrial membrane can constitute up to one third of the total cellular membrane content, carrying more than one fifth of the total mitochondrial protein. Two distinct inner membrane sub-regions can be distinguished: the inner boundary membrane closely apposed to the outer membrane, and the cristae membrane which represent invaginations of the inner membrane that are projecting into the matrix. Cristae membranes also show very rich shape variations, ranging from tubular, lamellar to triangle-shaped. The morphology of cristae membrane changes as well, with the differential mitochondrial activity (Reichert and Neupert, 2002). Outer and the inner membranes do not only differ in their appearance, but also in their lipid composition, permeability to various metabolites and integral membrane protein content, reflecting their different, highly specialized functions.

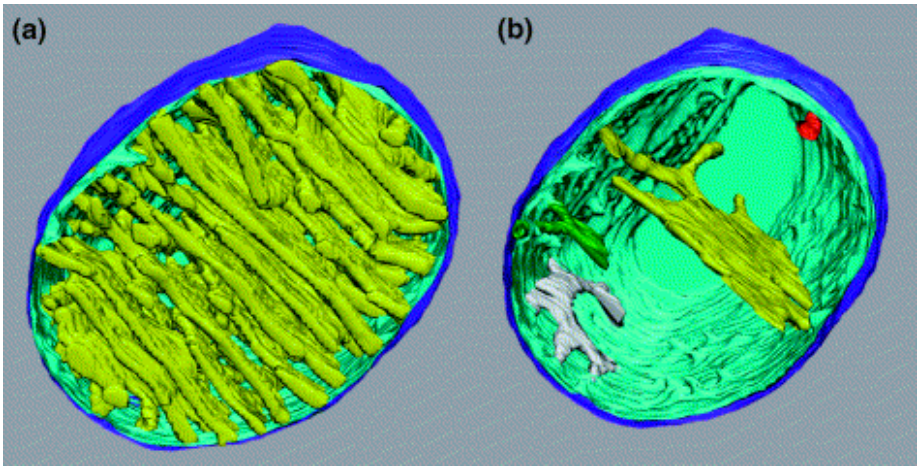

Figure 1. Computer models generated from segmented 3D tomograms of a mitochondrion in chicken cerebellum. **(A)** The entire model showing all cristae in yellow, the inner boundary membrane in light blue, and the outer membrane in dark blue. **(B)** Outer membrane, inner boundary membrane and four representative cristae in different colors

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The inner mitochondrial membrane contains components of the respiratory chain, ATP synthase complex, protein translocation machineries and many metabolite transporters (in *S. cerevisiae* 35 various members of the mitochondrial carrier family are present). It is impermeable to polar molecules and ions, safekeeping the electrochemical proton gradient, created by the action of the respiratory chain. The respiratory chain components pump protons from the matrix into the intermembrane space, with pH and voltage differentials ensuing. In the matrix, pH is more basic by about 0.4-1.4 pH units than in the intermembrane space and the inner membrane's surface facing the matrix is more negative than the one facing the intermembrane space, giving rise to a voltage gradient of about 0.14 volts. The energy of the described gradient is harnessed by the proton-transporting ATP synthase. This enzyme complex produces ATP from ADP and the inorganic phosphate, as the protons released into the mitochondrial matrix combine with reduced oxygen to form water.

The outer membrane is populated with highly abundant porins (voltage-dependent anion channel (VDAC)) which form large aqueous channels in the lipid bilayer, components of the protein translocation machinery, as well as proteins determining the organelle's morphology and mediating apoptosis. Due to the presence of porins, the outer membrane is permeable to water, inorganic ions and metabolites of molecular weight smaller than 5 kDa.

The mitochondrial matrix is the site of a large number of metabolic processes, and contains the mitochondrial genome (mtDNA) and special mitochondrial ribosomes. Mitochondrial nucleoids are covalently closed, circular (with some exceptions in certain algae and ciliates, where it is linear), multi-copy, double-stranded DNA molecules attached to the inner membrane. They differ from nuclear DNA in base composition, higher density upon separation by density gradient centrifugation and absence of histones. The mitochondrial genetic code displays certain deviations from the universal genetic code. Mitochondrial genes do not follow Mendelian rules of inheritance, being characterised by the non-mendelian (cytosolic) inheritance (Alberts *et al.*, 1994).

Mitochondrial protein synthesis generally differs from the cytosolic protein synthesis in several aspects: (i) N-formylmethionine is the first amino acid incorporated in a polypeptide chain, (ii) it is sensitive to antibiotics which inhibit bacterial protein synthesis and (iii) its ribosomes are of the 74S sedimentation coefficient species in fungi and 60S in metazoans. Although capable of sustaining their own translation, mitochondria do not possess large enough genomes to accommodate their protein repertoire in full: mtDNA codes only for eight of approximately 750 mitochondrial proteins identified in yeast (Sickmann *et al.*, 2003), and for 13 from more than a thousand proteins functioning in human mitochondria (Cotter *et*

al., 2004). On the whole, 20% of all cell proteins in eukaryotic cells are mitochondrial proteins (Model *et al.*, 2001).

The intermembrane space subcompartment harbours around 5% of total mitochondrial proteins. Among those are the proteins involved in maintenance of mitochondrial morphology (like Mgm1p; Herlan *et al.*, 2003), electron transport along the respiratory chain (cytochrome *c*; Alberts *et al.*, 1994), apoptosis (Smac, AIF, cytochrome *c*; Newmeyer and Ferguson-Miller, 2003), protein translocation (small Tim proteins; Neupert, 1997), copper transport (Cox17p; Beers *et al.*, 1997) and iron sulfur cluster biogenesis (Erv1p, Lange *et al.*, 2001).

1.1.2. Protein translocation in mitochondria of *N. crassa* and *S. cerevisiae*

1.2.2.1. Targeting of preproteins to mitochondria

Nuclear-encoded mitochondrial precursor proteins are synthesized in the cytosol on free ribosomes. During synthesis, they are bound by the cytosolic chaperones of the Hsp70 family which help to keep them in an import competent, unfolded or partially folded state. In mammals, mitochondrial import-stimulating factor (MSF) specifically recognizes and binds the signal sequences of mitochondrial precursors and stimulates their binding to mitochondria in an ATP dependent manner (Hachiya *et al*., 1994 and 1995). Although the majority of mitochondrial preproteins are imported posttranslationally, evidence for cotranslational import exists as well (Fujiki *et al.*, 1993).

Mitochondrial precursor proteins contain targeting and sorting sequences that determine the final destinations of proteins within mitochondria. Proteins destined for the matrix generally contain N-terminal cleavable presequences. These N-terminal extensions are rich in positively charged, hydrophobic and hydroxylated amino acid residues which form amphipathic α-helical structures and their lengths vary between *ca* 12 and 70 amino acid residues (von Heijne, 1986; von Heijne *et al* 1989; Roise, 1992; Roise and Schatz, 1988). Proteins that are to be inserted into the inner membrane display great versatility in their targeting signals (Table 1). Outer membrane proteins with single TMDs contain mitochondrial targeting information in their hydrophobic anchors and the flanking positively charged residues (Rapaport, 2002). The β-barrel proteins of the outer membrane possess internal targeting signals with no consensus sequences identified up to date.

The targeting signals of the intermembrane space proteins can be grouped into at least three classes. Class I consists of the N-terminal matrix-targeting sequences followed by the hydrophobic sorting sequences (bipartite presequences related to the signals of bacterial and eukaryotic secretory proteins), like those in cyt b_2 (Glick *et al.*, 1992a; Gärtner *et al*., 1995b). In class II, the signal is confined to an internal, highly hydrophilic part of the molecule rich in positively and negatively charged residues, like in cytochrome *c* heme lyase (CCHL, Lill *et al*., 1992; Segui-Real *et al.*,1993; Diekert *et al.*, 1999). In class III, represented by the small Tim proteins, the targeting signal has not yet been clearly defined, but the cysteine residues have been shown to be important for the import and assembly of a functional complex (Lutz *et al.*, 2003; Lu *et al.*, 2004)

There are proteins which localise to two subcompartments of mitochondria, like the Mcr1p which is found in the outer membrane, as well as in the intermembrane space (Hahne *et al.*, 1994), or Mgm1p, with the long isoform residing in the inner membrane and the short one in the intermembrane space (Herlan *et al.*, 2003). The targeting sequence of Mcr1p closely resembles that of the outer and the inner membrane proteins with single TMDs, and the one from Mgm1p consists of a presequence followed by two hydrophobic segments.

Table 1

Type of targeting signal	*Example*	*Reference*
Cleavable presequences combined with a hydrophobic anchor located downstream	CoxVa	Gärtner *et al*., 1995a
Cleavable presequences together with a downstream hydrophobic anchor, combined with a cluster of charged amino acids C-terminal to it	D-LDp	Rojo *et al.*, 1998
Internally positioned positively charged presequence-like stretches, often preceded by a TMD	BCS1p	Fölsch *et al.*, 1996; Stan *et al.*, 2003
Bipartite presequences	cyt c_1	Glick *et al.*, 1992a
Multiple internal targeting signals containing charged and non-charged parts in proteins with modular structure*	metabolite carriers	Kübrich *et al.*, 1998; Endres *et al.*, 1999; Wiedemann *et al.*, 2001

* these signals, contained in each of modules and capable of functioning independently for each module alone, exert a concerted action *in vivo* for highest import efficiency

Presequences which reach the matrix are, in the majority of cases, cleaved off by the mitochondrial processing peptidase MPP (Hawlitschek *et al.*, 1988; Gessert *et al.*, 1994; Gakh, Cavadini and Isaya, 2002) with a few exceptions, like the chaperonin 10 (Rospert et al., 1993; Jarvis *et al.*, 1995). A single cleavage by MPP is normally sufficient for the maturation of most matrix and inner membrane protein precursors, with the exception of the octapeptide-containing precursors that require two cleavages, sequentially carried out by MPP and mitochondrial intermediate peptidase (MIP), also localized to the matrix (Isaya *et al.*, 1991). The bipartite presequences are however cleaved by the heterodimeric inner membrane peptidase Imp1p-Imp2p (Nunnari *et al.*, 1993).

1.2.2.2. Translocases of the outer mitochondrial membrane

The outer membrane of mitochondria contains two major protein complexes involved in protein translocation, membrane insertion and assembly. All mitochondrial precursor proteins described up to date are recognised first by the components of the TOM complex (translocase of the outer mitochondrial membrane; Rapaport, 2002 and Paschen and Neupert, 2001). The TOM holo complex consists of the channel forming Tom40 subunit, three small Tom proteins Tom5, Tom6 and Tom7, and three receptor proteins, Tom22, Tom20 and Tom70 (Figure 2, Künkele *et al.*, 1998a). When purified without the receptor subunits Tom20 and Tom70, it is referred to as the TOM core complex, or the GIP (general import pore; Pfanner and Geissler, 2001). Both *N. crassa* and *S. cerevisiae* TOM complexes contain all these subunits.

Receptors of the TOM complex show differential substrate recognition. Tom20 is designated for binding presequence-carrying precursors, while Tom70 attends to the mitochondrial carrier family members (Söllner *et al.*, 1989 and 1990; Schlossmann *et al.*, 1994 and 1996; Brix *et al.*, 1997; Komiya *et al.*, 1997 and 1998). Tom22 binds all various kinds of precursors (van Wilpe *et al.*, 1999) and with the help of Tom5 (Dietmeier *et al.*, 1997) transfers them to the Tom40 which is most probably present in six copies per GIP complex. A pair of Tom40 molecules builds a channel with a pore diameter of *ca* 22 Å (Künkele *et al.*, 1998b; Schwartz et al., 1999), the size being sufficient to accomodate two α-helices. The channel has specific substrate-binding sites as well (Rapaport *et al.*, 1997; Hill *et al.*, 1998). Aside from Tom20, the cytosolic domain of Tom22, as well as parts of Tom5, all contain negatively charged, succeeding binding sites for the positively charged presequences. This “acid chain” of negatively charged patches across the outer membrane, was proposed to

drive the translocation of presequence-containing substrates from the *cis* to the *trans* side of the TOM complex (Komiya, 1998).

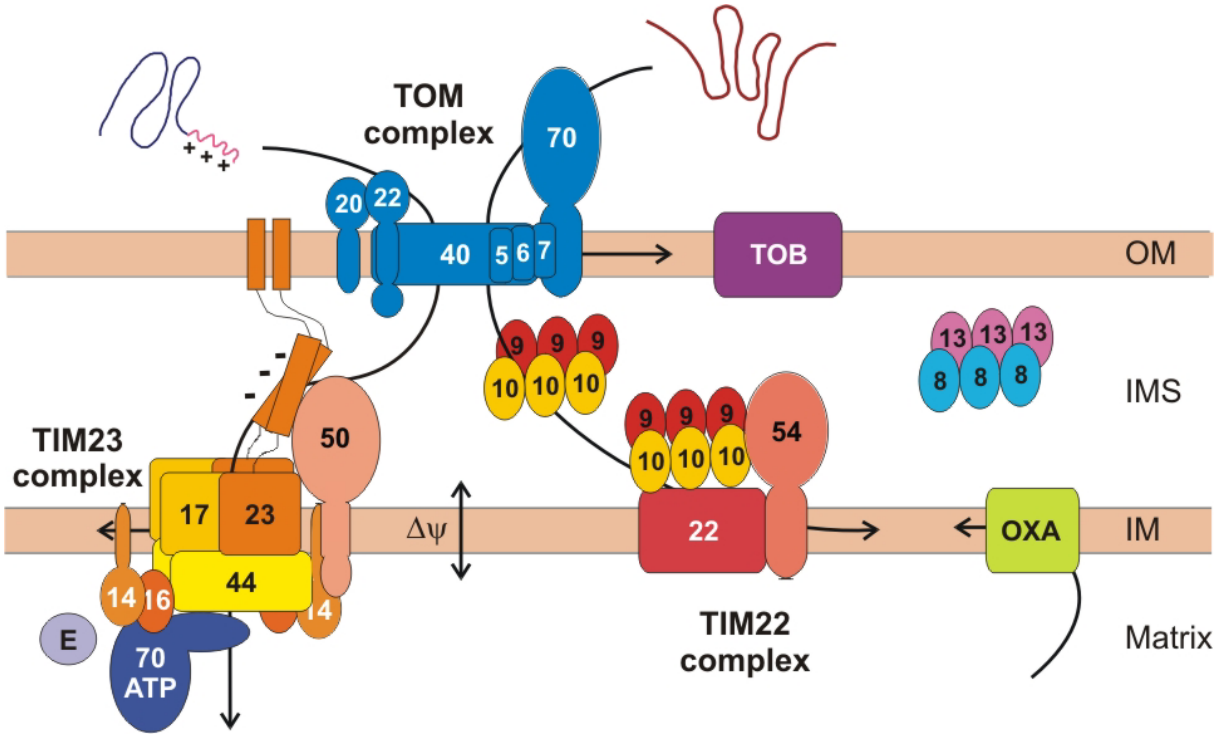


Figure 2. Import pathways in *N. crassa* mitochondria.

Meanwhile, it has been shown that the hydrophobic interactions also take place in the process of translocation across the outer membrane (Brix *et al.*, 1997 and 1999; Abe *et al.*, 2000; Meisinger *et al.*, 2001). Understanding of the translocation process across the outer membrane has therefore seen the acid chain hypothesis being recasted as the binding chain hypothesis that encompasses all different types of non-covalent interactions (Pfanner and Geissler, 2001).

An intriguing property of certain TOM complex members, namely Tom70 and Tom20, is a repetitive, degenerate motif of 34 amino acid residues, called the tetratricopeptide repeat (TPR) (Steger *et al.*, 1990; Iwahashi *et al.*, 1997; Young *et al.*, 2003). It is present in the cytosol-exposed domains of these proteins. This motif is a protein interaction module, often arranged in tandem arrays. It is found in unrelated proteins involved in quite diverse cellular processes. From data collected with various TPR-containing proteins, a common design seems to emerge. The module is usually structured into two anti-parallel α-helices, such that tandem arrays of TPR motifs generate a right-handed helical structure. This structure forms an amphipathic channel that should accommodate complementary regions of the binding partner proteins. It is therefore conceivable that the TPR motif has a vital role in

binding incoming precursors by the mentioned mitochondrial import receptors (Abe *et al.*, 2000).

At last, Tom6 and Tom7 proteins are involved in regulating the stability of the TOM complex (Dekker *et al.*, 1998).

The TOM complex is involved in transport of all nuclear-encoded mitochondrial proteins, regardless of their final destination within the organelle. It can insert proteins with α-helical folds into the outer membrane. For the integration of the β-barrel outer membrane proteins, the TOM complex cooperates with the other oligomeric outer membrane protein machinery, the TOB complex (for topogenesis of mitochondrial outer membrane beta-barrel proteins; Paschen *et al.*, 2003). It is also known as the SAM complex (sorting and assembly machinery; Wiedemann *et al.*, 2003). Up to now, the complex has been characterized in *S. cerevisiae* only. The TOB complex consists of the channel-forming subunit Tob55 (identified in *N. crassa* and in *S. cerevisiae*; Paschen *et al.*, 2003; Wiedemann *et al.*, 2003), and Mas37 (Gratzer *et al.*, 1995; Hachiya *et al.*, 1995). The latter component has been identified only in yeast thus far. This complex takes over the β-barrel precursor proteins from the TOM complex, but the mechanism of their insertion into the outer membrane is not yet resolved.

1.2.2.3. Translocases of the inner mitochondrial membrane

Proteins of the inner mitochondrial membrane are of dual origin: there are some encoded by the nuclear genes (for instance Tim17, Tim22, Tim23, Tim50, Tim54, Oxa1, AAC, etc.) and others, encoded by the mtDNA (cytochrome oxidase subunits Cox I, II and III, F_0F_1-ATPase subunits 6, 8 and 9, and apocytochrome *b*). Furthermore, subsets of nuclearly encoded precursors destined for the inner membrane differ significantly in their targeting signals. These facts make for their divergence as substrates of different inner membrane translocases.

The inner mitochondrial membrane contains three translocase complexes for insertion of precursor proteins encoded by the nuclear genes, all with different substrate specificities (for reviews see Neupert, 1997; Paschen and Neupert, 2001; Pfanner and Geissler, 2001; Jensen and Dunn, 2002). The TIM23 complex (for translocase of the inner mitochondrial membrane) has been characterised in much detail in *S. cerevisiae* and in *N. crassa*. This translocase is specialized for the precursor proteins which contain presequences. Substrates of the TIM23 translocase are destined mainly for the matrix, some for the intermembrane space and some for the inner membrane. The essential TIM23 translocase subunits embedded in the inner mitochondrial membrane are: Tim14 (Mokranjac *et al.*, 2003b), also termed Pam18

(from presequence translocase-associated motor; Truscott *et al.*, 2003), Tim17 (Kübrich *et al.*, 1994), the channel-forming Tim23 protein (Ryan *et al.*, 1993; Emtage *et al.*, 1993; Kübrich *et al.*, 1994) and the Tim50 receptor subunit (Geissler *et al*., 2002; Yamamoto *et al*., 2002; Mokranjac *et al.,* 2003a). The import motor of the Tim23 translocase (Neupert and Brunner, 2002; Voos and Röttgers, 2002; Okamoto *et al.*, 2002) is located in the matrix and it includes the essential subunits MIA1 (Tim16, Pam16; Kozany *et al.*, 2004; Frazier *et al.*, 2004), Tim44, mtHsp70 and Mge1 (Schneider *et al.*, 1996; Voos *et al*., 1996; Horst *et al.*, 1997). The only membrane-anchored component of this motor is the Tim14 protein. The transmembrane potential ($\Delta\psi$) and ATP are the general requirements for the productive action of the TIM23 translocase.

Two groups of the inner membrane proteins are exported from the matrix in a process mediated by the Oxa1 and Mba1 translocases, described up to date in *S. cerevisiae* (Bauer *et al.*, 1994; Bonnefoy et al., 1994; Herrmann *et al.*, 1997; Hell *et al.*, 1997; Preuss *et al.*, 2001) and *N. crassa* (Nargang *et al*., 2002). The first group contains some presequence-carrying proteins that are completely imported into the matrix from where they insert into the inner membrane in an export process. This pathway resembles insertion reactions of polytopic membrane proteins of bacterial origin and has been termed the conservative sorting pathway (Stuart, 2002; Herrmann and Neupert, 2003). The other group of Oxa1 and Mba1 substrates is composed of highly hydrophobic membrane proteins encoded by the mtDNA. During mitochondrial evolution transfer of their genes to the nucleus might have been prevented, because of their hydrophobic nature and the tendency to form unproductive aggregates in the cytosol. Therefore, they need to be inserted into the inner membrane co-translationally, before the aggregation takes effect.

Translocases mediating protein export from the matrix, Oxa1 and Mba1, overlap in substrate specificity and function. However, both are capable of performing their roles independently. The matrix-exposed C-terminus of Oxa1 forms an α-helical coiled-coil domain that binds mitochondrial ribosomes (Szyrach *et al.*, 2003) thereby tethering the precursor to the site of its integration into the lipid bilayer. Oxa1 is evolutionarily conserved – its homologues are found in mitochondria of all investigated species. Similarly, its homologues, YidC protein in the bacterial inner membrane and Alb3 protein in the chloroplast thylakoid membrane, mediate protein insertion into corresponding membranes (Kuhn *et al.*, 2003).

Two homologues, shown to be involved in the export translocation process coupled to assembly of the cytochrome oxidase, are the yeast Cox18 (Souza *et al.*, 2000), and the Oxa2 protein of *Neurospora crassa* (Funes *et al.*, 2004). Both proteins also bear significant degree

of homology to the Oxa1 protein, but lack the α-helical C-terminal ribosome-binding domain characteristic of Oxa1.

While the TIM23 complex inserts inner membrane proteins which contain only one TMD, the TIM22 complex is required for the insertion of all nuclear-encoded, inner membrane integral proteins characterised by multiple TMDs and the absence of the presequence. Metabolite carrier proteins are the major class of TIM22 substrates. They all reside in the inner membrane of mitochondria and have an approximate molecular mass of 30 kDa. Their distinctive attribute is the modular structure: six α-helical TMDs are tandemly organised in three related modules of ~100 amino acid residues (Figure 3), adapted from Pebay-Peyroula *et al.*, 2003).

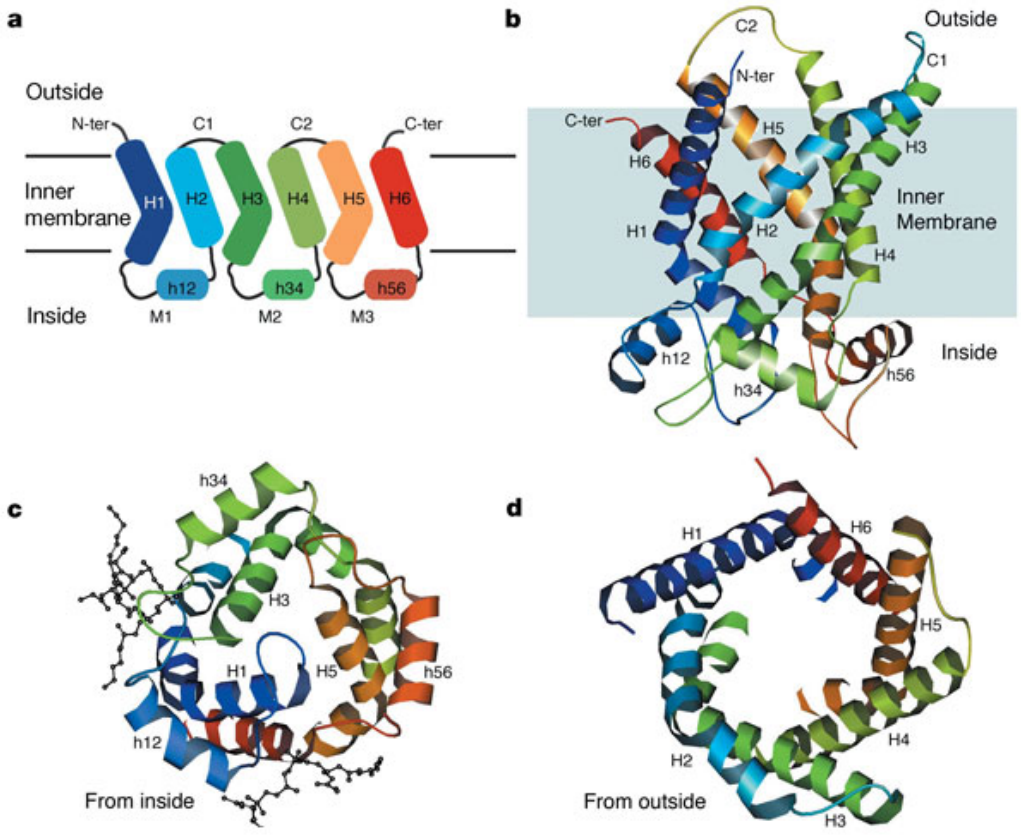


Figure 3. Architecture of the ADP/ATP carrier. (A) A schematic diagram of the carrier secondary structure. Transmembrane helices, surface helices, intermembrane space loops and matrix loops are labelled H, h, C or M, respectively. Inside and outside designate the matrix and the intermembrane space of mitochondria, respectively. **(B)** A ribbon diagram viewing the carrier from the side. The structure is coloured according to the sequence blue (N terminus) to red (C terminus). Membrane boundaries are drawn in agreement with the hydrophobic segments of the helices. **(C)** View from the "inside" (matrix). Two cardiolipins are represented in black as ball and sticks. **(D)** View from the outside (intermembrane space).

These tandem repeats are interrelated in different proteins, and probably have similar secondary structures: two transmembrane α-helices linked by an extensive hydrophilic region. Some members of the family have been well studied (Palmieri *et al.*, 2000), like the ADP/ATP carrier, the phosphate carrier, dicarboxylate and tricarboxylate carriers, the ornithine transporter, the folate transporter, the aspartate-glutamate transporter, the oxoglutarate carrier, the uncoupling protein, and many others, while some still await characterisation.

Other identified TIM22 translocase substrates include the Tim23, Tim17 and Tim22 proteins, all with four TMDs and possibly with secondary modular structure similar to that of carriers. The Tim22 precursor preferentially utilises the Tom20 receptor (Kurz *et al.*, 1999), but subsequently diverges from the TIM23 translocase substrates in joining the carrier import pathway. The Tim54 precursor also shows a peculiar behaviour on its import route, mirroring that of Tim22: it uses the Tom70 receptor which recognises its internal targeting signal(s), but then joins the pathway of the TIM23 complex substrates. In addition, Tim23 protein was reported to be bound by the Tim8·Tim13 complex on its journey through the intermembrane space when $\Delta\psi$ is dissipated (Paschen *et al.*, 2000; Davis *et al.*, 2000; Curran *et al.*, 2002b; Jensen and Dunn, 2002).

The TIM22 translocase encompasses several membrane-integrated subunits: Tim22 (Sirrenberg *et al.*, 1996; Kerscher et al., 1997), Tim54 (Kerscher *et al.*, 1997) and its only non-essential and for yeast unique component Tim18 (Kerscher *et al.*, 2000; Koehler *et al.*, 2000). Additional members of the TIM22 translocase reside in the mitochondrial intermembrane space. They are the small Tim proteins (Koehler, Merchant and Schatz, 1999). In yeast, there have been five members of the small Tim protein family identified. Homologues of Tim9 (Adam *et al.*, 1999; Koehler *et al.*, 1998b), Tim10 (Sirrenberg *et al.*, 1998), Tim8 (Davis *et al.*, 2000; Paschen *et al.*, 2000) and Tim13 protein (Davis *et al.*, 2000; Paschen *et al.*, 2000) are generally found in all species under investigation regarding mitochondrial TIM22 translocase. There exists one small Tim protein, Tim12 (Sirrenberg *et al.*, 1998, Koehler *et al.*, 1998a), which features a unique fifth cysteine residue in its primary sequence. The Tim9 and Tim10 proteins form one soluble heterohexameric complex in the intermembrane space, and Tim8 and Tim13 another, the first one being leastwise ten times more abundant. The main function of soluble small Tim complexes is to assist the transfer of the TIM22 translocase substrates across the intermembrane space from the outer to the inner membrane. Nonetheless, a small fraction of Tim9 and Tim10 proteins forms a 300 kDa

complex together with the membrane-associated Tim12 protein, and the membrane-integrated components of the TIM22 complex.

All small Tim proteins contain the 'twin CX_3C' motif, assumed to be involved in zinc binding. This last premise found its grounds in experimental findings that recombinant MBP-Tim10 and -Tim12 fusion proteins bind zinc, and that the interaction between Tim10 and AAC is inhibited by metal chelators (Sirrenberg *et al.*, 1998).

In yeast, Tim9, Tim10 and Tim12 proteins are essential, and Tim8 and Tim13 are not. However, human Tim8 homologue has been implicated in the occurrence of a recessive X chromosome-linked progressive neurodegenerative disorder. This rare disease is also known as the deafness dystonia or Mohr-Tranebjaerg syndrome (DFN-1/MTS; Tranebjaerg *et al.*, 1995; Jin *et al.*, 1996). It is caused by mutations in the DDP1 gene, resulting in a defective assembly of the DDP1/TIMM8a-TIMM13 complex (Koehler *et al.*, 1999). DDP1 is a designation for the human Tim8 homologue (stands for deafness dystonia peptide). The syndrome comprises various severe and progressive impairments, like the sensorineural deafness, cortical blindness, mental retardation, paranoia, dysphagia and dystonia.

Import orchestrated by the TIM22 translocase is also reliant on $\Delta\psi$.

The import of carrier proteins into mitochondria has been partitioned into several stages (Kübrich *et al.*, 1998; Endres *et al.*, 1999; Ryan *et al.*, 1999). Upon their synthesis in the cytosol, carrier molecules reach the mitochondria bound to cytosolic chaperons Hsp70 and MSF. This state is known as the stage I. At the outer membrane, each carrier module recruits one dimer of Tom70p receptor molecules and is concomitantly released from the chaperones in an ATP-dependent manner (stage II). Although Tom70 is proposed to be the major receptor for carrier proteins, in *tom70* null yeast strain the import of carriers resumes, albeit with considerably reduced efficiency and involving the Tom20 receptor (Steger *et al.*, 1990). Tom70 protects the carrier precursors from aggregation and hands them over to the Tom40 protein which forms a pore. The modules are inserted into the channel in stage IIIa and they are released from the outer membrane translocase through the action of the Tim9·Tim10 complex. In yeast, the Tim12 protein docks the soluble Tim9·Tim10 complex with bound substrate to the Tim22·Tim54 complex (stage IIIb). The Tim22 protein receives the precursor proteins and releases them into the inner membrane (stage IV) in a process which is strictly dependent on the presence of $\Delta\psi$. It is conceivable that the TIM22 translocase assembles carrier dimers in the inner membrane (stage V), but no proof has been offered hitherto to back this speculation.

1.2. Zinc fingers

In all organisms zinc is an essential element, a fact first established for eukaryotic plants in 1869 (Raulin, 1869). It is the second most abundant trace metal found in eukaryotes. If one subtracts the amount of iron present in haemoglobin, zinc becomes the most abundant trace metal in humans. The adult human body contains up to 3 g of ionic zinc (Berg *et al*., 1996). It occurs naturally as the divalent cation and has no redox activity under physiological conditions. Indispensable for growth, development and differentiation, it also exerts very important roles in the immune response, suppression of apoptosis, inhibition of cell transformation and in antioxidation.

Over the past 60 years more than 300 different enzymes have been identified involving zinc in the catalytic process. In addition, there exist hundreds of proteins in which zinc stabilizes certain structural motifs and/or plays a regulatory role (Cox *et al.,* 2000). Zinc is commonly coordinated to proteins via the thiol moieties of cysteine residues or the imidazole group of histidine residues, but other ligands, such as glutamate and aspartate residues, have also been identified (Lippard et al., 1994).

Four different primary types of zinc sites exist: structural, catalytic, cocatalytic and the protein interface site (Auld, 2001). In catalytic sites zinc is coordinated by any three N, O and S donors and one water molecule. Predominant amino acid ligands of these sites are the histidine residues. Structural sites contain no bound water, and cysteine is the most common amino acid found in them. Cocatalytic sites comprise 2-3 closely spaced metals, two being bridged by a side chain of Asp, Glu or His, or by a water molecule. These sites do not contain cysteine residues. Zinc ligands can also be provided by interfaces of two protein subunits forming a complex. These sites are usually grouped together with catalytic or structural types.

The first of many zinc-based protein motifs, termed the “zinc finger”, was identified in a transcription factor TFIIIA of *Xenopus*, less than 20 years ago (Miller *et al.*, 1985). It is estimated that the zinc finger transcription factors alone encompass about 2-3% of proteins encoded in the human genome (Maret, 2000; Matthews *et al.*, 2002). Zinc finger modules are small metal-binding domains found in nuclear hormone receptors, many gene regulatory proteins participating in transcriptional and translational processes, proteins involved in maintenance of metal ion homeostasis, peroxisomal biogenesis and signal transduction pathways, proteins with regulatory roles in apoptosis, proteins necessary for viral pathogenicity, chaperones and proteins which bind lipids (Laity *et al*., 2001; Saurin *et al.*,

1996). They perform their functions through binding to lipids, DNA, RNA and/or other proteins.

There exist numerous families of zinc finger proteins (well-characterized are currently fourteen) that contain multiple cysteine and/or histidine residues. Proteins are classified into different zinc finger families based on their different properties regarding:

- nature and arrangement of zinc-binding sites (in the simplest example CCCC, CCHC or CCHH variations, with different lengths of amino acid spacers between zinc-coordinating cysteine and histidine residues),
- subcellular localisation (nuclear, cytoplasmic, organellar),
- function of the protein, and
- protein folding patterns (most common one being the ββα fold of the classical zinc finger) (Wolfe *et al.*, 1999, Berg *et al.*, 1997).

The most common zinc finger families are listed in Table 2. Their specific zinc-binding patterns are indicated.

Table 2

Zinc finger family	*Zinc-binding motif*
Classical	C2H2
RING	C3HC4, C3H2C3, C2THC4, C3HC2EC or C3NC4*
LIM	C2(H/C)2C3(C/H/D)
FOG	C2HC
GATA-1	C4
FYVE	C8 or C7H
PHD/LAP	C4HC3
PINCH	C2HC4H

* T = threonine, Thr; N = asparagine, Asn.

New zinc-dependent protein folds are constantly being discovered owing to technological advances and accumulation of data on solved protein structures. Among those are the zinc bundle, zinc ribbon, treble clef (found in LIM, GATA and nuclear hormone receptor families, and a cross-braced treble clef variation, found in RING, FYVE, protein kinase C and PHD families), TAZ motif (four α-helices stabilized by 3 zinc ions), and extended V-shape fold characteristic of the DnaJ of *E. coli*; a few of these folds are shown in Figure 4 (adapted from Laity *et al.*, 2001).

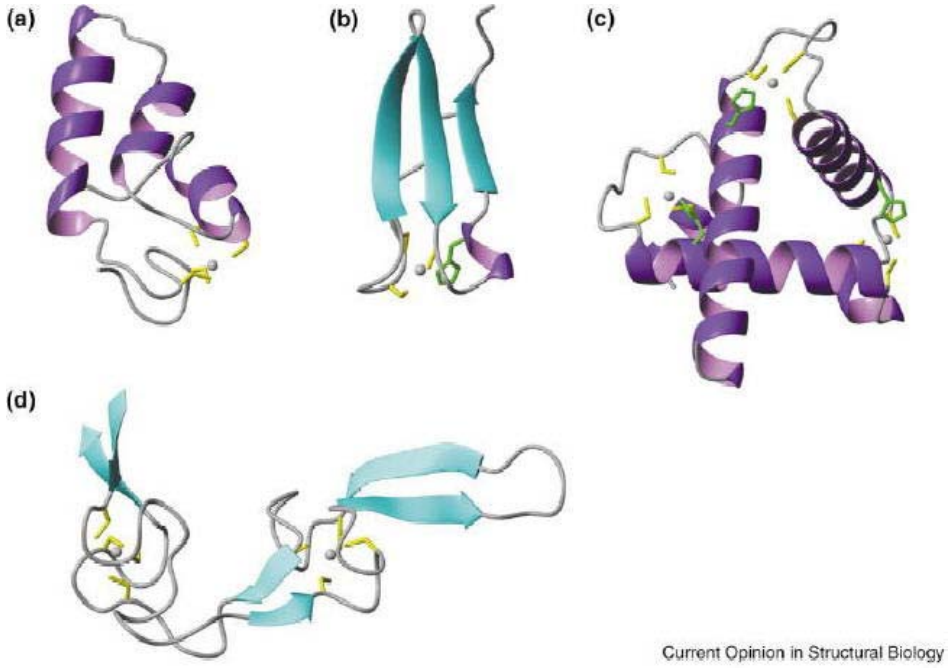


Figure 4. Ribbon diagrams of recently solved zinc finger structures (α helices and β strands are shown in magenta and cyan, respectively; Cys and His ligands are shown in yellow and green, respectively). **(A)** Zinc bundle of Rpb10 (PDB code 1EF4). **(B)** Zinc ribbon of L36 (PDB code 1DGE). **(C)** TAZ2 domain of CBP. **(D)** CR domain of DnaJ (PDB code 1EXK).

The so-called small Tim (translocase of the inner mitochondrial membrane) proteins also constitute one putative zinc finger family, of the simple C4 type. This protein family has been discovered quite recently, with not so many, yet some essential members (Sirrenberg *et al.,* 1998; Koehler *et al*., 1998a and 1998b; Adam *et al.*, 1999, Paschen *et al*., 2000). Relatively small proteins, with their molecular weights amounting to *ca.* 10 kDa, they reside and perform their biological functions in the mitochondrial intermembrane space. Homologues have been identified in mitochondria of organisms throughout the eukaryotic kingdom, but none in prokaryotes or other organelles with protein translocation machineries.

Peroxisomes harbour three proteins (Pex2, Pex10 and Pex12) involved in protein translocation that also contain a zinc-binding motif of the more complex RING type, known to be involved in protein scaffold organisation. In both small Tim and peroxisomal proteins zinc is presumbly involved in defining their final fold.

In the last decade, one especially peculiar group of zinc fingers has emerged. Its members are united by the ability to have their metal-coordinating cysteine residues oxidized with a functional purpose, rather than by simple similarity in their zinc-binding motifs. So far, the bacterial proteins Hsp33 and RsrA are the sole representatives of this group of proteins. These proteins use specific and reversible disulfide bond formation as a functional switch (Paget and Buttner, 2003). The conserved heat shock protein Hsp33 is a molecular chaperone

with a highly sophisticated mode of regulation. On the transcriptional level, its gene is under heat shock control, whereas on the posttranslational level Hsp33 protein stands under oxidative stress control. The redox sensor in Hsp33 is a four cysteine center that coordinates zinc under reducing, *i.e.* inactivating conditions and that forms two intramolecular disulfide bonds under oxidizing, *i.e.* activating conditions. As an oxidized dimer, Hsp33 is fully active in refolding proteins. Its activity appears to specifically protect proteins and cells from the otherwise deleterious effects of the oxidative stress. RsrA is another bacterial redox-sensitive, zinc-containing protein. It is a σ^R-specific anti-σ factor, comprising seven cysteines in its sequence. It binds the σ^R factor under reducing conditions, preventing it from activating transcription of its target genes. Disulfide stress induces formation of one disulfide bond in RsrA, causing it to release σ^R factor which then activates transcription of more than 30 genes and operons. One of the gene products reduces the oxidized RsrA, thereby restoring its σ^R-binding ability, and shutting off σ^R-dependent transcription, closing this physiological loop. By these means, cycling between reduced, zinc-bound and the oxidized states, featuring at least one disulfide bond, these zinc finger proteins are distinguished as key players in cellular responses to oxidative stress and in the overall thiol-disulfide redox balance.

1.3. Aims of the present study

The objective of this study was to establish the existence of homologues of *TIM9* and *TIM10* genes in *N. crassa*. This model system was then to be used to investigate the structural and functional features of *N. crassa* Tim9 and Tim10 proteins.

For the structural analyses to commence, specific requirements had to be met. Initial efforts were therefore directed towards isolation and purification of the *N. crassa* Tim9·Tim10 complex, in large quantities and of supreme purity. Certain properties of the purified Tim9·Tim10 complex that would constitute demands of any structural and for that matter also functional study attempt, had to be determined: its oligomeric state, potential zinc-binding ability, CD spectra, and functionality.

As to the functional characterization of the complex, this study aimed at elucidating two crucial aspects regarding the process of translocation of precursor proteins across the outer mitochondrial membrane mediated by the Tim9·Tim10 complex. In particular, those were: (i) the mechanism of substrate recognition by the Tim9·Tim10 complex, and (ii) the sufficiency of the Tim9·Tim10 complex and the TOM complex for the transfer of AAC precursor across the outer membrane. To answer the first of two questions, a screen of peptide libraries covering the primary sequences of putative substrates of the Tim9·Tim10 complex was performed. To resolve the second dilemma, the approach made use of a reconstituted system of the Tim9·Tim10 complex and the TOM complex.

2. Material and methods

2.1. Molecular biology methods

2.1.1. PCR (polymerase chain reaction)

DNA sequences were amplified through the polymerase chain reaction (PCR) using thermostable DNA polymerase, as described previously (Sambrook *et al*., 1989). DNA polymerases used were: *Taq* (isolated from *Thermus aquaticus*), and *Pfu* (isolated from *Pyrococcus furiosus*). Taq DNA polymerase has no proofreading ability, and therefore Pfu-polymerase was used when the PCR product needed to be used for subsequent cloning.

PCR mix (total volume of 100 µl) contained: 1-2 U DNA polymerase (*Taq*-polymerase and/or *Pfu*-polymerase), 10 µl 10x PCR-buffer (1% Triton X-100, 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris·HCl, pH 8.8), 2 µl [10 mM] dNTPs, 2 µl [50 µM] primers and 200 ng plasmid DNA template or 1 µg genomic DNA template.

The following program, with different modifications regarding primer annealing temperatures and length of elongation, was used:

1) 94ºC, 5 min,	nuclease inactivation and complete DNA denaturation;	
2) 30-40 cycles of:	DNA amplification:	
	94ºC, 1 min	DNA denaturation;
	45-65º C, 1 min	annealing of primers;
	72ºC, 1-6 min	new DNA synthesis (extension); duration of this step depends on the length of DNA fragment to be amplified; *Taq*-polymerase: 1 min/1 kb *Pfu*-polymerase: 2.5 min/1 kb;
3) 72ºC, 5-20 min	completion of the last reaction.	

Annealing temperature for primers was calculated by arithmetically adding the number of A and T nucleotides (in primer's sequence), multiplied by two, to the number of G and C nucleotides multiplied by 4 (and only for that part of primer which anneals with the template fully). Regions such as the restriction sites and possible Kozak sequences (Kozak, 1977 and 2003) contained in them were not taken into account, since they do not anneal. For a pair of

primers, temperature that is 5 degrees lower than the lowest calculated annealing temperature of the two primers was chosen. In some cases I also tested two additional temperature values (± 5ºC), to avoid occurance of possible non-specific PCR products.

2.1.2. DNA purification and analysis

2.1.2.1. Analytical and preparative gel electrophoresis

DNA fragments were separated according to their molecular weight through electrophoresis in horizontal 0.8-3% (w/v) agarose gels; the fragments ranged in size from 0.05 to 10 kb. Lower agarose percentage gels were used for separating larger DNA fragments, and higher agarose content gels for separating small DNA fragments. Agarose solutions were made by dissolving the desired amount of agarose in Tris-acetate-EDTA buffer (TAE), containing 1 mM EDTA and 40 mM Tris·acetate, pH 8.0, in a microwave oven. Ethidium bromide was added to a final concentration of 0.5 µg/ml (it allows visualization of DNA when the gel is exposed to UV light on a transilluminator). The agarose was stored at 65 ºC until use.

The samples were loaded onto gels in a loading buffer containing 6% (v/v) glycerol, 0.01% bromphenolblue and 0.01% xylencyanol. The electrophoresis was performed at RT in TAE buffer, with voltage set to U=60-70 mV. Commercially available molecular weight marker was used in each run.

DNA fragments to be further processed were excised from the gel with a clean scalpel and the DNA extracted from the gel using the "Gel extraction kit" protocol (Qiagen). Extracted DNA was routinely stored at –20ºC.

2.1.2.2. DNA concentration measurement

For DNA concentration measurements, the absorption of DNA solutions was measured at 260 nm. An OD of 1.0 corresponds to a concentration of 50 µg/ml of double stranded DNA, 33 µg/ml mono stranded DNA, 40 µg/ml RNA or 20 µg/ml oligonucleotides.

2.1.3. Cloning of DNA fragments

2.1.3.1. Enzymatic manipulation of DNA: restriction and ligation reactions

Digestion of DNA with restriction endonucleases

Plasmid DNA was digested with up to 5 U of specific restriction endonuclease enzyme for 1 µg of DNA. For preparative purposes up to 3 µg of DNA was digested in a 60 µl reaction volume, while for analytical ones, much smaller amounts in a 20 µl reaction volume were used. The buffer, incubation time (0.5-3 h) and temperature (usually 37ºC) of the reactions were chosen according to the manufacturer's recommendations. The obtained digested fragments were analyzed by agarose gel electrophoresis. For preparative purposes, desired DNA fragments were extracted from gels using Qiagen's "Gel extraction kit", and used for ligation reactions.

In those cases where plasmid DNA was cut with a single restriction enzyme, it was treated with calf intestinal alkaline phosphatase (CIP). This enzyme prevents vector's recircularization, through removal of its 5'-phosphate groups on linearised molecules. Digested vector DNA (10 µg for instance) was incubated in 100 µl reaction with 10 µl 10x CIP buffer (10 mM $ZnCl_2$, 10 mM $MgCl_2$, 100 mM spermidin, 0.5 M Tris·HCl, pH 9.0) and 0.1-0.5 units of alkaline phosphatase, for 30 min at 37ºC. The enzyme was inactivated through heating to 65ºC for 20 min in the presence of 5 mM EDTA, and separated from the DNA through agarose gel electrophoresis. DNA of interest was then extracted form the gel.

Ligation

Linearized DNA vector (50-200 ng) and a 5 fold molar excess of DNA fragment to be inserted, were incubated in a 10 µl reaction with 1 µl of 10x ligation buffer (10 mM $MgCl_2$, 5% (w/v) PEG-8000, 1 mM DTT, 1 mM ATP, 50 mM Tris·HCl, pH 7.6), and 0.5 µl (1 U) T4-DNA ligase (Gibco-BRL). Reactions were performed at 14ºC for 16 h and 0.5-1 µl of this mixture was used for *E. coli* cells transformation.

2.1.3.2. Preparation and transformation of *E. coli* competent cells

Preparation of competent cells

A small culture, usually 25 ml of LB^{amp}-medium, inoculated with a single colony of the corresponding *E. coli* strain (MH-1 or XL-1 Blue), was grown overnight at 37ºC under moderate shaking conditions. The following day, 1 l of liquid LB^{amp}-medium was inoculated

with the overnight culture. The bacterial cells were grown further until they reached the logarithmic growth phase (OD_{578} ~ 0.5). Then they were incubated on ice for 30 min, harvested by centrifugation (4,400 x g, 5 min, 4ºC) and washed sequentially with 400 ml, 200 ml, and 50 ml of 10% (v/v) glycerol. The competent cells were finally resuspended in 500 µl 10% (v/v) glycerol, aliquoted, and stored at –80ºC.

Transformation of competent cells through electroporation

To 40-60 µl of *E. coli* competent cells 0.5-1 µl of the ligation reaction mixture was added on ice. The cells were transferred to an ice-cold cuvette and the cuvette introduced into the electroporation Gene Pulser apparatus (BioRad) (settings: U=2.5 kV, R=400 Ω, C=25µF; time constant obtained τ was 7-8 ms). After a brief application of high electric voltage to the cells, the suspension was diluted with 1 ml of LB-medium, and incubated for 30-60 min at 37ºC under moderate shaking conditions (140 rpm), to allow cell recovery. The transformed cells were harvested by centrifugation (10,000 x g, 15 sec, RT) and resuspended in a small volume (up to 150 µl) of LB-medium. The cells were plated on LB-medium plates with ampicillin and incubated overnight at 37ºC.

2.1.4. *E. coli* strains used

Strain	*Genotype*	*Reference*
XL1-Blue	*supE*44, *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1, *lac*$^-$, F'[*proAB*$^+$, *lacI*^{q} *lacZ*ΔM15, Tn*10*(tet^r)]	commercially available from Stratagene
MH1	MC1061 derivative; araD139 lacX74 *galU galK hsr hsm*+ strA	Casadaban and Cohen, 1980

2.1.5. Small and large scale isolation of plasmid DNA from *E. coli*

Small scale preparation of plasmid DNA was performed according to a published procedure (Birnboim and Doly, 1979), through alkaline lysis. Small volume of LB-medium (2-5 ml) containing the appropriate antibiotic (ampicillin in majority of cases) was inoculated with a single bacterial colony picked out from a Petri dish, and incubated overnight at 37ºC, while shaking (140 rpm). The next day bacteria were harvested by centrifugation (8,000 x g, 30 sec, RT) and the resulting pellet resuspended in 300 µl of buffer E1 (10 mM EDTA, 50 mM Tris·HCl, pH 8.0) containing 100 mg/ml RNase. Cell lysis followed, through the addition of 300 µl of buffer E2 (0.2 M NaOH, 1% SDS). Samples were mixed by inverting the tubes 5

times and incubated 5 min at RT. Neutralization was accomplished by adding 300 µl of buffer E3 (3.1 M K-acetate, pH 5.5) and mixing the samples immediately afterwards, by inverting the tubes 5 times. They were then centrifuged (10,000 x g, 10 min, 2ºC), the supernatant transferred to new tubes and the DNA was precipitated through the addition of 600 µl of 96% isopropanol. Samples were then centrifuged again (10,000 x g, 40 min, 2ºC), washed with 85% cold ethanol, dried at RT, resuspended in 20-30 µl water and stored at –20ºC.

Large scale preparation of plasmid DNA (up to 0.5 mg) was performed using a "Jetstar" Midi-Kit (Genomed). LB-medium (50 ml) supplemented with ampicillin (or any other required antibiotic) was inoculated with bacteria carrying the plasmid to be isolated, and incubated overnight at 37ºC, while shaking at 140 rpm. Cells were harvested the next day by centrifugation (3,000 x g, 10 min, RT or 4ºC) and resuspended in 4 ml of buffer E1. Cell lysis was performed by adding 4 ml of buffer E2 and inverting the tubes 5 times; they were left for 5 min at RT. After neutralization by adding 4.4 ml of buffer E3, samples were centrifuged (17,418 x g, 10 min, 4ºC), and the supernatants immediately applied onto an anion-exchange column, previously equilibrated with 10 ml of buffer E4 (0.15% (v/v) Triton X-100, 0.6 M NaCl, 100 mM Na-acetate, pH 5.0). The column was washed with 20 ml of buffer E5 (0.8 M NaCl, 100 mM Na-acetate, pH 5.0) and the plasmid eluted into Corex tubes by adding 5 ml buffer E6 (1.25 M NaCl, 100 mM Tris·HCl, pH 8.5). DNA was precipitated through the addition of 3 ml of 96% isopropanol and one centrifugation step (12,000 x g, 30 min, 4ºC). It was then washed with 5 ml of 70% ethanol, re-centrifuged, and dried at RT. DNA was finally resuspended in up to 150 µl of ddH_2O, and the concentration was measured, before freezing it at –20ºC.

When a clone was propagated for the first time, 500 µl of the overnight culture was removed and added to 500 µl of sterilized solution of 50% LB medium mixed with 50% glycerol. It was then frozen at –80ºC, and stored as a glycerol stock for future propagation of the same clone.

2.1.6. Plasmids and genomic library clones used

Plasmid	*Reference*
pGEM4·*Nc*AAC	Endres *et al.,* 1999
pGEM4·AAC2	Lawson *et al.*, 1988
pGEM4·*Nc*Tim23	Mokranjac, PhD thesis
pGEM4·*Nc*Tim10	This thesis
pGEM4·*Nc*Tim10$_{his9}$	This thesis

pGEM4·*Nc*Tim9, clones 1, 3, and 5	This thesis
pGEM4·*Nc*Tim9$_{his9}$, clones 1, 3, and 5	This thesis
pGEM4·Su9(1-69)-DHFR	Gaume *et al.,* 1998
pMalcRI·*Nc*Tim10, clones M2, M4, X2, X5*	This thesis
pMalcRI·*Nc*Tim9, clones 3 and 31	This thesis
pCB1179·Pm·*tim10$_{his9}$*, clones 11, and 19	This thesis
pCB1179·Pm·*tim10$_{his9}$*·1kb, clones 3 and 8	This thesis
pQE30·*Nc*Tim10, clones M5, M6, X8, X9*	This thesis
Cosmid	*Reference*
pMOcosX#X20:A12	This thesis
pMOcosX#X25:B10	This thesis
pMOcosX#X12:C6	This thesis

* M=MH1; X=XL-1 bacterial clones

2.1.7. Cloning strategies

Constructs cloned for *in vitro* transcription and translation of mitochondrial preproteins comprised of cDNAs of relevant genes inserted into pGEM4 vector (Promega). Constructs for raising the antibodies consisted of cDNAs inserted into pMalcRI vector (NE Biolabs), creating maltose-binding protein (MBP) fusion proteins. Alternatively, cDNA was cloned into pQE30 vector (Qiagen), creating a his-tagged version of the gene of interest. For the expression of proteins in *N. crassa* wt background, genes encoding Tim9 and Tim10 proteins were cloned into pCB1179 vector. All plasmids were first transformed into *E. coli* XL-1 or MH1 strains for amplification and stock maintenance, and subsequently into *S. cerevisiae* or *N. crassa* cells.

pGEM4·*Nc*Tim10

The following primers were used:

N-terminal primer (containing a *BamHI* cutting site), called BamTIM10:

5'- AAT AAT GGA TCC ATG TTC GGA CTC GGC AGG -3',

C-terminal primer (containing a *SalI* cutting site), called TIM10Sal:

5'- AAT AAT GTC GAC TTA CAT GCC GAA GCC ACC -3'.

N. crassa cDNA(−) and cDNA(+) libraries (2.5 µl/50 µl PCR reaction) were used as templates. Three different annealing temperatures were tested till unspecific PCR products

were eliminated. (cDNA libraries marked plus and minus were obtained from *Neurospora* grown in the presence or absence of chloramphenicol, respectively).
The same primers were used to screen the genomic DNA library of *N. crassa* (1 µl of its 1:100 dilution/50 µl PCR reaction), and a 500 bp fragment contained in cosmids pMOcosX#X12:C6, X20:A12 and X25:B10 was identified.

pGEM4·*Nc*Tim10$_{his9}$
The following primers were used:
N-terminal primer, BamTIM10,
C-terminal primer (containing a *XbaI* cutting site), TIM10HisXba:
5'- TTT TTC TAG ATT AGT GAT GGT GAT GGT GGT GAT GGT GGT GCA TGC CGA AGC CAC CTC CAC C-3'.
N. crassa cDNA(−) and cDNA(+) libraries were used as templates.

pGEM4·*Nc*Tim9
Region homologous to that of *S. cerevisiae TIM9* gene was identified in a screen of *N. crassa* database, and primers for screening *N. crassa* cDNA and genomic libraries constructed. Positions of the starting methionine, as well as that of two introns were predicted, based upon the identification of the intron flanking sequences most commonly found in *N. crassa*, in the region corresponding to the *tim9 N. crassa* gene locus; these sequences are: G G T A^{77}/G A^{50}/C G T^{76}/C; C T A/G A C; A^{56}/T T^{52}/C A G G^{40} (numbers indicate the incidence with which the nucleotide is found in genes containing introns analyzed so far).

For screening the genomic *N. crassa* library, following primers were used:
N-terminal primer (containing an *EcoRI* cutting site), EcoMDGT9ge:
5'- CCG GAA TTC ATG GAT GGG TAA GCA AGA GAG-3',
C-terminal primer (containing a *HindIII* cutting site), ATTHindT9:
5'- TTC CCA AGC TTT TAC CGC CTC TGC ATC TCA GC -3'.
N. crassa genomic library (1 µl of its 1:100 dilution/50 µl PCR reaction) was used as a template.

For screening of the cDNA(−) and cDNA(+) libraries, following primers were used:
N-terminal primer (containing an *EcoRI* cutting site), EcoT9cDNA:
5'- CCG GAA TTC AAA TCG ACA ACA ATG GAT GGG -3',

C-terminal primer ATTHindT9.
N. crassa cDNA(−) and cDNA(+) libraries (2.5 µl/50 µl PCR reaction) were used as templates.

Upon comparison of sequenced cDNA (apprx. 300 bp) and genomic DNA (apprx. 800 bp) products of the PCR screens, the predictions of intron positions were verified.

pMalcRI·*Nc*Tim10
Same primers as for pGEM4·*Nc*Tim10 were used.

pMalcRI·*Nc*Tim9, clones 3 and 31
Same primers as for pGEM4·*Nc*Tim9 were used.

pCB1179·Pm·*tim10*$_{his9}$
The following primers were used:
N-terminal primer (containing an *EcoRI* cutting site), EcoTIM10P:
5'- TTT TGA ATT CCG CTC GGG CCG TTG TCT GC -3',
C-terminal primer, TIM10HisXba.
Cosmids pMOcosX#X20:A12, pMOcosX#X25:B10 and pMOcosX#X12:C6 were used as templates.

pCB1179·Pm·*tim10*$_{his9}$·1kb
The following primers were used to amplify the region 1 kb downstream from the *tim10* gene:
N-terminal primer (containing a *XbaI* cutting site), T10Xba1kb:
5'- TTT TCT AGA TTT TTT TGG ATT ACT GGA ACG G -3',
C-terminal primer (containing a *SacII* cutting site), T10Sac1kb:
5'- AAA CCG CGG CAG GAT CCA CAT ACC CGG -3'.
As templates cosmids pMOcosX#X20:A12, pMOcosX#X25:B10 and pMOcosX#X12:C6 were used. The resulting PCR product was inserted behind *tim10* promotor region and the *tim10* gene in the plasmid pCB1179·Pm·*tim10*$_{his9}$, using marked restriction sites.

pCB1179·P2·*tim10*$_{his9}$·1kb
To amplify a bigger promoter region of *tim10* together with *tim10* gene and to add a his-tag to it, the following primers were used:

N-terminal primer (containing an *EcoRI* cutting site), EcoTIM10P2:

5'- GGG AGT AGA TGA ATT CAT TAT TGC -3',

C-terminal primer, TIM10HisXba.

Cosmids pMOcosX#X20:A12, pMOcosX#X25:B10 and pMOcosX#X12:C6 were used as templates.

Resulting PCR products were cut with corresponding enzymes and exchanged against Pm·*tim10*$_{his9}$ fragment in pCB1179·Pm·*tim10*$_{his9}$·1kb construct.

pQE30·*Nc*Tim10

Same primers as for pGEM4·*Nc*Tim10 were used.

2.1.8. *S. cerevisiae* strains used

Strain	G*enotype*
D273-10B	ATCC 246557, *Mat α, Mal (rho+)*
W303-1A/-1B	*Mat a/α, ade2-1 ura3-1 his3-11 trp1-1 leu2-3 leu2-112 can1-100*; isogenic with RS 190 (ATCC 208354)
W334-a	*Mat a, leu2 ura3-52*
BY 4743	*Mat a/α, his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15/MET15Δ0 lys2Δ0/LYS2*
Δtom70	*tom70::KANmx3, Mat a/α, his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 ura3Δ0/ura3Δ0 met15/MET15Δ0 lys2Δ0/LYS2*
EJ11-6	*mrs11::HIS3 ade8 trp1 leu2 [pMRS11::URA3-CEN]*
tim10-1 (807 1B)	Koehler *et al.*, 1998
YPH501	*ade2-101 his3-Δ200 leu2-Δ1 ura3-52 trp1-Δ63 lys2-801*

2.1.9. Preparation of yeast DNA

Isolation of yeast DNA was performed as described previously by Rose *et al.,* 1990. YPD-medium (5 ml) was inoculated with *S. cerevisiae* cells and incubated overnight at 30ºC, while shaking (140 rpm). Cells were harvested by centrifugation, washed with 25 ml of water, and resuspended in 200 µl of breaking buffer (2% Triton-X100, 1% SDS, 100 mM NaCl, 1 mM EDTA, 10 mM Tris·HCl, pH 8.0). Phenol/chloroform/isoamyl alcohol (25:24:1) mix (200µl) and 0.3 g glass beads were added, and the samples vortexed for 2 min. The probes

were then centrifuged (36,670 x g, 5 min, RT) and the supernatant (the aqueous phase) transferred to new tubes. DNA was precipitated by adding 2.5 vol. of 100% ethanol. Samples were incubated for 10 min at –20°C, centrifuged (36,670 x g, 10 min, 2°C), and washed with 70% ethanol. Pellets were dried at RT, resuspended in 20 µl H_2O and stored at –20°C.

2.1.10. *N. crassa* strains used

Strain	*Description*	*Source*
74-OR23-1VA	wt	Fungal Genetic Stock Center #2489
TA2-1	contains $tim10_{his9}$	This thesis
TA2-14	contains $tim10_{his9}$	This thesis
TA2-14-$3^{1/2}$	contains $tim10_{his9}$	This thesis

2.1.11. Screening of *N. crassa* cosmid libraries

N. crassa genomic cosmid libraries screened in this study were prepared by Dejana Mokranjac (Mokranjac, PhD thesis, 2004). The cosmid library pMOcosX, screened for *N. crassa tim10* gene, comprises of 25 microtiter plates labeled pMOcosX#X1-25, each with 96 clones of the *Neurospora* genomic library (clones are labeled in a way standard for any microtiter plate, with the plate number preceeding the number of the clone; for example pMOcosX#X1:A1). Every microtiter plate has a corresponding 11 x 7 cm nylon membrane, created through a colony-hybridization method. Shortly, the colonies are lysed *in situ* upon replicating microtiter plates onto membranes, and the cellular debris washed off, leaving DNA bound to the mebranes (Dembowski, PhD thesis, 2001). Furhermore, all 96 clones from every plate are "pooled together" into 25 cultures, 25 midi-preps of DNA are made, and a single dot created for each of 25 plates on one 5 x 10 cm membrane. This method for creation of genomic libraries is referred to as the dot-blot method (Dembowski, PhD thesis, 2001). This particular membrane is the first membrane screened (later on referred to as the "primary" one), allowing identification of membranes corresponding to specific microtiterplates containing the clones of interest, which are to be screened in the second round.

In order to make a probe for screening a genomic library, PCR was performed using a PCR DIG (digoxigenin) Probe Synthesis Kit (Roche). Digoxigenin is a steroid, used to label probes in a PCR reaction. The labeled probe can then be easily detected with commercially available antibodies against digoxigenin. PCR reaction mixture (150 µl) contained: 110.25 µl ddH_2O, 15 µl 10x PCR buffer with $MgCl_2$, 15 µl 10x PCR DIG synthesis mix (dNTPs: dATP, dCTP, dGTP, 2mM each, and 1.3 mM dTTP and 0.7 mM DIG-11-dUTP), 3.75 µl [20 pM]

primer BamTIM10, 3.75 µl [20 pM] primer TIM10Sal, 2.25 µl enzyme mix and 1 µl genomic library as template. A control PCR with regular dNTP mix was performed as well. Conditions used were: initial denaturation: 94ºC for 5 min; 40 cycles of: 94ºC for 1 min, 60ºC for 1 min, 72ºC for 1 min; and the final elongation: 72ºC for 5 min. Expected PCR product size for genomic *tim10* clone is 500 bp, and with the label circa 600 bp. PCR product (1 µl) was run on a 2% agarose gel and the expected shift in size noted. The remaining 149 µl was heated to 94ºC for 5 min, and then cooled instantly by placing the probe in ice-cold water. It was then added to 35 ml (the volume is usually estimated based on the band intensity seen on 2% agarose gel) of standard hybridizing solution, containing 5x SSC (1x SSC: 150 mM NaCl, 15 mM Na-citrate, pH 7.0), 50% formamide, 0.1% Na-laurylsarcosin, 0.02% SDS and 2% blocking reagent.

The "primary" cosmid library membrane was preincubated for 2 h with the standard hybridizing solution at 42ºC, and then overnight at 42ºC with the generated probe, to allow for the hybridization between the digoxigenin-labeled probe and corresponding clones on the membrane to take place. The next day, solution with digoxigenin-labeled probe was poured off, and the membrane washed twice for 5 min in 2x SSC with 0.1% SDS solution at RT, and twice for 15 min with 0.1x SSC with 0.1% SDS solution at a higher temperature (circa 60ºC). Membrane was then incubated for 30 sec in P1 solution (150 mM NaCl, 0.3% Tween 20, 100 mM maleic acid, pH7.5) at RT, 30 min in 1x blocking solution (1% (w/v) blocking reagent in P1 buffer) at RT, and then left for 1 h at RT with αDIG-AP conjugate (an antibody against digoxigenin coupled to alkaline phosphatase, whose chemiluminescent substrate is disodium 3-(4-methoxyspiro (1,2-dioxetane-3,2'-(5'-chloro) tricycle [3,3.1.1$^{3.7}$]decan}-4-yl) phenyl phosphate (CSPD), diluted 1:10,000 in blocking solution. It was subsequently washed, twice for 15 min with P1 solution containing 0.03% Tween 20, and shortly twice with 10 ml of P3 buffer (100 mM NaCl, 100 mM Tris·HCl, pH 9.5). The membrane was placed between two sheets of plastic folia, excess P3 solution removed, 200 µl of the substrate CSPD, in 20 ml of solution P3 added, and the membrane incubated for 5 min at RT. Excess substrate was removed, the membrane sealed completely and incubated further for 10-15 min at 37ºC. Films were exposed for 1, 2, 3 and 4 hours.

To strip the membrane of the bound digoxigenin-labeled probe, it was shortly washed in ddH_2O, twice for 15 min in 0.2% NaOH, 1% (w/v) SDS at 37ºC, and 5 min in 2x SSC. The membrane was then dried and stored for future use in a sealed plastic bag, or subjected directly to further hybridization trials.

2.1.12. Southern blot

The agarose gel was incubated in 0.25 M HCl for 10 min to fragment the DNA through depurinization. It was further incubated twice for 30 min in solution Southern I (1.5 M NaCl, 0.5 M NaOH) to denature the DNA, and then washed twice for 30 min in Southern II solution (1.5 M NaCl, 1 M Tris·HCl, pH 7.4). Southern blot was assembled, according to Southern, 1975, using nylon membranes (Pall-Gelman). Transfer buffer used was 2x SSC. After 12 hours the membrane was washed in 6x SSC, dried and heated for 2 h at 120ºC.

2.1.13. Screening of clones through *in situ* colony-blotting

Clones were striken onto Petri dishes with appropriately supplemented LB medium, and grown overnight at 37ºC. The next day, circularly cut nitrocellulose membranes were gently pressed onto the Petri dishes, and after 2 min placed onto stacks of Whatmann 3MM paper soaked in different solutions. Membranes were soaked first for 10 min in 10 % (w/v) SDS solution, followed by 5 min in Southern I (denaturation), twice for 5 min in Southern II (neutralization) and twice for 15 min in 2x SSC solution. They were then washed twice in TBS, blocked in 5 % (w/v) skimmed milk in TBS solution and immunodecorated with appropriate antibodies.

2.2. Cell biology methods

2.2.1. *E. coli*: Media and culture

Media for E. Coli

LB-medium: 0.5% (w/v) yeast extract, 1% (w/v) Bacto-Tryptone, 1% (w/v) NaCl;

LB^{amp}-medium: LB-medium supplemented with 100 µg/ml of ampicillin.

Described media were used for preparing liquid cultures. To prepare plates with solid media, 2% (w/v) bacto-agar was added to the liquid culture solution. Bacto-agar, glucose and media were autoclaved separately (120ºC, 20-30 min), and the ampicillin was added after the media had been chilled to 50ºC.

2.2.2. *N. crassa*: Media and culture

2.2.2.1. Media and solutions for *N. crassa*

Trace elements solution: 50 g citric acid x H_2O, 50 g $ZnSO_4$ x 7 H_2O, 10 g $Fe(NH_4)_2SO_4$ x 6 H_2O, 2.5 g $CuSO_4$ x 5 H_2O, 0.5 g $MnSO_4$ x 1 H_2O, 0.5 g H_3BO_3 (water free), 0.5 g Na_2MoO_4 x 2 H_2O, dissolved in 1 l of H_2O. Chloroform (10 ml / 1 l) was added as a preservative, and the solution stored at RT.

Biotin solution: 100 ml ethanol, 100 ml H_2O, 20 mg biotin. It was stored at 4ºC.

50x Vogel's minimal medium (Vogel's salts; Vogel, 1964*)*: 150 g Na_3-citrate x 5 H_2O, 250 g KH_2PO_4 (anhyd.), 100 g NH_4NO_3, 10 g $MgSO_4$ x 7 H_2O, 5 g $CaCl_2$ x 2 H_2O, 5 ml trace elements-solution, 2.5 ml biotin solution, in 1 l of H_2O; 5 ml of chloroform was added as preservative, and the solution stored at RT.

Minimal medium: 2% (v/v) 50x Vogel's minimal medium, 2% (w/v) glucose.

Complete medium (Vogel's medium): 2% (v/v) 50x Vogel's minimal medium, 1% (w/v) sugar, 1% (w/v) glycerol, 0.2% (w/v) yeast extract, 0.1% (w/v) caseinhydrolysat.

Hygromycin stock solution: 50 mg/ml in water. It was stored at 4ºC.

Novozyme 234 stock solution: 5 mg/ml in 1 M sorbitol. It was filter sterilized and stored in 2 ml aliquots at −80ºC.

Solutions for the transformation – all were prepared fresh a day before use:

Polyethylene glycol-Tris-Calcium (PTC) solution in water: 40% (w/v) PEG 4000, 50 mM $CaCl_2$, 50 mM Tris·HCl, pH 8.0.

Sorbitol-Tris-Calcium (STC) solution: 1 M sorbitol, 50 mM $CaCl_2$, 50 mM Tris·HCl, pH 8.0.

pH was adjusted with 10 M NaOH to 8.0, prior to autoclaving. For both PTC and STC solutions, filter sterilised $CaCl_2$ was added to a final concentration of 50 mM after autoclaving, to prevent its precipitation.

10x FGS additive (per 500 ml, in water): 2.5 g (w/v) fructose, 2.5 g (w/v) glucose, 100 g (w/v) sorbose.

Heparin stock solution: freshly made 5 mg/ml in STC; it can also be stored at −80ºC.

Spermidin stock solution:

50 mM in water. It was stored at −20ºC.

Top (regeneration) agar: 10 ml (v/v) 50x Vogel's minimal medium, 91 g (w/v) sorbitol, 14 g (w/v) agar; the mixture was filled up with water to 450 ml, sterilized and 50 ml of prewarmed 10x FGS solution added. It was then split into 7-8 ml aliquots in 15 ml sterile Falcon tubes and stored at 4ºC. The prepared agar was prewarmed to 50ºC before use.

Bottom agar: 10 ml (v/v) 50x Vogel's minimal medium, 7.5 g (w/v) agar, 440 ml water. It was sterilised and cooled till 50-60ºC. Prior to pouring the bottom agar into Petri dishes (25 ml per standard plate), 50 ml of prewarmed 10x FGS additive and 2 ml of 50 mg/ml hygromycin stock solution (final concentration 200 μg/ml) were added. Hygromycin was added for the selection of strains generated in the scope of this thesis. Plates were left to dry and were stored at 4ºC.

Solutions for the microconidition:

Iodoacetic acid (IAA) stock solution: 208 mg IAA was dissolved in 10 ml of water to obtain a 100 mM stock solution. It was filter sterilized, and always used fresh.

Synthetic crossing (SC) medium (Westergard and Mitchell, 1947): 1 g (w/v) KNO_3, 0.7 g (w/v) K_2HPO_4, 0.5 g (w/v) KH_2PO_4, 0.5 g (w/v) $MgSO_4$ x 7 H_2O, 0.1 g (w/v) NaCl, 0.1 g (w/v) $CaCl_2$, 0.1 ml (v/v) trace element stock solution, 0.1 ml (v/v) biotin stock solution, brought to 1 l with water; pH was adjusted to 6.5 and the solution stored at 4ºC.

This medium was used for standard crosses, with various carbon sources, 1% (w/v) sucrose for instance.

For microconidiation slants, 2% (w/v) agar and 0.5% (w/v) sucrose were dissolved in 1:10 dilution of SC medium by heating - autoclaving for 20 min at 120ºC. When the solution was cooled to 50-60ºC, 1% (v/v) iodoacetic acid was added. Medium was distributed to glass tubes (16 x 150 mm), which were plugged with cotton, slanted and left to dry; they were stored up to 1 week at 4ºC.

Sorbose medium: 0.03 % (w/v) glucose, 2% (v/v) Vogel's minimal medium, 2% (w/v) agar, 1% (w/v) sorbose. Medium was autoclaved 15 min at 110ºC, cooled to 50-60ºC, and for TA2-14-3[2] strain hygromycin added to a final concentration of 150 μg/ml, prior to pouring plates.

SM buffer for isolation of N. crassa mitochondria: 250 mM sucrose, 10 mM MOPS·KOH, pH 7.2.

2.2.2.2. Growth of *N. crassa* hyphae

N. crassa hyphae were grown as described previously by Davis and Serres (1970).

N. crassa silica stocks preparation

Metal screw-capped tubes (13 x 50 mm) were filled with 4 ml of silica gel (Merck 7733), and the silica was activated and dry-sterilized through incubation for 3 h at 180ºC.

A culture of the strain of interest was grown for 5 days at 30ºC in 250 ml flask containing 40 ml of solidified medium. Skimmed milk (sterilized, 1% (w/v) in water, 10-20 ml) was poured carefully into the flask and the culture vortexed vigorously. The suspension of conidia was filtered through cotton-cloth funnel into sterile tubes and centrifuged shortly (10,000 x g, 15 sec, RT). Most of the supernatant was poured off, and the conidia were resuspended in the remaining small volume of supernatant. Using a 200 μl pipette, approximately 100 μl of air was taken in and then a 100 μl of conidial suspension. The tip was inserted into the sterile silica gel and the suspension slowly released into it, while stirring to disperse it more evenly into the gel. This procedure was repeated one more time. Tube's lid was loosely replaced and the silica stock dried in a desicator for 2-3 weeks at RT. Lid was then tightly closed and the stock placed at –20ºC.

Obtaining the conidia

Conidia were grown in 250 ml Erlenmeyer flasks with approximately 25 ml of solidified complete medium (Vogel's medium supplemented with 2% (w/v) agar). A few crystals of the silica gel-dried conidia, or conidia obtained from so-called primary cultures or A-flasks (inoculated with silica stocks of the appropriate strains), were inoculated in the centre of the flasks, and incubated for 6 and 3 days in the dark, respectively. This was followed by 3 days incubation at RT in daylight, to stimulate conidia formation. To collect conidia, 50 ml of sterile water was carefully poured into the Erlenmeyer, the suspension vortexed and after the airborne conidia in the flask had settled, the suspension poured into a new sterile flask. Concentration of the conidial suspension was determined by counting conidia using a haemocytometer under the light microscope. The conidia were stored at 4ºC (for 2-3 days maximally) before use.

Growth of hyphae

To isolate mitochondria from different *N. crassa* strains on a small scale, 1 l of liquid medium (containing 930 ml water, 20 ml 50x Vogel's minimal medium (Vogel, 1964) and 40 ml 1.4 M sucrose; all the components autoclaved separately) was inoculated with 10 ml of conidial suspension (10^8 conidia/ml). The culture was incubated for 15 h at 25ºC, aerated with sterile forced air and exposed to a visible and ultraviolet light source. The hyphae were collected by filtration.

To obtain large quantities of mycelia, 100 l cultures (containing 2 kg (w/v) sucrose and 2 l (v/v) 50x Vogel's minimal medium) were inoculated with 1 l conidia (10^8conidia/ml) and incubated for 24 h at 25ºC, under forced aeration and light and UV light exposure. The hyphae were collected and after wet weight determination, used for isolation of mitochondria.

Growth of hyphae in minimal medium with ^{35}S

For the isolation of *N. crassa* mitochondria containing radioactively labeled proteins, the cultures were grown with Vogel's salt solution, where $MgSO_4$ was exchanged with ^{35}S-labeled sulfate (1.56 mCi/l culture). Additionaly, 10 mg/l of unlabeled $MgSO_4$ was added to promote hyphal growth. Cultures (8 l) were grown in the isotop-laboratory, with the outcoming air being directed through two connected bottles filled with 1 M NaOH, in order to precipitate any radioactive sulfate present in this air. The subsequent growth and mitochondrial isolation were performed as described for non-radioactively labeled cultures.

2.2.2.3. Transformation of *N. crassa*

Preparation of spheroplasts

Supplemented Vogel's medium (150 ml) was inoculated with 1-2 x 10^9 conidia and incubated for 4-6 hours at 30ºC. The progress of germination was periodically examined, and when a level of 90% germination was achieved, germinated conidia were harvested in three 50 ml tubes via centrifugation (1,935 x g, 8 min, RT). Conidia were resuspended to a final volume of 50 ml (in water), and the centrifugation step was repeated twice. Final pellet was resuspended in 10 ml of 1 M sorbitol, transferred to a sterile 250 ml flask and 4 ml of 5 mg/ml Novozyme 234 (cell-wall-digesting preparation from *Trichoderma*) in 1 M sorbitol added. Suspension was then incubated for 30-60 min at 30ºC, while shaking (80-100 rpm). Degree of spheroplasting was examined after 30 min (over-digestion decreases their viability). This was done by placing 5 µl of the spheroplast suspension on a slide with cover slip. Water was added to one side and swelling and bursting of spheroplasts were observed under the microscope, which confirmed the loss of cell wall. After appropriate spheroplasting efficiency was ascertained, spheroplast suspension was gently poured over into a 50 ml conical tube, the flask rinsed with 1 M sorbitol which was added to the conical tube and the volume adjusted to 50 ml with the same sorbitol solution. Three centrifugation steps were performed, twice bringing the volume to 30 ml with 1 M sorbitol and once with STC solution (484 x g, 10 min, RT). Final pellet was resuspended in 7.5 ml of STC, 2 ml of PTC and 100 µl of DMSO, and mixed gently but thoroughly. Aliquots were made, and stored at –80ºC. *N. crassa* spheroplasts can be thawed and refrozen with no consequent lessening of their transformability.

Transformation of N. crassa – the classical method

An aliqout of spheroplasts was thawed and stored on ice, and 100 µl were added to a mixture of 1-5 µg of plasmid DNA, 2 µl spermidin and 5 µl heparin stock solution. After 30 min incubation on ice 1 ml of PTC solution was added, the suspension mixed carefully and incubated for 20 min at RT. The mixture was then transferred into pre-warmed top agar (equilibrated in a water bath at 54ºC), and immediately poured onto plates with bottom agar. Regeneration agar was left to solidify and the plates were then incubated at 30ºC for 2-3 days. Within first 24 h at 30ºC, some transformants had already grown a few hyphae; after two days they were visible to the naked eye, and within three days they were ready to be isolated with a spear-point needle into 13 x 100 mm culture tubes with 1-2 ml of minimal medium, supplemented appropriately (with hygromycin for instance). They were kept for three or more days at 30ºC in the incubator, subcultured once or twice, and then used for inoculation of 10 x

75 mm tubes containing complete medium, which were utilized for the generation of stocks (stored as conidial cultures at −20ºC). Typically, the efficiency of transformation was 10,000 transformants per μg of DNA used. In this way, the TA2-1 and TA2-14 strains were generated. To eliminate the non-transformed nuclei which accompany the transformed ones upon isolation, homokaryotic stable transformants were purified before use through a microconidiation method.

Microconidiation:

Selective induction of microconidiation in mycelia is used to obtain uninucleate microconidia for isolating homokaryotic derivatives. In present work it was employed to derive the TA2-14-3[2] strain, expressing equal amounts of the non-tagged and the tagged version of Tim10 protein. The procedure was done as described previously by Pandit and Maheshwari, 1993. The cultures were initiated with trace quantities of macroconidia placed in microconidiation slants, and kept for 7-12 days at 25 ºC in a room with day/night cycle. Macroconidia distinguish themselves from microconidia in size, and therefore a microscopic examination yields insight into microconidiation efficiency. Microconidia were harvested via addition of 2.5 ml sterile water to the microconidiation tubes, vortexing twice for 30-60 sec and filtration through a 5 μm filter (Millex®-SV, Durapor), which separated them from the macroconidia. Microconidia were counted by a haemocytometer and 150-200 of them plated on a Petri dish with sorbose medium. Plates were incubated at 34ºC for 2-3 days, and the new homokaryotic strains isolated by a spear-point needle. They were further grown in tubes with minimal medium, and later on in 10 x 75 mm tubes, as described previously for heterokaryotic transformants.

2.2.3. Isolation of mitochondria from *N. crassa* hyphae

The method was done as described by Sebald *et al.*, 1979. Hyphae grown as described above were collected from an overnight culture using a filter paper-covered funnel with a sieve connected to a vacuum pump. For every 10 g of wet weight hyphae 15 g of quartz sand and 20 ml of SMP (buffer SM supplemented with 1 mM PMSF) are added to the mortar and ground together for 1-2 min at 4ºC. The ground material is centrifuged twice (3,000 x g, 5 min, 4ºC) to get rid of cellular debrie, and the mitochondria sedimented (17,000 x g, 12 min, 4ºC). They were resuspended in 30 ml of SM buffer, sedimented again, resuspended in 0.5 ml of SM buffer and the protein concentration determined. The mitochondria were used within 1 h of preparation, as freezing them ruptures their outer membrane.

For purposes of Tim9·Tim10 complex purifications, large scale preparation of mitochondria were performed. Basically, the same protocol was followed, with some small modifications. Hyphae from a 100 l culture (1-2 kg) were mixed with SMP buffer (2 l for every 0.5 kg hyphae) and quartz sand (0.75 kg sand for every 0.5 kg hyphae), homogenized for 2 min in a blender, and passed through a mill. The mitochondria were isolated from the homogenate by differential centrifugation as above, but instead of two times, they were centrifugated just once at 17,700 x g, for 50 min. After the last centrifugation step mitochondria were resuspended in up to 150 ml of buffer containing 300 mM NaCl, 20 mM imidazole, 10 % glycerol, 50 mM MOPS·NaOH, pH 8.0, the protein concentration determined and 50 ml aliquots frozen at –20 or –80ºC .

2.2.4. Crude isolation of mitochondria from *N. crassa* ("mini" prep)

Hyphae from 18 ml overnight cultures were collected as described above and put into 2 ml tubes. SMP buffer (1 ml) and some quartz sand were added. Probes were subjected to homogenization in a Ribolyser (Hybaid) device, for 45 sec, setting 6.5, and subsequently centrifuged (1,000 x g, 10 min, 4ºC). Supernatants were transferred into new 1.5 ml tubes and recentrifuged. From supernatants of this centrifugation step, mitochondria were isolated through centrifugation (16,000 x g, 10 min, 4ºC). The pelleted mitochondria were dissolved in a small volume of SMP buffer and analysed further.

An alternative way was to finely grind collected mycelia (see above), in liquid nitrogen, with a small amount of quartz sand in a mortar. A certain amount of homogenized cells was transferred into two 2 ml tubes (to the 1 ml mark), which were then immersed in liquid nitrogen. SMP buffer (1 ml) was added to each probe and the samples shortly vortexed at 4ºC. A 10 min centrifugation step followed (1,000 x g). Supernatants were transferred into new tubes and mitochondria pelleted (10 min, 16,000 x g, 4ºC). Pellets were resuspended in 100 µl SM buffer and protein concentration determined. Probes were then analysed further.

2.2.5. *S. cerevisiae*: Culture and Media

2.2.5.1. Media for S.cerevisiae

YP-medium: 1% yeast extract, 2% peptone, pH 5.0 (adjusted with HCl);

YPD-medium: YP-medium supplemented with 2% glucose;

YPG-medium: YP-medium supplemented with 3% glycerol;

YPGal-medium: YP-medium supplemented with 2% galactose.

SC medium: 0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulphate, 1.5 g/l "drop-out mix" powder (mix containing equal weight of all amino acids; for selecting one auxothophic marker, the corresponding amino acid was left out), 2% glucose or 2% galactose or 3% glycerol as carbon source.

The described media were used for preparing liquid cultures. To prepare plates with solid media, 2% w/v bacto-agar was added. Bacto-agar, glucose, and media were autoclaved separately.

2.2.5.2. *S. cerevisiae* growth

S. cerevisiae growth was performed as described in Sambrook *et al*., (1989) in YPD complete medium or, when a selection on the auxotrophic marker was necessary, on SD-medium. The cells were incubated at 30ºC, under shaking conditions.

2.2.5.3. Transformation of *S. cerevisiae* (lithium acetate method)

The corresponding yeast strain was grown overnight in YPD-medium and diluted the next morning in 50 ml medium, to an OD_{578} of 0.2. Cells were grown further, till they reached an OD_{578} of 0.8. They were then transferred to a sterile centrifuge tube, and harvested by centrifugation (1,000 x g, 3 min, RT). After washing with 25 ml of sterile water, cells were harvested under the same conditions, resuspended in 1 ml 100 mM lithium acetate and transferred to an Eppendorf tube. Sample was centrifuged again (7,500 x g, 15 sec, RT) and the cells were resuspended in 400 µl 100 mM lithium acetate. For each transformation 50 µl

of the cell suspension was centrifuged (7,500 x g, 5 min, RT) and the supernatant removed. The following mixture was added to the cells in this particular order: 240 µl PEG 3350 (50% v/v), 36 µl 1 M lithium acetate, 5 µl single stranded salmon sperm DNA (10 mg/ml; previously incubated for 5 min at 95ºC), 70 µl H_2O containing 0.1-10 µg of DNA to be transformed. The mixture was vortexed for 1 min and incubated for 30 min at 30ºC, with moderate shaking (350-500 rpm), followed by another 20-25 min at 42ºC.

The cells were harvested by centrifugation (6,000-8,000 x g, 15 sec, RT), resuspended in a small volume of sterile water (150 µl), and spread on plates with the appropriate selective media. The plates were incubated for 2-4 days at 30ºC to recover transformants.

2.2.6. Dilution assay

To determine the growth characteristics of yeast strains, a dilutions assay was performed. The strains were grown in liquid culture to the logarithmic phase. Equal amounts of cells (0.5 OD_{578} units) from every culture were isolated, resuspended in 500 µl H_2O, and a series of 1:10 dilutions were made; 5 µl from each dilution was spotted on agarose plates. The results could be seen after 2-4 days incubation at the appropriate temperature.

2.2.7. Isolation of mitochondria from *S. cerevisiae*

Mitochondria were isolated from *S. cerevisiae* cultures as described by Herrmann *et al*., 1994. Yeast cells were grown to OD_{578} of 0.8-1.5, harvested by centrifugation (3,000 x g, 5 min, RT), washed with water and resuspended in a buffer containing 10 mM dithiotreitol (DTT), 100 mM Tris, pH unadjusted, to a concentration of 0.5 g/ml (2 ml of buffer is added for every gram of cell wet weight). Cell suspension was incubated for 15 min at 30ºC with moderate shaking, followed by the repeated centrifugation step and resuspended in 100 ml of 1.2 M sorbitol. To digest the cell wall and obtain spheroplasts, the cells were resuspended to a concentration of 0.15 g/ml in zymolyase buffer (1.2 M sorbitol, 20 mM KH_2PO_4·KOH, pH 7.4) and incubated with 4 mg zymolyase/g wet weight, for 30-60 min at 30ºC, under moderate shaking conditions.

The efficiency of cell wall digestion (spheroplasts generation) was tested after the first 30 minutes: 50 µl of cell suspension was diluted with 2 ml of water or with 2 ml of 1.2 M sorbitol. Formation of spheroplasts was complete when the OD of the water dilution amounted to 10-20% of the OD of the sorbitol dilution (the solution of spheroplasts in pure

water is clear, because spheroplasts burst under these conditions). All subsequent steps were performed at 4ºC.

The spheroplasts were isolated by centrifugation (3,000 x g, 5 min, 4ºC), resuspended (0.15 g/ml) in buffer for homogenization (0.6 M sorbitol, 1 mM EDTA, 0.2% (w/v) BSA, 1 mM PMSF, 10 mM Tris·HCl, pH 7.4), and dounced 10 times in a cooled douncer (homogenisor) on ice. Cell debris and intact cells were sedimented by a centrifugation step performed twice (2,000 x g, 5 min, 4ºC). The supernatant was centrifuged (17,400 x g, 12 min, 4ºC) to pellet down mitochondria. Sedimented mitochondria were resuspended in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS·KOH, pH 7.4), and separated from cell remnants through two centrifugation steps (2,000 x g, 5 min, 4ºC). Finally, mitochondria were resuspended in a small volume of the SEM buffer to a concentration of 10 mg/ml protein, aliquoted, frozen in liquid nitrogen, and stored at – 80ºC till use.

2.2.8. Isolation of crude mitochondria from *S. cerevisiae*

Cells corresponding to 20 OD units were harvested by centrifugation (3,000 x g, 5 min, RT) and washed with water. The cells were resuspended in 300 µl SHK^{80} (SH buffer with 80 mM KCl) or $SMNa^{100}$ buffer (SM buffer containing 100 mM NaCl) with 1 mM PMSF, and 0.3 g glass beads (diameter 0.3 mm) were added. The samples were vortexed four times 30 sec each, with 30 sec breaks in between (during this break the samples were incubated on ice). After centrifugation (1,000 x g, 3 min, 4ºC), the supernatant was transferred to a new tube and the protein concentration measured. The desired amount of protein (25-150 µg) was pelleted by centrifugation (10,000 x g, 10 min, 4ºC). The crude mitochondrial pellets were resuspended in 25 µl 2x sample buffer, shaken for 10 min at RT, and analyzed by SDS-PAGE.

2.3. Biochemical methods

2.3.1. Electrophoretic methods

2.3.1.1. SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

The proteins were separated under denaturing conditions via one-dimensional vertical slab SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described (Laemmli, 1970). The concentrations of acrylamide and bis-acrylamide in the separating gel were chosen

considering the molecular size of proteins to be separated. Glass plates of 160 x 140 mm and spacers of 1 mm thickness were used. The samples were dissolved in 25-60 µl sample buffer and if required, incubated at 95ºC for 3 min before loading. The electrophoresis was performed at 25-30 mA for 2.5 h for large gels and at 150 V for 1 h for minigels (BioRad). Protein molecular weight standards that contain protein bands at 116, 66, 45, 35, 25, 18 and 14 kDa (Peqlab), and at 17, 14, 10, 8, 6 and 2.5 kDa (Pharmacia) were used.

Buffers for SDS-PAGE:

Stacking gel: 5% (w/v) acrylamide, 0.03% (w/v) bis-acrylamide, 0.05% (w/v) APS, 0.25% (v/v) TEMED, 60 mM Tris·HCl, pH 6.8.

Separating gel: 10-16% (w/v) acrylamide, 0.07-0.3% (w/v) bis-acrylamide, 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.05% (v/v) TEMED, 385 mM Tris·HCl, pH 8.8.

Bottom gel: 2% (w/v) agar in running buffer.

Sample buffer: 2% (w/v) SDS, 10% glycerol, 0.02% (w/v) bromphenolblue, 5% β-mercaptoethanol, 60 mM Tris·HCl, pH 6.8.

Running buffer: 384 mM glycine, 0.1% (w/v) SDS, 50 mM Tris·HCl, pH 8.3.

2.3.1.2. High Tris-urea SDS-Polyacrylamide gel electrophoresis

The electrophoresis was performed at 35 mA, for 2 h 40 min.

Buffers for high tris-urea SDS-PAGE:

Stacking gel: 5% (w/v) acrylamide, 0.07% (w/v) bisacrylamide, 6 M urea, 0.1% (w/v) SDS, 0.5% (w/v) APS, 0.25% (v/v) TEMED, 125 mM Tris·HCl, pH 6.8.

Separating gel: 19% (w/v) acrylamide, 0.25% (v/v) bisacrylamide, 6 M urea, 0.1% (w/v) SDS, 0.05% (w/v) APS, 0.25% (v/v) TEMED, 0.75 M Tris·HCl, pH 8.8.

Bottom gel: as for regular SDS-PAGE

Sample buffer: as for regular SDS-PAGE

Running buffer: 50 mM Tris base, 200 mM glycin, 0.1 % SDS, pH 8.0.

2.3.1.3. Blue-Native gel electrophoresis (BNGE)

Blue-Native gel electrophoresis (BNGE) was used for the separation of proteins under non-denaturing (native) conditions as described (Schägger, 1991). Mitochondria were

solubilized at 4ºC in a desired buffer (20 µl) containing the appropriate amount of detergent, for up to 30 min in the overhead shaker. Most frequently used solubilization buffers contained 50 mM NaCl, 1 mM PMSF, 50 mM Na-phosphate·HCl, pH 8.0, with either 0.1-1% digitonin, 0.5-1% Triton X-100 or other detergent. A clarifying spin (20 min, 90.700 x g, 2ºC) followed. Glycerol was added to the protein samples to a final concentration of 10% (v/v), 3 µl (v/v) of 10x loading dye was added, and volume of all samples adjusted to 30 µl. They were then loaded onto 6%-13% or 6%-16.5% acrylamide gradient gels.

Buffers for BNGE:

Acrylamide 48%/Bis-acrylamide 1.5%

Acrylamide 30%/Bis-acrylamide 0.2%

Gel buffer: 1,5 M 6-amino-n-caproic acid, 0,15 M bis Tris·HCl, pH 7.0.

Sample buffer: 0.5% Coomassie-Brilliant-blue G250, 50 mM 6-amino-n-caproic acid, 10 mM bis-Tris·HCl, pH 7.0.

Cathode-Buffer: 50 mM Tricine, 15 mM bis-Tris, pH 7.0, with or without 0.02% Coomassie-Brilliant-blue G250.

Anode-Buffer: 50 mM bis-Tris·HCl, pH 7.0.

Gel was prepared and used the same day. Samples were usually neither heated (unless experiment requires otherwise), nor frozen. Electrophoresis was performed at 4ºC. In the first 1 h of electrophoresis, the gel was run with cathode buffer containing the Coomassie blue, voltage set to 100 V and amperage to 15 mA. Voltage was then increased to 500 V, and after the blue front had migrated to two thirds of the separation distance, the cathode buffer was replaced by the cathode buffer without the Coomassie-Brilliant-blue. Marker proteins used are thyreoglobulin (660 kDa), apoferitin (440 kDa), bovine serum albumine (BSA, monomer: 66 kDa, dimer: 132 kDa, trimer: 198 kDa) and alcohol dehydrogenase (ADH, monomer: 50 kDa, dimer: 100 kDa, trimer: 150 kDa).

2.3.1.4. 2D Blue-Native gel electrophoresis (BNGE)

The first dimension separation by BNGE was performed, protein lanes were cut out using a scalpel, and incubated twice for 5 minutes in 5 ml of sample buffer with β-mercaptoethanol. Excess solution was then soaked using Whatman filter paper and the cut lanes positioned onto glass plates (horizontally, in the area meant for the stacking gel) to be

assembled for running SDS-Gel electrophoresis of the second dimension. Upon plates assembly, bottom gel was poured, and shortly afterwards the separating one as well. Before pouring the stacking gel, spacers were inserted on both sides of the lane to be subjected to the second dimension run, one to load mitochondria proteins dissolved in sample buffer, second one for the marker proteins. The stacking gel was, however, prepared with two fold the amount of APS and three fold the amount of TEMED used usually. Further separation of proteins is as for the regular SDS-PAGE.

2.3.1.5. Coomassie blue staining of SDS gels

After SDS-PAGE, and removal of the bottom and stacking gels, separating gel was stained at RT for 30 min (or longer), with a solution containing 30% (v/v) methanol, 10% (v/v) acetic acid, and 0.1 (w/v) Coomassie-Brilliant-blue R250. The gel was then destained with destaining solution (30% (v/v) methanol, 10% (v/v) acetic acid) until the protein bands appeared against a clear background. The protein bands-background contrast was enhanced through post-destain 15 minutes incubation with 7% (v/v) acetic acid solution (RT). This step was followed by further 10-15 minutes incubation in 50% (v/v) methanol solution in water. The gel was dried overnight between two gel-drying-films (Promega) or placed onto two sheets of Whatman paper, covered with plastic wrap and dried for 1-2 h in a gel dryer at 80ºC.

2.3.1.6. Silver staining of SDS gels

Silver staining of gels was performed according to the published procedure (Bloom *et al*., 1987). Separating gel was incubated for 30-60 min in a fixation solution containing 50% (v/v) methanol, 12% (v/v) acetic acid, and 50 µl 37% HCHO per 100 ml. It was then washed twice for 10 min with 50% (v/v) ethanol, once for 1 min in 0.02% (w/v) $Na_2S_2O_3$ x $5H_2O$ and three times for 20 sec with water. The gel was then incubated for 15 min in 0.2% (w/v) $AgNO_3$ with 75 µl of 37% HCHO per 100 ml, and twice for 20 sec with water. It was finally developed with a solution containing 6% (w/v) Na_2CO_3, 2 ml 0.02% (w/v) $Na_2S_2O_3$ x $5H_2O$ and 50 µl 37% HCHO, for 1 to 15 minutes, depending on the desired intensity of staining. Subsequently, it was washed with water (20 sec), incubated for 15 min in 50 mM EDTA, washed once again with water and dried. The whole procedure was performed at RT, and solutions containing formaldehyde and silver-nitrate prepared fresh (the latter was kept in dark until use).

2.3.1.7. Transfer of proteins to nitrocellulose/PVDF membrane (Western-Blot)

Proteins separated by SDS-PAGE were transferred onto nitrocellulose membranes using a modified semi-dry method (Towbin, 1979; Kyhse-Anderson, 1984), whereas those analysed by BNGE to PVDF membranes.

PVDF membrane was activated before blotting through short incubation (up to 5 min) in methanol. It was then thoroughly washed, first with water, and then 5 min (or longer) with transfer buffer. Prior to blotting, separating part of the blue native gel, freed from the stacking and bottom gel parts, was equilibrated in blotting (transfer) buffer (20 mM Tris base (pH unadjusted), 150 mM glycine, 20% (v/v) methanol, 0.02% (w/v) SDS), by shaking for 15 minutes at RT or 4ºC. The nitrocellulose membrane, on the other hand, was incubated for three minutes in water and subsequently in transfer buffer prior to the blotting procedure. A respective membrane was placed onto transfer-buffer soaked three sheets of Whatman 3MM filter paper lying on the graphite anode electrode, the gel placed on top. It was then covered with another three soaked filter papers, and finally with the cathode graphite electrode. The transfer was performed at 1.5 mA/cm^2 for 1.5 h (for our gels of dimensions of apprx. 14 cm x 9 cm, it translates to 200 mA for 1 h 30 min). Electrotransfer onto PVDF membranes was generally performed at 220 mA for 1 h, and at 4ºC.

To verify transfer efficiency, and to visualize and label the marker proteins' bands, the nitrocellulose membranes were reversibly stained with Ponceau S solution (0.2% (w/v) Ponceau S in 3% (w/v) TCA), and the PVDF membranes with Coomassie solution. The PVDF membrane was then destained with destaining solution or simply with methanol, which was then removed by excessive washing with TBS buffer (150 mM NaCl, 10 mM Tris·HCl, pH 7.5). The membranes were then immunodecorated, or the radioactive material visualized by autoradiography.

2.3.2. Protein concentration determination

Protein concentrations were determined by the Bradford assay (Bradford, 1976). Protein solutions (1-10 µl) were diluted with 1 ml of 1:5 dilution of commercially available BioRad Bradford reagent and incubated for 10 min at RT. The absorbance was measured at 595 nm using a 1 cm-path length microcuvette. Protein concentration was calculated

according to the standard curve, obtained using the commercially available IgG proteins (BioRad).

2.3.3. Protein quantification by autoradiography and phosphorimaging

Dry nitrocellulose or PVDF membranes, with radiolabeled proteins blotted onto them, were exposed to Röntgen films (autoradiography). The films were scanned and the intensity of bands of interest quantified (densitometry). Alternatively, the membranes were exposed to phosphor imaging plates and the intensity of the bands was determined using our Phosphorimager (Fuji BAS 1500, TINA 2.0 software).

2.3.4. Synthesis of radioactively labelled proteins *in vitro*

Transcription/translation in a cell-free system

For *in vitro* synthesis of ^{35}S labelled proteins, constructs cloned into pGEM4 (Promega) plasmids first have to be transcribed into mRNA using SP6-RNA-polymerase (Melton, 1984; Sambrook, 1989). Transcription mixture (100 µl) contained: 20 µl 5x transcription buffer (200 mM Tris·HCl, 50 mM $MgCl_2$, 10 mM spermidine, pH 7.5), 10 µl 0.1 M DTT, 4 µl RNasin (40 U/µl), 20 µl 2.5 mM rNTP, 5.2 µl 2.5 mM m7G(5')ppp(5')G and 10-20 µg DNA. After addition of 3 µl of SP6-Polymerase (25 U/ml) the reaction mixture was incubated for 1 h at 37ºC. The RNA was precipitated by adding 10 µl of 10 M LiCl and 300 µl of absolute ethanol, centrifuged, and subsequently washed with 70% ethanol. After ethanol had evaporated, RNA was resuspended in H_2O (supplemented with 1 µl of RNasin), aliquoted and kept at –80ºC.

Translation was performed the following way: a mix containing 25 µl RNA, 3.5 µl amino acid mix (without methionine), 7 µl 15 mM Mg-acetate, 12 µl ^{35}S (10 mCi/ml) and 100 µl rabbit reticulocyte lysate (Promega) was incubated for 1 h at 30ºC. Rection was made 5 mM cold methionine and 250 mM sucrose, the probe was then centrifuged (90,700 x g, 1 h, 2ºC) to pellet down ribosomes, and 12 µl aliquots of the supernatant were frozen at –80ºC.

TNT coupled reticulocyte lysate system

This system combines transcription and translation in the same reaction mixture. TNT mix (50µl) contained: 25µl TNT rabbit reticulocyte lysate (Promega), 2 µl TNT reaction buffer, 1 µl TNT SP6 RNA polymerase, 1 µl amino acid mix without methionine, 2 µl ^{35}S

methionine (10 mCi/ml), 1 µl RNasin ribonuclease inhibitor (40 U/µl) and 2 µl DNA template (0.5 µg/µl). The reaction was incubated for 90 min at 30ºC, and further treatment was as described for the translation reaction alone.

2.3.5. Import of preproteins into isolated mitochondria

Import of radiolabelled precursor proteins into *N. crassa* and *S. cerevisiae* mitochondria was performed in either F5 import buffer (0.03-0.25% (w/v) BSA, 250 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 2.5 mM NADH, 10 mM MOPS·KOH, pH 7.2) or SI buffer (0.03% BSA (w/v), 0.6 M sorbitol, 80 mM KCl, 10 mM Mg-acetate, 2 mM K-phosphate, 2.5 mM $MnCl_2$, 2 mM ATP, 2.5 mM NADH, 50 mM HEPES·KOH pH 7.2), respectively. EDTA was not included in the import buffers, due to the potential zinc-binding capacity of the Tim9 and Tim10 proteins.

Import reactions were performed at various temperatures and time periods. In some cases the mitochondria and mitoplasts were protease treated before or after the import reaction. For removal of import receptors cytosolic domains before performing the import reaction, trypsin was used at a final concentration of 20 µg/ml, and the incubation was performed for 10 min on ice. It was then inhibited through the addition of 20x weight excess of soybean trypsin inhibitor, and the import performed as usual without further changes. Protease treatment after the import reaction was performed by incubation with proteinase K (PK) or trypsin for 15 min on ice. For mitochondrial protein concentrations of 1 and 0.1 mg/ml, final protease concentrations of 250 µg/ml and 50 µg/ml, respectively, were used. Proteinase K was inhibited through the addition of PMSF (1 mM), and trypsin was inhibited as desribed above. At the end of import reactions mitochondria were sedimented (12,000 x g, 10 min, 4ºC) and TCA precipitated or directly solubilized in sample buffer. Imported and bound proteins were analyzed by SDS-PAGE, autoradiography and phosphor imaging (Fuji BAS 1500, TINA software).

In some import reactions, the radiolabelled precursor proteins were preincubated with chemical amounts of Tim9·Tim10 complex for 10 min on ice.

To dissipate mitochondrial membrane potential prior to the import reaction, several different reagents were used: either 1 µM valinomycin, or 20 µM oligomycin, or 8 µM antimycin, or 1-50 µM CCCP, or a mixture of the first three reagents, termed the AVO mix, was used. All these reagents were made as 100x stock solutions in ethanol, which were kept at –20ºC.

For ATP depletion, a separate 10 min (RT) pre-incubation of both mitochondria and lysate with 25 U/ml of apyrase was applied. An ATP-regenerating system included: creatin kinase (final concentration 1 mg/ml) and creatin phosphate (final concentration 10 mM); sometimes, succinate and malate (both at 2.5 mM end concentration) were added, too.

2.3.6. Generation of mitoplasts ("swelling")

Mitochondria resuspended in SI buffer were diluted 10 times with buffer containing 1 mM ATP (empirically shown to increase the swelling efficiency), 20 mM HEPES·KOH, pH 7.2 and incubated on ice for 30 min. The mitoplasts were pelleted (17,400 x g, 10 min, 2ºC), and analysed by SDS-PAGE. To estimate the efficiency of outer membrane disruption, immunodecorations with antibodies against soluble proteins of the intermembrane space were performed. It is important to note that some intermembrane space proteins are associated with the inner membrane and can be extracted only by adding salt.

2.3.7. Trichloroacetic acid (TCA) precipitation of proteins

Proteins from aqueous solutions were precipitated by adding TCA to a final concentration of 12%. The samples were incubated for 30 min on ice or at –20ºC, and then centrifuged (30,000 x g, 20 min, 2ºC). The precipitated proteins were washed with acetone (kept at –20ºC), and re-centrifuged (30,000 x g, 10 min, 2ºC). Protein pellet was shortly dried at RT and dissolved in sample buffer.

2.3.8. Ammonium sulphate precipitation of proteins

Aqueous protein solutions were mixed at 4ºC with 2 volumes of saturated solution of ammonium sulphate, and incubated on ice for 30 min. This was followed by centrifugation (30,000 x g, 10 min, 4ºC). The supernatants were discarded, and the pellets containing precipitated proteins dissolved in the desired buffer. To denature proteins, a buffer containing 7 M urea, 50 mM TCEP, 30 mM Tris·HCl, pH 7.5 was used.

Saturated ammonium sulphate solution was prepared by dissolving 76.7 g of ammonium sulphate in 100 mM Tris·HCl pH 7.0 and chilling it to 4ºC, to allow ammonium sulphate crystals to sediment.

2.3.9. Carbonate extraction

Carbonate extraction (Fujiki *et al*., 1982) was used to investigate whether a protein is soluble or inserted into a membrane. For this purpose, pelleted mitochondria were usually resuspended in 50 µl of SH (0.6 M sorbitol, 20 mM HEPES·KOH pH 7.2) or any other appropriate buffer, and the same volume of 0.2 M Na_2CO_3 (pH 11.5) was added. Alternatively, pelleted mitochondria were resuspended directly in 100 µl of 0.1 M Na_2CO_3 (pH 11.5). The alkaline solution was incubated for 30 min on ice. That was followed by a centrifugation step (183,254 x g, 30 min, 2ºC). Supernatant containing soluble proteins was TCA precipitated, whereas the carbonate extraction pellet containing integral membrane proteins was resuspended directly in sample buffer for electrophoresis. The samples were then analyzed by SDS-PAGE.

2.3.10.Expression and purification of proteins

2.3.10.1 Purification of recombinant proteins expressed in *E. coli*

Purification of recombinant maltose binding protein (MBP, MW=42 kDa) fusion proteins MBP-Tim9 and MBP-Tim10 out of *E. coli* was done as described before (Guan *et al.*, 1987). The MH1 or XL-1 Blue *E. coli* colony containing the MBP-fusion protein cloned into pMalcRI vector was innoculated for overnight growth (37ºC, 140 rpm) in up to 20 ml of liquid LB^{amp}-medium. The next day, 5 ml of the overnight culture was diluted into 250 ml of LB^{amp}-medium. This culture was then further incubated till it reached an OD_{600} of 0.5. At this stage, 1-2 ml were removed from the culture (uninduced-cells control), the cells were pelleted (10.000 x g, 15 sec, RT) and resuspended in 100 µl of sample buffer/ OD unit/ ml. The rest was induced by adding isopropyl-β,D-thiogalactoside (IPTG) to a final concentration of 1 mM. Bacteria were grown further for 2-3 hours, OD_{600} measured again, and 1-2 ml taken for the induced-cells sample, which was treated as described above.

Cells were then harvested by centrifugation (3,000 x g, 10 min, 4ºC), washed with H_2O, resuspended in 15 ml of column buffer (200 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 1 mM PMSF, 20 mM HEPES·NaOH, pH 7.4). To degrade the cell walls and to obtain spheroplasts lysozyme was added to a final concentration of 1 mg/ml and the suspension incubated for 30 min at 0ºC, while rolling. The spheroplasts were sonicated 10 times for 12 sec, with 48 sec breaks in between, on ice, utilizing Branson sonicator 450 (settings: *timer*: hold; *output control*: 4; *duty cycle*: 80 %). A column was packed with 5-7 ml

of amylose resin, washed with several column volumes (CV) of water, and then with 7 CV of column buffer. The sonicated suspension was centrifuged (39,000 x g, 25 min, 4ºC) and the supernatant applied onto the equilibrated amylose column with a flow rate of 0.2 ml/min. Flow-through was collected, column washed with 7 CV of column buffer and the bound proteins eluted with up to 20 ml of elution buffer (10 mM maltose in column buffer). Fractions of 1 ml volume were collected, protein concentration determined and the fractions frozen at –80ºC until further use.

A slightly modified protocol was used to purify the $_{his6}$Tim10 recombinant protein. Ni-NTA (Qiagen) column was used instead of an amylose one. For cell lysis and loading the column, buffer A containing 300 mM NaCl, 0.01 mg/ml lysozyme, 10 mM imidazole, 10% glycerol, 1 mM PMSF, 50 mM Na_2HPO_4·NaOH, pH 8.0 was used. For washing and eluting the column, buffer A with 20 mM imidazole and no lysozyme, and buffer A without lysozyme and with 300 mM imidazole were used, respectively.

2.3.10.2 Purification of immunoglobulin G (IgG)

Desired antiserum (4 ml) was centrifuged (20,000 x g, 20 min, 4ºC) and the white film of aggregated lipids that forms on the surface removed with a pipette-tip. The antiserum was diluted with 10 ml of buffer A (100 mM KPi, pH 8.5), filtered, and loaded onto a 5 ml Protein A Superose column (Amersham Pharmacia) equilibrated with buffer A. After washing the column with 5 CV of buffer A, bound IgGs were eluted with buffer B (100 mM citric acid, pH 3.0). The eluate was immediately neutralized with 2 ml of 2 M Tris·HCl, pH 8.0, dialyzed overnight against 5 l of H_2O and lyophilized. The IgGs were resuspended in 10 mM MOPS·KOH, pH 7.2 to a final protein concentration of 10-50 mg/ml, aliquoted, and stored at –20ºC.

2.3.10.3 Purification of Tim9·Tim10 complex from *N. crassa* mitochondria

Mitochondria isolated from *N. crassa* strain TA2-14-3[2] were resuspended to a final concentration of 10 mg/ml in solubilization buffer (300 mM NaCl, 20 mM imidazole, 10% glycerol, 1% TX-100, 1 mM TCEP, 1 mM PMSF, 50 mM MOPS·NaOH, pH 8.0), and incubated for 1 h at 4ºC, while mixing. The unsolubilized material was sedimented (15,900 x g, 30 min, 4ºC), the supernatant filtered through filter paper and loaded onto a Superflow Ni-NTA agarose (Qiagen) column (2 ml Ni-NTA beads per 10 g mitochondrial protein) at a flow rate of 5.5 ml/min, using the Äkta-Prime unit (Pharmacia). The column was then washed with

6 CV of high salt wash buffer (essentially same as the solubilization buffer, but with 40 mM imidazole and no TX-100 and PMSF), and with 14 CV of low salt wash buffer (10 mM K^+-acetate, 40 mM imidazole, 10% glycerol, 1 mM TCEP, 50 mM Tris·acetate, pH 8.0), at a 2 ml/min flow-rate. The column was eluted with 3-4 CV of 300 mM imidazole, 10% glycerol, 1 mM TCEP, 25 µM Zn^{2+}-acetate, 20 mM Tris·acetate, pH 7.5, at a flow-rate of 1 ml/min.

Fractions containing the highest protein concentration were pooled together and loaded onto a 1 ml Resource Q or Q Sepharose ion-exchanger column, flow-rate 0.5 ml/min, using Äkta-Purifier (Pharmacia). The column was washed with 2 CV of buffer A containing 20 mM Tris·acetate pH 7.5, 10 % glycerol, 1 mM TCEP, 25 µM Zn^{++}-acetate, and eluted with 4 CV of buffer A supplemented with 1 M K^+-acetate, applying the step-gradient. Flow-through fractions of this column contained the Tim9·Tim10 complex, while the contaminants eluted only with high salt elution buffer. Fractions containing Tim9·Tim10 complex were pooled together, dialyzed against 20 mM Tris·acetate, pH 7.5, 1 mM TCEP, 25 µM Zn^{++}-acetate, using 3-15 ml Slide-A-Lyzer® Cassettes (Pierce) and concentrated using omega Microsep® 10K or omega Macrosep® 10K centrifugal devices – concentrators (PALL). Protein concentration was determined, and a small amount of diluted probe analyzed for purity via high-Tris urea SDS-PAGE, followed by Coomassie or silver staining of the gel(s). Tim9·Tim10 complex was stored at 4ºC till use.

2.3.11. Gel filtration

Superdex 75 HR 10/30 column (Amersham Pharmacia) was used to perform size-exclusion chromatography of the purified *N. crassa* Tim9·Tim10 complex (up to 40 µg), and Superose 12 HR 10/30 and Superose 6 HR 10/30 columns (both Amersham Pharmacia) for the separation of detergent-solubilized mitochondria (1 mg). The buffers used were: buffer A, containing 0.5% (w/v) digitonin, 50 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole and 50 mM Na_2HPO_4·NaOH, pH 8.0; buffer B, comprising 0.5% ß-dodecyl maltoside, 50 mM NaCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 10% (v/v) glycerol, 20 mM HEPES·, pH 7.4, or buffer C: 20 mM HEPES, 200 mM K-acetate, 10% (v/v) glycerol, 1 mM TCEP and 25 µM Zn-acetate. Mitochondria were solubilized at a final concentration of 0.5 mg/ml, for 30 min at 4ºC, in buffers A or B containing 1 mM PMSF. The unsolubilized material was pelleted (90,700 x g, 20 min, 2ºC) and the supernatant loaded onto a Superose12 or Superose 6 size-exclusion chromatography column connected to the Äkta-Purifier (Pharmacia).

Tim9·Tim10 complex was loaded in buffers A or C, but not containing detergent, nor PMSF. Flow rate was set to 0.2 ml/min, detergent concentration in the buffer reduced (0.05%

(w/v) digitonin or ß-dodecyl maltoside) and 0.25 or 0.5 ml fractions collected. They were TCA precipitated and submitted to SDS-PAGE, Western blot analysis and immunodecoration. Standard markers used were: 25 µg thyreoglobulin (660 kDa), 25 µg BSA (66 kDa), 50 µg alcohol dehydrogenase (50 kDa), 100 µg carboanhydrase (29 kDa) and 200 µg cytochrome c (12.5 kDa). All marker protein solutions were ultracentrifuged (20 min, 90,700 x g, 2ºC) prior to performing gel filtration runs. Following SDS-PAGE analysis, fractions containing markers were stained with Coomassie blue.

2.3.12. Digitonin fractionation

The method allows selective solubilization of the outer mitochondrial membrane, while keeping the inner one intact. Essentially, it was performed as described (Rojo, 1998), with minor modifications. Mitochondria (125 µg, at a final concentration of ~5 µg/µl mitochondrial protein), in either SM, SMK^{80} or $SMNa^{300}$, were incubated with proteinase K (end concentration 250 µg/ml) in same buffers with various end concentrations of digitonin. As a control, PK was added to mitochondria fully solubilized with 0.1% (w/v) SDS. The samples were incubated for 15 min on ice, diluted with 96 µl of buffer without PK, and incubated further on ice for 30 min. Finally, TCA precipitation was performed. Samples were analyzed by SDS-PAGE, blotting and immunodecorations.

2.3.13. Thin layer chromatography (TLC) for determination of detergent traces in protein preparations

Glass plates coated with a thin layer of a solid adsorbent (silica, SiO_2) were used. Samples of 15 µl buffer (1 mM TCEP, 25 µM Zn^{++}-acetate, 20 mM Tris·acetate, pH 7.5) containing various detergent concentrations (0.005-1% (v/v) TX-100) were deposited onto plates, and the plates were left to dry. Sepharose Q elution fractions containing purified *N. crassa* Tim9·Tim10 complex (5 µl containing up to 35 µg total protein), Ni-NTA elution fractions containing purified Tim9·Tim10 complex used as the load for the Sepharose Q column (5 µl as well) and some of Ni-NTA load (15 µl corresponding to 150 µg total protein) were spotted onto a second TLC plate. The TLC plates were then placed for less than 1 h in a shallow pool (of approximately 0.5 cm) of running solution (60:39:1 of $CHCl_3$: methanol : water) in a developing chamber. When the solvent reached the top of the plates, the plates were removed from the developing chamber, dried, and the separated components of the mixtures visualized by placing the plate in iodine vapor.

2.3.14. Chemical cross-linking

Cross-linking of ^{35}S-labeled precursors of AAC, Su9(1-69)DHFR and Tim23 was performed by adding 100-500 µM of various cross-linkers (most frequently used was m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS)) during incubation of the precursor with de-energized mitochondria in F5/0.03% (w/v) BSA buffer. After 30 min at 15°C excess cross-linker was quenched by the addition of glycine pH 8.8 to a final concentration of 100 mM, mitochondria solubilised and immunoprecipitation performed. Antibodies against *N. crassa* Tim9, Tim10, Tim8 and the preimmune serum of the rabbit producing antibodies against Tim9 protein were used, and the outcome analysed through SDS-PAGE, blotting and autoradiography.

For ascertaining the oligomeric state of the purified Tim9·Tim10 complex, cross-linking experiments with glutaraldehyde were performed. Various amounts of the purified Tim9·Tim10 complex (ranging from 0.1 to 30 µg total protein per reaction) were incubated at 25°C with different amounts of glutaraldehyde (0.03-0.1%) for different time periods (1, 3, 9 and 30 min), in a reaction volume of 40.5 µl. Excess cross-linker was quenched as above. As a negative control, denatured protein complex in buffer containing 0.5% SDS was subjected to the same procedure. Samples were then analysed by SDS-PAGE, blotted onto a nitrocellulose membrane, and immunodecorated with antibodies against the histidine-tag, Tim9 and Tim10 proteins.

To investigate the influence of zinc on Tim9·Tim10 complex oligomeric state maintenance, same experiments were performed in buffers containing either chelating reagents (o-Phe, EDTA and EGTA, alone or in combination, to a final concentration of up to 10 mM each) or a sulfhydryl reagent N-ethylmaleimide (NEM), alone or in combination with EDTA, both used at 5 mM final concentrations.

2.3.15. Screening of peptide libraries with the purified Tim9·Tim10 complex

Cellulose-bound peptide libraries covering complete primary structures of *S. cerevisiae*AAC2, *N. crassa* Tim17, *N. crassa* Tim22, *N. crassa* Tim23, *N. crassa* Flx1 and *H. sapiens* UCP1 were prepared by automated spot synthesis by Jerini AG, Berlin (Frank, 1992; Kramer and Schneider-Mergener, 1998). Peptides of 13 amino acid residues and with an overlap of 10 residues, corresponding to the sequences of indicated proteins, were C-terminally linked to the cellulose membrane via a $(\beta\text{-Ala})_2$ spacer. Screening of peptide

libraries with purified Tim9·Tim10 complex was done as described before (Brix *et al.*, 1999). Dry membrane was incubated once in methanol and three times in washing buffer (100 mM KCl, 30 mM Tris·HCl, pH 7.6) at RT for 10 min. For analysis of protein binding activity, the membrane was incubated with 20 ml of 150 nM solution of the isolated Tim9·Tim10complex in binding buffer (100 mM KCl, 5% (w/v) sucrose, 0.05% (v/v) Tween 20, 0.5% (w/v) BSA, and 30 mM Tris·HCl, pH 7.6) for 1 h at 25ºC, while shaking.

After washing (3 min, RT) the peptide-bound Tim9·Tim10 proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using cathode buffer (75 mM Tris base, 120 mM 6-aminohexanoic acid, and 0.01% SDS) and anode buffers AI and AII (AI containing 90 mM Tris base and AII containing 300 mM Tris base, pH unadjusted). Blotting procedure was similar to the one described above, with some differences: three Whatman 3MM filter papers were soaked in AII and positioned onto anode graphite electrode of the blotting chamber, another three in AI and put on top of first three, followed by the PVDF membrane washed in AI. This membrane was then covered with the one containing peptide libraries (peptides facing the PVDF membrane) and incubated in cathode buffer, followed by another three filter paper sheets soaked in the cathode buffer as well, which were then covered with the cathode graphite electrode. Semi-dry blotting conditions used were: 1mA/cm^2, 2 h (for our membrane dimensions of 13cm x 8cm, this translates to 104 mA). Bound Tim9 and Tim10 proteins were detected with antibodies against the corresponding proteins. Obtained data was analyzed by scanning laser densitometry and quantified utilizing TINA software.

For regeneration of the peptide library, the membrane was washed for 10 min in 0.5% SDS in TBS solution, three times shortly with water, and finally twice for 10 min with TBS.

2.3.16.Pull-down assay

To demonstrate a direct interaction between the Tim9·Tim10 complex and its substrates, a pull-down assay was used. Eppendorf tubes to be used for this experiment were precoated with 0.1% BSA in 10 mM Tris·HCl pH 7.5, at RT, for 1-10 min, while shaking.

Protein A sepharose beads were washed with up to 5 volumes of each water, 10 mM Tris·HCl pH 7.5, and F5/0.03% (w/v) BSA. They were then split into required number of tubes and antibodies against Tim9, Fis1p and Tim9 preimmune serum were added and incubated with Protein A Sepharose beads for 1.5 hours. Purified Tim9·Tim10 complex (90 µg) was incubated for 30 min on ice with 3 µg of purified either AAC2 or MBP-Fis1(1-98) as a control protein, in F5/0.03% BSA buffer, in the presence of 1 mM ATP and 0.1% TX-100. Similarly, 40 µg of the Tim9·Tim10 complex were incubated for 1 min at RT with 20 µl of

radioactively labeled precursors of AAC2, *N. crassa* AAC and Su9(1-69)DHFR, in the same buffer. Samples were then diluted 1:150 and 1:300 with F5/0.03% (w/v) BSA/0.05% TX-100 buffer, for non-labeled and ^{35}S-labeled Tim9·Tim10 substrates respectively, to a final volume of 1.5 ml. Each probe was then split (3 x 450 µl) into tubes with Protein A Sepharose beads with prebound preimmune serum, Tim9 or Fis1p antibodies. From each sample 45 µl was kept as 10% input. Binding proceeded for 3 h at 4ºC in an overhead roller, and the beads were then sequentially washed with F5 with 0.03% (w/v) BSA and 0.05% TX-100, F5 with 0.05% TX-100 and 10 mM Tris·HCl pH 7.5 buffer, for 3 min at 4ºC. Elution was performed with 2x sample buffer for 3 min at 95ºC. The bound material was analyzed by SDS-PAGE and immunodecoration or autoradiography.

2.3.17.In-gel digestion of proteins for sequencing

The method was used to identify Tim9 protein in the purified Tim9·Tim10 complex, in collaboration with Dr Lutz Eichacker.

Digestion buffer: 15 µl (25 µg/250 µl 1 mM HCl), 40 µl $H_2{}^{18}O$, 10 µl H_2O, 50 µl 0.1 M NH_4HCO_3, 5 µl 1% (w/v) $CaCl_2$, making up a total volume of 120 µl.

15 µl of [1 µg/µl] Resource Q flow-through fraction, containing the purified Tim9·Tim10 complex, was loaded on a high Tris-urea gel and electrophoresis was performed. The gel was stained with Coomassie blue and washed twice for 10 min with water. The protein bands of interest (of molecular weights 10 and 12 kDa), and a piece of gel of the same size in an area not containing proteins (control), were excised with a clean scalpel, cut into 1 x 1 mm cubes, and transferred into 1.5 ml eppendorf tubes. Gel particles were then washed for 5 min with 100-150 µl of water, a mixture of water and CH_3CN (v/v) of 1:1, and a 1:1 mixture of 0.1 M NH_4HCO_3:CH_3CN (all solutions removed via vacuum application).

The gel pieces were then shrunk with 300 µl acetonitrile, and dried in a vacuum centrifuge. They were further swollen in 150 µl (enough to cover them) of 10 mM DTT/0.1 M NH_4HCO_3, by incubating them for 45 min at 56ºC. The gel particles were subsequently washed twice with acetonitrile and the same volume of 55 mM iodoacetamid/0.1 M NH_4HCO_3 solution added (iodoacetamid was freshly weighed), and an incubation of 30 min at RT in dark followed. They were then shortly washed, once with 0.1 M NH_4HCO_3, and once with acetonitrile. Then they were shrunk again with acetonitrile. If necessary, the washing cycle was repeated, until Coomassie stain was completely removed.

After drying the gel particles completely, they were rehydrated in a digestion buffer at 4ºC. After 10-15 min, some more of digestion buffer was added, because it got absorbed. Incubation for 45 min at 4ºC insued, the remaining supernatant removed, and replaced with 5-10 µl of the same buffer without trypsin. The gel pieces were shaken for further 3 h or overnight at 37ºC.

The peptides were then extracted from the gel. The gel pieces were incubated for 15 min at 37ºC, while shaking, with 25 µl of 25 mM NH_4HCO_3 added. The gel pieces were spun down and 1-2 volumes of acetonitrile added. Another 15 min-incubation at 37ºC followed, and after the gel pieces had been spun down, the supernatant was collected; 40-50 µl of 5% (v/v) formic acid was added and the probe incubated for 15 min at 37ºC, with shaking. The gel was spun down, the supernatant not removed, and 1-2 volumes of acetonitrile added, followed by shaking for 15 min at 37ºC. The gel particles were spun down and the extracts pooled together. Sample was then dried in a speed vac centrifuge, and dissolved in 5% (v/v) formic acid in ddH_2O, final volume 10 µl. A GC column equilibrated with 400 µl methanol and subsequently with 5% (v/v) formic acid was loaded with the sample, and washed with 5% (v/v) formic acid. It was then eluted with 1.4 µl of the 60% (v/v) methanol, 5% (v/v) formic acid solution. The eluate was transferred into the needle for the mass spectrometry (MS) and subjected to the MS analysis. The analysis was further kindly perfomed and the results analysed by the members of Dr Lutz Eichacker's group.

2.4. Immunological methods

2.4.1. Generation of specific antibodies against *N. crassa* Tim9 and Tim10 proteins in rabbits

N. crassa intronless versions of *tim9* and *tim10* genes were cloned into pMalcRI vector, yielding MBP-*tim9* and MBP-*tim10* fusion constructs, which were transformed into MH1 and XL-1 *E. coli* cells. The expressed fusion proteins were purified and separated from remaining contaminants via SDS-PAGE. The gels were blotted and the bands, corresponding in size to fusion constructs, excised from the nitrocellulose membranes. Excised protein bands (the amount of total protein apprx. 200 µg) were dissolved in 200 µl DMSO by vortexing for circa 3 min. TiterMax adjuvant (200 µl) was added and the emulsion injected subcutaneously into rabbits in their neck area. For all subsequent injections, that took place every four weeks, TiterMax adjuvant was replaced with Freundsches one. The rabbits were bled 10-12 days

after each injection cycle except for the first one. Approximately 10-30 ml of blood was bled each time, and it was left at RT to coagulate, when it was centrifuged twice (5 min at 3,000xg and 15 min at 20,000xg, RT), and the resulting antiserum (supernatant of centrifugation) heated to 56ºC for 20 min. It was then aliquoted and frozen at −20ºC.

2.4.2. Affinity purification of antibodies against Tim9 and Tim10 proteins

Since the antisera contained antibodies recognizing unrelated antigens (the so-called cross-reactivity), affinity purification had to be performed. For that purpose, the MBP-fusion proteins that served as antigens were coupled to the activated Sepharose. PD10 column (Amersham Pharmacia Biotech) was equilibrated with 50 ml of 0.5 M NaCl, 0.1 M $NaHCO_3$, pH 8.3 (the "coupling" buffer). An amylose-column elution fraction of MBP-fusion protein (Tim9 or Tim10), containing not more than 9 mg of protein, was loaded onto this PD10 column, and 0.5 ml fractions were collected. Protein concentration was determined and three peak fractions pooled together. CNBr-activated Sepharose^{TM} 4B (Amersham Biosciences) (0.3 g) was added onto and mixed with 10 ml of 1 mM HCl, pH 2.8 poured into a glass, and left for 2-5 min for the sepharose to swell. The suspension was mixed again and poured onto a sintered glass filter, connected to a vacuum-pump for washing with 100 ml of 1 mM HCl. The matrix was transferred into a disposable 10 ml plastic column (Biorad), drained from HCl and the outlet was closed with the supplied cap. Fraction containing MBP-fusion protein was added, the column closed with parafilm and incubated for 1 h at RT, while gently rolling. It was then positioned into a standard stand, the content allowed to settle, and the outlet opened. Unbound material was collected, the column washed with 6 ml of coupling buffer, and drained completely, before adding 6 ml of 1 M ethanolamine pH 8.0. When 4 ml of ethanolamine buffer have passed through, the outlet was closed and the column left for 2 h at RT. It was drained, washed in three cycles with 6 ml of 0.5 M NaCl, 0.1 M Na-acetate, pH 4.0 and 0.5 M NaCl, 0.1 M Tris·HCl, pH 8.0 and left overnight with 3 ml of 0.05% NaN_3 in water.

The day after, column was equilibrated with 10 ml of 10 mM Tris·HCl, pH 7.5, and 6 ml of the antiserum to be affinity purified, premixed with 24 ml of 10 mM Tris·HCl, pH 7.5, 150 µl of 200 mM PMSF, 15 µl of 1 M o-Phe and 30 µl of 1 mg/ml leupeptin, loaded onto it. The flow-through was reloaded twice. The column was washed with 10 ml of 10 mM Tris·HCl, pH 7.5, and then with 10 ml of 0.5 M NaCl, 10 mM Tris·HCl, pH 7.5.

For the elution, column is subjected to alternating pH through application of following buffers, in given order (10 ml each): 10 mM Na-citrate, pH 4.0, 100 mM glycine·HCl, pH 2.5, and 100 mM Na-phosphate, pH 11.5. Fractions of 1 ml are collected and neutralized immediately, with 200 µl 1 M Tris·HCl, pH 8.8, in the case of first two buffers, and with 100 µl glycine, pH 2.2 in the case of the phosphate one. The column was equilibrated to alkaline pH with 10 ml of 10 mM Tris·HCl, pH 8.8, between the glycine and phosphate buffer elutions. It was washed with 10 mM Tris·HCl, pH 7.5, and left in 0.05% NaN_3 at 4ºC.

From each of the affinity purified antisera, their "load", flow-through, wash and elution fractions 1-3, diluted 1:250 in 5% (w/v) skimmed milk in TBS, were checked by immunodecoration. From this decoration the set of elution fractions containing the desired purified antibody (citrate, glycin or phosphate) were identified, and all ten in that set were then tested as described.

2.4.3. Immunodecoration

Proteins blotted onto nitrocellulose or PVDF membranes were visualized by immunodecoration. Non-specific binding sites were blocked by incubating the membrane with either 5% (w/v) skimmed milk in TBS buffer, 3% BSA (w/v) in TBS buffer or NET-gelatin solution (150 mM NaCl, 5 mM EDTA, 0.05% TX-100, 0.25% (w/v) gelatine, 50 mM Tris·HCl, pH 7.5), at RT for 1 h. Then the membranes were incubated with specific primary antibody (1:200 to 1:1.000 dilutions in one of the above mentioned solutions) for 1 h at RT, or overnight at 4ºC. The membrane was then washed 3 times (each wash lasts 5-10 min), with TBS, TBS/0.05% (w/v) Triton X-100 and again with TBS, and incubated for 1 h with horseradish peroxidase coupled to secondary goat anti-rabbit-IgG or anti-mouse-IgG (diluted 1:10.000 in one of the above solutions). The membrane was again washed (as already described) and treated with ECL reagents: luminol (2.5 mM 3-aminophtalhydrazide and 0.4 mM p-cumaric acid in 0.1 M Tris·HCl, pH 8.5), diluted 1:1 with 0.018% (v/v) H_2O_2 (in 0.1 M Tris·HCl, pH 8.5). The luminescence reactions were detected with Röntgen films (Fuji NewRX).

2.4.4. Immunoprecipitation and co-immunoprecipitation

Eppendorf cups were precoated with BSA by washing them for 5 min at RT with 0.1% BSA in 10 mM Tris·HCl, pH 7.5. Desired amount of Protein A Sepharose™ CL-4B (Amersham Biosciences) beads slurry was washed with water, followed by 0.1% BSA in 10

mM Tris·HCl pH 7.5, and then with desired buffer. Appropriate amounts of antiseras (25-100 µl per 25 µl of beads), and required buffers are added (so that the final volume is no less than 500 µl), and incubates for at least 3 h at 4ºC, while rotating the cups overhead. The beads were then washed from the unbound antiserum.

Mitochondrial protein samples to be co-immunoprecipitated (final protein concentration 2.5 mg/ml) were dissolved in lysis buffer (0.05% BSA, 0.5% digitonin, 50 mM NaCl, 1 mM PMSF, 25 µM Zn-acetate and 50 mM Na-phosphate·HCl, pH 8.0), and incubated 1 h at 4ºC while gently shaking. Mitochondria with radioactively imported and cross-linked proteins were solubilized at a final protein concentration 2 mg/ml in 100 mM NaCl, 2 mM PMSF, 1% SDS, 50 mM Na-phosphate·HCl, pH 8.0, and incubated for 15 min at RT, while vigorously shaking. The latter samples were subsequently diluted 20 times with a TBS buffer that contains 0.2% TX-100. After a clarifying spin (90.700xg, 20 min, 2ºC), the supernatants were added to antibodies pre-coupled to Protein A Sepharose beads. The samples were incubated further at 4ºC for additional 3 h, while rotating overhead, the beads were then washed with 500 µl lysis buffer with no PMSF, then with lysis buffer with decreasing amount of detergent (0.1% digitonin, 0.05% TX-100) and no BSA, and finally with 500 µl of 10 mM Tris·HCl pH 7.4 (3 min each wash, rotating overhead, 4ºC). Bound proteins were eluted with 2x Laemmli sample buffer via incubation for 3 min at 95ºC or 10 min at 56ºC, and analyzed by SDS-PAGE.

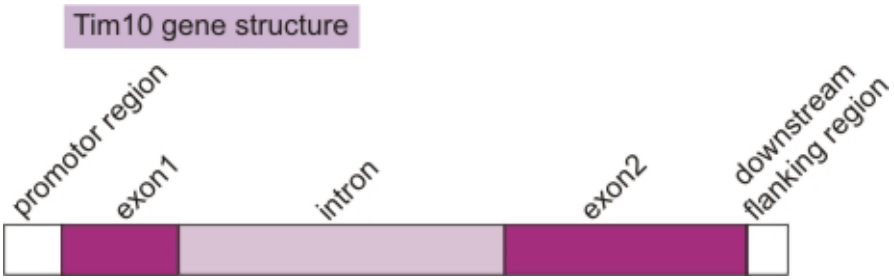
3. Results

3.1. Identification of the N. crassa tim9 and tim10 genes

3.1.1. Identification of the *N. crassa tim10* gene

The amino acid sequence of the *N. crassa* Tim10 protein was determined based on a BLAST search using the sequence of the *S. cerevisiae* Tim10 protein as a query.

A



B

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NcTIM10      ....MFGLGRPQPTSAEKIAAVENELKVVAEMHSRM      32
ScTIM10      MSFLGFGGGQPQLSSQQKIQAAEAELDLVTDMFNKL      36
AtTIM10      ....MASPIPVGVTKEQAFSMAQTEMEYRVELFNKL      32
DmTIM10      ....MALPQISTADQAKLQLMQEMEIEMMSDLYNRM      32

NcTIM10      VKICTLKCIDKSYREGDLSKGESVCLDRCAAKFFET      68
ScTIM10      VNNCYKKCINTSYSEGELNKNESSCLDRCVAKYFET      72
AtTIM10      AQTCFNKCVDKRYKEAELNMGENSCIDRCVSKYWQV      68
DmTIM10      TNACHKKCIPPRYSESELGKGEMVCIDRCVAKYLDI      68

NcTIM10      HQKISDQLQKETQARGGGGFGM.........          90
ScTIM10      NVQVGENMQKMGQSFNAAGKF..........          93
AtTIM10      NGMVGQLLS.........AGKPPV.........        83
DmTIM10      HEKIGKKLT.........AMFMQDEELMKKMSS        92
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Figure 5. The *tim10* gene of *N. crassa*. (A) Structure of the *N.crassa tim10* gene. **(B)** Protein sequence alignment. The sequences of *N. crassa* Tim10 protein with homologues from other organisms are presented (Nc, *Neurospora crassa*; Sc, *Saccharomyces cerevisiae*; At, *Arabidopsis thaliana*; Dm, *Drosophila melanogaster*). Identical residues occurring in all organisms are indicated in darker violet, 75% similar in light blue and 50% similar in pink.

Primers were constructed based on sequences obtained from the data base (http://www.broad.mit.edu/annotation/fungi/ neurospora/), and the intronless version of the *tim10* gene of around 300 bp was PCR-amplified using *N. crassa* cDNA(−) library as a template. When cDNA(+) library was used as the template, yield of the PCR product was much smaller.

Using these same primers, the *N. crassa* genomic library was screened and a 500 bp PCR product was obtained. Another PCR reaction, with the same primers and digoxigenin-labeled uracyl nucleotides was performed. A digoxigenin-labeled product of slightly increased molecular weight (circa 600 bp) was obtained. This probe was used to screen the *N. crassa* cosmid library (pMOcosX) and three cosmids containing the *tim10* gene were identified: X20:A12, X25:B10 and X12:C6.

Using these cosmids as templates, the *tim10* gene was amplified by PCR, inserted into pCB1179 vector and sequenced. This revealed that the gene is interrupted by one intron (gene structure is given in Figure 5A). The DNA sequence was deposited in the Genebank (GENBANK accession number AF343077). The *N. crassa* Tim10 protein comprises 90 amino acid residues and has 44% identity to the yeast Tim10p (Figure 5B).

The intronless version of the gene was further cloned into pGEM4 vector for the synthesis of radioactively labelled Tim10 precursor. Moreover, his- and MBP-tagged Tim10 proteins were expressed in *E.coli*, purified and injected as antigens into rabbits. Serum obtained from the rabbit injected with MBP-Tim10 fusion construct was found to decorate Tim10 in the mitochondrial preparations isolated from the *N. crassa* mycelium.

3.1.2. Identification of the *N. crassa tim9* gene

In the early stages of this study, the full genome sequence of *N. crassa* was not yet available. Thus, the initial BLAST searches performed with the sequence of Tim9 protein from yeast as bait were unsuccessful in identifying the sequence of the *N. crassa* Tim9 protein. To tackle this task, other approaches were attempted.

The ^{35}S-labeled *N. crassa* wild type mitochondria were isolated, solubilised in buffers containing different detergents, and a coimmunoprecipitation with an antibody against Tim10 protein was performed (Figure 6). Upon solubilization with TX-100, a wide range of proteins of variable molecular weight coimmunoprecipitated with the Tim10 protein. The pattern of protein bands was however indistinguishable for the preimmune and the Tim10 antisera, aside from the intensity gain of the band equal in size to Tim10 protein.The two most prominent

protein bands were detected by autoradiography in probes solubilized with SDS (control), one of 10 kDa and the other of 20 kDa, presumed to correspond to the Tim10 protein, and a protein of 20 kDa of unknown function, rich in histidine residues. The unspecific cross-reactions of the Tim10 antiserum imposed serious obstacles to identifying the Tim9 protein via this aproach.

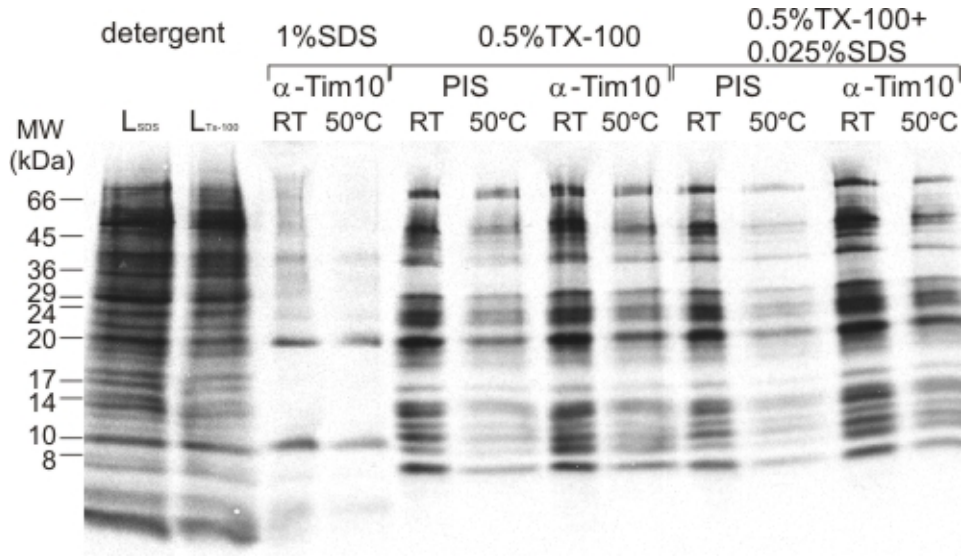


Figure 6. Coimmunoprecipitation of the ^{35}S-labeled wt *N. crassa* mitochondria. Antibodies against Tim10 protein and the corresponding preimmune serum were used (RT, room temperature; PIS, preimmune serum). Marked temperatures describe conditions used for elution; all probes were first eluted at RT, followed by elution at 50ºC.

As an alternative method to identify the Tim9 protein, a heterokaryotic strain comprising the histidine-tagged version of Tim10 protein in addition to the wild type protein was constructed (for details see section 3.3). The TA2-1 transformant was selected for further experiments among a range of different transformants. Mitochondria isolated from this strain were solubilized in the sodium-phosphate buffer containing 1% TX-100, and a pull-down assay with the Ni-NTA beads was performed. The bound fractions contained the Tim10 protein migrating at 10 kDa, and the $Tim10_{his9}$ protein at 12 kDa. No additional band in the small molecular weight range that would correspond to the Tim9 protein was detected (Figure 7, lane 1). Similar results were obtained when mitochondria were solubilized in various other buffers (data not shown), or when the Ni-NTA elution fractions of the aforementioned purification were run on the long high-Tris urea gel with better resolution of the low molecular weight range (data not shown).

To exclude the possibility that the Tim9 protein migrates together with the $Tim10_{his9}$ protein on high-Tris urea gels, the Ni-NTA purification procedure was modified, by adding

one elution step with 8 M urea, prior to the elution with imidazole. Urea denatures the proteins, but it does not destabilize the interaction of the histidine tag with the column matrix. This allowed the dissociation of all proteins without a histidine-tag from the column. No band of about 12 kDa, which would correspond to a protein released by urea was detected (Figure 7, lane 2).

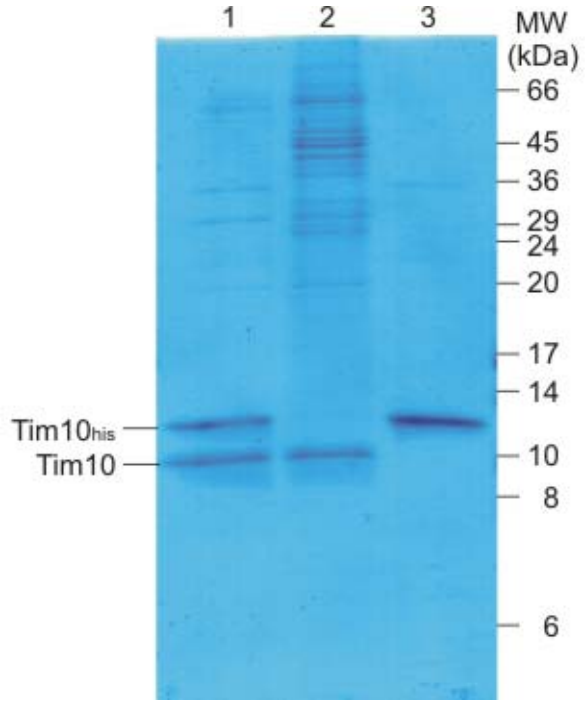


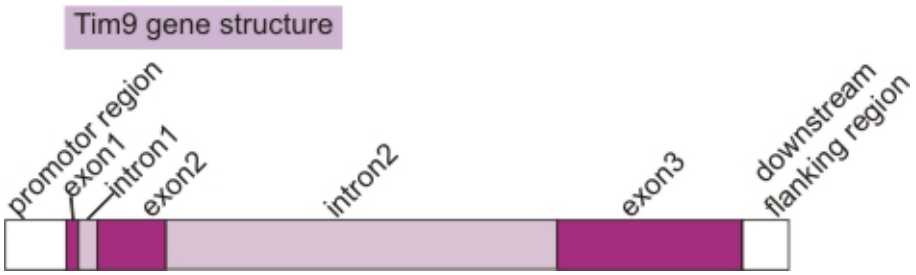
Figure 7. Purification of the Tim10·Tim10$_{his9}$ complex. Elution of the Ni-NTA columns was preformed under native (control, lane 1), and denaturing conditions (lane2). After elution with 8 M urea, the same Ni-NTA column was subjected to the elution with 300 mM imidazole (lane 3).

To test whether the *N. crassa* Tim9 protein is "hidden" on the gels by co-migrating with the Tim10 protein, the 10 kDa protein band was digested with trypsin and the tryptic fragments were sequenced. Indeed, several peptides, not belonging to the Tim10 protein, were identified (data not shown). Combining the sequences of these peptides yielded a stretch of amino acid residues long enough to perform a BLAST search of the meanwhile extended database of *N. crassa* genome.

The segment identified in this search, allowed the identification of a DNA sequence corresponding to the region of the alleged *tim9* gene. Inspection of a larger DNA segment around the identified region allowed the construction of the primers for *tim9* exons, making an educated guess regarding the position of introns. Simultaneous screens of the *N. crassa* cDNA and genomic libraries produced the PCR products of the expected size which confirmed the presumed gene organization of *tim9*. Meantime, sequencing of the *N. crassa* genome was

concluded, and based on comparison of the *tim9* cDNA to the corresponding region on the chromosome the existence of the two introns was confirmed (Figure 8A). The *N. crassa tim9* gene encodes a protein of 88 amino acid residues which is 40% identical to the yeast Tim9p (Figure 8B). The gene sequence was deposited in the Genebank (GENBANK accession number AY141127).

A



B

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NcTIM9      .........MDGLTAAESRELDQRLQKRQVKEFMSVF     28
ScTIM9      .........MDALNSKEQQEFQKVVEQKQMKDFMRLY     28
AtTIM9      MDASMMAGLDGLPEEDKAKMASMIDQLQLRDSLRMY     36

NcTIM9      GNLVDNCFTACVDDFTSKALSGRESGCISRCVLKSM     64
ScTIM9      SNLVERCFTDCVNDFTTSKLTNKEQTCIMKCSEKFL     64
AtTIM9      NSLVERCFVDCVDSFTRKSLQKQEETCVMRCAEKFL     72

NcTIM9      STQTRLGERFGELNAAMTAEMQRR.                88
ScTIM9      KHSERVGQRFQE..QNAALGQGLGR                87
AtTIM9      KHTMRVGMRFAELNQNAPTQD....                93
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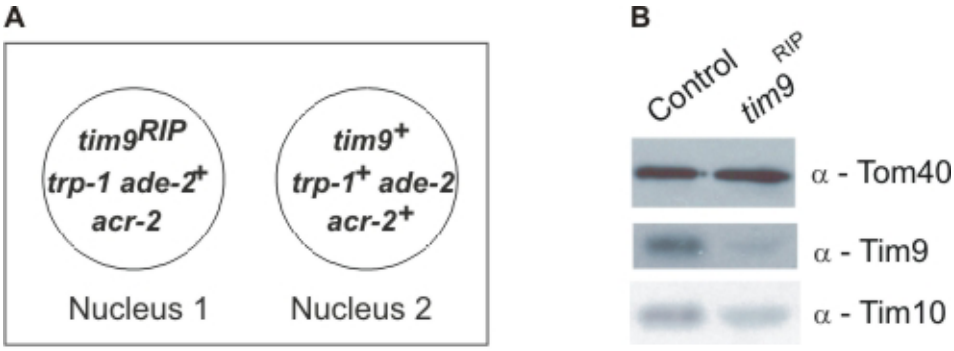
Figure 8. *tim9* gene of *N. crassa*. (A) Structure of *N.crassa tim9* gene. **(B) Protein sequence alignment of *N. crassa* Tim9 with homologues from other organisms.** (Nc, *Neurospora crassa*; Sc, *Saccharomyces cerevisiae*; At, *Arabidopsis thaliana*). Identical residues occurring in all organisms are indicated in darker violet and 50% similar in pink.

The coding sequence of the *tim9* gene was cloned into pGEM4 vector for the production of radioactively labelled Tim9 protein, and into pMalcRI plasmid for the expression of MBP-fusion proteins, used for the generation of antibodies. Import of radioactively labelled Tim9 and Tim10 proteins showed no dependency on ATP, transmembrane potential, and the common import receptors (data not shown).

3.1.3. Tim9 is an essential protein in *N. crassa*

Since *TIM9* was shown to be an essential gene in yeast (Koehler *et al.*, 1998b; Adam *et al.*, 1999), our research collaborator Frank Nargang and his coworkers used the sheltered repeat-induced point mutation (RIP) procedure to generate the *tim9* mutants in *N. crassa.* The product of the procedure was a heterokaryotic strain with the *tim9* gene in one type of nucleus inactivated by RIP, and a wild type copy of the gene in the other (Figure 9A).



C

Colonies examined	Ade-requiring homokaryons	Trp-requiring homokaryons	Hetero-karyons
160	47	0	113

Figure 9. (A) Sheltered heterokaryon containing the *tim9*^{RIP} mutant. The box depictes the heterokaryon, circles represent genetically distinct nuclei which make up the heterokaryon. Nucleus 1 contains only the nonfunctional RIPed version of *tim9*, whereas nucleus 2 contains a wt copy of the gene. **(B) Level of Tim9 protein is decreased in cultures where the nucleus containing the RIPed gene is increased in number.** The sheltered heterokaryon (panel A) and an acriflavine resistant control strain (Host III) were grown in the presence of acriflavine and tryptophan and mitochondria were isolated. Mitochondrial proteins were separated by SDS-PAGE and blotted to nitrocellulose. The blot was decorated with antibodies against the indicated proteins. **(C) Scoring of single colonies from the heterokaryotic strain decribed in A.** (ade, adenine; trp, tryptofan).

Growth of the heterokaryon in the presence of acriflavine and tryptophan resulted in the RIPed nucleus gaining numerical superiority, since it provided the drug resistance and the addition of tryptophan attended to its nutritional requirements. Under these conditions the cells showed a slowed growth rate. The level of Tim9 protein was drastically decreased in the mitochondria of the heterokaryotic strain, whereas the level of the Tim10 protein was slightly reduced in these cells (Figure 9B).

In order to determine whether *tim9* is an essential gene in *N. crassa*, conidiaspores produced by the heterokaryon were streaked onto medium containing all nutritional

requirements of both nuclei in the strain (Figure 9A). Testing of nutritional requirements of individual colonies produced from these conidia disclosed the *tim9*^{RIP} nucleus as inviable (Figure 9C). To confirm that the effects of RIP are to be attributed to the *tim9* gene, the sheltered heterokaryon was transformed with a bleomycin resistance plasmid containing a wild type copy of the *tim9* gene. Viable tryptophan-requiring homokaryotic strains were recovered (data not shown). Therefore, *tim9* is an essential gene in *N. crassa*.

The *tim10* gene has proven an unsuitable candidate for the RIP mutagenesis because of the proximity of *tim10* to a potential tRNA-synthetase gene and the size of the duplication required to act as a RIP substrate.

3.2. The Tim9 and Tim10 proteins form a heterooligomeric complex in the intermembrane space of mitochondria

To investigate the subcellular localization of the *N. crassa* Tim9 and Tim10 proteins, subcellular fractionation by differential centrifugation of cellular homogenate was performed. Both proteins were found to be exclusively localised in the mitochondria (data not shown). Their intramitochondrial location was further examined via the digitonin fractionation procedure (Hartl *et al.*, 1986).

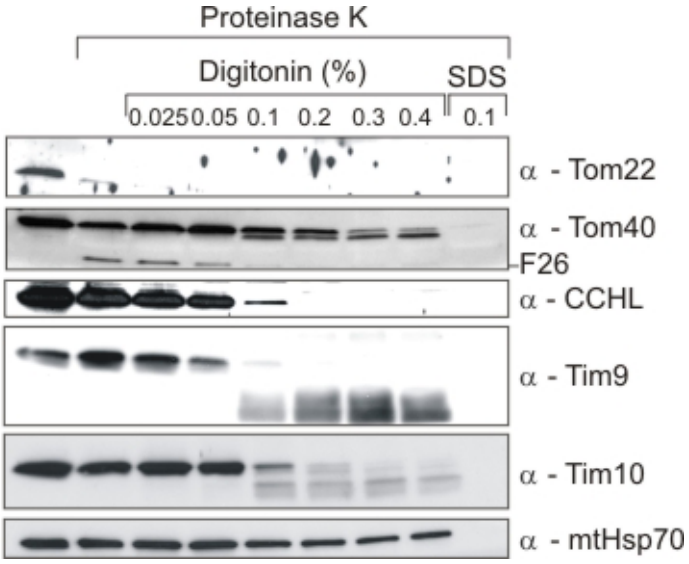


Figure 10. Tim9 and Tim10 localize to the mitochondrial intermembrane space. Mitochondria were subfractionated in the presence of PK (0.25 µg/µl) by adding increasing amounts of the detergent (0.025-0.4%). The samples were TCA precipitated and analysed by SDS-PAGE and immunodecoration with the antibodies against indicated proteins (F26, typical N-terminal fragment of Tom40, indicative of opening of the outer membrane).

The isolated mitochondria of *N. crassa* were treated with increasing amounts of the mild detergent digitonin. Upon this treatment solubilization of the outer membrane occurred first, followed by that of the inner membrane. Fractionation was performed in the presence of proteinase K to monitor for the degree of accessibility of various proteins. The degradation of both Tim9 and Tim10 proteins occurred at the same digitonin concentrations as that of one representative of the intermembrane space proteins, like CCHL, and of the N- and C-termini of Tom40 which are also in the intermembrane space (Künkele *et al.*, 1998) (Figure 10). This indicates that Tim9 and Tim10 proteins of *N. crassa* reside in the mitochondrial intermembrane space. A proteinase K-resistant core structure observed in both Tim9 and Tim10 proteins is suggestive of a tight fold of this domain (Figure 10).

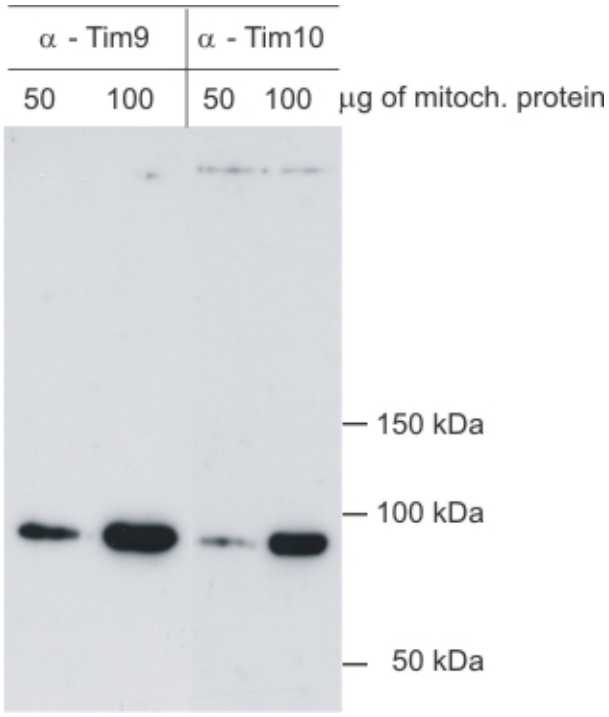


Figure 11. Analysis of the Tim9·Tim10 complex by BNGE. Mitochondria were solubilized in a buffer containing 0.5 % (w/v) digitonin and subjected to analysis via BNGE (with the marked amount of total mitochondrial protein loaded per lane), and immunodecoration with antibodies against either Tim9 or Tim10 protein. Molecular weight of ADH used as a marker protein is indicated on the righthand side.

To ascertain the oligomeric state of the Tim9 and Tim10 proteins, mitochondria were solubilized in a buffer containing either 0.5% digitonin or 0.5% ß-dodecyl maltoside (DDM), and subjected to the analysis by blue native gel electrophoresis (BNGE). Both proteins were present in a 70-80 kDa complex as revealed by the decoration with antibodies against both Tim9 and Tim10 proteins (Figure 11).

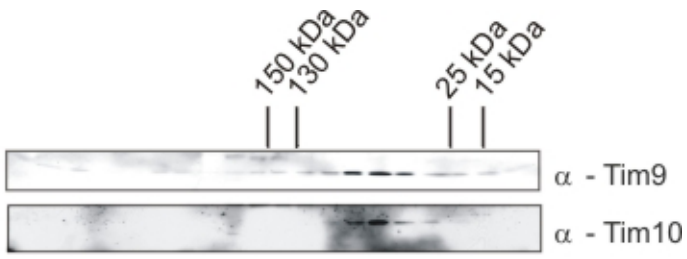


Figure 12 Analysis of the Tim9·Tim10 complex by size exclusion chromatography over a Superose 6 gel-filtration column. Mitochondria were lysed in a buffer containing 0.5% DDM and subjected to a gel filtration trial. Elution peaks of marker proteins are indicated as their respective molecular masses.

Size exclusion chromatography of the solubilized mitochondria confirmed that both proteins constitute the 70 kDa complex (Figure 12). To further affirm that the two proteins do indeed make up the same complex, the mitochondria were solubilized in a buffer containing 0.5% digitonin, and the coimmunoprecipitation with antibodies against both proteins was performed. Antibodies against the Tim9 protein efficiently precipitated both Tim9 and Tim10 proteins. Similarly, the antibodies against the Tim10 protein immunoprecipitated the Tim9 and Tim10 proteins (Figure 13). On the other hand, they were not immunoprecipitated by antibodies against the inner membrane protein Tim23 or the membrane associated matrix protein Tim44 (not shown). Antibodies against the Tim22 protein precipitated a negligible amount of Tim9 and Tim10 proteins under the conditions used (Figure 13). It seems as if the small Tim proteins of *N. crassa* do not associate stably with the Tim22·Tim54 complex. In conclusion, *N. crassa* Tim9 and Tim10 proteins interact to form a heteromeric complex in the intermembrane space.

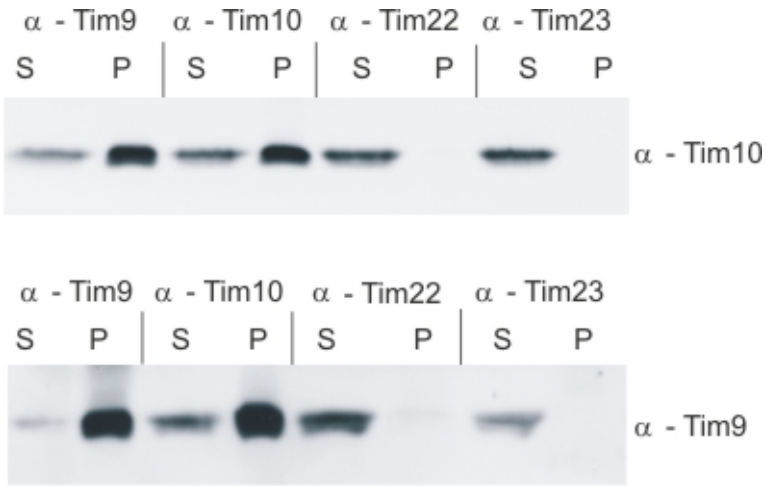


Figure 13. Analysis of the Tim9·Tim10 complex by coimmunoprecipitation. Mitochondria (75µg/lane) were solubilised in a buffer containing 1% digitonin, and added to protein A Sepharose beads with prebound antibodies raised against indicated proteins. Proteins that were not bound (supernatant, S) and that were bound (pellet, P) were analysed by SDS-PAGE and immunodecorated with indicated antibodies.

3.3. Isolation of the Tim9·Tim10 complex

For the purification of the Tim9·Tim10 complex, we inserted a sequence encoding the Tim10 protein tagged with nine histidine residues under the control of its endogenous promotor into the pCB1179 plasmid. The expression construct was then transformed into wild type *N. crassa* strain 74A. This procedure yielded heterokaryotic transformants, all expressing a nanohistidinyl-tagged version of the Tim10 protein, in addition to the wild-type protein. These two versions of the Tim10 protein were present in different ratios in different strains, and hence, transformants were examined for expression levels of the tagged *vs*. the untagged protein. Two heterokaryonic strains (TA2-1 and TA2-14) with the favourable expression of the $Tim10_{his9}$ protein were chosen among a large number of transformants. These two strains were further subjected to a microconidiation procedure and from all the homokaryonic strains originating from these microconidia the TA2-14-3 strain was selected. This strain is a homokaryon expressing roughly equal amounts of the Tim10 and $Tim10_{his9}$ proteins. It grew comparably to the parental wild type strain (data not shown). Mitochondria isolated from the mycelium of this strain were used to purify the Tim9·Tim10 complex, combining metal-affinity and ion-exchange chromatography technics.

To optimize the Ni-NTA purification procedure, following parameters were tested: sodium phosphate, Tris, potassium acetate, HEPES and MOPS buffers, various pH values, different imidazole, zinc and salt concentrations in washing buffers, and different reducing agents. Protein yields and the purity of the Tim9·Tim10 complex released from mitochondria by solubilization with Triton X-100 to those obtained upon disruption of the mitochondrial membrane integrity through sonication were also compared. Although both methods produced complexes of comparable purity, the yield was significantly higher when TX-100 was used. The optimal conditions, established and used from then on, are noted in the "Material and methods" section.

A large scale purification procedure was also established. Different amounts of the loaded protein with respect to column volume were evaluated and subsequently set at 5 g of total protein per 1 ml of the Ni-NTA column matrix. To minimize the possibility of protein degradation during purification steps, flow rate was increased to 5.5 ml/min. This reduced the time required for the loading of the solubilized material but still allowed for complete protein binding as determined by analysis of the flow-through fractions Because of a higher column pressure resulting from the increased flow rate, it was necessary to use the Superflow matrix,

which endures pressures up to 1 MPa, instead of the conventional Ni-NTA Agarose (both from Qiagen).

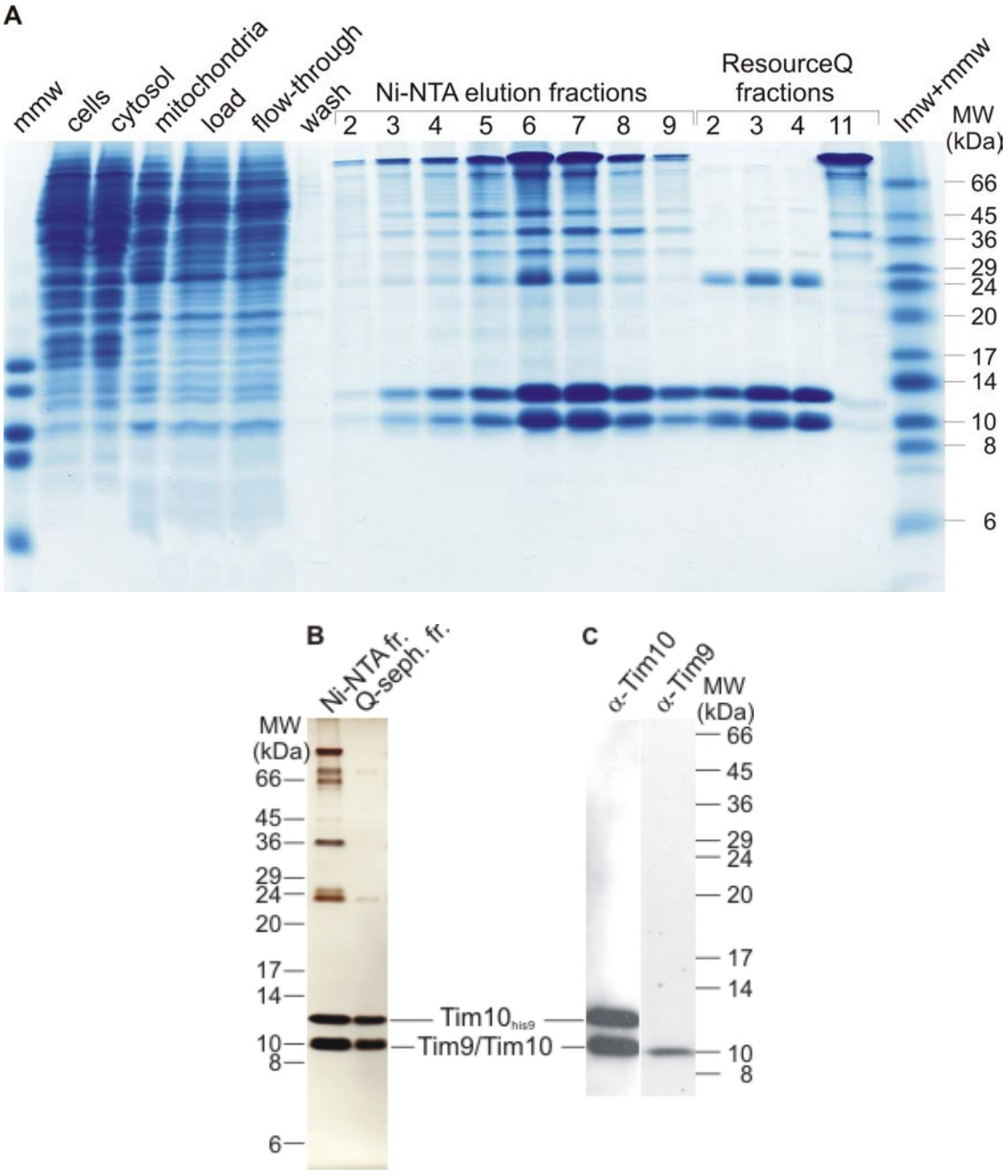


Figure 14. Purification of the Tim9·Tim10 complex. (A) Tim9·Tim10 complex was isolated from TA2-14-3 *N.crassa* mitochondria lysed in sodium phosphate buffer containing 1% (v/v) Triton X-100. The solubilized material was subjected to metal-affinity chromatography; fractions 5-8 from the Ni-NTA column were loaded onto a Resource Q ion-exchange chromatography column. Samples of the entire purification procedure were analysed by high Tris urea SDS-PAGE, and the gel was stained with Coomassie Blue. (lmw, low molecular weight marker; mmw, mini molecular weight marker; MW molecular weight). **(B)** Tim9·Tim10 complex isolated over a Ni-NTA column in MOPS buffer, was loaded onto a Q Sepharose ion exchanger; the respective fractions were analysed by high Tris urea PAGE, and the gel was stained with silver nitrate. **(C)** Proteins of fraction three of the Resource Q ion-exchanger were analysed by SDS-PAGE and immunodecoration with antibodies against Tim9 and Tim10.

The Tim9·Tim10 complex eluted from the Ni-NTA column contained several contaminating proteins (Figure 14A). Therefore, we went on to investigate possibilities for segregating the complex from these protein contaminants. The size exclusion chromatography turned out to be an undependable tool. The loss in protein amount due to weak, yet undeniably present, unspecific interactions of the Tim9·Tim10 complex with the gel filtration column's matrices, overshaddowed the overall modest improvements gained in purity-level. The ion exchange chromatography was tested next. Fractions eluted from a Ni-NTA column were pooled together and subjected to either cation- or anion-exchange chromatographies (Figure 14A). The cation-exchange chromatography was found to be inadequate, since the Tim9·Tim10 complex could not be eluted with high salt buffers at all pH values tested (pH 6-10, at half-unit increments). The anion-exchangers, on the contrary, showed excellent potential for improving the Tim9·Tim10 complex purification procedure. When using the pH values from 6.0 till 8.0, the complex was in the flow-through of the anion-exchange column, while the contaminants remained bound. The only exception was pH 6.0, where some contaminants were also found in the flow-through. At pH values equal to and higher than 9.0, Tim9·Tim10 complex was bound to the ion-exchanger. However, for all subsequent purifications more physiological pH values were selected, closer to that of the mitochondrial intermembrane space (pH 7.5-8.0). Replacing the Resource Q with Q Sepharose anion exchanger enabled me to substantially decrease both protein amount losses, caused by unspecific binding to the ion-exchanger matrix, and the amount of contaminating proteins (compare A to B in Figure 14).

The protein bands of the purified complex were identified by mass spectroscopy (data not shown) and immunodecoration (Figure 14C).

To essay whether there are some residual TX-100 traces in the samples containing the Tim9·Tim10 complex, I have performed a thin-layer chromatography (TLC) of these probes. Every step of the purification procedure was monitored via this method, which can detect TX-100 concentrations as low as 0.005% (v/v). Minor amounts of TX-100 were found in the washing fractions of the Ni-NTA column. In the Q Sepharose flow-through fractions, dialysed against buffers without TX-100, the detergent was completely eliminated (data not shown). To syllogise, *N. crassa* Tim9 and Tim10 proteins are the sole constituents of the purified complex.

3.4. Structural organization of the purified Tim9·Tim10 complex

To prove the authenticity of the Tim9·Tim10 complex purified from mitochondria expressing $Tim10_{his9}$, it was compared to the complex set free from the wild type mitochondria. Both complexes displayed an apparent molecular weight of 70-80 kDa when analyzed by BNGE (Figure 15). The identical results were obtained using antibodies against Tim9 and Tim10 proteins, confirming that the 70-80 kDa complex contains both proteins. Thus, the purified complex possesses the same composition as the native one.

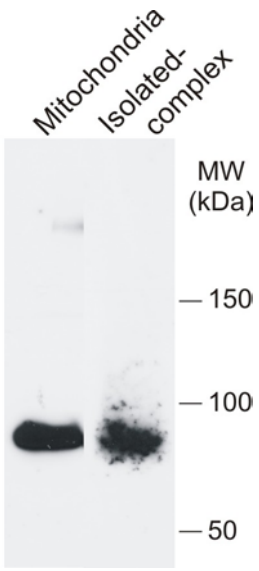


Figure 15. *N. crassa* Tim9 and Tim10 proteins form a hetero-hexamer. Mitochondria solubilized in 0.5% digitonin and the purified Tim9·Tim10 complex were analyzed by blue native gel electrophoresis, followed by immunodecoration with antibodies against Tim9.

To study the oligomeric organization of the purified Tim9·Tim10 complex, the chemical cross-linking assay was used. The purified complex was incubated with glutaraldehyde, and the cross-linking products analyzed by high-tris urea/SDS-PAGE and immunostaining with antibodies against either Tim9 or Tim10 proteins (Figure 16).

In the absence of glutaraldehyde, the Tim9 and Tim10 proteins were found to be monomers. The bands corresponding to the oligomeric species (dimer to hexamer) were observed when the complex was treated with the cross-linker (Figure 16A). As a control, no cross-linking adducts were observed when the cross-linking was performed in the presence of 0.5% SDS (Figure 16B). Upon decoration with the antibodies against the Tim10 protein, at least two different X-linking species of a dimer product could be more readily observed (Figure 16A, right panel). The same bands were observed upon decorations with the antibody

against the histidine-tag (data not shown). Thus, the cross-linking adduct corresponding to the higher molecular weight dimer contains the $Tim10_{his9}$ protein. This suggests that this variation of the Tim10 protein is also a part of the oligomeric structure.

In conclusion, the isolated Tim9·Tim10 complex is a heterohexamer of an approximate molecular weight of 70-80 kDa, equivalent in size and oligomeric state to the native complex.

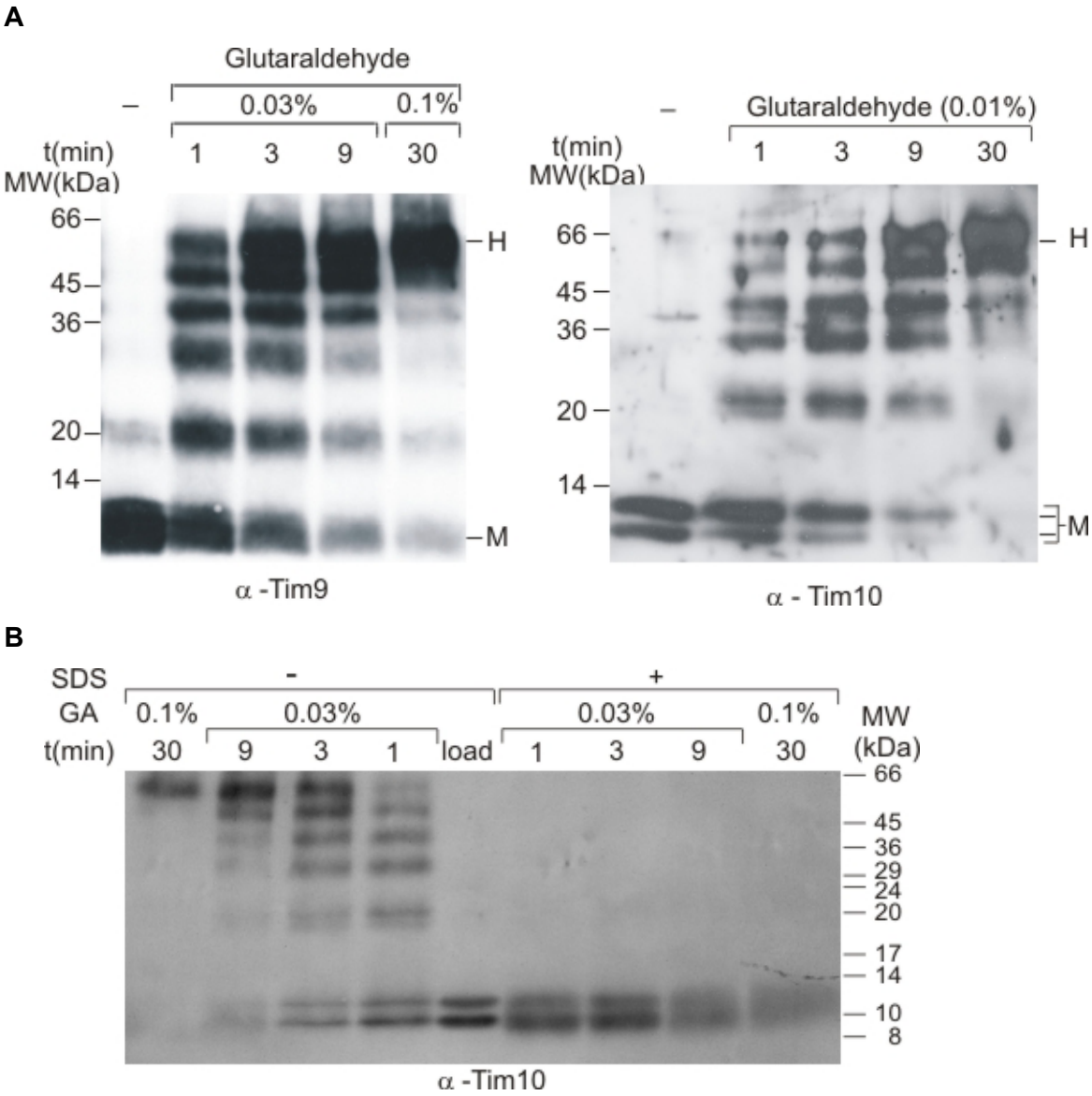


Figure 16. The Tim9 and Tim10 proteins form a heterohexamer. (A) The purified Tim9·Tim10 complex was incubated with glutaraldehyde, at indicated concentrations, for indicated time periods. Cross-linked products were analyzed by SDS-PAGE, followed by immunodecoration with antibodies against Tim9. (M, monomer; H, hexamer). **(B)** The purified complex was treated as above; in some of the samples, as control, the same X-linking procedure was performed in the presence of 0.5% SDS. After high Tris-urea PAGE, the immunodecoration was performed using antibodies against Tim10.

3.5. Initial trials for the crystallisation of the Tim9·Tim10 complex

One of the initial goals was to obtain a three-dimensional structure of the Tim9·Tim10 complex. To investigate the disposition of the complex to form ordered crystals, we have attempted to measure circular dichroism (CD) spectra of the purified complex. However, the complex adhered to the glass of the cuvette of the CD spectropolarimeter and subsequently partially aggregated. Therefore, we were unable to obtain any conclusive data on the secondary structure of the purified Tim9·Tim10 complex. Nevertheless, we probed the ability of the pure fractions of the isolated Tim9·Tim10 complex to crystalise. Using the hanging drop vapour diffusion crystallisation method (Crystal Screen^{TM} kit, Hampton Research, CA) we tested various crystallisation conditions. As none of the tested crystallisation conditions yielded crystals of the Tim9·Tim10 complex, we abandoned this line of research.

3.6. The influence of zinc on the integrity of the purified Tim9·Tim10 complex

It was previously reported that the yeast Tim10p as a recombinant MBP-Tim10p fusion protein binds Zn^{2+} (Sirrenberg *et al.*, 1998). To determine if Zn^{2+} is genuinly essential for the oligomeric state of the purified *N. crassa* Tim9·Tim10 complex, the ability of the complex to refold in the presence of reducing reagents or metal chelators was examined. The treated samples were analysed using BNGE technique. When incubated at low temperatures, the complex proved stable in the presence of metal chelator EDTA or the reducing agent TCEP (Figure 17A). When it was heated to 95°C for 10 min in the presence or absence of EDTA and left to cool on ice, it refolded to its native structure. In contrast, the presence of the reducing reagent TCEP during the procedure led to its dissociation, demonstrated by the disappearance of the bands corresponding to the complex and detection of the monomeric Tim9 and Tim10 proteins (Figure 17A).

As a control, dimerization of the cytosolic domain of the yeast Tom70p which contains three cysteine residues was analysed. The dimer resumed its native conformation after heat denaturation in the presence of both EDTA and TCEP (Figure 17B). Thus, the observed effect of the reducing agent on the stability of the Tim9·Tim10 complex is not a common feature of every protein containing multiple cysteine residues. These results argue that the complex purified from mitochondria isolated under non-reducing conditions does not

require Zn^{2+} for the maintenance of its oligomeric structure. The cysteine residues are rather engaged in the formation of the disulfide bonds.

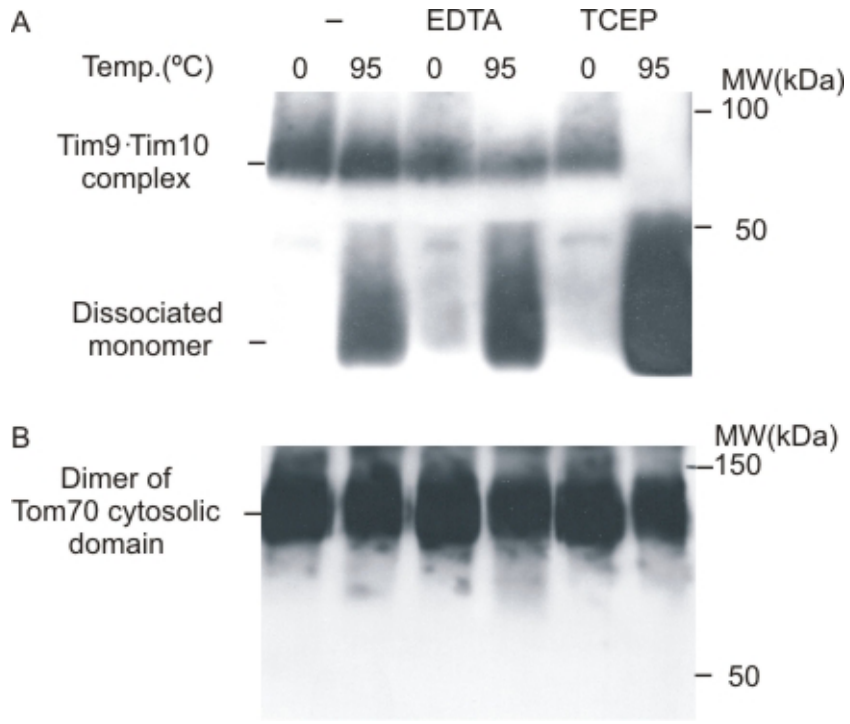


Figure 17. Oligomeric structure of the purified Tim9·Tim10 complex depends on redox conditions. (A) Purified Tim9·Tim10 complex was incubated for 10 min at either 0°C or 95°C in the absence or presence of EDTA (2 mM) or TCEP (1 mM). The samples were kept on ice for further 10 min, and were then analyzed by BNGE followed by blotting and immunodecoration with antibodies against Tim10. **(B)** Purified cytosolic domain of Tom70p was treated as described in A, and the resultant corresponding membrane immunodecorated with antibodies against Tom70p.

Furthermore, determination of zinc content in the purified Tim9·Tim10 complex by atomic absorption spectrometry revealed substoichiometrical zinc to protein ratio of 0.20-0.24 (data not shown). Of note, the complex was purified in the presence of zinc in all buffers employed. These results might indicate that the complex formation and/or maintenance do not require zinc. We can not exclude however the possibility that the zinc-comprising complex of Tim9 and Tim10 proteins can not be purified using the histidine affinity tag approach.

The cross-linking experiments of the purified Tim9·Tim10 complex were repeated in the presence of a cysteine-derivatizing reagent N-ethylmaleimide (NEM) and/or different metal chelators (EDTA, EGTA, o-Phe). These treatments did not affect the formation of the covalently linked hexameric species.

Taken together, these results suggest that the integrity of the Tim9·Tim10 hetero-hexamer does not depend on the coordinative binding of metal ions.

3.7. The purified Tim9·Tim10 complex is functional in binding its substrate proteins

Studies of the yeast Tim9p and Tim10p proteins suggested that they are generally involved in import of the carrier proteins into the mitochondrial inner membrane (Sirrenberg *et al.*, 1998; Koehler *et al.*, 1998a and 1998b). To assert the functionality of the purified *N. crassa* Tim9·Tim10 complex, its ability to interact with its physiological substrate, the precursor of ADP/ATP carrier (AAC), was examined. For that purpose, the ability of excess amounts of the purified complex to compete the *in vitro* import of radiolabelled precursors of AAC2p, and of control pSu9(1-69)DHFR proteins, was tested. The Tim9·Tim10 complex of *N. crassa* inhibited the import of the carrier protein almost entirely, while the import of the matrix-destined protein was not affected (Figure 18A). A slight increase in the import of pSu9(1-69)DHFR precursor in the presence of *N. crassa* Tim9·Tim10 complex did not result from the partial loss of the outer membrane integrity, since the intermembrane space proteins D-lactate dehydrogenase and the Tim13p protein were not degraded by the added protease, demonstrated through immunodecorations (data not shown).

The Tom70p protein was found to be the main receptor for the AAC precursor. In the absence of the Tom70p receptor, AAC2p is imported via a considerably less efficient bypass pathway (Hines *et al.*, 1990). Therefore, the significant inhibition in the AAC import observed in our experiments could have resulted from the possible interaction of the *N. crassa* Tim9·Tim10 complex with the Tom70p receptor. Furthermore, the addition of the purified antibodies against *N. crassa* Tom70 protein to the isolated *N. crassa* mitochondria prior to the *in vitro* import significantly reduced the import of the radioactively labelled AAC2p precursor, but neither of the yeast Tim13p nor the *N. crassa* Tim10 proteins (data not shown). However, import into isolated yeast mitochondria from a strain lacking this receptor protein was also competed by the *N. crassa* Tim9·Tim10 complex (Figure 18B).

Additional evidence for a direct interaction between the *N. crassa* Tim9·Tim10 complex and its substrate came from the immunoprecipitation experiments. Significant amounts of the *in vitro* synthesized, radioactively labelled AAC protein, preincubated with the *N. crassa* Tim9·Tim10 complex, were immunoprecipitated with the antibody against the *N. crassa* Tim9 protein (data not shown). No specific precipitation of control protein pSu9(1-69)DHFR was observed. Moreover, upon incubation of the complex with a mixture of porin and AAC2p isolated from yeast cells (Panneels, 2003), the antibodies against *N. crassa* Tim9

protein specifically precipitated the AAC2p protein, but not porin (Figure 19 and data not shown).

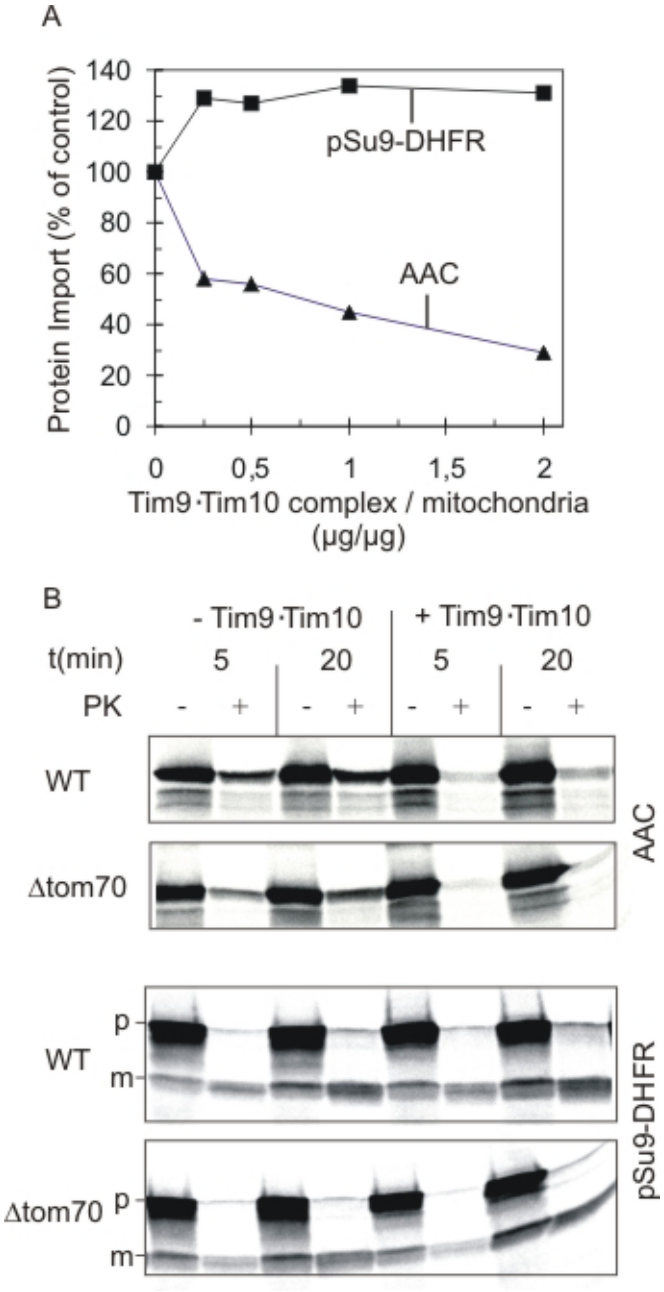


Figure 18. The purified Tim9·Tim10 complex externally added to mitochondria inhibits import of the AAC precursor *in vitro.* (A) Indicated amounts of purified Tim9·Tim10 complex were incubated for 10 min at 0°C with radiolabeled AAC precursor, and as a control, pSu9-DHFR. These mixtures were then added to import buffer containing 40 µg of wild type mitochondria. After incubation for 10 min at 25°C proteinase K was added. Imported proteins were analyzed by SDS–PAGE. The amount of import in the absence of added Tim9·Tim10 complex was taken as 100%. **(B)** Mitochondria from wild type and from a Tom70p null strain were incubated either with purified Tim9·Tim10 complex or mock treated for 2 min at 0°C (lanes +/-Tim9·Tim10). They were then added to import buffer containing radiolabeled AAC, and as a control, pSu9-DHFR. After incubation for 5 or 20 min, mitochondria were reisolated, resuspended in SH buffer and kept on ice. One half of each sample was then treated with proteinase K (+PK) and the other left untreated (-PK). Imported proteins were analyzed by SDS–PAGE. (p, precursor; m, mature form).

When the Tim9·Tim10 complex was omitted from the reaction mixture, and when the preimmune serum or an antibody against an unrelated protein Fis1p was used, the immunoprecipitation did not surpass background levels (Figure 19 and data not shown). To summarise these results, the purified Tim9·Tim10 complex specifically recognizes its physiological substrates.

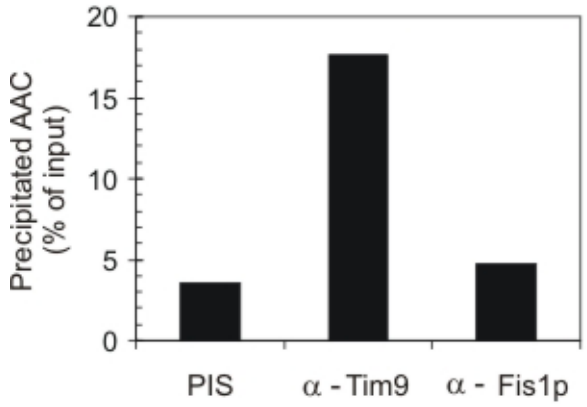
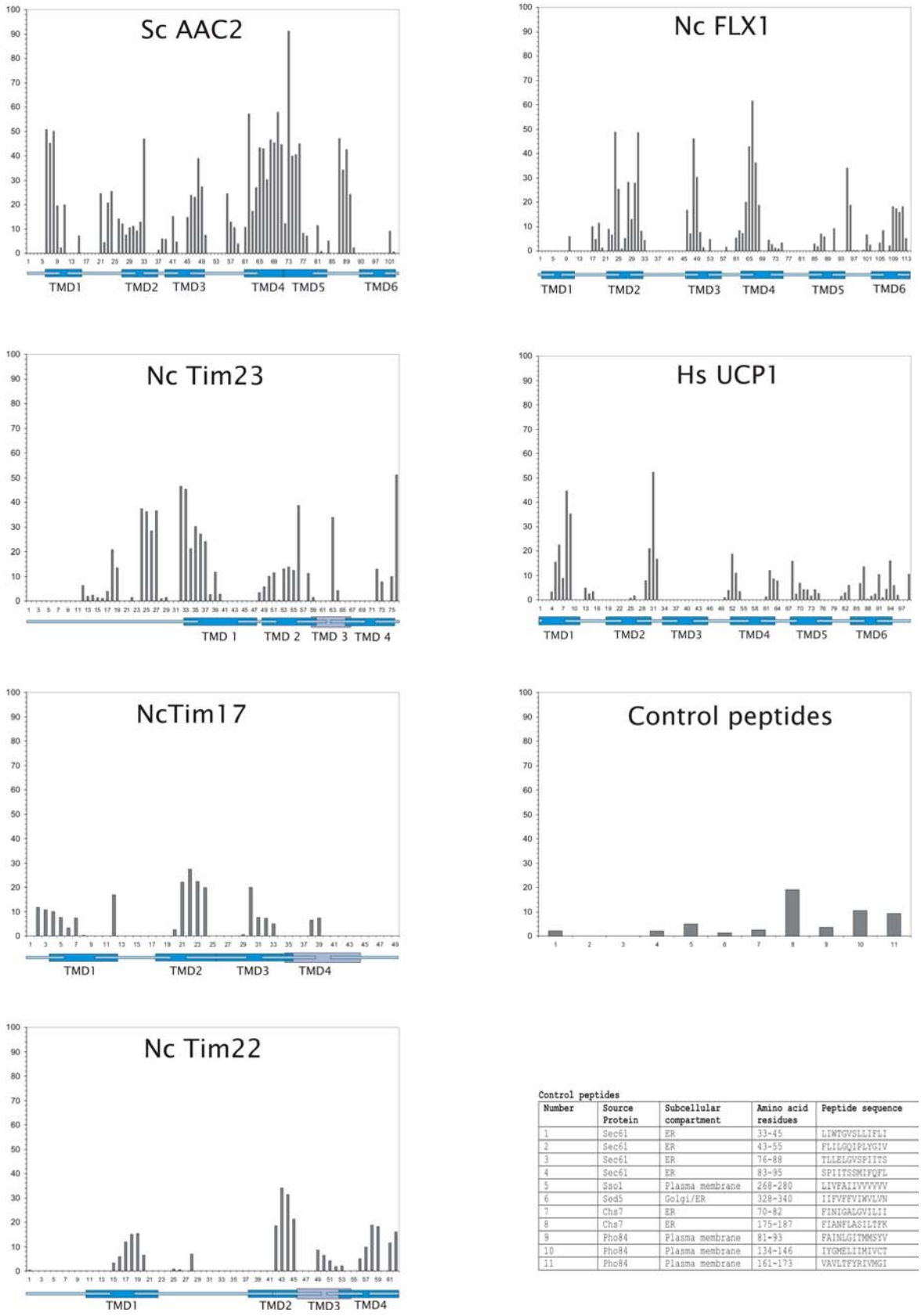


Figure 19. The purified Tim9·Tim10 complex binds the AAC precursor. Purified Tim9·Tim10 complex was incubated with a mixture of porin and AAC2p for 30 min at 4°C. The mixture was then split into three aliquots which were added to protein A-coupled sepharose beads containing prebound antibodies from preimmune serum or antibodies against either *N. crassa* Tim9 or *S. cerevisiae* Fis1p. After incubation for 3 hr at 4°C, the beads were pelleted and proteins in the pellets were subjected to SDS-PAGE, blotting and immunodecoration with antibodies against AAC2p. The amount of added AAC2p was taken as 100%.

3.8. Identification of the sequences in protein substrates recognised by the Tim9·Tim10 complex

Next, we wanted to determine what sequences the *N. crassa* Tim9·Tim10 complex can bind within its various substrates. Previously, it was suggested that the complex recognizes a conserved sequence motif found in each of the three intermembrane space loops of the carrier proteins (Sirrenberg *et al.*, 1998; Endres *et al.*, 1999). Alternatively, it was proposed that the *S. cerevisiae* Tim9p·Tim10p complex could function as a chaperone in the intermembrane space by binding the exposed hydrophobic sequences of the unfolded precursor proteins (Koehler, 1999).

To map the exact substrate regions to which *N. crassa* Tim9·Tim10 complex binds, peptide libraries of the following proteins were screened: AAC2p from *S. cerevisiae*, Tim17, Tim22, Tim23 and FLX1 proteins (a homologue of the yeast FLX1, a carrier-type FAD-transporter) from *N. crassa*, and the human UCP1 (uncoupler protein) (Figure 20).



Control peptides

Number	Source Protein	Subcellular compartment	Amino acid residues	Peptide sequence
1	Sec61	ER	33-45	LIWTGVSLLIFLI
2	Sec61	ER	43-55	FLILGQIPLYGIV
3	Sec61	ER	76-88	TLLELGVSPIITS
4	Sec61	ER	83-95	SPIITSSMIFQFL
5	Ssol	Plasma membrane	268-280	LIVFAIIVVVVVV
6	Sed5	Golgi/ER	328-340	IIFVFFVIWVLVN
7	Chs7	ER	70-82	FINIGALGVILII
8	Chs7	ER	175-187	FIANFLASILTFK
9	Pho84	Plasma membrane	81-93	FAINLGITMMSYV
10	Pho84	Plasma membrane	134-146	IYGMELIIMIVCT
11	Pho84	Plasma membrane	161-173	VAVLTFYRIVMGI

Figure 20. Substrate recognition by the Tim9·Tim10 complex. A peptide library on a cellulose membrane, corresponding to the indicated proteins and control peptides, was incubated with purified Tim9·Tim10 complex. Bound proteins were blotted to PVDF membranes and decorated with antibodies against Tim9. Binding was quantified by scanning densitometry of three independent experiments. The intensity of the strongest spot from each experiment was set to 100. Below each peptide, it is indicated whether its sequence covers a transmembrane domain (TMD), a loop or if its sequence stretches over both types of structure.

These librarires commonly comprise of 13mer peptides covering the whole primary sequence of a given protein, with 10 residues-overlaps. The yeast carrier was chosen to allow the comparison with experiments performed with the yeast small Tim proteins (Curran *et al.*, 2002a). The radioactively labelled precursor of *S. cerevisiae* AAC2p was efficiently imported into the isolated *N. crassa* mitochondria (data not shown), supporting combining of translocons of different taxonomic groups as feasible. Control peptides covering transmembrane domains of various non-mitochondrial proteins were also included in the screen (origin and sequences of control peptides are given in Figure 20). All peptides were spotted onto one membrane allowing direct comparison of different proteins.

Analysis of the binding sequences of the *N. crassa* Tim9·Tim10 complex revealed a strong general inclination towards peptides corresponding to transmembrane domains (TMD) and their flanking regions. Among the analyzed proteins, the highest affinity was observed towards the AAC2p protein (Figure 20). The strongest interaction was with the peptides covering parts of its fourth TMD, followed by the TMDs 1, 3 and 5.

Binding to Tim22 protein was relatively weak and it occurred mainly in regions covering its TMDs, rather than the intermembrane space loops. Interaction of the Tim9·Tim10 complex with the human Ucp1 was less restricted to TMDs, yet the highest affinity observed was still towards the putative TMDs 1 and 2.

One unexpected finding of the screen was the significant binding of the complex to two segments of the *N. crassa* Tim23 protein (Figure 20). A possible interaction of the yeast Tim9p and Tim10p proteins with the yeast Tim23p protein is still under debate (Leuenberger *et al.*, 1999; Davis *et al.*, 2000). Peptides covering segments of the *N. crassa* Tim23 protein identified in the screen to be bound by the purified Tim9·Tim10 complex with considerable affinity were the putative intermembrane space domain (peptides 24-27, residues 70-91) and the interface between the intermembrane space domain and the first predicted TMD (peptides 33-37, residues 97-121) (Figure 20). Both regions are highly conserved in the Tim23 proteins from different organisms (Figure 21).

Binding to the hydrophobic non-mitochondrial control peptides was weak (Figure 20). Moreover, from 20 peptides displaying the highest affinity in both AAC2p (Figure 22) and FLX1 (data not shown), nineteen comprised at least one charged residue within their sequence. The Tim9·Tim10 complex displayed the highest affinity prevalently towards peptides covering interface regions between the membrane spanning segments and the soluble loops (Figure 22) and not those covering midsections of the putative TMDs.

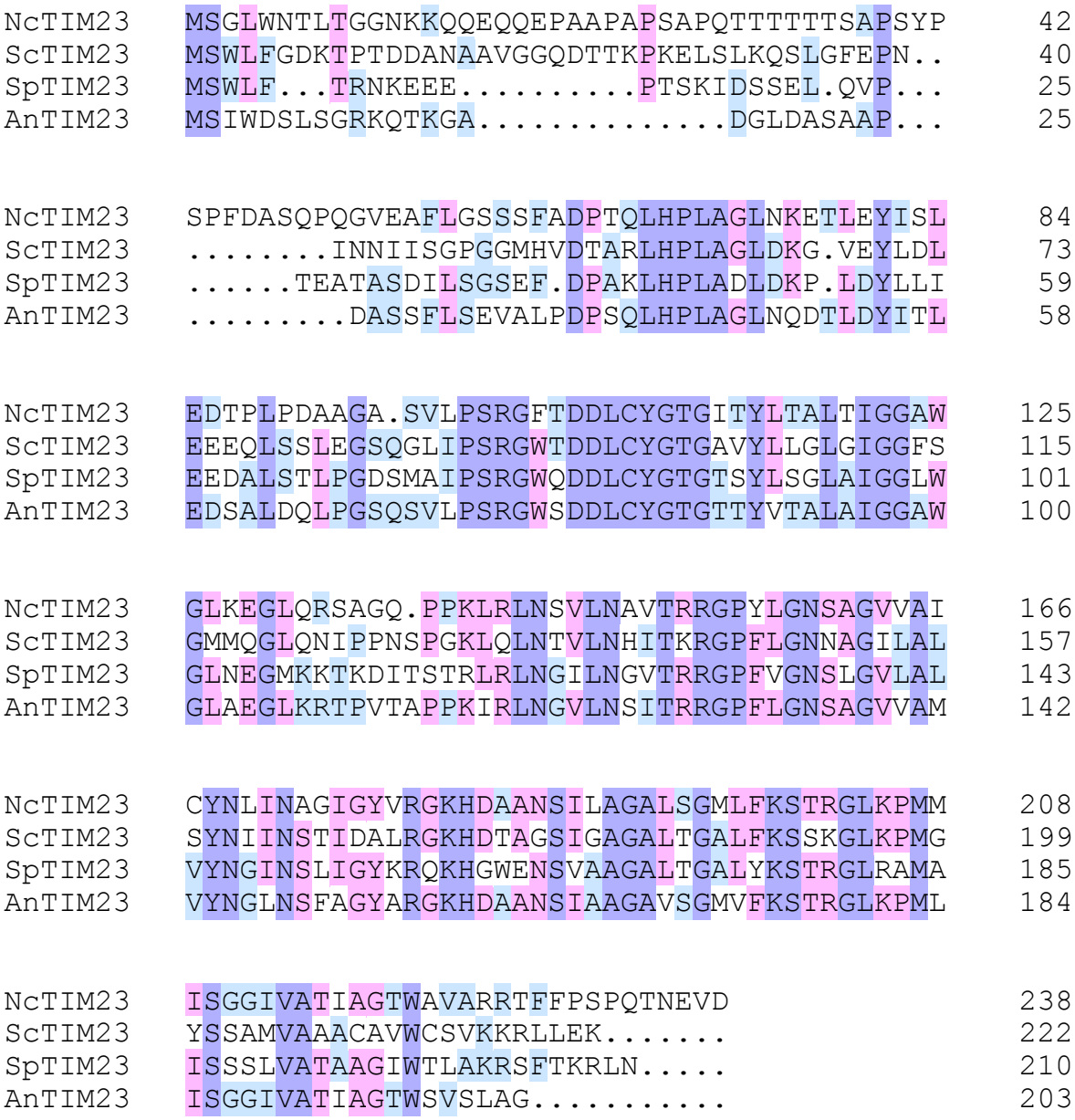


Figure 21. Protein sequence alignment of Tim23 proteins from different organisms. Identical residues occurring in all organisms are indicated in darker violet, 75% similar in light blue and 50% similar in pink (An, *Aspergillus nidulans*; Nc, *Neurospora crassa*; Sp, *Saccharomyces pombe*; Sc, *Saccharomyces cerevisiae*). The segments to which Tim9·Tim10 complex binds with highest affinity cover amino acid residues 70-91 and 97-121 in *N. crassa* Tim23 protein.

Among AAC2p-derived peptides, *N. crassa* Tim9·Tim10 complex demonstrated the highest binding towards a segment covering a part of an intermembrane space loop and the beginning of the fifth TMD. A peptide covering the same region in the phosphate carrier was found to be among several internal segments that interact with Tom receptors and was the only peptide to interact with the purified Tim22p (Brix *et al.*, 1999; Kovermann *et al.*, 2002).

Throughout the mitochondrial carrier family, amino acid sequences related to this stretch are found to be conserved (Nelson *et al.*, 1998).

Hence, it ensues that hydrophobicity is not a crucial parameter for substrate recognition by the Tim9·Tim10 complex. Segments to which Tim9·Tim10 complex preferentially binds are instead characterised by the comprisal of at least one polar residue and cover transition regions between transmembrane domains and the neighbouring loops.

Peptides AAC2

No.	Sequence	%	No.	Sequence	%	No.	Sequence	%
1.	MSSNAQVKTPLPP	0	36.	**AF**KDKIKAMFGFK	0	71.	YDSLKPLLLTGSL	45
2.	NAQVKTPLPPAPA	0	37.	DKIKAMFGFKKEE	1	72.	LKPLLLTGSLEGS	12
3.	VKTPLPPAPAPKK	0	38.	KAMFGFKKEEGYA	6	73.	LLLTGSLEGS**FLA**	91
4.	PLPPAPAPKKESN	0	39.	FGFKKEEGYAKWF	6	74.	TGSLEGS**FLASFL**	40
5.	PAPAPKKESNFLI	0	40.	KKEEGYAKWFAGN	0	75.	LEGS**FLASFLLGW**	41
6.	APKKESNFLID**FL**	51	41.	EGYAKWFAGN**LAS**	15	76.	S**FLASFLLGWVVT**	45
7.	KESNFLID**FLMGG**	45	42.	AKWFAGN**LASGGA**	5	77.	**ASFLLGWVVTTGA**	8
8.	NFLID**FLMGGVSA**	50	43.	FAGN**LASGGAAGA**	0	78.	**LLGWVVTTGASTC**	7
9.	ID**FLMGGVSAAVA**	19	44.	N**LASGGAAGALSL**	0	79.	**WVVTTGASTCSYP**	0
10.	**LMGGVSAAVAKTA**	2	45.	**SGGAAGALSLLFV**	15	80.	**TTGASTCSYPL**DT	0
11.	**GVSAAVAKTAASP**	19	46.	**AAGALSLLFVYSL**	24	81.	**ASTCSYPL**DTVRR	11
12.	**AAVAKTAASPI**ER	0	47.	**ALSLLFVYSL**DYA	23	82.	**CSYPL**DTVRRRMM	1
13.	**AKTAASPI**ERVKL	0	48.	**LLFVYSL**DYARTR	39	83.	**PL**DTVRRRMMMTS	0
14.	**AASPI**ERVKLLIQ	0	49.	**VYSL**DYARTRLAA	27	84.	TVRRRMMMTSGQA	5
15.	**PI**ERVKLLIQNQD	7	50.	**L**DYARTRLAADSK	8	85.	RRMMMTSGQAVKY	0
16.	RVKLLIQNQDEML	0	51.	ARTRLAADSKSSK	0	86.	MMTSGQAVKYDGA	0
17.	LLIQNQDEMLKQG	0	52.	RLAADSKSSKKGG	0	87.	SGQAVKYDGAFDC	47
18.	QNQDEMLKQGTLD	0	53.	ADSKSSKKGGARQ	0	88.	AVKYDGAFDCLRK	34
19.	DEMLKQGTLDRKY	0	54.	KSSKKGGARQFNG	0	89.	YDGAFDCLRKIVA	43
20.	LKQGTLDRKYAGI	0	55.	KKGGARQFNGLID	0	90.	AFDCLRKIVAAEG	24
21.	GTLDRKYAGILDC	25	56.	GARQFNGLIDVYK	24	91.	CLRKIVAAEGVGS	2
22.	DRKYAGILDCFKR	4	57.	QFNGLIDVYKKTL	13	92.	KIVAAEGVGSLFK	0
23.	YAGILDCFKRTAT	21	58.	GLIDVYKKTLKSD	11	93.	AAEGVGSLFK**GCG**	0
24.	ILDCFKRTATQEG	25	59.	DVYKKTLKSDGVA	4	94.	GVGSLFK**GCGANI**	0
25.	CFKRTATQEGVIS	0	60.	KKTLKSDGVAGLY	0	95.	SLFK**GCGANILRG**	0
26.	RTATQEGVISFWR	14	61.	LKSDGVAGLYR**GF**	11	96.	K**GCGANILRGVAG**	0
27.	TQEGVISFWR**GNT**	12	62.	DGVAGLYR**GFLPS**	57	97.	**GANILRGVAGAGV**	0
28.	GVISFWR**GNTANV**	8	63.	AGLYR**GFLPSVVG**	17	98.	**ILRGVAGAGVISM**	0
29.	SFWR**GNTANVIRY**	11	64.	YR**GFLPSVVGIVV**	27	99.	**GVAGAGVISMY**DQ	0
30.	R**GNTANVIRYFPT**	11	65.	**FLPSVVGIVVYRG**	43	100.	**GAGVISMY**DQLQM	0
31.	**TANVIRYFPTQAL**	9	66.	**SVVGIVVYRGLYF**	43	101.	**VISMY**DQLQMILF	9
32.	**VIRYFPTQALNFA**	13	67.	**GIVVYRGLYFGM**Y	30	102.	**MY**DQLQMILFGKK	1
33.	**YFPTQALNFAF**KD	47	68.	**VYRGLYFGM**YDSL	47	103.	DQLQMILFGKKFK	0
34.	**TQALNFAF**KDKIK	0	69.	**GLYFGM**YDSLKPL	45			
35.	**LNFAF**KDKIKAMF	0	70.	**FGM**YDSLKPLLLT	59			

Figure 22. Binding of the *N. crassa* Tim9·Tim10 complex to the peptide library covering AAC2p. The sequences of 103 peptides covering primary structure of AAC2p are presented; amino acid residues belonging to the transmembrane domains are underlined and bold. Intensity of binding to each peptide is given in percentages; binding levels higher than 30% of the maximal value are indicated by bigger digits.

3.9. Tim23 protein is a substrate of the Tim9·Tim10 complex in N. crassa

To substantiate the proposed interaction of the Tim9·Tim10 complex with the Tim23 precursor protein, we used the chemical cross-linking approach. When the radiolabelled precursor of Tim23 was incubated with de-energized mitochondria, an import intermediate was formed. It was accessible to external protease, but partially translocated into the intermembrane space, thereby being equivalent to the well defined stage III intermediate of the AAC precursor (Pfanner and Neupert, 1987). Under these conditions, the addition of the bifunctional cross-linking reagent m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) resulted in the formation of the distinct cross-linking adducts that specifically immunoprecipitated with the antibodies against Tim9, Tim10 and Tim8 proteins (Figure 23). The precursor bound to mitochondria was degraded by the externally added proteinase K, as opposed to the cross-linking adduct which was inaccessible to it.

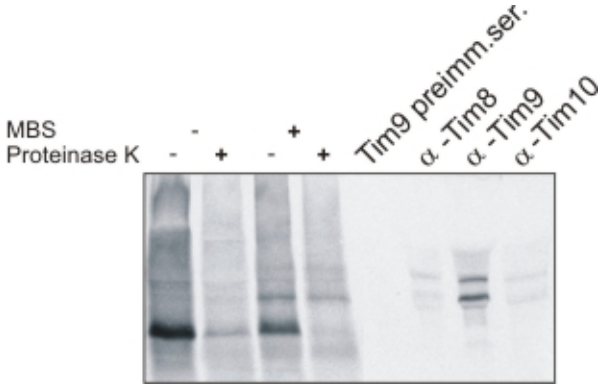


Figure 23. The Tim23 protein is in the vicinity of Tim9, Tim10 and Tim8 proteins on its mitochondrial translocation pathway. The radiolabelled Tim23 precursor was incubated with de-energized mitochondria in the presence or absence of the chemical cross-linker MBS for 30 min at 15°C. Reactions were then quenched by the addition of glycine (pH 8.8) to a final concentration f 100 mM, and one half of the sample was treated with proteinase K. The mitochondria of all four samples were pelleted and subjected to SDS-PAGE. Another sample treated with MBS but not with PK (+MBS, -PK) was subjected to immunoprecipitation with antibodies against the indicated TIM proteins or with preimmune serum. The immunoprecipitates were solubilized in sample buffer and analyzed by SDS-PAGE and autoradiography.

These results indicate that the Tim23 precursor molecules associated with the Tim9·Tim10 complex were translocated across the outer mitochondrial membrane. Thus, while crossing the intermembrane space on its way into the inner membrane the precursor of *N. crassa* Tim23 protein interacts with both Tim9·Tim10 and Tim8·Tim13 complexes.

3.10. The TOM core complex and the Tim9·Tim10 complex are sufficient for the import of the ADP/ATP carrier across the outer membrane of mitochondria

The most competent way to certify the utter sufficiency of certain components in a particular translocation process across biological membranes *in vitro* is to use the reconstituted system. We employed the reconstituted *in vitro* system to examine if some as yet unknown components in the internal compartments of mitochondria are required for translocating the AAC precursor across the mitochondrial outer membrane. Detergent solutions of the purified TOM core complex (Ahting *et al.*, 1999) and *E. coli* lipids were combined, the detergent was removed by polystyrene beads and thereby the proteoliposomes were formed. In certain cases, the enzyme lactate dehydrogenase (LDH) was also enclosed in the vesicles and its activity was used to essay the pore forming ability of the reconstituted TOM complex. When the substrates of LDH, NADH and pyruvate, were added to vesicles containing either the reconstituted TOM core complex or porin (a control pore forming protein), free influx of the externally added LDH-substrates was observed (data not shown) (Künkele *et al.*, 1998a). The Tim9·Tim10 complex was added to some of the reconstitution reactions, resulting in its enclosure within the proteoliposomes. In Figure 24A the protein composition of the lipid vesicles containing the TOM core complex alone or in combination with the enclosed Tim9·Tim10 complex is depicted.

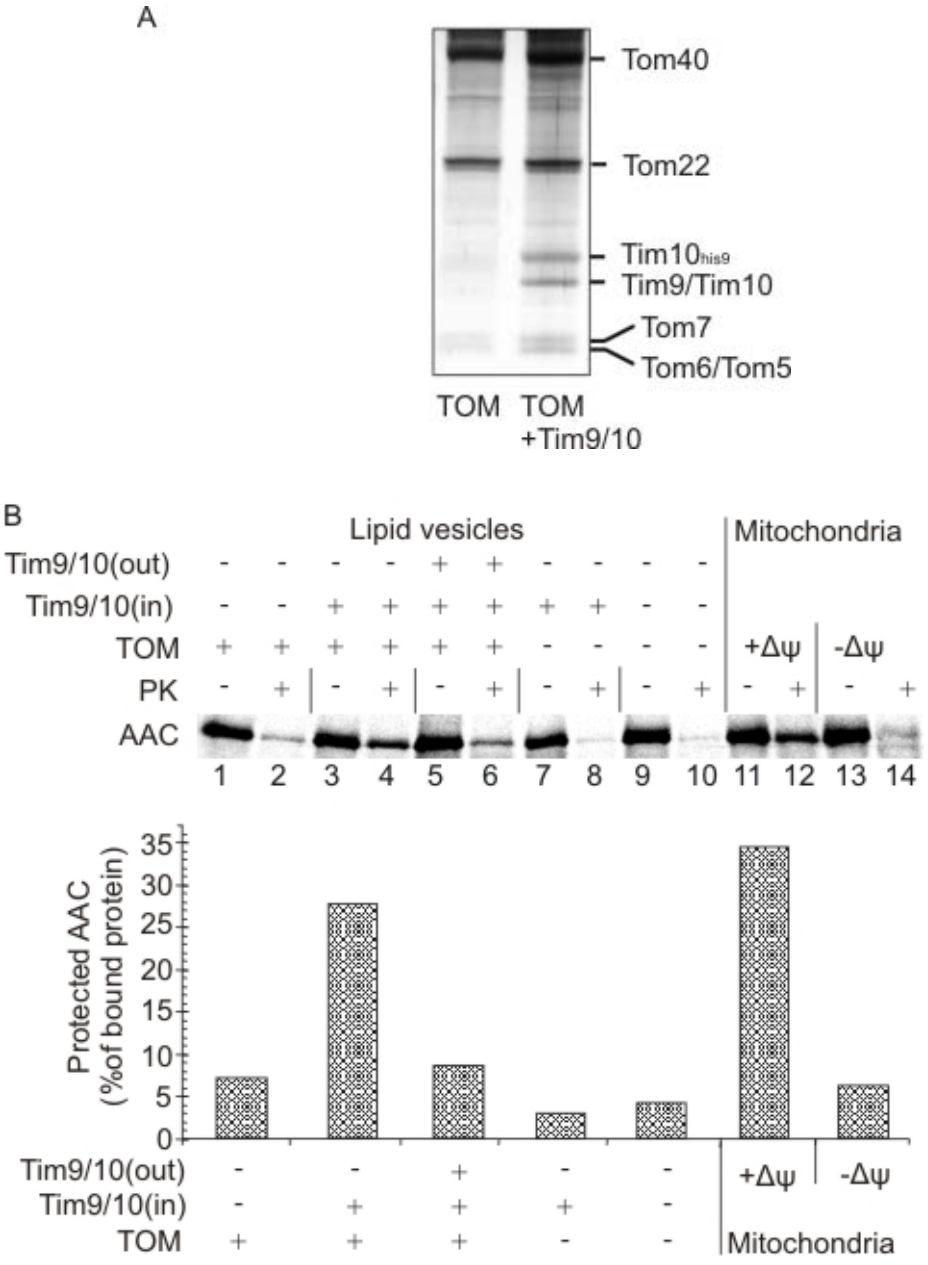
Translocation of the radiolabelled carrier precursor across the membranes of proteoliposomes was assessed through resistance to the externally added protease. Some unspecific association of the AAC protein with the pure lipid vesicles was observed, due to overall hydrophobic nature of the carrier protein (Figure 24B, lanes marked -PK). With the TOM core or the Tim9·Tim10 complex reconstituted into vesicles separately, the amount of the protease-protected carrier protein was negligible (Figure 24B, lanes 2 and 8, respectively). However, the amount of protected material increased by about four fold to levels similar to those observed with intact mitochondria, when vesicles containing both the TOM complex and the Tim9·Tim10 complex were used (Figure 24B, lanes 4 and 12). The specificity of this observation was supported by several control experiments:

i. import of the carrier precursor was competed out with the externally added Tim9·Tim10 complex (Figure 24B, lane 6);

ii. complete degradation of the imported AAC precursor by proteinase K (PK) occurred upon solubilization of the proteoliposomes by Triton-X100 (not shown);

iii. the degree of protection of the AAC precursor against PK was of the background level (data not shown), when porin was reconstituted into lipid vesicles containing the enclosed Tim9·Tim10 complex.

In conclusion, the TOM core complex and the Tim9·Tim10 complex meet all the requirements for the productive transfer of the ADP/ATP carrier across the outer membrane of mitochondria.



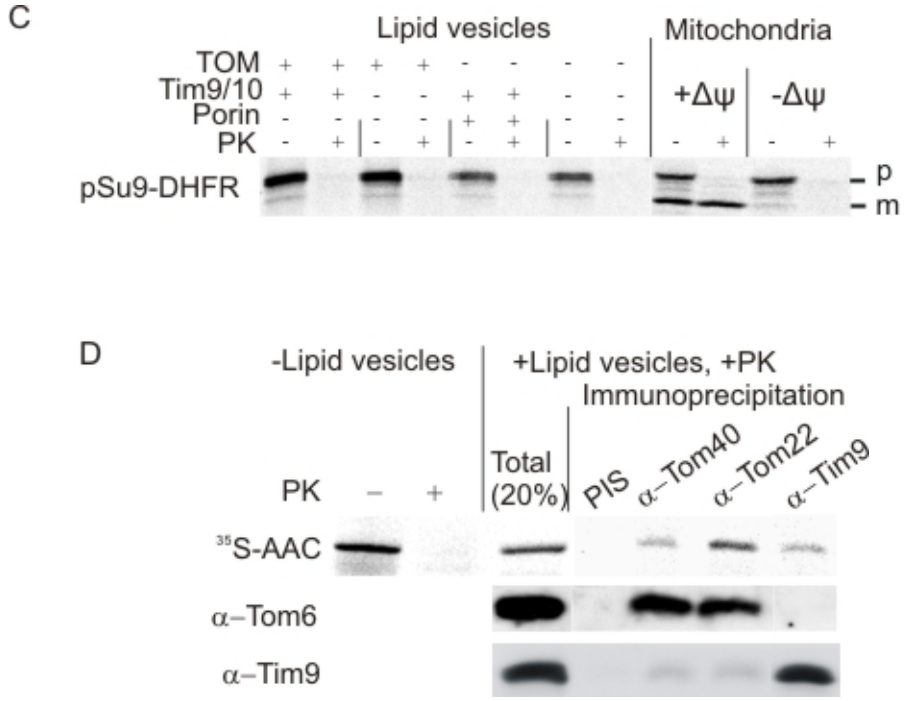


Figure 24. Translocation of the AAC precursor into proteoliposomes containing the TOM core complex and the Tim9·Tim10 complex. (A) Proteins of proteoliposomes containing either only the TOM core complex (TOM) or in addition Tim9·Tim10 complex (TOM+Tim9/10) were analyzed by SDS-PAGE and silver staining. **(B)** Import of AAC into proteoliposomes containing TOM core complex and Tim9·Tim10 complex. Radiolabeled AAC precursor was incubated with proteoliposomes (50 µg lipids) in the absence (lanes 1-4, and 7-10) or presence (lanes 5-6) of externally added Tim9·Tim10 complex. The following proteoliposomes were used: lanes 1-2, liposomes containing TOM core complex; lanes 3-6, liposomes containing TOM complex and enclosed Tim9·Tim10 complex; lanes 7-8, liposomes containing enclosed Tim9·Tim10 complex; and lanes 9-10, liposomes without reconstituted proteins. Import of AAC precursor into intact mitochondria in the absence (+ΔΨ, lanes 11–12) or presence (-ΔΨ, lanes 13-14) of valinomycin is shown for comparison. After import, vesicles and mitochondria were reisolated by centrifugation, resuspended in buffer and halved. One half was left untreated (-PK) while the other was treated with 50 µg/ml proteinase K (+PK). The samples were precipitated with TCA and subjected to SDS–PAGE and autoradiography (upper panel). The proportions of bound AAC precursor which were resistant to proteinase K treatment were determined (lower panel). **(C)** Import of pSu9-DHFR into proteoliposomes containing TOM core complex and Tim9·Tim10 complex. Radiolabeled pSu9-DHFR precursor was incubated with proteoliposomes or with mitochondria as described above (p, precursor; m, the mature form). **(D)** AAC precursor interacts simultaneously with TOM and Tim9·Tim10 complexes. Radiolabeled AAC precursor was incubated for 30 min at 25°C with proteoliposomes containing the TOM core and the Tim9·Tim10 complexes. After the import reaction, proteolipisomes were treated with proteinase K and re-isolated. Vesicles were solubilized in digitonin (1%) and immunoprecipitation was performed. Precipitated proteins were analyzed by SDS-PAGE, blotting and autoradiography (^{35}S-AAC) and immunodecoration with the antibodies indicated at the left side.

4. Discussion

Members of the mitochondrial protein translocation machinery have been identified and subjected to detailed studies in yeast, *Neurospora*, higher plants and mammals. Homologues of *TIM9* and *TIM10* genes have been identified in genomes of all investigated eukaryotic organisms (Bauer *et al.*, 1999; Kayingo *et al.*, 2000), yet only in *S. cerevisiae* they have been the subject of elaborate research. In this study, we have identified and isolated the Tim9 and Tim10 mitochondrial intermembrane space proteins of *N. crassa* and investigated their structural and functional properties. Their role in the translocation of precursors of the mitochondrial metabolite carrier family members across the mitochondrial outer membrane was the major focus of this study. We identified the specific segments within the sequences of substrates of the Tim9·Tim10 complex which are recognised by this complex, and defined the minimal machinery sufficient for the translocation of its major substrate, the ADP/ATP carrier (AAC), across the mitochondrial outer membrane. These data allowed us to propose a working model for the translocation of carrier proteins across the outer membrane.

4.1. Structural organization of the N. crassa Tim9·Tim10 complex

The Tim9 and Tim10 proteins of *N. crassa*, identified in the scope of this study, are soluble proteins of the mitochondrial intermembrane space, organised in an oligomeric complex of around 70-80 kDa. To characterise it more closely, we isolated and purified the complex of Tim9 and Tim10 proteins from the intermembrane space of *N. crassa* mitochondria. Chemical cross-linking experiments elucidated the 70 kDa oligomer as a hexamer. Such mode of oligomeric organisation of small Tim proteins is conserved, which infers its importance for the fulfilment of their function. A complex similar to the Tim9·Tim10 complex of *N. crassa* has also been characterised in yeast (Sirrenberg *et al.*, 1998; Koehler *et al.*, 1998; Adam *et al.*, 1999). Furthermore, a 70 kDa complex comprising Tim9 and Tim10a proteins has recently been identified in human mitochondria as well (Mühlenbein *et al.*, 2004). All these 70 kDa complexes are most probably composed of three Tim9 and three Tim10 molecules, as experimentally verified with the yeast components (Luciano *et al.*, 2001; Curran *et al.*, 2002a; Vial *et al.*, 2002). The other two members of the conserved family of small Tim proteins in *N. crassa*, Tim8 and Tim13, pair exclusively with each other, constituting another hetero-oligomeric 70 kDa complex in the intermembrane

space (Hoppins *et al.*, 2004). Yeast and human counterparts of these two proteins are also characterised by a similar mode of structural arrangement (Leuenberger *et al.*, 1999; Koehler *et al.*, 1999; Paschen *et al.*, 2000; Rothbauer *et al.*, 2001).

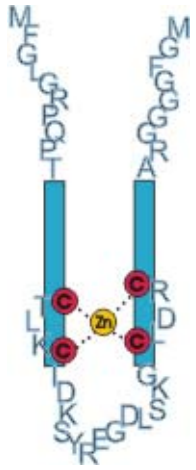


Figure 25. Hypothetical model of the secondary structure of the Tim10 protein. (Program used to predict the secondary structure of the Tim10 protein can be found on the web site: **http://www.expasy.org/tools/#secondary**).

The purified Tim9·Tim10 complex seems to be quite stable under variety of different conditions, like high salt concentrations, sonication, and relatively high detergent amounts, and even to be able to refold into a hexamer upon exposure to extreme temperatures (95ºC). This, together with the relatively simple secondary structure predictions for the small Tim proteins (Figure 25), and the potential stabilization of the ensuing 3D structure through zinc binding, led us to attempt to obtain crystals and solve the crystal structure of the Tim9·Tim10 complex. Attempts to confirm the secondary structure predictions through CD spectrometry failed due to the unspecific adherence to equipment and consequential aggregation of the Tim9 and Tim10 proteins. Also, under all different conditions of crystallization screens which we tested, we did not observe crystal formation.

4.2. Zinc binding and the redox state of Tim9 and Tim10 proteins

The mitochondrial intermembrane space is considered to be in a complete redox equilibrium with the reducing cytoplasm. Therefore, the small Tim proteins were also assumed to be in a reduced state. Due to the presence of two CX_3C motives in their primary sequence, which are conserved in all identified small Tim proteins (Sirrenberg *et al.,* 1998; Koehler *et al*., 1998; Adam *et al.*, 1999, Koehler *et al.*, 1999; Leuenberger *et al.*, 1999; Paschen *et al*., 2000; Mühlenbein *et al.*, 2004), they were suggested to bind zinc.

However, although we included zinc in all steps of the purification procedure, only substoichiometric zinc to protein ratios in the purified *N. crassa* Tim9·Tim10 complex were measured. Moreover, formation of their hexameric cross-linking species was neither affected by the treatment with a variety of metal chelators, nor with the cysteine alkylating reagents. We observed the inhibition of refolding of the complex into hexameric structures in the presence of a reducing reagent after the heat-treatment. These data strongly pointed towards the possibility that the cysteines of the purified Tim9·Tim10 complex were in an oxidized state. This however, might also be the result of an inability to reverse their oxidation which could have occurred during the mitochondrial isolation procedure, despite the presence of the reducing reagent throughout the subsequent purification procedure of the Tim9·Tim10 complex.

In recent publications (Curran *et al.*, 2002a; Curran *et al.*, 2002b; Lu *et al.*, 2004a; Lu *et al.*, 2004b), the same four cysteine residues of the purified yeast recombinant Tim9p·Tim10p and Tim8p·Tim13p complexes have been found to be involved in intramolecular disulfide bond formation. In short, the authors purified the recombinant Tim9p·Tim10p and Tim8p·Tim13p complexes and employed the thiol-trapping method for ascertaining the redox status of the cysteins of small Tim proteins. When the cysteine residues were reduced, they were available for the alkylation reaction to take place, and they therefore showed decreased electrophoretic mobility. The used alkylating reagent (4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid, AMS) adds 0.5 kDa to the molecular weight of each thiol group (which amounts to 2 kDa per small Tim protein). As controls, they pretreated some samples with either hydrogen peroxide or a reducing reagent. They found the subunits of their purified complexes to migrate as the same molecular weight species as the proteins from the hydrogen peroxide-pretreated control sample. Moreover, one other research group (Lu *et al.*, 2004a) has even shown that once oxidized, cysteines of the Tim9p and Tim10p proteins are buried in the structure beyond the reach of a reducing reagent, i.e., they are no longer eligible for reduction. They argued that both reduced and oxidized forms of the Tim9p·Tim10p complex have a certain physiological relevance, and that *in vivo*, the structure of the complex may be redox dependent. In the reducing environment of the cytoplasm, and on their route to mitochondria, yeast small Tims are reduced and largely unstructured. Upon their import into mitochondria, intramolecular bonds are proposed to be formed, which induce conformational changes of monomeric Tim proteins into assembly-competent forms. So far, there have been no reports of the purified zinc-containing native complexes of the small Tim proteins, from yeast and *Neurospora* (Curran *et al.*, 2002a, Vasiljev *et al.*, 2004). The purified

complexes of the recombinant yeast small Tim proteins without a tag or with a GST tag, from the groups of Koehler (Curran *et al.*, 2002a and 2002b) and Tokatlidis (Lu *et al.*, 2004a and 2004b), did not demonstrate any zinc-binding either.

For the yeast Tim13p protein, Lutz *et al.* (2003) have on the other hand shown that it is largely reduced *in vivo*. This time, the aforementioned thiol-trapping approach was applied to yeast spheroplasts isolated under nitrogen atmosphere. Virtually all endogenous Tim13p was found to be modified by the AMS reagent, and hence to be in a reduced state. However, a counterclaim has recently been made from Lu *et al*. (2004b), as they reported the small Tim proteins to be fully oxidized in the TCA-precipitated whole yeast cell extracts.

Zinc-binding trials with the MBP-fusion proteins of yeast small Tim proteins have shown that the zinc ions were found bound to small Tim proteins only when the zinc was present throughout the purification procedure (Sirrenberg *et al.*, 1998; Rothbauer *et al*., 2001; Lutz *et al.*, 2003).

Taken together, the question whether the small Tim proteins are zinc-binding proteins is still an open one. Aside from the small Tim proteins, there exist no other zinc fingers of the C4 type characterised by similar spacings of exactly three amino acid residues in both metal-chelating pairs of cysteines. Zinc finger proteins that exceptionally contain one CX_3C motif are extremely rare (for instance ribosomal protein L24E (Grishin, 2001)). The overwhelming majority of the zinc finger proteins are characterised by the CX_2C patterns, or with numbers of residues in the intercysteine spacer exceeding three. The reported dissociation constants Kd for eukaryotic structural zinc-binding domains are quite consistent, regardless of whether the site comprises CCHH, CCHC or CCCC pattern. They all fall into the range of 10^{-11} to 10^{-9} M for naturally occurring zinc sites (Payne *et al*., 2003). This means that most zinc finger proteins bind their zinc ions relatively tightly, and that once folded they are very stable (Luciano *et al.*, 2001). There exists a special group of bacterial zinc finger proteins, like Hsp33 and RsrA (Paget and Buttner, 2003), which use specific and reversible disulfide bond formation as functional switches. For these two proteins, it has been shown that both the zinc-bound reduced and the zinc-free oxidized forms have distinct roles in the cell. In Hsp33, the C4 motif, which tightly binds one Zn^{2+} ion, blocks the dimerization surface of this protein, keeping the protein in its inactive, monomeric state. It has been empirically shown that zinc ion enhances the proteolytic stability of this protein and that the zinc-loaded protein can be activated through hydrogen peroxide treatment much faster than the zinc-free reduced Hsp33 protein. In small Tim proteins, zinc also stabilises the reduced state against proteolysis, without significantly affecting the secondary structure (Lu *et al.*, 2004a). Ultimately, zinc

increases the reactivity of the cysteine residues in Hsp33 by as yet unknown means, whereas in RsrA zinc has been shown to protect the cysteines from oxidation. Clearly, more experiments are required to resolve the question of the classification of small Tim proteins as actual zinc finger proteins.

4.3. Import of small Tim proteins across the outer membrane

The import of Tim9 and Tim10 precursors into mitochondrial intermembrane space was also investigated in the course of this study. The results of experiments concerning import requirements of small Tim proteins of *N. crassa* corroborated that, for their import, they do not require (i) the known import receptors, (ii) ATP, and (iii) the transmembrane potential across the inner mitochondrial membrane.

These results are in accordance with the general observations regarding the import of proteins into the intermembrane space. The yeast small Tim proteins were imported *in vitro* into mitochondria isolated from *Δtom20* and *Δtom70* strains as efficiently as into wt yeast mitochondria (Kurz *et al*., 1999; Lutz *et al.*, 2003). Removal of these receptors through trypsin-treatment of isolated yeast mitochondria prior to the import, does not affect their import into the intermembrane space (Kurz *et al.*, 1999; Lutz *et al.*, 2003).

Import of the radioactively labelled small Tim proteins into mitochondria tends to show an overall low efficiency. This might be the result of their improper partial folding outside the mitochondria. Lutz *et al.* (2003) have proposed that zinc acquisition might stabilize the folding on the *trans* side of the TOM complex and trap the small Tim proteins in the intermembrane space, thereby providing the driving force for their translocation across the outer membrane. This model is in agreement with the general observation, that in zinc finger proteins, zinc binding results in the folding and the enhanced rigidity of the protein (Berg and Godwin, 1997). However, not all metalloproteins have their folding coupled to their cofactor acquisition. One extreme example are the blue copper proteins (Nar *et al.*, 1992), completely folded even in the absence of metal. Their metal-binding sites are fully preorganized and the metal binds with little structural change.

Somewhat similar mechanism of import into the intermembrane space was reported for the apocytochrome *c* (Diekert *et al.*, 2001). Previously, it was believed that this protein might undergo spontaneous insertion into lipid bilayers (Snel *et al.*, 1994), since it interacts with negatively charged phospholipids at the outer surface of the mitochondrial outer

membrane, and does not exert requirements for the import receptors either (Mayer *et al*., 1995). In a reconstituted system containing the proteoliposomes with the TOM complex and with the enclosed antibodies against apocytochrome *c* in them, it was shown that this precursor did indeed use the TOM pore. Surprisingly, saturating the TOM complex with a matrix-destined precursor did not affect translocation of the apocytochrome *c* across the membrane of such vesicles (Diekert *et al.*, 2001). The authors proposed a model where the acquisition of haem by the apocytochrome *c* in a process mediated by the cytochrome *c* heme lyase (CCHL) provides the driving force which shifts the equilibrium towards the imported species, i.e. the holocytochrome *c*.

For CCHL itself, *trans* side receptors have not yet been identified. This intermembrane space protein also does not require ATP, transmembrane potential and import receptors for import, and it also does not carry a charged presequence (Lill *et al.*, 1992). Its import signal is rather located in the third quarter of the protein sequence (Diekert *et al*., 1999). It was suggested that association of CCHL with either alleged components of the intermembrane space, high affinity binding sites within Tom40p, or the surface of the inner mitochondrial membrane, provides the requisite means for its translocation across the outer membrane.

Lu *et al.* (2004b) advocate another mechanism for the import of the small Tim proteins across the mitochondrial outer membrane. Their model relies on the oxidative folding of small Tim proteins in the mitochondria. It involves import of the subunits in a cysteine-reduced and unfolded state, followed by the folding into an assembly-competent structure maintained by the intramolecular disulfide bonding, and assembly of the oxidised, zinc-devoid subunits into a functional complex. An enzyme in the intermembrane space, Erv1p (Lange *et al.*, 2001), might be responsible for the disulfide bond formation in small Tim proteins.

In conclusion, the driving force for the import of small Tim proteins could be the interaction with a protein in the intermembrane space which (i) delivers the zinc ion to them, or (ii) introduces the disulfide bond formation in them, thereby promoting their folding.

4.4. Function of the small Tim proteins

The yeast Tim10p and Tim12p proteins were initially identified as suppressors of a defect in mitochondrial RNA splicing (Jarosch *et al.*, 1995 and 1997). Later on, it was established that these essential proteins, together with the Tim9p protein, are all involved in the import of the metabolite carrier precursor proteins (Sirrenberg *et al.,* 1998; Koehler *et al*., 1998; Adam *et al.*, 1999). The ADP/ATP carrier (AAC) is the most abundant member of the

carrier family. Detailed *in vitro* import studies of its import pathway revealed that AAC could be arrested at several distinct stages during import (Kübrich *et al.*, 1998; Endres *et al*., 1999; Ryan *et al*., 1999). Addition of the AAC preprotein (stage I) to isolated mitochondria in the absence of ATP leads to its arrest at the outer membrane (termed stage II) where it is bound to the receptor Tom70p. Each of the modules of the AAC recruits one Tom70p dimer (Wiedemann *et al*., 2001). The AAC precursor can then be chased across the outer membrane by the addition of ATP. In the absence of the transmembrane potential across the inner mitochondrial membrane, it is arrested at the inner face of the TOM machinery (stage III). Recently, it has been shown that the carrier inserts into the TOM pore in a loop conformation (Wiedemann *et al.*, 2001), in stage IIIa. It is subsequently released from the TOM complex into the intermembrane space in an event mediated by the Tim9p·Tim10p complex (stage IIIb, Koehler *et al.*, 1998; Adam *et al*., 1999). This, however, still does not imply that a soluble AAC-Tim9·Tim10 import intermediate exists. The complex of small Tim proteins might insure that the AAC does not aggregate in the transition through the intermembrane space compartment, thereby exerting chaperone-like behaviour (Vial *et al.*, 2002). The transition from stage II to stage IIIa, and from IIIa to IIIb requires a fully functional Tim9p·Tim10p complex (Truscott *et al.*, 2002). In the fully energized mitochondria, the AAC is inserted into the inner membrane through the action of the TIM22 translocase with the involvement of the Tim12p protein (stage IV, Sirrenberg *et al.*, 1996; Kerscher *et al*., 1997 and 2000; Koehler *et al*., 1998 and 2000), where it assembles into its dimeric form (stage V).

4.4.1. *N. crassa* Tim9·Tim10 complex - the mode of substrate recognition

The ability of Tim9p and Tim10p proteins to facilitate the translocation of the carrier proteins across membranes (Kübrich *et al.*, 1998; Endres *et al.*, 1999; Ryan *et al.*, 1999), brought up a question of the nature of the interactions of the Tim9p·Tim10p complex with their various substrates. It was suggested that the complex recognizes a conserved, carrier-signature sequence motif found in each of the three matrix loops of carrier proteins (Sirrenberg *et al.*, 1998; Endres *et al.*, 1999). However, some of the substrates of the Tim9·Tim10 complexes lack sequence similarity to the carrier proteins, making the sequence-specific recognition an unlikely option. Alternatively, it was proposed that the Tim9p·Tim10p complex could function as a chaperone in the intermembrane space by binding exposed

hydrophobic sequences of unfolded precursor proteins (Koehler, 1999). Conventional chaperones which might fulfil such a role have not been found in the mitochondrial intermembrane space.

Other studies support the view of the small Tim proteins as chaperons. A recombinant Tim9p·Tim10p complex was reported to have a moderate general chaperone activity (Vial *et al.*, 2002). In another study, screen of a library of peptides covering the ADP/ATP carrier with the yeast Tim9p·Tim10p complex was performed (Curran *et al.*, 2002a). In the latter study, it was published that the Tim9p·Tim10p complex interacts preferentially with peptides covering membrane spanning domains of AAC2p.

This data was however obtained in the yeast system only, and the study analyzed binding to just one substrate protein, so it was left an open question whether these findings can be extrapolated to other substrates and to the Tim9·Tim10 complexes of other species.

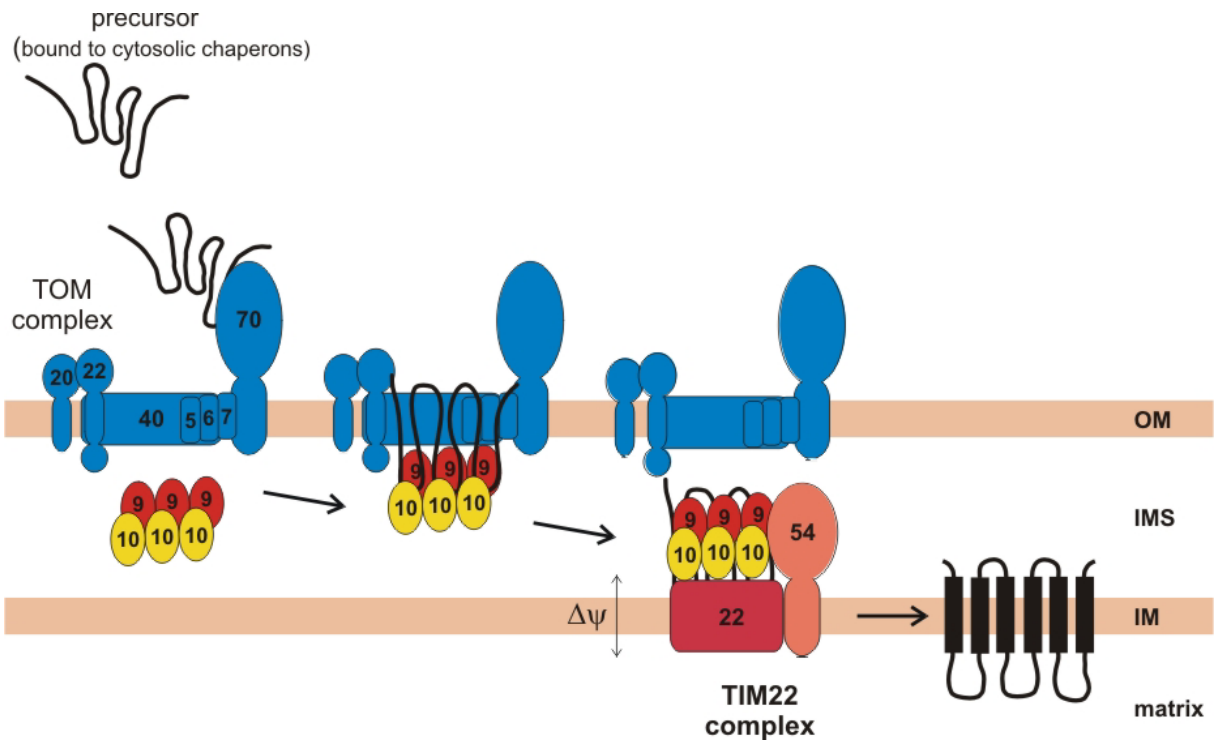


Figure 26. Model of the mitochondrial import of the carrier precursor facilitated by the Tim9·Tim10 complex of *N. crassa*

To address this question, we have screened a library of peptides covering a variety of precursor proteins of the mitochondrial inner membrane using the native *N. crassa* Tim9·Tim10 complex. The complex exerted the highest affinity of binding towards peptides concomitantly comprising residues of both transmembrane domains and the loops between

them. Advantages of overwhelming binding to such regions over exclusive binding to the membrane-spanning segments are evident. Such mode of binding allows the trapping of segments which emerge into the intermembrane space from the TOM complex, while most of the precursor is still protected from this aqueous environment by remaining in the TOM complex. In this manner, the transmembrane domains are able to undergo transfer from the TOM to the Tim9·Tim10 complex without having their hydrophobic stretches exposed to the intermembrane space (Figure 26).

Furthermore, the model of the carrier translocation involving a soluble intermembrane space intermediate can be considered unlikely for following reasons. Up to date no such soluble import intermediate of Tim9p·Tim10p and AAC2p has been identified in and isolated from the mitochondrial intermembrane space. This further suggests that a translocation contact site might form, where the outer and inner membrane come together and allow the carrier precursor to slip from the TOM pore into the Tim22p protein channel (Kovermann *et al*., 2002). This proposition is consistent with the *N. crassa* Tim9·Tim10 recognition pattern and binding to the parts of the carrier protein which are also left unconcealed when the carrier is finally integrated into the lipid bilayer of the inner membrane (Pebay-Peyroula *et al*., 2003). These segments are most probably concealed during import as well, by being buried inside the TOM channel and/or the TIM22 translocase. Binding to domains along the ends of the hydrophobic segments of the carrier would in this way prevent any retrograde movement along its import pathway, simultaneously supporting the vectorial progress towards the completion of import.

4.4.2. Tim23 - a novel Tim9·Tim10 complex substrate in *N. crassa*

The Tim23p protein inserts into the inner membrane with the help of the membrane-embedded TIM22 complex (Kerscher *et al.*, 1997; Kaldi *et al.*, 1998; Leuenberger *et al.*, 1999). Upon *in vitro* import of the Tim23p precursor into the isolated de-energised mitochondria, the major cross-linking species of the Tim23p precursors to Tim8p and Tim13p proteins form, and very little if any, cross-linking products to the Tim9p and Tim10p proteins (Leuenberger *et al.*, 1999; Paschen *et al.*, 2000). Interestingly, a weak interaction of the Tim9p·Tim10p complex with the COOH-terminal hydrophobic domain of Tim23p was reported from another research team. They suggested that the Tim9p·Tim10p complex might play an important role in the import of Tim23p precursor (Davis *et al.*, 2000; Jensen and Dunn, 2002). In this study, the Tim23 protein was identified as another substrate of the *N.*

crassa Tim9·Tim10 complex. Results of both our peptide screen and the cross-linking experiments in *N. crassa* revealed the existence of an interaction between Tim23 and the native Tim9·Tim10 complex. This interaction of the Tim23 precursor with the Tim9·Tim10 complex might occur concomitantly with its interaction with the Tim8·Tim13 complex, or at a later stage of its import pathway.

In the peptide screens made by Koehler group using the yeast model system, it was suggested that peptides representing Tim23p were bound by the Tim8p·Tim13p complex, but not the Tim9p·Tim10p complex (Curran *et al.*, 2002a; Curran *et al.*, 2002b). A plausible explanation of these differences between *Neurospora* and yeast might be the absence of Tim12p in *N. crassa*. An essential protein in yeast, it facilitates the transfer of the substrate proteins from the Tim9p·Tim10p to the TIM22 complex (Koehler *et al.*, 1998a; Sirrenberg *et al.*, 1998), but it has not been found in any organism other than *S. cerevisiae*. In *N. crassa* a similar function may be fulfilled by the Tim9·Tim10 complex itself.

4.4.3. The minimal machinery for the translocation of AAC across membranes

Recent studies have indicated the demand for Tim9p and Tim10p proteins in the process of the carrier preprotein release from the TOM complex and its insertion into the inner membrane (Luciano *et al.*, 2001; Truscott *et al.*, 2002). Yet, the explicit facts of whether they accomplish the precursor transfer across the outer membrane with the help of additional, yet unknown component(s) or whether the Tim9p·Tim10p complex and the TOM complex are sufficient for mediating this process were not dealt with. To address this issue, we reconstituted the TOM core complex into lipid vesicles with the purified *N. crassa* Tim9·Tim10 complex entrapped, and performed import of the ADP/ATP carrier precursor.

Following this line of research, we made several observations. First, the TOM core complex (lacking import receptors) can facilitate the transfer of the AAC precursor to the internal side of the outer membrane. Whereas in the absence of the Tim9·Tim10 complex parts of the AAC precursor molecule are still exposed to the cytosol, the presence of a functional Tim9·Tim10 complex at the internal side of the outer membrane was sufficient to pull the AAC precursor further across the outer membrane. Evidently, the TOM core complex and the Tim9·Tim10 complex form the minimal machinery for the translocation of the ADP/ATP carrier across the mitochondrial outer membrane.

The observation that the AAC is found still associated with the receptors Tom70p and Tom20p when the function of Tim10p protein is impaired, support our conclusion (Wiedemann *et al.*, 2001; Truscott *et al*., 2002). In other words, whereas the initial insertion of AAC into the import channel is independent of the Tim9p·Tim10p complex, completion of the translocation process across the outer membrane, including the release from the TOM complex, requires a functional Tim9p·Tim10p complex. It appears that neither proteins in the mitochondrial inner membrane or the intermembrane space, nor structural elements like the import contact sites are required for this translocation step. Rather, the interaction of the small Tim proteins with the incoming precursor is sufficient to prevent its retrograde sliding out of the translocation machinery and to ensure a vectorial movement along the import pathway.

4.4.4. Potential involvement of the small Tim proteins in the biogenesis of the β-barrel proteins

Recently, certain reports have been published suggesting an additional, though non-essential function of the small Tim proteins, both in *N. crassa* and *S. cerevisiae*. Their role in the biogenesis of β-barrel proteins of the mitochondrial outer membrane has been reported (Hoppins *et al*., 2004; Wiedemann *et al*., 2004).

Hoppins *et al.* (2004) have isolated *tim8* and *tim13* mutants in *N. crassa* and have shown that mitochondria lacking the Tim8·Tim13 complex were mildly impaired in the import of Tom40 and porin, which are both β-barrel proteins. Their cross-linking studies demonstrated the existence of an interaction between Tom40 precursor and the Tim8·Tim13 complex. The reported cross-linking products formed exclusively in the initial import stages. Also, formation of an early assembly intermediate of the Tom40 precursor was slower in the mitochondria lacking the Tim8·Tim13 complex, than in the wild type mitochondria. Wiedemann *et al*. (2004) investigated the yeast mitochondria with defects in small Tim proteins and also found them impaired in the early stages of the Tom40p assembly pathway. Deficiency in the Tim9p·Tim10p complex seemed to have a more profound effect on the biogenesis of the mitochondrial β-barrel proteins, than the one of the impaired Tim8p·Tim13p complex.

However, all the observed effects were very moderate. Furthermore, the endogenous levels of β-barrel proteins of the mitochondrial outer membrane were not affected. There were also no phenotypic repercussions observed.

Using our reconstituted system, we could not detect any protease protected porin species upon its incubation with the proteoliposomes with the Tim9·Tim10 complex entrapped. This was not surprising since the TOB complex was not present in our trials. Nevertheless, this system might prove useful for acquiring further insights into the biogenesis of β-barrel proteins of the mitochondrial outer membrane.

5. Summary

Mitochondria are essential cellular organelles of eukaryotic organisms, which import most of their proteinaceous constituents from the cytoplasm. Two mitochondrial membranes contain different translocation machineries which are involved in the import and proper sorting of mitochondrial precursor proteins. The TIM22 translocase in the inner mitochondrial membrane mediates the import of polytopic proteins into this membrane. In addition to the membrane integrated components Tim22 and Tim54, the TIM22 translocase possesses components in the intermembrane space, termed Tim9 and Tim10.

In the present study, the *tim9* and *tim10* genes of the TIM22 translocase of *N. crassa* were identified. The structural and functional characteristics of the corresponding gene products, the Tim9 and Tim10 proteins, were examined. Tim9 was demonstrated to be an essential protein. The Tim9 and Tim10 proteins were shown to build a 70-80 kDa heterohexameric complex in the mitochondrial intermembrane space. The isolated Tim9·Tim10 complex had the same oligomeric structure as the native one, and it proved fully functional in interacting *in vitro* with its physiological substrate, the ADP/ATP carrier (AAC).

Peptide library screens were performed to determine the structural determinants of the substrates that are recognised by the Tim9·Tim10 complex. Efficient binding to the regions covering residues of the hydrophobic membrane spanning domains and of the connecting hydrophilic loops was observed. In this way, Tim9 and Tim10 proteins interact with their substrates, while the hydrophobic regions of the substrates are still present in the TOM complex and thereby protected from the aqueous environment of the intermembrane space compartment. Furthermore, when enclosed into proteoliposomes containing the reconstituted TOM complex, Tim9·Tim10 complex specifically promoted the translocation of the AAC precursor. Hence, the Tim9·Tim10 complex and the TOM complex are both necessary and sufficient to facilitate translocation of carrier proteins across the outer mitochondrial membrane. Finally, peptide screens and chemical cross-linking experiments were used to identify the precursor of *N. crassa* Tim23 protein as a novel substrate of the Tim9·Tim10 complex.

6. Abbreviations

AAC	ADP/ATP carrier
Ac	acetate
ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
amp	ampicillin
APS	ammonium peroxodisulfate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
β-ME	β-mercaptoethanol
BSA	bovine serum albumin
BNG(E)	blue native gel (electrophoresis)
Ci	Curie
CCCP	carbonyl cyanide m-chlorphenylhydrazone
CCHL	cytochrome *c* haem lyase
cDNA	complementary DNA
CIP	calf intestinal alkaline phosphatase
CNBr	cyanogen bromide
CSPD	3-(4-methoxyspiro (1,2-dioxetane-3,2'-(5'-chloro) tricycle [3,3.1.1$^{3.7}$]decan}-4-yl) phenyl phosphate, disodium salt
CV	column volume
DDM	n-dodecyl-β-maltopyranosid
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxid
dNTP	deoxyribonucleoside triphosphate
DNA	desoxyribonucleic acid
DTT	dithiotreitol
$\Delta\psi$	membrane potential
E. coli	*Escherichia coli*
EDTA	ethylendiamine tetraacetate
ER	endoplasmatic reticulum
GIP	general import pore
HEPES	N-2 hydroxyl piperazine-N`-2-ethane sulphonic acid

Hsp	heat shock protein
IgG	immunoglobulin G
IM	inner membrane
IMS	intermembrane space
IP	immunoprecipitation
IPTG	isopropyl-β,D-thiogalactopyranoside
KAN	kanamycin
kDa	kilodalton
LB	Luria Bertani
m7G(5`)ppp(5`)G	7-methylguanosine triphospate
MBP	maltose-binding protein
MBS	m-maleimidobenzoyl-N-hydroxysuccinimide ester
MOPS	N-morpholinopropane sulphonic acid
MPP	mitochondrial processing peptidase
MSF	mitochondrial import stimulating factor
MTS	matrix targeting signal
MW	molecular weight
N-	amino-
N. crassa	*Neurospora crassa*
NADH	nicotine amide adenine dinucleotide
NADPH	nicotine amide adenine dinucleotide phosphate
Ni-NTA	nickel-nitrilotriacetic acid
ODx	optical density at x nm
OM	outer membrane
OMVs	outer membrane vesicles
PAGE	polyacrylamide gel electrophoresis
PAS	Protein A Sepharose
PCR	polymerase chain reaction
PEG	polyethylene glycol
PIS	preimmune serum
PK	Proteinase K
PMSF	phenylmethylsulfonyfluoride
PVDF	polyvinylidene difluoride
RIP	repeat-induced point mutation

RNase	ribonuclease
RNasin	ribonuclease inhibitor
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulfate
STI	soybean trypsin inhibitor
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TCEP	Tris-(2-carboxyethyl) phopshine
TEMED	N,N,N‘,N‘-tetramethylene diamine
TIM	translocase of the inner mitochondrial membrane
TLC	thin layer chromatography
TOM	translocase of the outer mitochondrial membrane
Tris	tris-(hydroxymethyl)-aminomethane
TX-100	Triton X-100
Vol.	volumes
v/v	volume per volume
w/v	weight per weight
wt	wild type

7. References

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