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**Role of the Hep1 chaperone in the *de novo* folding
and the prevention of aggregation
of the mitochondrial Hsp70 chaperone Ssc1**

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Erklärung

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1. Introduction

1.1. Protein folding problem

Proteins are the most versatile and complex biomolecules, playing an active role in the vast majority of biological processes. Although around 20 % of eukaryotic proteins remain intrinsically disordered *in vivo* (Dunker et al. 2008), a great number of proteins need to adopt a proper three-dimensional structure, which is critical for their physiological function. In the 1960s, Chris Anfinsen, the pioneer of protein folding, postulated that the native three-dimensional structure of a protein is a thermodynamically stable structure, determined only by the amino acid sequence and the solution conditions (Anfinsen 1973). Nevertheless, the process of obtaining the native structure by a protein is very complex. Although it has been extensively studied, it still remains one of the most crucial biological problems.

An unfolded polypeptide chain has an enormously high number of degrees of freedom. According to the Cyrus Levinthal paradox, there are too many available conformations in the conformational space for an unfolded protein to find the native conformation by a random search and in a biologically relevant timescale (Levinthal 1969). Thus, proteins could not randomly explore all possible conformational states. They fold on a funnel-shaped free-energy ‘landscape’, obtaining the native structure by reaching the global free energy minimum (Bryngelson et al. 1995; Wolynes 2005). During this process, multiple transient states based on local interactions within a polypeptide chain are formed and promote the acquisition of the native state. These interactions include mostly hydrophobic forces, hydrogen bonds and for certain proteins also ion-pairing (Onuchic and Wolynes 2004). Free-energy landscapes are unique for every protein under particular conditions and are defined by both thermodynamic and kinetic properties of the polypeptide chain.

An unfolded protein retains high entropy and can populate a large ensemble of conformations; therefore it can usually follow several folding pathways down the energy funnel to obtain the final conformation (Radford et al. 1992). For the small single-domain proteins of up to 100 amino acids, the folding landscapes are usually smooth (Fig. 1.1a), allowing very rapid, often two-state transition from the ensemble of unfolded states to the final native conformation (Fersht 2000; Onuchic and Wolynes 2004). In the case of larger

proteins, comprising around 90 % of all cellular proteins, the folding landscapes are much more rugged, leading to the formation of folding intermediates (Brockwell and Radford 2007) (Fig. 1.1b). Such intermediate states are based on native and non-native interactions and can represent partially folded on- or off-pathway steps in the folding process. The on-pathway intermediates proceed eventually towards the native conformation, whereas the off-pathway intermediates are usually kinetically stable and may have to overcome a high free-energy barrier to break the non-native interactions and finally acquire the native fold (Jahn and Radford 2005; Gianni et al. 2007; Baldwin 2008). Thus, the formation of intermediate states can either promote folding or kinetically trap the protein in a metastable form, hindering the folding process.

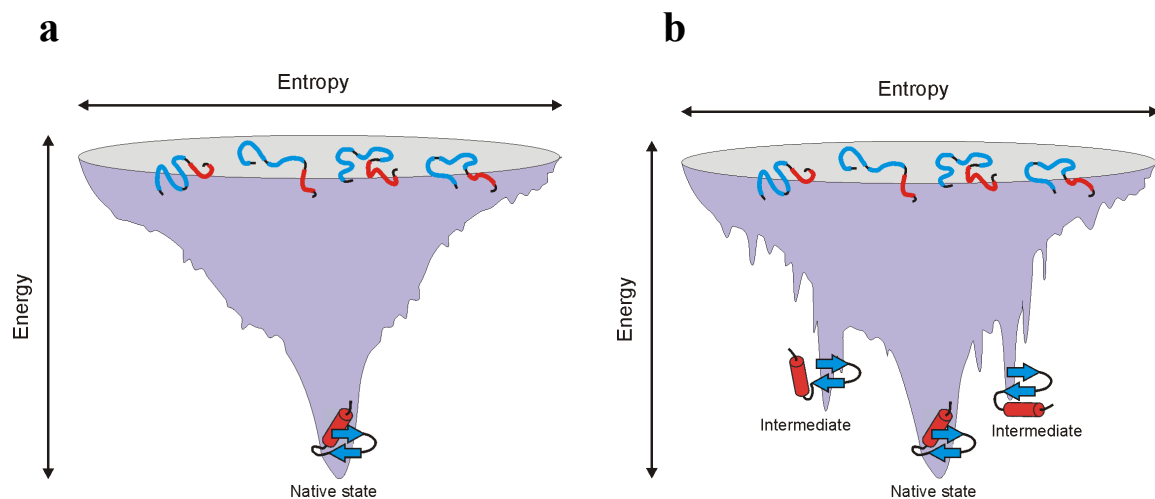


Figure 1.1. Smooth and rough energy folding landscapes – a schematic view.

A polypeptide chain occupies in its denatured state a broad ensemble of structures. During the folding process, the polypeptide chain is funneled to the native state either directly, in the case of a smooth energy landscape (a), or it requires navigation, possibly via intermediate states, in the case of a rough energy landscape (b).

Misfolded or partially folded conformers, especially those kinetically stable, often have a tendency to aggregate (Stefani and Dobson 2003; Wright et al. 2005). Aggregation is largely driven by the hydrophobic intermolecular interactions and such folding intermediates usually expose their hydrophobic residues that should be buried inside the native structure. This leads to the association of the molecules in either amorphous heterogeneous structures, or, in contrast, in the highly ordered stable fibrils, known as amyloids (Eichner et al. 2011). *In vivo*, the formation of aggregates is usually highly toxic for the cells and may lead to deleterious disorders (Chiti and Dobson 2006). Therefore,

several mechanisms assisting protein folding, preventing protein aggregation and dealing with the already formed aggregates exist in the cell and are necessary for normal cell functioning.

1.2. Molecular chaperones

1.2.1. Role of molecular chaperones

In vitro, the process of protein folding might take microseconds, for some small proteins (Kubelka et al. 2004), or minutes or even hours, for the large, multidomain proteins (Herbst et al. 1997). Many proteins fail to fold *in vitro* under the conditions tested. Under *in vivo* conditions, protein folding is far more difficult, due to the high density of the cellular environment, where the protein concentration reaches 300–400 g/l (Zimmerman and Trach 1991). Such molecular crowding enhances the chances of intramolecular interactions, including those of partially folded and misfolded proteins causing deleterious aggregation. Therefore, a versatile network of molecular chaperones, together with co-chaperones that regulate chaperone activity and substrate specificity, has been developed within the cell. Molecular chaperones are defined as proteins that interact with, stabilize or assist other protein in acquiring native conformation, not being a part of the protein's final structure (Hartl 1996). Molecular chaperones fulfill *inter alia* a multitude of protein folding-related functions. Firstly, they assist *de novo* folding of the proteins, so that the folding is accomplished on a biologically relevant timescale. During protein synthesis they bind and escort the nascent polypeptide chain before it gets completely released from the ribosome (Frydman 2001; Kaiser et al. 2006). After protein synthesis is completed, molecular chaperones facilitate the folding of nascent proteins. They can passively prevent protein aggregation, by shielding the hydrophobic regions of unfolded and partially folded proteins (holdases) (Ganea 2001; Hoffmann et al. 2004; Apetri and Horwich 2008) but also actively force the folding of the polypeptide chain (foldases), preventing the formation of off-pathway intermediates (Langer et al. 1992; Tang et al. 2006; Sharma et al. 2011). Secondly, molecular chaperones play a crucial role in repair mechanisms, actively dissolving protein aggregates and refolding misfolded species (Goloubinoff et al. 1999; Mogk et al. 1999; Liberek et al. 2008; Iosefson et al. 2012). They help mutated proteins to obtain a stable structure, providing a buffer system that is critical for the evolution of new protein functions (Rutherford and Lindquist 1998; Maisnier-Patin et al. 2005). Thirdly,

molecular chaperones are involved in several proteolytic pathways. They facilitate the clearance of aggregated and misfolded protein species that are resistant to the repair mechanisms, and regulate the normal level of protein turnover (Kriegenburg et al.; Wagner et al. 1994; Rottgers et al. 2002). All these functions are absolutely crucial for protein quality control and the maintenance of protein homeostasis (proteostasis). Thus, defects in the correct functioning of the molecular chaperone network result in detrimental consequences at the cellular as well as systemic level.

1.2.2. Classes of molecular chaperones

Molecular chaperones are grouped in numerous, structurally and mechanistically unrelated chaperone classes. The vast majority of chaperones are known as heat-shock proteins (Hsps), since they are strongly up-regulated under cellular stress conditions, when the amount of misfolded and aggregated proteins is dramatically increased (Schlesinger 1990). Heat-shock proteins are classified according to their molecular weight: Hsp60 and the chaperone Hsp10, Hsp70 and the co-chaperone Hsp40, Hsp90, Hsp100 and the small Hsps.

In bacteria, the first chaperone interacting with the nascent polypeptide chain emerging from the ribosome is the trigger factor (Ferbitz et al. 2004). The trigger factor does not belong to any of the heat-shock protein classes. The trigger factor interacts strongly with the hydrophobic regions of the nascent chain and it gets released upon burial of the hydrophobic residues inside the folding protein (Baram et al. 2005; Schlunzen et al. 2005; Merz et al. 2008). The release of the trigger factor might facilitate further folding or a transfer of the protein to the downstream chaperone systems such as DnaK (Teter et al. 1999; Agashe et al. 2004). In eukaryotes, the role of the Trigger factor is taken over by two chaperone complexes called RAC, the Ribosome-Associated Complex (Gautschi et al. 2001; Otto et al. 2005) and NAC, the Nascent chain-Associated Complex (Wiedmann et al. 1994; Gautschi et al. 2002).

The Hsp70 chaperone class is one of the biggest and most versatile chaperone families and it is comprised of both constitutively expressed as well as stress-inducible proteins. In eukaryotes, Hsp70 chaperones are present in various cellular compartments, including the cytosol, nucleus, ER, mitochondria and chloroplasts, and many of those chaperones are essential for the cell viability. The Hsp70 chaperones interact with the substrate proteins in an ATP-dependent cycle of binding and release (Szabo et al. 1994; Laufen et al. 1999;

Mayer et al. 2001; Mayer and Bukau 2005; Jiang et al. 2007). Hsp70 chaperones work as primary folding assistants for the newly synthesized polypeptides. This is the case for the best characterised representative of the Hsp70 chaperone class, bacterial DnaK, which is the main folding helper for nascent bacterial proteins (Bukau and Horwich 1998). Substrates that fail to obtain the final structure with the help of Hsp70 cycling may be transferred to the chaperonin (Hsp60) system for further folding assistance (Langer et al. 1992). Apart from functioning in *de novo* folding, Hsp70 proteins are involved in a number of other processes, like protein transport across the organellar membranes (Kang et al. 1990), disassembly of huge oligomeric protein complexes (Schlossman et al. 1984; Rothnie et al. 2011) and degradation of terminally misfolded proteins (Kriegenburg et al.; Voos 2009). Hsp70 chaperones are able to fulfill all those various functions due to (i) the high number of general as well as specialized members of this chaperone class (Hageman et al. 2011); (ii) the co-chaperones that determine substrate specificity and are selectively employed by Hsp70 chaperones depending on the cellular process (Silver and Way 1993; Kampinga and Craig 2010) and (iii) the cross-talk with the other chaperone systems like Hsp60, Hsp90 and Hsp100 (Bukau et al. 2006; Hartl et al. 2011).

The second main class of molecular chaperones is formed by chaperonins. Chaperonins are 0.8–0.9 MDa cage-forming complexes that encapsulate substrate proteins in an ATP-dependent fashion for folding inside their cavity (Kerner et al. 2005; Yebenes et al. 2011). Chaperonins can be divided into two groups (Horwich et al. 2007). Group I chaperonins, known as Hsp60s, are present in bacteria, mitochondria and chloroplasts. They consist of heptameric rings and work together with the heptameric Hsp10 proteins that form the lid for the folding cage (Braig et al. 1994). The best studied representative of this group is the bacterial GroEL, with GroES as an Hsp10 lid (Langer et al. 1992). Group II chaperonins are present in archaea (thermosome) and in eukaryotes (TRiC/CCT). They are comprised of octa- or nonameric rings and are independent of Hsp10, since their lid is formed by α -helical extensions of the chaperonin ring (Douglas et al. 2011; Munoz et al. 2011). Substrates of the chaperonins are usually proteins with highly frustrated folding landscapes and which tend to form kinetically trapped aggregation-prone intermediates such as actin and tubulin (Horwich and Fenton 2009; Munoz et al. 2011). Encapsulation by chaperonins lowers the effective concentration of aggregation-prone species, passively preventing aggregation, a mechanism known as ‘kinetic partitioning’ (Apetri and Horwich 2008). In some cases encapsulation can, however, actively accelerate the folding rates via

destabilizing misfolded states and promote the formation of native conformations (Brinker et al. 2001; Lin et al. 2008).

Hsp90 chaperones represent another chaperone class, acting downstream from the Hsp70 system. They cooperate with a plethora of co-factors that regulate Hsp90 activity and provide substrate specificity and crosstalk with other chaperone systems (Scheufler et al. 2000; Zhao and Houry 2007). Similarly to Hsp70 proteins and chaperonins, Hsp90 undergoes an ATP-dependent cycle in which it interacts with client proteins (Shiau et al. 2006); however, the exact function and the mechanism of action of this chaperone are not well-understood. Various types of Hsp90 clients include transcription factors, such as p53, and signal-transduction molecules, such as steroid receptors and kinases (Wandinger et al. 2008). Thus, the Hsp90 system is involved in a number of crucial cellular processes, such as secretory pathways, cellular transport, cell-cycle progression and apoptosis (McClellan et al. 2007). Due to its role in cancer development, Hsp90 serves as a potential target for anticancer therapy (Neckers 2007; Workman et al. 2007). On the other hand, Hsp90 functions in buffering the effects of mutations that destabilize proteins, thereby allowing the evolution of new protein functions and traits (Rutherford and Lindquist 1998).

Hsp100 chaperones are members of the large AAA+ ATPase superfamily. These chaperones are able to unfold stably folded proteins or even dissociate large aggregates of misfolded proteins (Lee et al. 2003; Martin et al. 2005). They consist of one (class II) or two (class I) hexameric rings forming a polypeptide translocation channel. In an ATP-dependent manner, loose regions of the folded/misfolded protein are pulled through that channel, thereby unraveling the rest of the protein. A protein unfolded in this way is either subjected to a refolding process catalyzed by other chaperone systems, or directed to degradation by a protease (P) that is in many cases coupled to the Hsp100 chaperone (ClpAP, ClpXP) (Burton and Baker 2005; Inobe and Matouschek 2008). The ClpB/Hsp104 subfamily of Hsp100 class I chaperones forms unique coiled-coil propeller domains, which are crucial for protein recovery from the large aggregates (Lee et al. 2003). Hsp100 chaperones play a key role in protein quality control, in proteolytic pathways as well as under stress conditions (Schmitt et al. 1996; Lee et al. 2001; Weibezahn et al. 2005).

The small Hsps comprise a large and poorly conserved family of proteins that build shell-like complexes of 0.2–0.8 MDa and consist of 12–24 small subunits. Small Hsps share a conserved region called α -crystallin domain that is involved in subunit interactions (Ganea 2001). The expression of these chaperones, or their transition toward the active state is, in the most cases, induced upon different types of stress (Kappe et al. 2003). Small

Hsp chaperones act by passive stabilization of stress-denatured proteins, thereby preventing them from aggregation (Haslbeck et al. 2005). They are involved *inter alia* in the inflammatory response, in the protection against oxidative stress and aging, in anti-apoptotic processes, as well as in the neuroprotection (Latchman 2002; Hsu et al. 2003; Haslbeck et al. 2005).

Elements of the chaperone machinery, despite its diverse mechanisms, pathways and specificity, form complex cellular networks and cooperate with each other in a highly regulated manner. This complicated and dynamic functional interplay is, to date, only poorly understood and may vary to a great extent in different cell types and depending on environmental conditions.

1.3. Mechanism of Hsp70 chaperone system

The mechanism of action of the Hsp70 chaperones is determined by their structure, which is generally well conserved through all species. The Hsp70 chaperones are composed of an N-terminal ATPase domain of 45 kDa, which binds nucleotides and hydrolyses ATP and a C-terminal 25 kDa peptide binding domain (PBD), which consists of β -sandwich and α -helical lid subdomain and transiently interacts with the substrate proteins (Flaherty et al. 1990; Zhu et al. 1996; Liu and Hendrickson 2007; Chang et al. 2008). The domains are connected via a highly conserved hydrophobic interdomain linker that is crucial for interdomain communication and thus for the correct functioning of the Hsp70 protein (Han and Christen 2003; Jiang et al. 2005; Vogel et al. 2006; Swain et al. 2007) (Fig. 1.2).

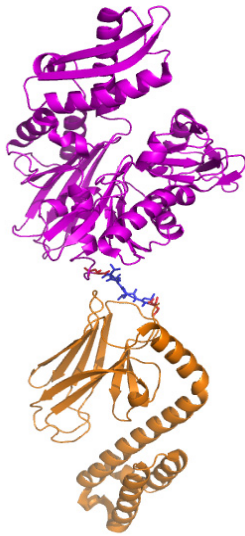


Figure 1.2. Structure of the bacterial Hsp70 chaperone, DnaK, from the wild-type *E. coli* K-12

The ATPase domain is shown in magenta, PBD shown in orange and the interdomain linker shown in blue using the stick representation. Structure view was generated using PyMOL program. Protein Data Bank accession code 2KHO (Bertelsen et al. 2009).

The mechanism of Hsp70 action is based on an ATP-dependent cycle of binding and release of the extended segments of unfolded proteins that usually consist of five to seven hydrophobic residues (Rudiger et al. 1997). It depends on the cooperation of the ATPase domain with the PBD. In the ATP-bound state of the ATPase domain, the PBD remains in an open conformation leading to the high on- and off-rates of substrate binding. Upon ATP hydrolysis, the PBD closes, which causes an increase in the affinity to the substrate and prevents its dissociation. Exchange of ADP for the new ATP molecule enables opening of the PBD and release of the substrate, completing the ATP-driven reaction cycle (Fig. 1.3). Thus, the nucleotide state of the ATPase domain determines the substrate affinity of the PBD. On the other hand, the substrate binding to the PBD stimulates ATP hydrolysis by the ATPase domain (Szabo et al. 1994; Buchberger et al. 1995; McCarty et al. 1995; Woo et al. 2009). This constant coupling of the conformational changes in both domains is mediated by the interdomain linker, which binds to the hydrophobic cleft in ATPase domain and is alone able to stimulate its activity (Vogel et al. 2006; Swain et al. 2007).

As the basal ATPase activity of Hsp70 proteins is relatively low ($0.018\text{--}1\text{ min}^{-1}$), and the substrate stimulation ranges only between 2 to 10-fold (Mayer et al. 2000; Mayer et al. 2000), Hsp70 proteins require two types of co-chaperones that accelerate the ATPase cycle: the J domain-containing proteins, classified also as Hsp40s and the nucleotide exchange factors (Liberek et al. 1991; Rungeling et al. 1999) (Fig. 1.3). The J domain-containing proteins (J proteins) stimulate the ATP hydrolysis of Hsp70 up to 1000-fold (Cyr et al. 1994; Karzai and McMacken 1996; Laufen et al. 1999). Additionally, they may themselves recognize and bind substrate proteins and target them to the Hsp70 chaperone,

thereby determining substrate specificity (Han and Christen 2003; Summers et al. 2009; Kampinga and Craig 2010). The nucleotide exchange factors interact with the ATPase domain of Hsp70 proteins and trigger opening of the nucleotide-binding cleft, facilitating ADP release and thus re-binding of new ATP molecule (Schonfeld et al. 1995; Skowrya and Wickner 1995; Brehmer et al. 2004). Thus, nucleotide exchange factors control the lifetime of the Hsp70-substrate complex.

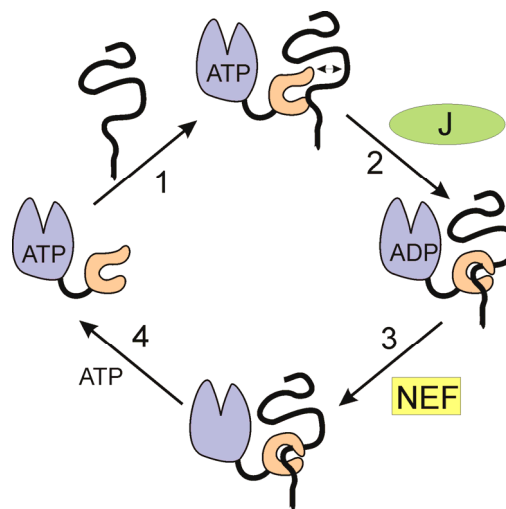


Figure 1.3. ATPase cycle of an Hsp70 chaperone

(1) Unfolded substrate binds weakly to the Hsp70 in an ATP-bound (open) state. (2) ATP hydrolysis stimulated by a J protein leads to Hsp70 lid closure and substrate entrapment. (3) Nucleotide exchange factor (NEF) facilitates ADP release. (4) New ATP binds and triggers lid opening and subsequent substrate release.

1.4. Role of the mitochondrial chaperones in the biogenesis of mitochondria

As one of the most crucial cellular compartments, mitochondria, the endosymbiotic organelles, play an indispensable role for the cell functioning. Apart from energy production and biosynthesis of essential metabolites, mitochondria are involved in a number of cellular pathways and processes like signaling, cell cycle, stress response and apoptosis. Thus, a large set of proteins resides in this organelle, including important enzyme complexes, proteins responsible for the mitochondrial morphology, as well as a

variety of molecular chaperones that regulate and maintain the correct protein level, activity and turnover (Voos and Rottgers 2002; Voos 2009).

The molecular chaperones play an indispensable function in the biogenesis of the mitochondrial proteins. They are involved in the complicated process of protein import into the mitochondria, together with a myriad of other factors. Moreover, since the mitochondrial proteins are translocated in an unfolded manner, they need to fold *de novo* upon reaching the final destination. Molecular chaperones assist in the folding process of those proteins and of mitochondrially encoded proteins, and help many of them to assemble into functional complexes.

1.4.1. Mitochondrial import pathways

Being highly integrated into the metabolism of the host cell, mitochondria have transferred the vast majority of their genes into the nuclear genome. Therefore, apart from a few proteins still encoded in the mitochondrial chromosome, mitochondrial proteins are synthesized on the cytoplasmic ribosomes as precursor proteins and subsequently translocated into the correct mitochondrial subcompartment: the outer mitochondrial membrane, the inner membrane divided into the cristae membrane and the inner boundary membrane, the intermembrane space (IMS) and the innermost matrix.

Mitochondrial protein transport is carried out by the large protein complexes spanning the mitochondrial membranes called translocases (Endo et al. 2003; Koehler 2004; Rehling et al. 2004; Neupert and Herrmann 2007). Virtually all mitochondrial precursor proteins enter the mitochondria through the TOM complex (the translocase of outer membrane), consisting of receptor proteins recognizing the mitochondrial precursors and a protein-conducting channel (Ahting et al. 1999; Rapaport 2005; Perry et al. 2008). The TOM complex is sufficient for the import of many outer membrane and IMS proteins. Some outer membrane proteins require assistance of the soluble small TIM complexes (translocase of the inner membrane) and enter the outer membrane via the TOB/SAM complex (topogenesis of outer membrane β -barrel proteins/sorting and assembly machinery) (Kozjak et al. 2003; Wiedemann et al. 2004; Paschen et al. 2005; Endo and Yamano 2010). The small cysteine-containing intermembrane space proteins use the disulfide relay system of Mia40-Erv1 that oxidatively folds them, thereby trapping them in the intermembrane space (Herrmann and Riemer; Hell 2008; Sideris and Tokatlidis 2010). The large hydrophobic inner membrane proteins, such as the carrier family members, are

escorted by the small TIM complexes to the TIM22 translocase that inserts them into the inner membrane (Sirrenberg et al. 1996; Bauer et al. 2000; Rehling et al. 2003).

After entering the mitochondria through the TOM pore, the great majority of mitochondrial proteins are imported further via the so-called general import pathway, passing through the TIM23 translocase (Marom et al. 2010; Mokranjac and Neupert 2010; van der Laan et al. 2010). Those proteins are directed either into the mitochondrial matrix or into the inner membrane and usually contain a cleavable mitochondrial targeting sequence located in most cases at the N terminus. Their translocation is driven by the ATP-hydrolysis by the mitochondrial Hsp70. The inner membrane proteins are either laterally released from the TIM23 complex via the stop-transfer mechanism or are first completely imported into the matrix and then inserted into the membrane with the assistance of OXA1 complex. The OXA1 pathway, known also as a ‘conservative sorting pathway’ due to its origin from the bacterial ancestors, is also used by the mitochondrially-encoded proteins destined to the inner membrane (Hell et al. 1998; Hell et al. 2001; Ott and Herrmann 2009). The mitochondrial import machinery and the different mitochondrial import pathways are schematically presented in Fig. 1.4.

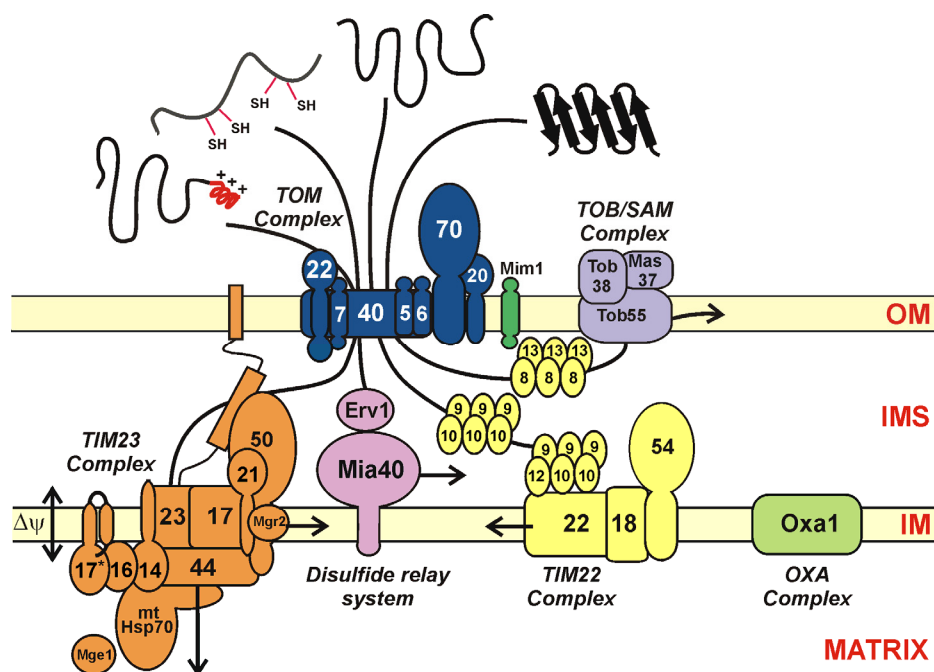


Figure 1.4. An overview of pathways of pre-protein import and sorting into the mitochondria. OM, outer membrane, IMS, intermembrane space, IM, inner membrane.

Adapted from (Mokranjac and Neupert 2008).

Mitochondrial proteins, due to the size of the translocating channels, are, as mentioned above, transported in an unfolded state. Therefore, the imported proteins have to fold inside the mitochondria and may require the help of molecular chaperones in that process.

In the mitochondrial matrix, there are several chaperone systems acting as folding assistants. The mitochondrial Hsp70 chaperone system and the Hsp60 chaperonin are the main folding helpers of mitochondria (Voos and Rottgers 2002; Voos 2009). Since mitochondrial Hsp70 interacts with the precursor proteins being imported into the mitochondrial matrix, it is also the first chaperone that mediates folding (Kang et al. 1990; Liu et al. 2001). Many mitochondrial proteins are subsequently transferred from mitochondrial Hsp70 to the Hsp60 chaperonin where they undergo further folding steps inside the chaperonin cavity (Endo 1991; Rospert et al. 1996; Heyrovská et al. 1998). The third chaperone system in the mitochondrial matrix, the mitochondrial Hsp78 complex, belongs to the Hsp100 chaperone class and is a homologue of the bacterial ClpB chaperone (Moczko et al. 1995; Schmitt et al. 1995; Krzewska et al. 2001). The Hsp78 chaperone displays mostly disaggregation and refolding activity and is particularly required under stress conditions (Schmitt et al. 1996; von Janowsky et al. 2006).

1.4.2. Mitochondrial Hsp70 chaperone systems

In the mitochondrial matrix of the yeast *Saccharomyces cerevisiae* there are three members of Hsp70 family: Ssc1p, Ssq1p and Ecm10 (Fig. 1.5). The most abundant mitochondrial Hsp70, known as mtHsp70 or Ssc1p, is the major mitochondrial folding helper that assists in *de novo* folding and prevents aggregation of newly imported proteins (Craig et al. 1989; Kawai et al. 2001; Liu et al. 2001). Additionally, a fraction of Ssc1 is associated with the TIM23 translocase and it plays a key role in the general import pathway of the mitochondrial precursors (Kang et al. 1990; Manning-Krieg et al. 1991; Schneider et al. 1994; Ungermann et al. 1994; Horst et al. 1997; Liu et al. 2003; D'Silva et al. 2004). The Ssc1p homologue, Ssq1p is involved in the biogenesis of FeS cluster-containing proteins that takes place in mitochondria (Lutz et al. 2001; Dutkiewicz et al. 2003; Dutkiewicz et al. 2004). Although the exact mechanism of Ssq1 is not completely understood, it most probably functions in the transfer of the assembled FeS cluster to the recipient apoproteins (Dutkiewicz et al. 2006). The third member of the mitochondrial Hsp70 subfamily, called Ecm10 or Ssc3p, shares over 82 % sequence identity with Ssc1. However, it has been shown not to overlap functionally with Ssc1 due to its decreased

stability and differences in interaction with co-chaperones (Pareek et al. 2011). *In vivo*, expression levels of Ecm10 are very low and since no phenotype upon *ECM10* gene deletion has been observed, the physiological function remains to be determined (Baumann et al. 2000).

Structures of all three mitochondrial Hsp70s resemble the structure of other Hsp70 proteins. They consist of a highly conserved N-terminal ATPase domain and a less conserved PBD built of the β -stranded substrate binding pocket and the α -helical lid (Bukau and Horwich 1998). The domains are connected via an interdomain linker that mediates domain crosstalk and consists of four hydrophobic amino acid residues (VLLL) (Han and Christen 2003; Jiang et al. 2005; Vogel et al. 2006; Swain et al. 2007).

All three mitochondrial Hsp70 proteins interact with the co-chaperones regulating their activity and specificity. Ssc1 cooperates with two distinct J proteins: Mdj1p in the processes of protein folding (Rowley et al. 1994), and Tim14 during the import reaction (D'Silva et al. 2003; Mokranjac et al. 2003; Truscott et al. 2003). Ssq1 requires another J protein, Jac1, that interacts with the FeS cluster scaffold protein Isu1 (Lutz et al. 2001; Voisine et al. 2001). Mitochondrial nucleotide exchange factor Mge1p seems to be shared by all three members of mtHsp70 subfamily (Miao et al. 1997; Schmidt et al. 2001; Pareek et al. 2011).

In higher eukaryotes, only one or two isoforms of Hsp70 protein are present in the mitochondria (Wadhwa et al. 1993). In human, the only isoform is called mortalin, due to its involvement in cell death and tumorigenesis (Wadhwa et al. 1993; Burbulla et al. 2010). Mortalin fulfills the functions of both Ssc1 and Ssq1 and was reported to be additionally present in other cellular compartments, where it interacts with p53 and proteins of the Ras-Raf pathways (Kaul et al. 1993; Ran et al. 2000; Wadhwa et al. 2002). However, the function and relevance of the extramitochondrially localized mortalin remain unclear to date.

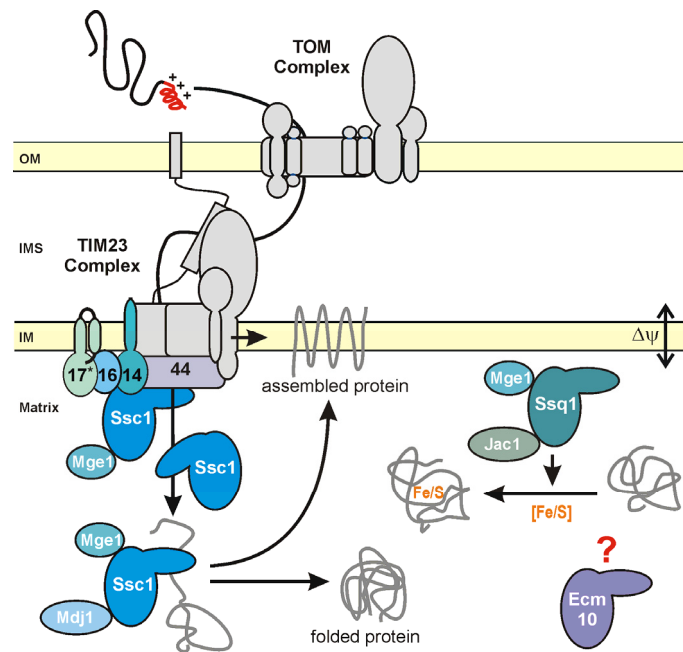


Figure 1.5. Mitochondrial Hsp70 chaperone systems in *S. cerevisiae*. See text for details.

1.4.3. Role of the mitochondrial Hsp70 chaperone Ssc1 in protein import and folding

Ssc1 plays a critical role for the functioning of mitochondria and is essential for the cell viability under all conditions (Craig et al. 1987). It is involved in two independent processes underlying the biogenesis of mitochondrial proteins: pre-protein import into mitochondria and *de novo* folding of proteins in the mitochondrial matrix.

Ssc1 is a main constituent of the import motor of the TIM23 translocase, thereby driving protein translocation in the general import pathway. The import motor is associated with the core of the TIM23 translocase and consists of Tim44, Ssc1 and the co-chaperones Tim14/Pam18, Tim16/Pam16 and Mge1 (Neupert and Brunner 2002; Marom et al. 2010; van der Laan et al. 2010). Tim44 plays a role as an adapter protein and it docks Ssc1 onto the membrane (Kronidou et al. 1994; Rassow et al. 1994; Schneider et al. 1994). Ssc1 is the only ATP-consuming unit of the import motor. The cycles of ATP hydrolysis by Ssc1 are coupled to the vectorial translocation of the polypeptide chain into the mitochondrial matrix. The hydrolysis rates are stimulated by Tim14 which is a J protein and by the nucleotide exchange factor Mge1 (Laloraya et al. 1994; Nakai et al. 1994; Westermann et al. 1995; Deloche and Georgopoulos 1996; D'Silva et al. 2003; Mokranjac et al. 2003;

Truscott et al. 2003). In the absence of incoming pre-protein, the stimulatory function of Tim14 is inhibited by Tim16 associated with it (Frazier et al. 2004; Kozany et al. 2004; Li et al. 2004; Mokranjac et al. 2006).

The suggested mechanism of translocation is called a trapping mechanism and is based on the Brownian motion of the pre-protein in transit (Ungermann et al. 1994; Neupert and Brunner 2002; Okamoto et al. 2002; Liu et al. 2003; Yamano et al. 2008; Marom et al. 2010). In the first step of translocation, the membrane potential drives transport of the positively charged presequence across the inner mitochondrial membrane. When the pre-protein emerges from the TIM23 channel on the matrix side, it is recognized by Tim44 and transferred to Ssc1. Upon pre-protein binding, Ssc1 dissociates from Tim44, allowing for a new Ssc1 molecule to bind. ATP hydrolysis stimulated by Tim14 leads to entrapment of pre-protein by the PBD of Ssc1 and prevents the pre-protein from backsliding. Via Brownian motion, a part of pre-protein remaining outside of mitochondria gradually unfolds and moves deeper into the matrix. There, it gets bound by another Ssc1 molecule associated with Tim44, and the cycle repeats. Mge1 helps Ssc1 to exchange ADP to ATP which causes the release of pre-protein and allows Ssc1 to bind to Tim44 again. In the end, the cycling of Ssc1 leads to the complete unidirectional translocation of the pre-protein into the mitochondrial matrix in a hand-over-hand manner

An alternative model of the pre-protein translocation, called the power stroke model, was also suggested (Glick 1995; Voos et al. 1996; Horst et al. 1997; Matouschek et al. 1997; Voisine et al. 1999; Krayl et al. 2007). In this model, the ATP hydrolysis by Ssc1 triggers its conformational change that leads to an active pulling of the pre-protein into the mitochondrial matrix. That causes a global unfolding of the pre-protein remaining on the cytosolic side and drives the translocation process. It is likely that both mechanisms are used for the pre-protein translocation, depending on the pre-protein itself and its state of folding outside of the mitochondria (van der Laan et al. 2010).

Apart from the function in the import of pre-proteins, Ssc1 is also one of the main folding helpers of mitochondria (Kang et al. 1990; Liu et al. 2001). Ssc1 interacts with newly imported, unfolded proteins, prevents their aggregation and assists in folding. Moreover, it helps in the folding of mitochondrially encoded proteins and their assembly into macromolecular complexes (Herrmann et al. 1994). In addition, Ssc1 cooperates with the Hsp78 chaperone on refolding and reactivation of misfolded and aggregated proteins (Krzewska et al. 2001). In all these processes, Ssc1 employs a soluble J protein, Mdj1, that stimulates the ATP hydrolysis of Ssc1, and, like during the import reactions, nucleotide

exchange factor Mge1 (Rowley et al. 1994; Westermann et al. 1996; Miao et al. 1997; Schmidt et al. 2001; Pareek et al. 2011). The Mdj1 protein, specific for the protein folding action of Ssc1, also shows a chaperone activity by itself and is involved in inheritance of the mitochondrial genome (Prip-Buus et al. 1996; Duchniewicz et al. 1999). The role of the Ssc1 chaperone system in protein folding was demonstrated both *in vivo* and in a reconstituted system using model substrates, such as Su9-DHFR and luciferase (Kang et al. 1990; Manning-Krieg et al. 1991; Rospert et al. 1996; Liu et al. 2001).

1.4.4. Hep1 – a chaperone for a chaperone

Although Ssc1 prevents protein aggregation and is involved in dissociation of already aggregated proteins, it has itself the propensity to aggregate. Therefore, to be fully active *in vivo* and *in vitro*, Ssc1 requires the helper protein, the chaperone Hep1 (Sanjuan Szklarz et al. 2005; Sichting et al. 2005).

Hep1, also known as Tim15/Zim17 (Burri et al. 2004; Yamamoto et al. 2005), was identified in *Saccharomyces cerevisiae* as an ATP-dependent interaction partner of Ssc1 (Sichting et al. 2005). Hep1 is a small 17 kDa protein, which binds zinc ions (Burri et al. 2004; Sichting et al. 2005; Yamamoto et al. 2005). It is present throughout the eukaryotic kingdom, however only in mitochondria and chloroplasts. To date, no bacterial Hep1 counterpart was found.

Deletion of the *HEP1* gene in *S. cerevisiae* causes a pleiotropic phenotype, including a temperature-sensitive growth defect, impairment of the pre-protein import into the mitochondria via the general import pathway, decreased activity of FeS cluster-containing proteins, mitochondrial and nuclear genome instability and altered mitochondrial morphology (Burri et al. 2004; Sanjuan Szklarz et al. 2005; Sichting et al. 2005; Yamamoto et al. 2005; Diaz de la Loza et al. 2011). All those defects could be explained by the impaired activity of the mitochondrial Hsp70 proteins. Indeed, in the absence of Hep1, Ssc1 and Ssq1 were found to be aggregated and partially non-functional (Sanjuan Szklarz et al. 2005; Sichting et al. 2005). Therefore, Hep stands for Hsp70 escort protein, as it prevents aggregation and keeps mitochondrial Hsp70s in a functional state. The importance of Hep1 for Ssc1 was additionally shown in the bacterial system. Only upon co-expression with Hep1, Ssc1 was recovered in a soluble form (Sichting et al. 2005). The same protective effect was observed towards the human mtHsp70 (HSPA9) and the chloroplast HSP70B from the algae *Chlamydomonas reinhardtii* upon bacterial co-

expression with the corresponding Hep proteins (Willmund et al. 2008; Zhai et al. 2008). Additionally, Hep1 prevented oligomerisation and loss of activity of the purified Ssc1 *in vitro* (Sichting et al. 2005). All these findings suggest that the main function of Hep proteins is the maintenance of the structure and function of the cognate Hsp70 chaperones.

It was also put forward that yeast Hep1 is a constituent of the import motor of TIM23 translocase, possibly functioning as a ‘fractured’ J protein, that contributes a zinc finger domain to the Tim14 *in trans* (Burri et al. 2004; Yamamoto et al. 2005). As such a component, Hep1 would present the incoming substrate to the Ssc1 associated with the translocase. However, Hep1 was found not to be associated with the other translocase components. Therefore, this hypothesis remains unlikely (Sichting et al. 2005).

The NMR structure of the core part of Hep1 revealed it to be an L-shaped molecule, with two conserved zinc-finger motifs located at one end (Fig. 1.6). Binding of the zinc ions seems to be critical for the overall structure of the protein (Momose et al. 2007). Although the zinc-finger motifs of Hep1 resemble those of other J proteins, the overall fold of Hep1 is different from the cysteine-rich domain of DnaJ, the bacterial representative of J proteins (Martinez-Yamout et al. 2000; Momose et al. 2007). On the convex face of the L, a large acidic groove is present. The concave face of the L contains an aspartate residue (Asp111) and two positively charged residues, Arg106 and His107, in close proximity to the zinc-finger motifs. Mutational analysis revealed Asp111, Arg106 and His107 to be crucial for the *in vivo* function of Hep1 and for the interaction with Ssc1 *in vitro*. This suggests that the concave face of Hep1 is the potential binding site for Ssc1 (Momose et al. 2007). The mechanism of the function of the Hep1 protein as well as the structural determinants of Ssc1, critical for the interaction with Hep1, are, however, still unclear.

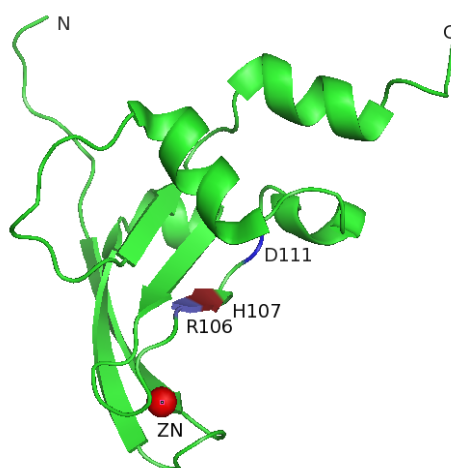


Figure 1.6. Structure of Hep1 core from *S. cerevisiae*

Amino acid residues important for the Hep1 function are indicated. The zinc ion is shown as a red sphere. Structure view generated using PyMOL program. Protein Data Bank accession code 2E2Z (Momose et al. 2007).

1.5. Aim of the present study

The yeast mitochondrial Hsp70 chaperone Ssc1 is a crucial component of the mitochondrial chaperone network, essential for the function of mitochondria and, therefore, for the survival of the cell. Despite being involved in protein folding and prevention of protein aggregation, Ssc1 itself has propensity to aggregate. Thus, a helper protein, the chaperone Hep1 is required to maintain the structure and function of the Ssc1 chaperone. Why a chaperone has the propensity to aggregate and how Hep1 prevents the aggregation process, remained unclear. To get insight into the mechanism and function of Hep1 in the prevention of aggregation of Ssc1, it was the first aim of this study to analyse the structural determinants of Ssc1 for the propensity to aggregate and for the interaction with Hep1.

Based on the phenotypes observed in the absence of Hep1, one could expect Hep1 to be involved in the *de novo* folding of Ssc1. The biogenesis of molecular chaperones, in particular the Hsp70 chaperones, including their folding *de novo*, has been poorly studied. The second aim of the present study was to characterise the *de novo* folding of the Ssc1 chaperone and to elucidate a potential chaperone function of Hep1 in this process, establishing and employing both *in organello* and *in vivo* approaches. In addition, the folding process should be reconstituted with purified components, to provide more insights into the folding process of Ssc1 on the molecular level.

2. Materials and methods

2.1. Molecular biology methods

2.1.1. Isolation of plasmid DNA from *E.coli*

Plasmid DNA from *E. coli* cells was isolated using the PureYield Plasmid Midiprep System kit (Promega). Bacteria carrying the plasmid of interest were inoculated in 40–50 ml LB-Amp medium and grown overnight (37 °C with shaking at 140 rpm). Cells were harvested by centrifugation (10000 x g, 10 min, RT) and resuspended in 6 ml of Cell Resuspension Solution. Next, cells were lysed for 3 min with 6 ml of Cell Lysis Solution and neutralized with 10 ml of Neutralization Solution. Precipitated material was removed by centrifugation (10000 x g, 10 min, 4°C), and the supernatant was applied to a clarifying column and an anion-exchange column to bind DNA and placed onto a vacuum manifold. Next, the anion-exchange column was washed with 5 ml of Endotoxin Removal Wash and then with 20 ml of the Column Wash Solution. The column was dried under vacuum for 30 sec, plasmid DNA was eluted with 600 µl of deionized water and DNA concentration was measured by the NanoDrop 2000c (Thermo Scientific). Plasmid DNA was stored at –20 °C.

2.1.2. Amplification of DNA sequences by Polymerase Chain Reaction (PCR)

A PCR reaction was used to amplify DNA fragments used for subsequent cloning. A typical mix for the PCR reaction (total volume of 100 µl) contained 1.25 U of GoTaq® DNA Polymerase (Promega), 20 µl of 5X Green GoTaq® Reaction Buffer (Promega), 2 µl dNTPs (10 mM each), 1 µl of forward and reverse primer (50 µM each) and 50–150 ng plasmid DNA as a template. The following PCR program, with small variations depending on the DNA sequence, was used:

Cycle step	Temperature	Time	No. of cycles
Initial denaturation	94 °C	4 min	1
Denaturation	94 °C	1 min	30
Primer annealing	60–68 °C	30 sec	
Primer extension	72 °C	1– 2.5 min	
Final extension	72 °C	10 min	1
Cooling	4 °C	-	-

DNA fragments obtained by PCR were analysed by agarose gel electrophoresis (2.1.5).

2.1.3. Restriction digestion of DNA

DNA was digested with 1 µl (2–5 U) of the appropriate restriction endonucleases (New England Biolabs) per 1 µg of DNA in a buffer specific for the restriction enzyme. Reactions were carried out in 37 °C for 1 h in a total volume of 100 µl. Digested DNA was analysed by agarose gel electrophoresis (2.1.5).

2.1.4. Ligation of DNA fragments

A DNA fragment and a target vector after digestion with the same or compatible restriction enzymes were ligated together using T4 DNA ligase (Promega). 80–200 ng of vector DNA and 3–10-fold molar excess of DNA fragment were incubated in ligase buffer with addition of 1 U of T4 DNA ligase in a final volume of 10 µl. Ligation was carried out at 25 °C for 1–3 h. 1 µl of the ligation mix was transformed into electrocompetent MH1 *E. coli* cells (2.1.8).

Ligase buffer

30 mM TRIS-HCl pH 7.8,
10 mM MgCl₂,
10 mM DTT,
1 mM ATP

2.1.5. Agarose gel electrophoresis of DNA

DNA fragments were separated according to their sizes by horizontal agarose gel electrophoresis. Agarose gels were prepared in TAE buffer with addition of 0.5 µg/ml ethidium bromide in a pre-casted container. The DNA sample was supplemented with a 10 x loading buffer and loaded on a 0.8–1.5 % (w/v) agarose gel, depending on the fragment size of the DNA. Gels were run in TAE buffer at 90–150 V depending on the gel sizes. Separated DNA fragments were visualized under UV light.

TAE buffer

40 mM TRIS-acetate pH 7.5,
20 mM Na-acetate,
1 mM EDTA

10 x loading buffer

40 % (v/v) glycerol,
0.25 % (w/v) bromphenol-blue,
0.25 % (w/v) xylencyanol

2.1.6. Isolation of DNA fragments from agarose gel

Selected DNA fragments were cut out from the gel with a scalpel under UV light. DNA was extracted from the excised agarose block using the Wizard SV® Gel and PCR Clean-Up System kit (Promega). One volume of Membrane Binding Solution was added to the sample and the sample was incubated at 65 °C until the agarose block completely dissolved. Next, the sample was applied to the DNA-binding silica column. After a double wash with the Membrane Washing Solution, the column was quickly dried by a short centrifugation step. Bound DNA was eluted with 30–50 µl of nuclease-free H₂O. The concentration of the isolated DNA was measured with the NanoDrop 2000c.

2.1.7. Overview of *E. coli* strains used

Strain	Genotype	Reference
MH1	MC1061 derivative; <i>araD139</i> , <i>lacX74</i> , <i>galU</i> , <i>galK</i> , <i>hsr</i> , <i>hsm</i> ⁺ , <i>strA</i>	(Casadaban and Cohen 1980)
XL1Blue	<i>supE44</i> , <i>hsdR17</i> , <i>recA1</i> , <i>endA1</i> , <i>gyrA96</i> , <i>thi-1</i> , <i>relA1</i> , <i>lac</i> ⁻ , F'[<i>proAB</i> ⁺ , <i>lacI</i> ^q , <i>lacZ</i> ΔM15, Tn10(<i>tet</i> ^r)]	Commercially available from Stratagene
BL21(DE3)	F ⁻ , <i>ompT</i> , <i>gal</i> , <i>dcm</i> , <i>lon</i> , <i>hsdS</i> _B (<i>r</i> _B ⁻ <i>m</i> _B ⁻), λ(DE3[<i>lacI lacUV5-T7</i> gene 1 <i>ind1 sam7 nin5</i>])	Commercially available from Novagen
BL21(DE3) ΔDnaK	F ⁻ , <i>ompT</i> , <i>gal</i> , <i>dcm</i> , <i>lon</i> , <i>hsdS</i> _B (<i>r</i> _B ⁻ <i>m</i> _B ⁻), λ(DE3[<i>lacI lacUV5-T7</i> gene 1 <i>ind1 sam7 nin5</i>]) Δ <i>dnaK52::Cm</i> ^r	Kind gift from M. Mayer and B. Bukau, University of Heidelberg

2.1.8. Preparation of electrocompetent *E. coli* cells

500 ml of LB culture was inoculated with 1 ml of the overnight culture of *E. coli* cells. The culture was grown at 37°C with shaking at 140 rpm until OD₆₀₀ = ca. 0.5 and then incubated on ice for 30 min. Next, cells were harvested by centrifugation for 5 min at 4400 x g at 4 °C and resuspended in 500 ml of sterile, ice-cold 10 % (v/v) glycerol. The procedure of harvesting and resuspending was repeated with 250 and 50 ml ice-cold 10 % (v/v) glycerol. Finally, cell pellet was resuspended in 500 µl of ice-cold 10 % (v/v) glycerol, cells were aliquoted (40 µl per test-tube) and stored at -80 °C.

2.1.9. Transformation of *E. coli* cells by electroporation

The ligation mixtures or the plasmid DNA were transformed into electrocompetent *E. coli* cells by electroporation. A 40 µl aliquot of electrocompetent cells was thawed on ice and 1 µl of ligation mixture or plasmid DNA (10–50 ng) was added and incubated for 1 min on ice. Next, cell suspension was transferred to the 0.2 cm-thick cooled electroporation cuvette and the cuvette was placed in the electroporation apparatus ‘Gene Pulser’ (BioRad). The apparatus was set to 1.5 kV, 400 Ω, 25 µF, and time constant 8–9 ms and the cell suspension was treated with a short electrical pulse. Next, cells were diluted with 1 ml of ice-cold SOC medium, transferred to a test-tube and incubated with shaking at 37 °C

for recovery. Cells were then harvested by short centrifugation, resuspended in 100 µl of SOC medium and plated on LB plates supplemented with 100 µg/ml ampicillin. Plates were incubated at 37 °C overnight. To check whether obtained bacterial colonies contained the right construct, 5–20 colonies were tested by PCR. To this end, a little amount of bacterial material was used in the reaction instead of template DNA and the procedure was performed as described in 2.1.2.

2.1.10. Overview of plasmids used

Plasmid	Description	Reference
pYX142-ATPase-his	For over-expression of C-terminally his-tagged ATPase domain in yeast mitochondria	(Blamowska et al. 2011)
pYX142-pSu9-PBD-his	For over-expression of C-terminally his-tagged PBD in yeast mitochondria	(Blamowska et al. 2011)
pYX142-Ssc1-his	For over-expression of C-terminally his-tagged Ssc1 in yeast mitochondria	(Blamowska et al. 2011)
pYX142-ATPaseLinker-his	For over-expression of C-terminally his-tagged ATPaseLinker in yeast mitochondria	This thesis
pYX142-ATPaseA4-his	For over-expression of C-terminally his-tagged ATPaseA4 in yeast mitochondria	This thesis
pYX142-ATPaseLinker	For over-expression of non-tagged ATPaseLinker in yeast mitochondria	This thesis
pGEM4-Ssc1	For <i>in vitro</i> transcription and translation of Ssc1	(Moro et al. 2002)

pGEM4-Cytb ₂ (1-167)DHFR	Template for cloning of b ₂ (87–167) DHFR DNA sequence into pGEM4 vector	(Schneider et al. 1994)
pGEM4-b ₂ (87–167)DHFR	Acceptor vector for cloning of single Ssc1 domains as fusion proteins into pGEM4 vector	This thesis
pGEM4-ATPase-b ₂ (87–167)DHFR	For <i>in vitro</i> transcription and translation of ATPase-DHFR fusion protein	This thesis
pGEM4-pSu9-PBD-b ₂ (87–167)DHFR	For <i>in vitro</i> transcription and translation of PBD-DHFR fusion protein	This thesis
pGEM4-ATPaseLinker-b ₂ (87–167)DHFR	For <i>in vitro</i> transcription and translation of ATPaseLinker-DHFR fusion protein	This thesis
pGEM4-ATPaseA4-b ₂ (87–167)DHFR	For <i>in vitro</i> transcription and translation of ATPaseA4-DHFR fusion protein	This thesis
pGEM4-Ssc1A4	For <i>in vitro</i> transcription and translation of Ssc1A4 mutant	This thesis
pETDuet-Ssc1 _{mat} +his-Hep1 _{mat}	For co-expression of mature Ssc1 and N-terminally his-tagged mature Hep1 in <i>E. coli</i>	(Sichting et al. 2005)
pETDuet-ATPaseLinker _{mat} +his-Hep1 _{mat}	For co-expression of mature ATPaseLinker and N-terminally his-tagged mature Hep1 in <i>E. coli</i>	(Blamowska et al. 2011)
pETDuet-PBD-his	For expression of C-terminally his-tagged PBD in <i>E. coli</i>	(Blamowska et al. 2011)
pQE30-his-Hep1 _{mat}	For expression of N-terminally his-tagged mature Hep1 in <i>E. coli</i>	(Sichting et al. 2005)

pQE30-his-MgeI _{mat}	For expression of N-terminally his-tagged mature MgeI in <i>E. coli</i>	(Horst et al. 1997)
pETDuet-his-TEV-DnaJ	For expression of N-terminally his-tagged DnaJ in <i>E. coli</i>	Kind gift of D. Mokranjac, LMU München

2.1.11. Cloning strategies

Plasmids generated in this thesis were cloned using the following strategies. Sequences of the generated constructs were confirmed by sequencing by Eurofins MWG GmbH (Ebersberg).

pYX142-ATPaseLinker-his

The coding sequence for the ATPase domain with the subsequent interdomain linker of Ssc1 (amino acid residues 1–415) was amplified by PCR using primers 5'EcoRI-Ssc1 and 3'Ssc1dC239-his-XhoI and the plasmid pYX142-Ssc1-his as a template. The obtained DNA fragment was cloned into the vector pYX142 using *EcoRI* and *XhoI* restriction sites encoded in the primers.

5'EcoRI-Ssc1:

5' - ATAT GAATTC ATG CTT GCT GCT AAA AAC ATA C - 3'

3'Ssc1dC239-his-XhoI:

5' - ATAT CTCGAG TTA ATG GTG ATG GTG ATG GTG TAA TAA TAA GAC GTCAG - 3'

pYX142-ATPaseA4-his

The coding sequence for the ATPase domain of Ssc1 (amino acid residues 1–411) with the additional four alanine residues was amplified by PCR using primers 5'EcoRI-Ssc1 and 3'Ssc1dC239-A4-his-XhoI and the plasmid pYX142-Ssc1-his as a template. The obtained DNA fragment was cloned into the vector pYX142 using *EcoRI* and *XhoI* restriction sites encoded in the primers.

3'Ssc1dC239-A4-his-XhoI:

5' - ATAT CTCGAG TTA ATG GTG ATG GTG ATG GTG AGC TGC AGC GGC GTC AGT AAC CTC - 3'

pYX142-ATPaseLinker

The coding sequence for the ATPase domain with the subsequent interdomain linker of Ssc1 (amino acid residues 1–415) was amplified by PCR using primers 5'EcoRI-Ssc1 and 3'Ssc1dC239-XhoI and the plasmid pYX142-Ssc1-his as a template. The obtained DNA fragment was cloned into the vector pYX142 using *EcoRI* and *XhoI* restriction sites encoded in the primers.

3'Ssc1dC239-XhoI:

5'- ATAT CTCGAG TTA TAA TAA TAA GAC GTC AG - 3'

pGEM4-b₂(87–167)DHFR

The coding sequence for the yeast cytochrome *b₂* (amino acid residues 87–167) together with the sequence of mouse dihydrofolate reductase (DHFR) was amplified by PCR using primers 5'XbaI-cytb2_aa87 and 3'DHFR-PstI and the plasmid pGEM4-cytb2(1–167)DHFR as a template. The obtained DNA fragment was cloned into the vector pGEM4 using *XbaI* and *PstI* restriction sites encoded in the primers.

5'XbaI-cytb2_aa87:

5'- CGAT TCTAGA AAT AAA CAA AAG ATT TCG CC - 3'

3'DHFR-PstI:

5'- ATAT CTGCAG TTA GTC TTT CTT CTC GTA GAC - 3'

pGEM4-ATPase-b₂(87–167)DHFR

The coding sequence for the ATPase domain of Ssc1 (amino acid residues 1–411) was amplified by PCR using primers 5'EcoRI-Ssc1 and 3'ATPase_nostop-XbaI and the plasmid pYX142-Ssc1-his as a template. The obtained DNA fragment was cloned into the vector pGEM4-b₂(87–167)DHFR using *EcoRI* and *XbaI* restriction sites encoded in the primers.

3'ATPase_nostop-XbaI:

5'- ATAT TCTAGA AAC CTC ACC GGA CAA GAC AGC - 3'

pGEM4-pSu9-PBD-b₂(87–167)DHFR

The coding sequence for the PBD of Ssc1 (amino acid residues 410–654) together with the coding sequence for the presequence of subunit 9 of the F₁F₀ ATPase of *Neurospora crassa* on the 5' end was amplified by PCR using primers 5'EcoRI-Su9-PBD and 3'Ssc1_nostop-XbaI and the plasmid pYX142-pSu9-PBD-his as a template. The obtained

DNA fragment was cloned into the vector pGEM4-b₂(87–167)DHFR using *EcoRI* and *XbaI* restriction sites encoded in the primers.

5'EcoRI-Su9-PBD:

5'- ATAT GAATTC ATG GCC TCC ACT CGT GTC - 3'

3'Ssc1_nostop-XbaI:

5'- ATAT TCTAGA CTG CTT AGT TTC ACC AGA TTC G - 3'

pGEM4-ATPaseLinker-b₂(87–167)DHFR

The coding sequence for the ATPase domain with the subsequent interdomain linker of Ssc1 (amino acid residues 1–415) was amplified by PCR using primers 5'EcoRI-Ssc1 and 3'Ssc1d239_nostop-XbaI and the plasmid pYX142-Ssc1-his as a template. The obtained DNA fragment was cloned into the vector pGEM4-b₂(87–167)DHFR using *EcoRI* and *XbaI* restriction sites encoded in the primers.

3'Ssc1d239_nostop-XbaI:

5'- ATAT TCTAGA TAA TAA TAA GAC GTC AGT AAC C - 3'

pGEM4-ATPaseA4-b₂(87–167)DHFR

The coding sequence for the ATPase domain of Ssc1 (amino acid residues 1–411) with the additional four alanine residues was amplified by PCR using primers 5'EcoRI-Ssc1 and 3'Ssc1d239A4_nostop-XbaI and the plasmid pYX142-ATPaseA4-his as a template. The obtained DNA fragment was cloned into the vector pGEM4-b₂(87–167)DHFR using *EcoRI* and *XbaI* restriction sites encoded in the primers.

3'Ssc1d239A4_nostop-XbaI:

5'- ATAT TCTAGA AGC TGC AGC GGC GTC AGT AACC - 3'

pGEM4-Ssc1A4

The Ssc1A4 mutant was generated by an overlap extension PCR method. The coding sequences for Ssc1 (amino acid residues 1–411 and 416–654) were amplified by PCR using primer pairs 5'EcoRI-Ssc1/3'Ssc1A4_REV and 5'Ssc1A4_FOR/3'Ssc1-Sac1 and the plasmid pYX142-Ssc1-his as a template. The primers 3'Ssc1A4_REV and 5'Ssc1A4_FOR contained sequences encoding four alanine residues. The obtained DNA fragments were used as template for the final PCR reaction with primers 5'EcoRI-Ssc1 and 3'Ssc1-Sac1. The obtained DNA fragment was cloned into the vector pGEM4 using *EcoRI* and *SacI* restriction sites encoded in the primers.

5'Ssc1A4_FOR:

5' - CT GAC GCC GCT GCA GCA GAT GTT ACC CCA TTG - 3'

3'Ssc1A4_REV:

5' - GT AAC ATC TGC TGC AGC GGC GTC AGT AAC CTC ACC - 3'

3'Ssc1-Sac1:

5' - CCC GAGCTC TTA CTG CTT AGT TTC ACC AGA TTC G - 3'

2.1.12. Overview of yeast *S. cerevisiae* strains used

Strain	Description	Reference
FY1679-11a	Wild-type strain corresponding to <i>hep1</i> deletion strain	Euroscarf (10000R)
$\Delta hep1$ (<i>hep1::KanMX</i>)	<i>hep1</i> deletion strain (parental strain: FY1679-11a)	This thesis
<i>mif4</i>	Hsp60-defective temperature- sensitive mutant strain	(Cheng et al. 1989)
[<i>pGALOTC</i>]RP11	Wild-type strain corresponding to <i>mif4</i> strain	(Cheng et al. 1989)
$\Delta hsp78$ (<i>hsp78::URA3</i>)	<i>hsp78</i> deletion strain	(Schmitt et al. 1995)
PK82	Wild-type strain corresponding to $\Delta hsp78$ strain	(Gambill et al. 1993)
$\Delta mdj1$ (<i>mdj1::KanMX</i>)	<i>mdj1</i> deletion strain (parental strain: W303-1A)	Martin Sichtung (unpublished work)
W303-1A	Wild-type strain corresponding to $\Delta mdj1$ strain	Kind gift from R. Rothstein, Columbia University, New York

2.1.13. Deletion of *HEP1* gene in FY 1679-11a yeast strain – generation of Δ *hep1* strain

HEP1 gene deletion was done by homologous recombination of a PCR product exchanging the gene, in the haploid yeast strain FY 1679-11a. The PCR product contained a *KANMX* cassette encoding for resistance against kanamycin and its analogues and short flanking sequences homologous to the flanking regions of *HEP1* locus, encoded in primers. The PCR product was produced using primers 5'*hep1_KO* and 3'*hep1_KO* on the plasmid pFA6KANMX4 as a template (Wach et al. 1997). The PCR product was transformed into yeast and became stably integrated into the chromosome via the regions homologous to the *HEP1* gene. Positive clones were isolated on YPG medium containing 500 µg/ml G418 (kanamycin analogue) and the *HEP1* gene depletion was tested by fast mitochondria isolation (2.2.5.1) and immunodecoration against *hep1* protein (2.4.1).

5'*hep1_KO*:

5'- TAA TTG TAA TTT TTT TTT CTT GCA TTT TCG CGT GAT ATC AAA ATT CGT ACG
CTG CAG GTC GAC - 3'

3'*hep1_KO*:

5'- CGG TTA TAT ATC TAT CTA TGT ACA TGC CTG TGC ATG AGC TCT TCA TAT CGA
TGA ATT CGA GCT CG - 3'

2.1.14. Transformation of yeast *S. cerevisiae* with recombinant DNA

Yeast cells were transformed with DNA using the lithium acetate method (Gietz et al. 1992). 50 ml yeast culture was grown at 30 °C with 140 rpm shaking until OD₆₀₀ = ca. 0.5. Cells were harvested by centrifugation for 5 min at 1500 x g and washed once with sterile deionized H₂O. The cell pellet was resuspended in 1ml 100 mM Li acetate and transferred into a 1.5 ml test tube. Cells were harvested again by short centrifugation and resuspended in 400 µl 100 mM Li acetate. The cell suspension was divided into 50 µl aliquots (one aliquot per transformation). Cells were briefly pelleted, and overlaid with 240 µl 50 % (w/v) PEG 3550, 36 µl 1 M Li acetate, 5 µl heat-denatured, ice-cold salmon-sperm DNA (10 mg/ml), 60 µl H₂O and 5 µl DNA (0.1–10 µg plasmid DNA or PCR product). Samples were vigorously mixed until resuspended and then incubated with shaking at 30 °C for 30 min and heat-shocked at 42 °C for 25 min. Cells were then briefly pelleted, resuspended in 150 µl sterile deionized H₂O and plated on selective media. The plates were incubated at

24 °C for 2–10 days until the appearance of single yeast colonies. Transformants were tested with PCR and with fast mitochondria isolation, SDS-PAGE and immunodecoration.

2.2. Cell biology methods

2.2.1. Media for *E. coli* culture

Liquid LB medium (Lysogeny broth)

0.5 % (w/v) yeast extract,
1 % (w/v) bacto-tryptone,
1 % (w/v) NaCl

Liquid LB-Amp medium

LB medium supplemented with 100 µg/ml of ampicilin

Liquid SOC medium

2 % (w/v) bacto-tryptone
0.5 % (w/v) yeast extract
8.56 mM NaCl
2.5 mM KCl
10 mM MgCl₂
20 mM glucose

All media were prepared in double distilled water and autoclaved. In order to prepare solid media (LB or LB-Amp agar plates), liquid LB medium was supplemented with 1.7 % (w/v) bacto-agar, autoclaved (120 °C, 20 min) and in the case of LB-Amp plates, 100 µg/ml of ampicilin was added afterwards.

2.2.2. Cultivation of *E. coli* cells

The liquid medium (usually LB-Amp, or LB in the case of cultivation of electrocompetent *E. coli*) was inoculated with a single *E. coli* colony from the plate and grown overnight at 37 °C in the case of MH1, XL1Blue and BL21(DE3) strains and at 30 °C in the case of BL21(DE3) ΔDnaK strain with shaking at 140 rpm. Then culture was diluted to the desired

volume and grown further as described, or directly used for the plasmid isolation or preparation of electrocompetent cells.

2.2.3. Media for yeast *S. cerevisiae* culture

YP medium (non-selective)

1 % (w/v) bacto-peptone,

1 % (w/v) yeast extract,

pH adjusted to 5.5

YPG medium (non-selective)

YP medium supplemented with 3 % glycerol

YPGal medium (non-selective)

YP medium supplemented with 2 % galactose

Lactate medium (non-selective, semi-synthetic)

3 g yeast extract,

1 g KH_2PO_4 ,

1 g NH_4Cl ,

0.5 g $\text{CaCl}_2 \times 2\text{H}_2\text{O}$,

0.5 g NaCl ,

1.1 g $\text{MgSO}_4 \times 6\text{H}_2\text{O}$,

0.3 ml 1 % FeCl_3 ,

22 ml 90 % lactic acid,

H_2O added to 1 l,

pH adjusted to 5.5 (using KOH)

SD medium (selective)

0.17 % yeast nitrogen base,

0.5 % $(\text{NH}_4)_2\text{SO}_4$,

2 % glucose,

appropriate amount of auxotrophic markers,

H_2O added to 1 l

SLac medium (selective)

0.17 % yeast nitrogen base,
0.5 % (NH₄)₂SO₄,
22 ml 90 % lactic acid,
appropriate amount of auxotrophic markers,
H₂O added to 1 l,
pH adjusted to 5.5 (using KOH)

Auxotrophic markers used for selective media

20 mg/l adenine sulfate
20 mg/l uracil
20 mg/l L-tryptophan
20 mg/l L-histidine
30 mg/l L-leucine
30 mg/l L-lysine

All media were prepared in double distilled water and autoclaved. Glucose, glycerol, galactose and selection markers were autoclaved separately and added afterwards to the autoclaved medium. In order to prepare corresponding solid media, liquid medium was supplemented with 2 % (w/v) bacto-agar autoclaved separately.

2.2.4. Cultivation of *S. cerevisiae* cells

Agar plates with yeast cultures were obtained from yeast transformation or inoculated from the glycerol stocks (yeast cell suspension in 15 % (v/v) glycerol, stored in -80 °C). Liquid cultures were inoculated from the agar plates and grown in appropriate medium usually at 24 °C, unless otherwise stated, with shaking at 140 rpm. The generated *Δhep1* strain, *Δhsp78* strain and the corresponding wild-type (wt) strains were usually grown on lactate medium. *Δhep1* strain and the corresponding wt strain over-expressing Ssc1 constructs from the pYX142 vector were grown on selective SD medium lacking L-leucine (auxotrophic marker). The *mif4* strain and the corresponding wt strain were grown on YPG medium at 24 °C and shifted to 37 °C 2h prior to isolation of mitochondria. *Δmdj1* strain and the corresponding wt strain were grown on YPGal medium. Prior to mitochondria

isolation, cells were grown for at least 60 h and diluted accordingly to avoid the OD₆₀₀ exceeding 1.5.

2.2.5. Isolation of yeast mitochondria

2.2.5.1. Fast (crude) mitochondria isolation

The yeast culture was inoculated from a single colony in 50 ml of the appropriate medium and grown overnight at 24 °C with 140 rpm shaking. Yeast cells corresponding to 10 OD units were collected by centrifugation at 3000 x g for 5 min (RT), washed with deionized water and resuspended in 300 µl SH-KCl buffer containing 1 mM PMSF. Then, 200 µl of glass beads of diameter of 0.5–1 mm were added and samples were vigorously vortexed four times for 30 sec, with 30 sec intervals between each mixing on ice. Nuclei, broken cell debris and glass beads were collected by low-speed centrifugation (1000 x g, 3 min, 4°C) and the supernatant, containing crude mitochondria, was transferred to a new test-tube. Mitochondria were collected by high speed centrifugation (17400 x g, 10 min, 4°C). The supernatant, containing cytosolic proteins, was treated with trichloroacetic acid to precipitate the proteins. Mitochondrial pellet was directly suspended in 10–50 µl of Laemmli buffer, unless otherwise stated, and incubated at 95 °C for 5 min. Mitochondrial as well as cytosolic protein fractions were analysed by SDS-PAGE and immunodecoration.

SH-KCl buffer

0.6 M sorbitol,

20 mM HEPES-KOH pH 7.4,

80 mM KCl

2.2.5.2. Large scale isolation of yeast mitochondria

4–6 litres of yeast culture were grown until the OD₆₀₀ reached 0.8–1.5. Cells were collected by centrifugation (3000 x g, 5 min, RT) and washed once with deionized water. After the measuring of their wet weight, cells were resuspended in 100 mM TRIS base, 10 mM DTT to a concentration of 0.5 g cells/ml, to facilitate spheroplast formation. Cells were incubated at 30 °C for 15 min with 140 rpm shaking. Next, cells were collected by centrifugation (3000 x g, 5 min, RT), washed once with 1.2 M sorbitol and resuspended in zymolyase buffer to a concentration of 0.15 g cells/ml, in order to digest the yeast cell wall and form spheroplasts. Cells were incubated in zymolyase buffer for 30–60 min at 30 °C

with 140 rpm shaking. Then, spheroplasts were collected by centrifugation (3000 x g, 5 min, 4 °C), resuspended in ice-cold homogenization buffer to a concentration of 0.15 g cells/ml and homogenized 10 times in a cooled glass dounce homogenizer on ice. Homogenized suspension was centrifuged two times (2000 x g, 5 min, 4 °C) to get rid of cell debris, unbroken cells and nuclei. The supernatant, containing mitochondria, was poured to a new tube and the mitochondria were collected by centrifugation (17400 x g, 12 min, 4 °C). Mitochondrial pellet was resuspended in ice-cold SH buffer and centrifuged two times at a low speed (2000 x g, 5 min, 4 °C), to get rid of remaining impurities. Finally, isolated mitochondria were collected by high-speed centrifugation (17400 x g, 12 min, 4 °C), the mitochondrial pellet was resuspended in 0.5–1 ml of ice-cold SH buffer and the mitochondrial protein concentration was determined by the Bradford assay (2.3.5). The protein concentration was adjusted to 10 mg/ml with SH buffer and the mitochondrial solution was aliquoted (30–50 µl per test-tube), frozen in liquid N₂ and stored at -80 °C.

Zymolyase buffer

1.2 M sorbitol,
20 mM KH₂PO₄-KOH pH 7.4
4 mg zymolyase per 1 g of cells

SH buffer

0.6 M sorbitol,
20 mM HEPES-KOH pH 7.4

Homogenization buffer

0.6 M sorbitol,
10 mM TRIS-HCl pH 7.4,
1 mM EDTA,
0.2 % (w/v) BSA (fatty acid free),
1 mM PMSF

2.2.6. Sucrose gradient fractionation of mitochondrial proteins

Isolated mitochondria containing 200 µg of proteins were solubilized in lysis buffer at a concentration of 1 mg/ml for 30 min at 4 °C and subjected to a clarifying spin of 10,000 x g for 5 min. The pellet after the clarifying spin was directly suspended in 1 x Laemmli buffer and incubated for 5 min at 95 °C. Supernatant, containing mitochondrial lysate, was layered onto 4 ml of a continuous sucrose gradient (10 % to 30 % of sucrose) and centrifuged for 40 min at 485,000 x g at 4 °C using the SW 60 Ti rotor (Beckman Coulter). The gradient was fractionated into 500 µl fractions, starting from the bottom-most fraction, using a custom-made fractionation device. The proteins of the fractions were precipitated

by TCA and the samples were analysed by SDS-PAGE and immunodecoration with the indicated antibodies.

Lysis buffer

20 mM TRIS-HCl pH 7.4,
80 mM KCl,
1 % (v/v) Triton X-100,
1 mM PMSF

2.2.7. Protein aggregation assay

To determine the solubility of mitochondrial proteins, an aggregation assay was performed. Isolated mitochondria were pre-incubated for 15 min at 30°C and then solubilized in the lysis buffer (2.2.6) at a protein concentration of 1 mg/ml for 30 min on ice. Half of the sample was removed as control of total protein. The other half was centrifuged at 36000 x g for 10 min at 4°C to separate the soluble (Supernatant) and insoluble (Pellet) fractions. Total and supernatant were precipitated with TCA (2.3.4). Samples corresponding to 20–30 µg of mitochondrial proteins were analysed by SDS-PAGE and immunodecoration against the indicated proteins.

2.2.8. Aggregation assay of Hsp60

To analyse the solubility of Hsp60 in the *mif4* and the corresponding wild-type strain, cells were grown at 24 °C until mid-logarithmic phase, then shifted to 37 °C and grown further for 2.5 h. Next, cells corresponding to 3 OD units were collected by centrifugation at 3000 x g for 3 min, washed once with water and resuspended in 20 mM TRIS-HCl pH 7.4, 80 mM KCl, 1 mM PMSF. Then, 200 µl of glass beads of diameter 0.5–1 mm were added and samples were vigorously vortexed four times for 30 sec with 30 sec intervals on ice between mixings. The glass beads were collected by low-speed centrifugation (1000 x g, 5 sec), the broken cell suspension was transferred to a fresh tube and 1 % (v/v) of Triton X-100 was added. Cells were lysed on ice for 30 min. After a clarifying spin at 1000 x g, cell lysate was transferred to the fresh tube and centrifuged at 15000 x g for 15 min at 4 °C to separate the soluble and insoluble fraction. The soluble fraction was precipitated with TCA (2.3.4). The insoluble fraction was directly suspended in 1 x Laemmli buffer. Samples

were analysed by SDS-PAGE and immunodecoration with antibodies against bacterial GroEL that recognize yeast Hsp60.

2.2.9. Chemical cross-linking of mitochondrial proteins *in organello*

To analyse the interactions between mitochondrial proteins, the proteins were chemically cross-linked using DSG (disuccinimidylglutarate), a membrane-permeable, lysine-specific cross-linking reagent. Mitochondria were suspended in the standard import buffer (2.2.10) lacking BSA, at a protein concentration of 1 mg/ml, with addition of 10 U/ml apyrase and 10 μ M oligomycin to deplete ATP. The samples were incubated for 10 min at 37°C. DSG dissolved in DMSO was added to the samples to a final concentration of 150 μ M. Samples were incubated for 30 min on ice and then the remaining cross-linking reagent was quenched by addition of 0.1 M glycine, pH 8.8 for 10 min on ice. Mitochondria were re-isolated and cross-linked adducts were analysed by SDS-PAGE and immunodecoration. When the adducts were to be isolated via his-tag on NiNTA beads, 150 μ g of re-isolated mitochondria were solubilized in the solubilization buffer, at a protein concentration of 1 mg/ml, for 30 min on ice. After a clarifying spin at 125000 x g for 20 min at 4 °C, an aliquot of total (5 %) was taken out and the rest was added to 60 μ l (bed volume) of NiNTA-agarose beads. After 2 h of incubation at 4 °C with gentle mixing, the beads were pelleted, a sample (5 %) of non-bound material was taken out and the rest of the supernatant was removed. The beads were washed 3 times with 500 μ l of the washing buffer. The bound proteins and protein adducts were eluted by 1 x Laemmli buffer supplemented with 300 mM imidazole and heating at 95 °C for 5 min. Total (5 %), non-bound (5 %) and eluted material (100 %) was analysed by SDS-PAGE and immunodecoration.

Solubilization buffer

20 mM TRIS-HCl pH 7.4,
80 mM K-acetate,
20 mM imidazole,
10 % (v/v) glycerol,
1 % (v/v) Triton X-100,
1 x Complete Protease Inhibitor Cocktail
(Roche)

Washing buffer

20 mM TRIS-HCl pH 7.4,
80 mM K-acetate,
20 mM imidazole,
10 % (v/v) glycerol,
0.05 % (v/v) Triton X-100,
1 x Complete Protease Inhibitor Cocktail
(Roche)

2.2.10. Import of radiolabelled pre-proteins into isolated mitochondria and folding assay

Mitochondria were resuspended to a final protein concentration of 0.5 mg/ml in the standard import buffer supplied with 5 mM NADH, 2.5 mM ATP, 10 mM creatine phosphate and 100 µg/ml of creatine kinase. After pre-incubation for 3 min at 25 °C, 1–3 % (v/v) of radiolabelled pre-protein lysate was added and the import reaction was performed for various time periods at 25 °C. Pre-protein import was stopped by addition of 9 volumes of ice-cold SH buffer (2.2.5.2) containing 2 µM valinomycin. Non-imported material was digested by 50 µg/ml trypsin for 15 min on ice. Protease treatment was stopped by addition of 20-fold excess of soybean trypsin inhibitor (STI) and incubation for 10 min on ice. Mitochondria were re-isolated by centrifugation at 26500 x g for 12 min at 4 °C. Mitochondrial pellets were washed once with 500 µl of SH-KCl buffer (2.2.5.1), and solubilized with the lysis buffer for 15 min on ice at a protein concentration of 1 mg/ml. The samples were halved, one half was mock-treated and the other half was digested with 50 µg/ml of trypsin for 10 min on ice to assess the folding state of the imported protein. The trypsin reaction was stopped by TCA precipitation and the samples were analysed by SDS-PAGE and autoradiography.

Standard import buffer

600 mM sorbitol,
50 mM HEPES-KOH pH 7.2,
80 mM KCl,
0.01 % (w/v) BSA (fatty acid free),
10 mM Mg-acetate,
2.5 mM EDTA,
2 mM K-phosphate,
1 mM MnCl₂

Lysis buffer

20 mM TRIS-HCl pH 7.5
80 mM KCl
1 % (v/v) Triton X-100

2.2.11. *In vivo* labelling

Ahep1 and wild-type cells were grown in lactate medium until the OD₆₀₀ = 0.5–1. Cells were harvested by centrifugation at 3000 x g for 5 min at 25 °C (5 OD units per sample) and resuspended in Slac medium at a concentration of 5 OD units/ml. Then 0.5 % (v/v) of ³⁵S-methionine (activity of 10 mCi/ml) was added and cells were grown for a further 10

min at 25°C. Next, 100 µg/ml of cycloheximide to stop the protein synthesis and 10 mM cold methionine were added and cells were further grown for the indicated time periods. Cells were harvested by centrifugation at 10000 x g for 1 min at 25 °C, resuspended in SH-KCl buffer (2.2.5.1) supplemented with 1 mM PMSF and disrupted by shaking with glass beads of a diameter of 0.5–1 mm at 4°C. Crude mitochondria were isolated as described in 2.2.5.1 and solubilized in the lysis buffer (2.2.10) for 15 min on ice. Samples were halved; one half was mock-treated, the other half was digested with 2.5 µg/ml of trypsin for 10 min on ice. Digestion was stopped by TCA precipitation and protein pellet was resuspended in 10 µl of 1 % (m/v) SDS, 100 mM TRIS-HCl pH 7.4 and incubated at 95 °C for 5 min. Next, samples were diluted 20-times with IP buffer (2.4.2) and, after a clarifying spin at 125000 x g for 20 min at 4 °C, subjected to immunoprecipitation with antibodies against Ssc1 (2.4.2). Samples were analysed by SDS-PAGE, autoradiography and quantification.

2.3. Protein biochemistry methods

2.3.1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

A one-dimensional, vertical, discontinuous SDS-PAGE system was used to separate proteins according to their molecular sizes under denaturing conditions (Laemmli 1970). Depending on the size of a protein of interest, various concentrations of acrylamide and bis-acrylamide were used to prepare the separating gel. Gel dimensions were approximately 14 cm x 9 cm x 0.1 cm for the separating gel and 14 cm x 1 cm x 0.1 cm for the stacking gel. Protein samples were suspended in 10–50 µl of 1 x Laemmli buffer and incubated at 95 °C for 5 min prior to loading. In order to estimate the size of separated proteins, unstained protein molecular weight marker (Fermentas) was used. Electrophoresis was carried out for 90–120 min at 35 mA. Separated proteins were stained with Coomassie Brilliant-blue solution (2.3.3) or transferred onto a nitrocellulose membrane (Western blot) (2.3.2).

Separating gel

8–16 % (w/v) acrylamide,
 0.16–0.33 % (w/v) bis-acrylamide,
 375 mM TRIS-HCl pH 8.8,
 0.1 % (w/v) SDS,
 0.05 % (w/v) APS,
 0.05 % (v/v) TEMED

Stacking gel

5 % (w/v) acrylamide,
 0.1 % (w/v) bis-acrylamide,
 60 mM TRIS-HCl pH 6.8,
 0.1 % (w/v) SDS,
 0.05 % (w/v) APS,
 0.05 % (v/v) TEMED

Electrophoresis buffer

50 mM TRIS base,
 384 mM glycine,
 0.1 % (w/v) SDS,
 pH 8.3 (not adjusted)

1 x Laemmli buffer

60 mM TRIS-HCl pH 6.8,
 2 % (w/v) SDS,
 10 % glycerol,
 5 % (v/v) β -mercaptoethanol,
 0.05 % (w/v) bromphenol-blue

2.3.2. Protein transfer onto the nitrocellulose membrane (Western blot)

Proteins separated by SDS-PAGE were transferred electrophoretically onto the nitrocellulose membranes using a semi-dry transfer method. The nitrocellulose membrane (Protran, Whatman), blotting paper (Macherey-Nagel, MN 440 B) and the polyacrylamide gel after SDS-PAGE were soaked in the blotting buffer. Next, a blotting ‘sandwich’ was assembled in the custom-made blotting chamber consisting of graphite electrodes, in the following order: one sheet of blotting paper, the nitrocellulose membrane, the polyacrylamide gel and one sheet of blotting paper. Electrotransfer was carried out at 250 mA for 70 min. After the transfer, the membrane was briefly washed in water and stained with the Ponceau S solution, to visualize transferred proteins. Transferred proteins were further analysed by immunodecoration or, in the case of radioactive proteins, by autoradiography.

Blotting buffer

20 mM TRIS base,
 150 mM glycine,
 20 % (v/v) methanol,
 0.08 % (w/v) SDS

Ponceau S solution

0.2 % (w/v) Ponceau S,
 3 % (w/v) TCA

2.3.3. Coomassie Brilliant-blue staining of SDS-polyacrylamide gels

Separating gels after SDS-PAGE were washed with deionized water. Then, the staining solution was added. Gels were incubated in the staining solution for 1–3 h, washed with deionized water and incubated in several portions of destaining solution until the protein bands were clearly visible and the rest of the gel was colorless.

<u>Staining solution</u>	<u>Destaining solution</u>
30 % (v/v) methanol,	30 % (v/v) methanol
10 % (v/v) acetic acid,	10 % (v/v) acetic acid
0.1 % (w/v) Coomassie-Brilliant-blue G-250	

2.3.4. Protein precipitation with TCA (trichloroacetic acid)

Proteins were precipitated from aqueous solutions by addition of TCA to a final concentration of 12 % (v/v). Samples were incubated at -20 °C for at least 30 min, thawed on ice and centrifuged at 39000 x g for 20 min at 4 °C. The pellets of precipitated proteins were washed with -20 °C acetone, dried for 2 min at 30 °C, dissolved in 1 x Laemmli buffer and heated at 95 °C for 5 min.

2.3.5. Determination of protein concentration

Protein concentration was determined using the Bradford assay (Bradford 1976). The protein solution (1–10 µl) was added to 1 ml of 5-fold diluted Protein Assay reagent (Bio-Rad). The samples were mixed and incubated for 10 min at RT. The absorbance of the samples was measured at 595 nm in a 1 cm path-length cuvette. The protein concentration was determined from a calibration curve prepared with a solution of bovine IgG proteins (Bio-Rad) of known concentration as a standard.

2.3.6. NiNTA pull-down of proteins from mitochondria

To analyse the interactions between mitochondrial proteins, pull-down experiments of proteins containing a his-tag were carried out. Isolated mitochondria (150 µg) were suspended in the standard import buffer (2.2.10) with addition of 10 U/ml apyrase and 10 µM oligomycin to deplete ATP. Samples were incubated for 10 min at 37°C. Then, mitochondria were re-isolated and solubilized at a concentration of 1 mg/ml in

solubilization buffer for 30 min at 4 °C. After a clarifying spin at 125000 x g for 20 min at 4 °C, an aliquot of total (20 %) was taken out and the rest of the solubilized material was incubated with 50 µl (bed volume) of NiNTA-agarose beads for 2h at 4 °C with gentle turn-over mixing. The beads were then pelleted, and a fraction of non-bound material (FT, 20 %) was taken out. The rest of the supernatant was removed, beads were washed 3 times with 500 ml of washing buffer and bound proteins were eluted with 1 x Laemmli buffer containing 300 mM imidazole and heating at 95 °C for 5 min. Total (20 %), supernatant (20 %) and elution (100 %) fractions were then subjected to SDS-PAGE and immunodecoration.

Solubilization buffer

20 mM TRIS-HCl pH 8.0,
80 mM K-acetate,
20 mM imidazole,
10 % (v/v) glycerol,
1 % (v/v) Triton X-100,
1 mM PMSF

Washing buffer

20 mM TRIS-HCl pH 8.0,
80 mM K-acetate,
20 mM imidazole,
10 % (v/v) glycerol,
0.05 % (v/v) Triton X-100,
1 mM PMSF

2.3.7. *In vitro* synthesis of radiolabelled pre-proteins

The coding sequences for pre-proteins were cloned into the pGEM4 vector under the control of *Sp6* promoter. These coding sequences were transcribed into mRNA using SP6 RNA polymerase and subsequently translated using rabbit-reticulocyte lysate in the presence of ³⁵S-methionine.

The transcription mixture was prepared on ice and incubated for 1 h at 37°C. Following addition of 10 µl 10 M LiCl and 600 µl absolute ethanol, the sample was incubated at -20 °C for 30 min and precipitated mRNA was isolated by centrifugation at 36670 x g for 30 at 4°C. The RNA pellet was washed with ice-cold 70 % (v/v) ethanol, dried for 3 min at 30 °C, resuspended in 50 µl H₂O supplemented with 0.6 µl RNAsin and used for *in vitro* translation or stored at -80 °C.

The translation mixture was prepared on ice and incubated for 1 h at 30 °C. Then, 8 µl of 58 mM non-labelled methionine (chase) and 16 µl of 1.5 M sucrose were added. Next, the sample was centrifugated at 90700 x g for 45 min at 2°C to remove ribosomes and aggregated proteins. The supernatant was divided into aliquots and stored at -80°C.

Premix:

40 mM HEPES-KOH pH 7.4
6 mM Mg-acetate
2 mM spermidin
0.1 mg/ml BSA
10 mM DTT
0.5 mM ATP, CTP, GTP, UTP each

Transcription mixture

60 µl Premix,
10 µl 2.5 mM m⁷G(5')ppp(5')G,
20 µg plasmid DNA,
2.3 µl RNasin (40 U/µl),
0.8 µl Sp6 polymerase
H₂O added to 100 µl

Translation mixture

70 µl rabbit reticulocyte lysate (Promega),
2 µl RNasin (20 U),
2 µl amino acid mix (1mM each, without methionine),
8 µl ³⁵S-methionine,
16 µl mRNA

2.3.8. Detection and quantification of radiolabelled proteins

Radiolabelled proteins after SDS-PAGE and Western blot were detected by autoradiography. The dry nitrocellulose membrane with radioactive signal was exposed to an X-ray film (Kodak Bio Max MM) for a desired time period, depending on the signal intensity (1–120 days). Then, the film was developed in a developing machine (Gevamatic 60, AGFA Gevaert). The films were scanned and the intensity of bands of interest was quantified by densitometry using Image Master 1D Elite software (Amersham).

2.3.9. Purification of recombinant his-tagged proteins

Recombinant his-tagged proteins were purified using NiNTA affinity chromatography. His-tagged mature Hep1 was over-expressed in XL1Blue strain of *E. coli* and his-tagged PBD and his-tagged mature Mge1 were over-expressed in BL21 (DE3) ΔDnaK strain of *E. coli*.

A 50 ml LB-Amp starter culture was inoculated from a single bacterial colony transformed with the appropriate plasmid and grown overnight at 37 °C. Starter culture was used to inoculate 500 ml of LB-Amp to an OD₆₀₀ of 0.05–0.12. Bacteria were grown at 37 °C with shaking until OD₆₀₀ = 0.5 and then expression of the protein of interest was induced by

adding 0.5–1 mM IPTG. After 3 h of induction, bacteria were collected by centrifugation at 4400 x g for 15 min at 25 °C, washed once with water and the bacterial pellet was frozen at -20 °C. After 30 min of incubation at -20 °C, bacterial pellet was thawed and resuspended in 20 ml of buffer A. To destroy the cell wall, 1 mg/ml of Lysozyme (Sigma) was added and the solution was incubated for 45 min at 4 °C with mild shaking. Next, cells were disrupted by sonication in an ice-bath using a Branson Sonifier (10 x 12 sec, 10 sec pause, setting 4, 80 % duty cycle). Unbroken cells and cell debris was removed by centrifugation at 27200 x g for 15 min at 4 °C and the supernatant, containing over-expressed proteins, was loaded onto the previously prepared NiNTA column. To prepare the column, 0.75 ml of NiNTA-agarose beads were loaded into the empty column, left to settle and washed with 20 ml of deionized water. The column was then equilibrated with 20 ml of buffer A. After passing the bacterial lysate through the column at a flow rate of 1 ml/min, the column was washed with 15 ml of buffer A and the bound his-tagged proteins were eluted with buffer A supplemented with 300 mM imidazole. The eluate was collected in 1 ml aliquots, the fractions containing the most protein were pulled together and the buffer was exchanged to the storage buffer using PD-10 Desalting column (GE Healthcare). After determination of the protein concentration (2.3.5), the protein solution was divided into aliquots, frozen in liquid nitrogen and stored at -80 °C.

Buffer A

20 mM HEPES-KOH pH 7.4,
250 mM KCl,
20 mM imidazole,
2 mM β -mercaptoethanol,
10 % (v/v) glycerol,
1 mM PMSF

Storage buffer

20 mM HEPES-KOH pH 7.4,
100 mM KCl,
5 mM $MgCl_2$,
5 % (v/v) glycerol

2.3.10. Purification of recombinant Ssc1 and the ATPaseLinker domain

To purify mature recombinant Ssc1 and ATPaseLinker, the Mge1-affinity column was used. To prepare this column, BL21 (DE3) Δ DnaK cells over-expressing his-tagged Mge1 protein were grown, induced, harvested and treated as described in 2.3.9. Similarly, Mge1-containing bacterial lysate was passed through the NiNTA-agarose column and washed with 15 ml of buffer A (2.3.9). Bound Mge1 protein was, however, not eluted from the

column. Instead, the column was washed with 15 ml of buffer D and with 15 ml of buffer E. Finally, it was re-equilibrated with 20 ml of buffer A. Such prepared Mge1-affinity column was ready for Ssc1 or ATPaseLinker purification.

Mature, recombinant Ssc1 and ATPaseLinker were over-expressed in BL21 (DE3) Δ DnaK strain of *E. coli*. 50 ml LB-Amp pre-culture was inoculated from a single bacterial colony transformed with the appropriate plasmid and grown overnight at 30 °C. Pre-culture was used to inoculate 1 litre of LB-Amp to an OD₆₀₀ of 0.05–0.12. Bacteria were grown at 30 °C with shaking until the OD₆₀₀ = 0.5, and then expression of the protein of interest was induced by addition of 1 mM IPTG. After 3 h of induction, bacteria were collected by centrifugation at 4400 x g for 15 min at 25 °C, washed once with water and the bacterial pellet was frozen at -20 °C. After 30 min incubation at -20 °C, bacterial pellet was thawed and resuspended in 40 ml of buffer A. To destroy the cell wall, 1 mg/ml of Lysozyme (Sigma) was added and the solution was incubated for 45 min at 4 °C with mild shaking. Next, cells were disrupted by sonication in an ice-bath using a Branson Sonifier (10 x 12 sec, 10 sec pause, setting 4, 80 % duty cycle). Unbroken cells and cell debris were removed by centrifugation at 27200 x g for 15 min at 4 °C and the supernatant containing the over-expressed protein was passed two times through the Mge1-affinity column at a flow rate of 1 ml/min. The column was then washed with 15 ml of buffer A and with 20 ml of buffer D. Finally, the bound Ssc1 or ATPaseLinker was slowly eluted with buffer E (flow rate 0.2 ml/min). The eluate was collected in 1 ml aliquots, the fractions containing the most protein were pulled together and the buffer was exchanged to the storage buffer (2.3.9) using PD-10 Desalting column (GE Healthcare). After determination of the protein concentration (2.3.5), the protein solution was divided into aliquots, frozen in liquid nitrogen and stored at -80 °C.

Buffer D

20 mM HEPES-KOH pH 7.4,
250 mM KCl,
2 mM β -mercaptoethanol,
10 % (v/v) glycerol,
1 mM PMSF

Buffer E

20 mM HEPES-KOH pH 7.4,
250 mM KCl,
2 mM β -mercaptoethanol,
10 % (v/v) glycerol,
1 mM PMSF
10 mM MgCl₂,
5 mM ATP

2.3.11. Purification of recombinant his-tagged DnaJ

Recombinant his-tagged DnaJ was over-expressed in BL21 (DE3) Δ DnaK strain of *E. coli* and purified using NiNTA affinity chromatography. 500 ml of bacterial culture was grown, induced and collected as described for his-tagged proteins (2.3.9). The bacterial pellet was frozen at -20 °C, thawed after 30 min and resuspended in 20 ml of sonification buffer. To destroy the cell wall, 1 mg/ml of Lysozyme (Sigma) was added and the solution was incubated for 45 min at 4 °C with mild shaking. Next, cells were disrupted by sonication in an ice-bath using a Branson Sonifier (10 x 12 sec, 10 sec pause, setting 4, 80 % duty cycle). Cell debris containing DnaJ was collected by centrifugation at 27200 x g for 30 min at 4 °C and the supernatant was discarded. Pellet was resuspended in 20 ml of buffer AJ and incubated for 1 h at 4 °C with mild agitation. Cell debris was pelleted again at 27200 x g for 30 min at 4 °C and 0.5 ml of previously washed NiNTA-agarose beads were added to the supernatant, containing extracted DnaJ. The mixture was incubated for 2 h at 4 °C while shaking, and then beads were used to pack an empty column and the flow-through was collected. The column was subsequently washed with 10 ml of buffer BJ and with 10 ml of buffer CJ. Bound DnaJ was eluted with 10 ml of buffer EJ. Eluate was collected in 0.5 ml aliquots, the fractions containing the most protein were pulled together and the buffer was exchanged to the storage buffer J using PD-10 Desalting column (GE Healthcare). After determination of the protein concentration (2.3.5), the protein solution was divided into aliquots, frozen in liquid nitrogen and stored at -80 °C.

Sonification Buffer

50 mM TRIS-HCl pH 8.0,
10 mM DTT,
10 mM EDTA,
0.6 % (m/v) Brij 58

Buffer AJ

50 mM TRIS-HCl pH 8.0,
2 M urea,
5 mM DTT,
500 mM KCl,
20 mM imidazole,
0.1 % Triton X-100,

Buffer CJ

50 mM TRIS-HCl pH 8.0,
5 mM DTT,
500 mM KCl,
20 mM Imidazole,
0.05 % (m/v) Brij 58,
10 % (v/v) glycerol

Buffer EJ

50 mM TRIS-HCl pH 8.0,
5 mM DTT,
500 mM KCl,
0.05 % (m/v) Brij 58,

10 % (v/v) glycerol,
1 mM PMSF

10 % (v/v) glycerol,
100 mM EDTA

Buffer BJ

50 mM TRIS-HCl pH 8.0,
5 mM DTT,
500 mM KCl,
20 mM imidazole,
0.1 % Triton X-100,
10 % (v/v) glycerol

Storage buffer J

20 mM HEPES-KOH pH 7.4,
250 mM KCl,
5 mM DTT,
0.05 % (m/v) Brij 58,
10 % (v/v) glycerol

2.3.12. Unfolding of recombinant proteins by urea

Recombinant protein to be unfolded was first precipitated by ammonium sulfate. 2/3 of volume of saturated solution of ice-cold ammonium sulfate was added 3 times to the solution of recombinant protein, with vigorous vortexing in between. The sample was incubated on ice for 30 min and the precipitated protein was collected by centrifugation at 15000 x g for 12 min at 4 °C. The supernatant was removed and the protein pellet was resuspended in urea buffer to the final concentration of 6 mg/ml. Samples were stored at -20 °C and incubated for 10 min at 25 °C each time prior to use.

Saturated solution of ammonium sulfate

35.2 g ammonium sulfate,
H₂O added to 50 ml,
pH 7.0 adjusted with KOH

Urea buffer

8 M urea,
20 mM TRIS-HCl, pH 7.4

2.3.13. Refolding assay

Recombinant proteins were precipitated with ammonium sulfate and unfolded by resuspension in the urea buffer (2.3.12). Samples were diluted 20-fold in the refolding buffer to an unfolded protein concentration of 0.3 mg/ml. 0.1 mg/ml of yeast cytochrome *c*

(Sigma) was added to block unspecific interactions. When stated, Hep1 or Mge1 (the amounts indicated) and/or 2.5 mM of the indicated nucleotides were added. Samples were incubated at 25°C for the indicated time periods to allow protein refolding. Aliquots containing 6 µg of the refolding protein were withdrawn, diluted in one volume of ice-cold refolding buffer, placed on ice and 5 µg/ml of trypsin was added, where indicated. Input samples were mock-treated, as a control. After 15 min at 4°C, Soybean trypsin inhibitor (STI) was added to all samples, and a clarifying spin at 39000 x g for 10 min at 4 °C was carried out. Supernatants were diluted in 1 x Laemmli buffer and heated for 5 min at 95 °C. Samples were analysed by SDS-PAGE and Coomassie staining.

Refolding buffer

20 mM HEPES-KOH pH 7.4,
100 mM KCl,
5 mM MgCl₂,
5 % (v/v) glycerol

2.3.14. ATPase activity assays

2.3.14.1. Enzymatic ATPase assay

The ATPase activity of recombinant native Ssc1 and Ssc1 after refolding was measured using a colorimetric assay based on an ATP-regenerating enzyme system (Norby 1988). In this assay, ATP consumption is coupled to the oxidation of NADH to NAD⁺ that can be monitored photometrically at 360 nm. Ssc1 (4.3 µM) was incubated for 20 min at 25°C in refolding buffer with 8.3 mM ATP and 0.3 mg/ml BSA, and, where indicated, 8.6 µM Hep1, in a total volume of 60 µl. Samples were diluted 4.3-fold in refolding buffer (3.13) equilibrated for 3 min at 25 °C, containing 0.12 mM NADH, 3 mM phosphoenolpyruvate, 10 U of lactate dehydrogenase (Sigma), 15 U of pyruvate kinase (Sigma), 2 µM Mge1 and 2 µM DnaJ. The change of absorbance at 360 nm at 25°C was monitored over time by a spectrophotometer (JASCO, Gross-Umstadt, Germany), and converted to the consumption of mol of ATP per mol of Ssc1 per min.

2.3.14.2. Radioactive ATPase assay

5 µg of Ssc1 (native or unfolded) was incubated in a total volume of 20 µl of ATPase buffer, in the presence or absence of 2.5 µg recombinant Hep1, at 25 °C for 20 min to allow potential Ssc1 refolding. Then, 3.38 µg of recombinant Mge1, 6.08 µg of recombinant DnaJ and γ -³²P-ATP (final conc. of 0.1 mM) was added and samples were further incubated at 25 °C. After 5, 10 and 20 min, 3 µl aliquots were taken out and the reaction was stopped by adding 3 µl of stop buffer and cooling on ice. Finally, 2 µl of samples were spotted on PEI-Cellulose F Thin Layer Chromatography plates and the samples were developed in 1 M LiCl for 45 min at 25 °C. Afterwards, plates were dried, covered with plastic foil and exposed to phosphoimager plates. The signal of radiolabelled ATP, ADP and released phosphate was visualised and quantified by phosphoimager (Fuji Bas3000, Aida Image Analyser).

ATPase buffer

50 mM HEPES-KOH pH 7.4,
100 mM KCl,
5 mM MgCl₂,
400 mM urea (in the case of folded Ssc1)
5 mM ATP (unlabelled)

Stop buffer

100 mM EDTA pH 8.0
10 mM ATP (unlabelled)
10 mM ADP (unlabelled)

2.3.15. Crosslinking of recombinant proteins

Chemical crosslinking *in vitro* was performed using the non-specific crosslinking reagent glutaraldehyde. Unfolded Ssc1 (3 µM) was initially incubated for 20 min at 30°C in crosslinking buffer in a final volume of 20 µl. Where indicated, 6 µM Hep1, 6 µM Mge1 and/or 2.5 mM of the indicated nucleotide were added to the sample prior to the initial incubation. In the case of chase experiments, 2.5 mM of indicated nucleotides were added after the initial incubation and samples were further incubated for 10 min at 30 °C. Next, glutaraldehyde was added to a final concentration of 1 mM and samples were incubated for 30 min at 30 °C. Finally, samples were suspended in 1 x Laemmli buffer containing 200 mM of glycine to quench the remaining glutaraldehyde, and samples were heated to 95 °C for 5 min and analysed by SDS-PAGE and immunodecoration.

Crosslinking buffer

50 mM HEPES-KOH pH 7.4,
200 mM NaCl,
100 mM KCl,
5 mM MgCl₂

2.4. Immunology methods

2.4.1. Immunodecoration

Proteins transferred to the nitrocellulose membrane (2.3.2) were visualized by immunodecoration with specific antibodies. The membrane was first incubated for 30 min in blocking solution to block all non-specific binding sites. Then, the membrane was incubated in primary antibody solution (diluted 100–10000-fold in blocking solution) with gentle shaking/rolling for 1.5 h at 25 °C or, alternatively, at 4 °C overnight. The antibody solution was collected and stored at -20 °C to be re-used. The membrane was washed subsequently with TBS, TBS containing 0.05 % (v/v) Triton X-100 and TBS, each washing step for ca. 5–10 min. Afterwards, a fresh solution of secondary antibody conjugated with horseradish peroxidase (HRP) (diluted 10000 times in blocking solution) was prepared and the membrane was incubated in it for 30–60 min at 25 °C or, alternatively, at 4 °C overnight. The membrane was washed similarly as after the primary antibody treatment and covered with the fresh chemiluminescent substrate (ECL reagent 1 and 2 mixed in 1:1 ratio) of peroxidase. Chemiluminescence was detected on X-ray films (Fuji New RX) and the films were developed in a developing machine (Gevamatic 60, AGFAGevaert).

TBS

135 mM NaCl,
10 mM TRIS-HCl pH 7.5

Blocking solution

5 % (m/v) milk powder
TBS

ECL reagent 1

100 mM TRIS-HCl pH 8.5,
0.44 mg/ml luminol,
67 µg/ml p-coumaric acid

ECL reagent 2

100 mM TRIS-HCl pH 8.5,
0.03 % (v/v) H₂O₂

2.4.2. Immunoprecipitation

40 μ l (bed volume) of Protein A-Sepharose CL-4B beads (GE Healthcare) were pre-washed 3 times with 1 ml of TBS (4.1). Then, antibodies against Ssc1 (50 μ l) were coupled to the beads by 30 min of incubation at 4 °C with gentle agitation. Finally, beads were washed 2 times with 1 ml of TBS and once with 1 ml of IP buffer.

Protein pellets after TCA precipitation were resuspended in 1 % (w/v) SDS, 100 mM TRIS-HCl pH 7.4 and incubated at 95 °C for 5 minutes. Next, samples were diluted 30-times in IP buffer and, after a clarifying spin at 125000 x g for 20 min at 4 °C, added to the washed protein A-sepharose beads with prebound antibodies against Ssc1. After 2 h of gentle agitation at 4°C, beads were washed 4 times with 500 μ l of IP buffer and bound proteins were eluted with 1 x Laemmli buffer and heating up at 95 °C for 5 min. Samples were analysed by SDS-PAGE and autoradiography.

IP buffer

20 mM TRIS-HCl pH 7.4,

300 mM NaCl,

0.2 % (v/v) Triton X-100,

0.5 mM PMSF,

1 x Complete Protease Inhibitor Cocktail (Roche)

3. Results

3.1. Aggregation of the Ssc1 chaperone

3.1.1. Ssc1 forms highly heterogeneous complexes in the absence of Hep1

As previously reported, the mitochondrial Hsp70 chaperone Ssc1 has a tendency to aggregate (Sanjuan Szklarz et al. 2005; Sichting et al. 2005). In the absence of its chaperone Hep1, Ssc1 assembles irreversibly into oligomeric species of high molecular mass. Do these oligomeric species represent heterogeneous aggregates or homogenous complexes of distinct molecular mass? To test this, a sucrose gradient analysis was carried out. Mitochondria isolated from wild-type cells (wt) and cells lacking Hep1 (*Δhep1*) were solubilized in a Triton X-100-containing buffer and a short clarifying spin at 10000 x g was performed. Next, the extracts were separated on a continuous sucrose gradient. In the case of *Δhep1* mitochondrial extract, a large portion of Ssc1 was found in the pellet fraction after the clarifying spin, indicating the presence of large molecular mass aggregates of Ssc1 (Fig. 3.1, right panel). The soluble portion of Ssc1 was detected in all fractions of the gradient. This reflects the presence of heterogeneous oligomeric Ssc1 species of various sizes besides monomeric Ssc1 (Fig. 3.1, right panel). These species presumably represent intermediates in the aggregation process. In contrast, Ssc1 from the mitochondrial extracts of wild-type cells, as well as the control proteins from both mitochondrial extracts were detected in the top gradient fractions, indicating the presence of non-aggregated species (Fig. 3.1, left panel).

In conclusion, Ssc1 tends to form heterogeneous, large aggregates of non-native Ssc1 species in the absence of Hep1.

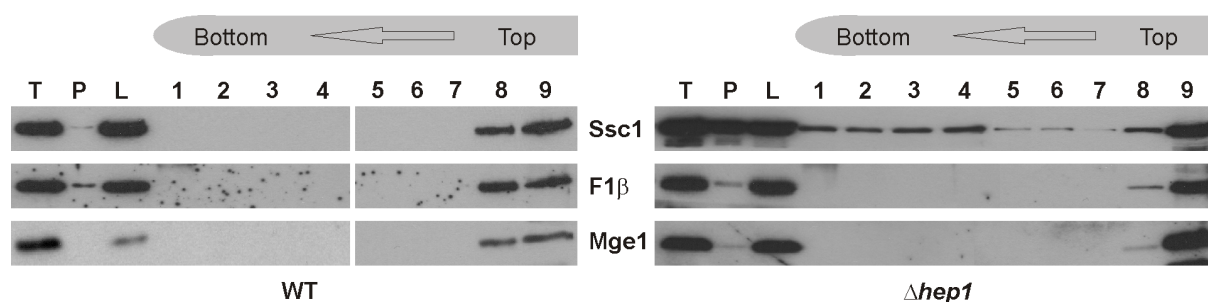


Figure 3.1. Separation of mitochondrial protein extracts from wild-type and Δ hep1 cells by sucrose gradient centrifugation

Mitochondrial extracts from wild-type (WT) and Δ hep1 cells were subjected to a clarifying spin and soluble fractions were layered on a continuous sucrose gradient (10 % to 30 % of sucrose) and centrifuged for 40 min at 485,000 x g. The gradient was fractionated and the protein content of the fractions was analysed by SDS-PAGE and immunodecoration with the antibodies against the indicated proteins. Total (T), pellet (P) and the soluble fraction (load, L) after clarifying spin represent 10 % of the material loaded onto the sucrose gradient.

3.1.2. The ATPase domain with interdomain linker is responsible for aggregation of Ssc1 in the absence of Hep1

In order to characterise the molecular mechanism of the aggregation of Ssc1 and its counteraction by Hep1, it was required to identify the structural determinants in Ssc1 that make it prone to aggregation. To this end, the individual domains of Ssc1 were expressed as his-tagged variants in the wild-type yeast strain and in Δ hep1 strain and tested for solubility. In contrast to the full-length Ssc1, neither ATPase domain alone, nor PBD with the interdomain linker at its N-terminus, hence further referred to as PBD, was present in the fraction of aggregated material in the Δ hep1 strain (Fig. 3.2a). These observations are consistent with the results obtained previously by M. Sighting (Sighting 2007). Apparently, neither the ATPase domain nor the PBD has the tendency to form aggregates. It could be the interdomain communication that triggers the formation of the aggregation-prone conformation in Ssc1. Since the interdomain linker is known to mediate the interdomain communication within Ssc1 via the interaction with the ATPase domain, a variant consisting of the ATPase domain with the interdomain linker (ATPaseLinker) was tested for aggregation. This variant was found to be soluble in wild-type mitochondria, but it was

partially present in the pellet fraction upon expression in the *Δhep1* strain (Fig. 3.2b). These findings suggest that the ATPase domain together with the interdomain linker is responsible for the tendency of Ssc1 to aggregate. The specific amino-acid residues VLLL of the interdomain linker are critical for Ssc1 aggregation, since the ATPaseLinkerA4 variant, in which the linker amino-acid residues were replaced by 4 alanine residues, was soluble in the presence and absence of Hep1 (Fig. 3.2b). Taken together, the ATPase domain of Ssc1 in combination with the interdomain linker adopts an aggregation-prone conformation, which leads to the formation of Ssc1 aggregates in the absence of Hep1.

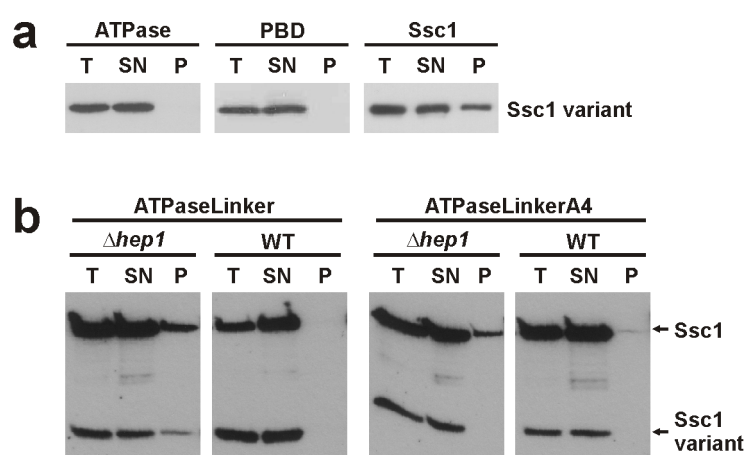


Figure 3.2. The ATPaseLinker aggregates in the absence of Hep1

(a) Mitochondria from *Δhep1* strains expressing his-tagged versions of ATPase domain, PBD or full-length Ssc1 were solubilized. Soluble (SN) and aggregated (P) proteins were separated by centrifugation. These fractions as well as total material (T) were subjected to SDS-PAGE and immunodecoration with antibodies against Ssc1. (b) Mitochondria from the *Δhep1* and the wild-type (WT) strains expressing his-tagged variants of the ATPaseLinker and the ATPaseLinkerA4 were solubilized. Aggregation of the protein variants was assayed as described in (a).

3.1.3. The ATPase domain with interdomain linker is the smallest entity within Ssc1 that interacts with Hep1

To investigate the mechanism of the protective function of Hep1, the interaction between Hep1 and Ssc1 was analysed. Previous results by M. Sichtung demonstrated that the ATPase domain in the context of a full-length Ssc1 is required for the interaction with Hep1 (Sichtung et al. 2005; Sichtung 2007). To find out whether the ATPase domain alone

is sufficient for this interaction, the full-length Ssc1, as well as its individual domains containing C-terminal his-tags, were analysed for the interaction with Hep1 by NiNTA pull-down. Since Ssc1 was reported to interact with Hep1 upon ATP depletion, isolated mitochondria expressing the aforementioned variants were depleted of ATP, solubilized and incubated with NiNTA beads. Non-bound and bound protein fractions were analysed by SDS-PAGE and immunodecoration. Hep1 was co-purified with the full-length Ssc1, however, it was not found in the bound fraction neither with the ATPase domain nor with the PBD (Fig. 3.3 upper panel). Apparently, the ATPase domain alone is necessary but not sufficient to interact with Hep1. Since the presence of the interdomain linker triggered the aggregation of ATPase domain in the absence of Hep1, it was expected that the ATPase domain together with the interdomain linker would be able to interact with Hep1. To test this hypothesis, an interaction of the ATPaseLinker variant with Hep1 upon depletion of ATP in wild-type mitochondria was analysed by the NiNTA pull-down. Indeed, Hep1 was found in the bound fraction together with the ATPaseLinker, indicating that it associates with the ATPaseLinker *in organello* (Fig. 3.3 lower panel). This interaction was specific, as Hep1 did not bind to the NiNTA beads when a non-his-tagged ATPaseLinker variant was used. Moreover, the interaction depended on the specific sequence of the interdomain linker, since Hep1 did not associate with the ATPaseLinkerA4 variant (Fig. 3.3 lower panel). Thus, the ATPase domain with the interdomain linker is necessary and sufficient for the interaction of Ssc1 with Hep1.

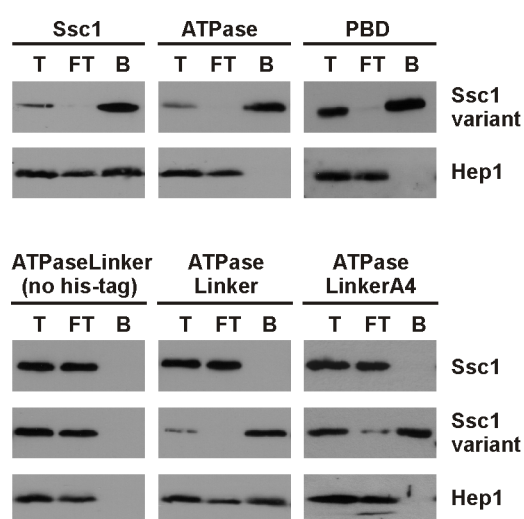


Figure 3.3. Hep1 interacts with the ATPaseLinker

Mitochondrial extracts from strains expressing the indicated Ssc1 variants with his-tags (unless otherwise stated) were depleted of ATP and subjected to the pull-down assay with NiNTA beads. T, total (20 %), FT, non-bound material (20 %) and B, bound fraction (100 %) were analysed by SDS-PAGE and immunodecoration with antibodies against Ssc1 and Hep1.

To corroborate these findings, a chemical crosslinking of mitochondrial proteins with DSG upon depletion of ATP was performed as described before (Sichting et al. 2005). Samples were analysed by SDS-PAGE and immunodecoration with α -Hep1 antibodies. As expected, a crosslinked adduct of full-length Ssc1 and Hep1 was observed in all crosslinking agent-treated samples (Fig. 3.4a). However, when the ATPaseLinker variant was tested, an additional adduct corresponding from its size to the ATPase domain with the interdomain linker crosslinked to Hep1 was detected. This adduct bound to the NiNTA beads in the pull-down assay, confirming that the adduct indeed contained the his-tagged ATPaseLinker beside Hep1 (Fig. 3.4b).

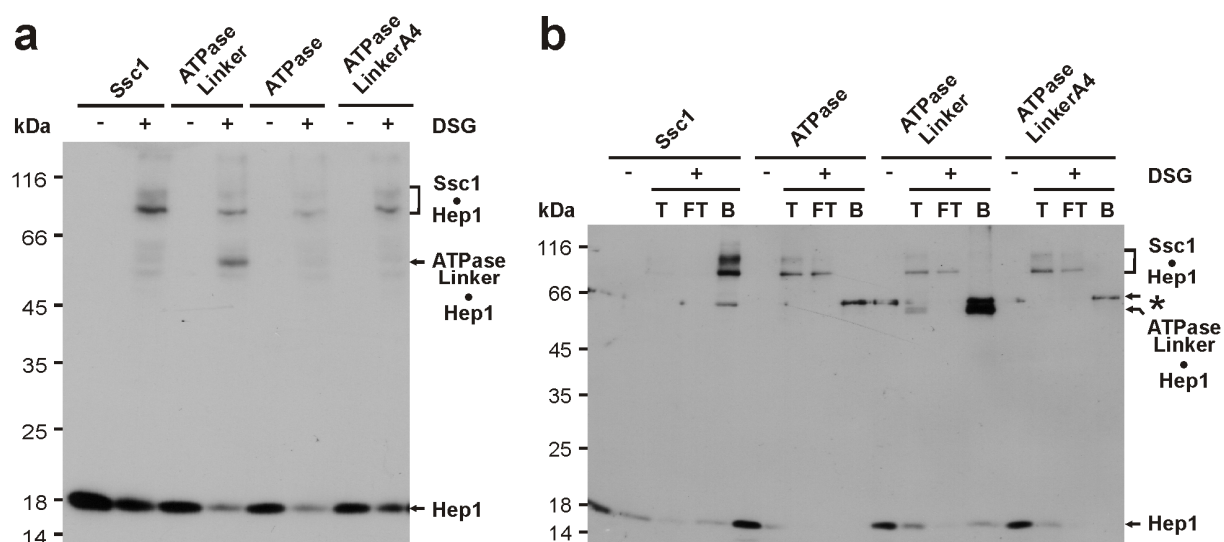


Figure 3.4. The ATPaseLinker forms a crosslinked adduct with Hep1

(a) Mitochondria from strains expressing the indicated Ssc1 variants were depleted of ATP and subjected to chemical crosslinking with DSG. Samples were analysed by SDS-PAGE and immunodecoration with antibodies against Hep1. **(b)** Mitochondria were subjected to crosslinking as in **(a)**, then they were solubilized and incubated with NiNTA beads. Non-treated control samples (- DSG, 5 %), and DSG-treated samples (total, T (5 %), non-bound material, FT (5 %) and bound proteins, B (100 %)) were analysed by SDS-PAGE and immunodecoration with antibodies against Hep1. The adduct of Ssc1 and Hep1, as well as the adduct of the ATPaseLinker and Hep1, is depicted. The asterisk indicates a cross-reaction of the antibodies.

Taken together, the ATPase domain in combination with the interdomain linker forms the smallest entity of Ssc1 that is recognized by Hep1. In the absence of Hep1, this entity has the tendency to aggregate and is responsible for aggregation of the full-length Ssc1.

3.2. *De novo* folding of the Ssc1 chaperone in organello and *in vivo*

3.2.1. Ssc1 and the individual Ssc1 domains fold rapidly *in organello*

To characterise the *de novo* folding of Ssc1, a folding assay based on the *in vitro* import of radiolabelled precursor into isolated mitochondria was established. In this assay, the folding state of the imported Ssc1 is assessed by trypsin treatment upon solubilization of mitochondria, since folded Ssc1 is trypsin-resistant, whereas the non-folded species are degraded. Ssc1 was rapidly imported into mitochondria, and almost all imported material was resistant to trypsin, indicating that the imported Ssc1 became folded (Fig. 3.5a). The kinetics of both import and folding of Ssc1 were comparable, demonstrating that the imported Ssc1 folds rapidly *in organello* (Fig. 3.5b).

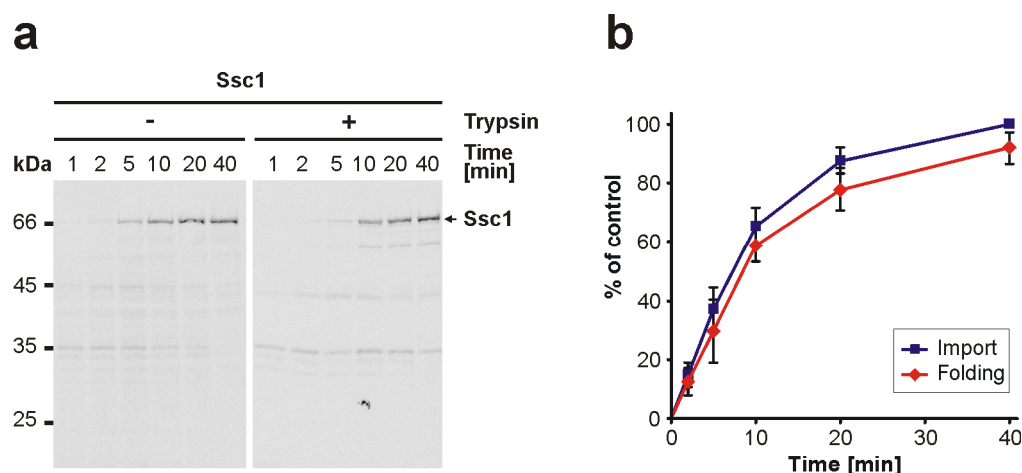


Figure 3.5. The chaperone Ssc1 folds rapidly *in organello*

Radiolabelled Ssc1 precursor was imported into wild-type mitochondria for the indicated time periods. Non-imported material was digested with trypsin. Mitochondria were re-isolated and solubilized. Half of each sample was mock treated (-) to assess the import efficiency, the other half was digested with trypsin (+) to assess the folding state of the imported Ssc1. Samples were analysed by SDS-PAGE and autoradiography (a) and quantification (b). The amount of Ssc1 imported at the latest time point was set to 100 %. The graph shows mean values of five independent experiments +/- standard deviation.

3.2.2. The ATPase domain, in contrast to the PBD, requires the interdomain linker for folding

To investigate the folding mechanism of Ssc1, the *de novo* folding of the individual Ssc1 domains in the context of the full-length protein was tested. To this end, constructs of the peptide binding domain (PBD) and the ATPase domain fused to the mouse dihydrofolate reductase (DHFR), from now on referred to as PBD-DHFR and ATPase-DHFR, respectively, were generated. To exclude steric hindrance, the domains and DHFR were separated by a spacer of 80 amino acid residues (residues 87–167) of the yeast cytochrome *b*₂ (Fig. 3.6).

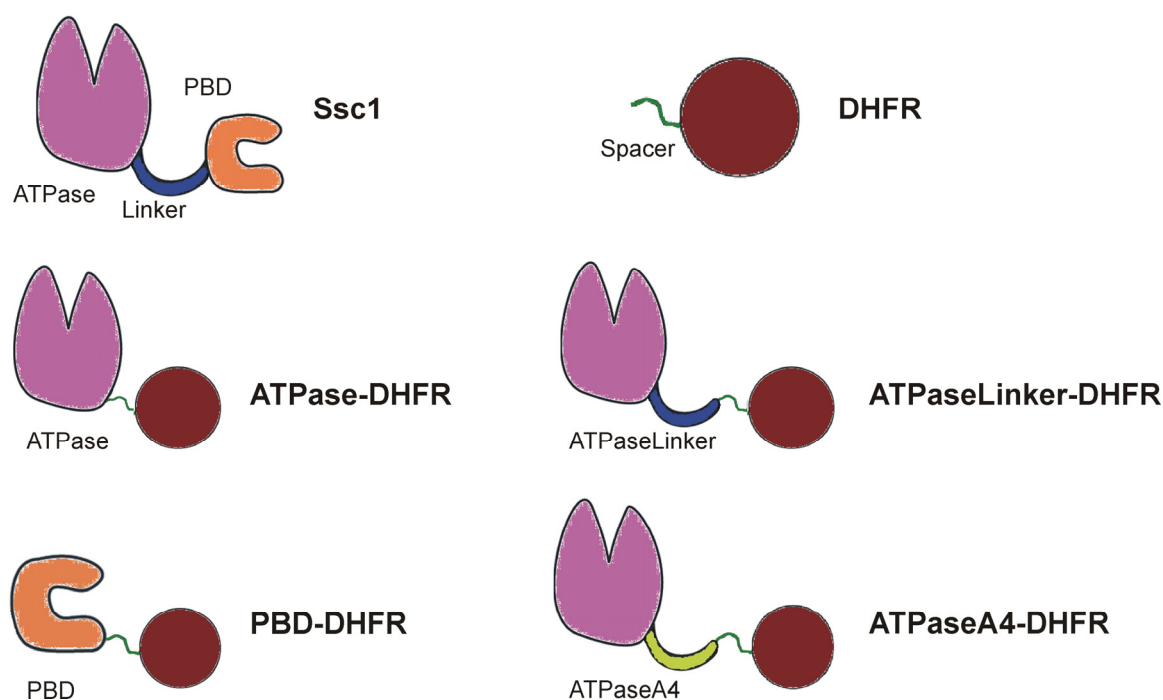


Figure 3.6. Schematic view of the Ssc1 variants used in the folding assay

To analyse the folding of Ssc1, the depicted variants of Ssc1 were generated, in which the individual Ssc1 domains were fused to mouse DHFR via a spacer (residues 87–167 of cytochrome *b*₂), to mimic the double domain organisation of the full-length protein.

The variants were imported into mitochondria and their folding state was assessed by trypsin digestion. The digestion of PBD-DHFR generated two protease-resistant fragments, corresponding in size to the folded PBD and DHFR (Fig. 3.7a). The kinetics of import and folding of the PBD-DHFR variant were virtually identical, indicating that the folding of this protein occurs immediately after it reaches the mitochondrial matrix. Notably, digestion of the imported ATPase-DHFR variant revealed only one protease-resistant fragment corresponding to the folded DHFR, whereas almost all of the ATPase domain molecules were trypsin-sensitive (Fig. 3.7b). This shows that the ATPase domain cannot fold in the context of the fusion protein. In contrast, the PBD folds rapidly, independently of the protein context.

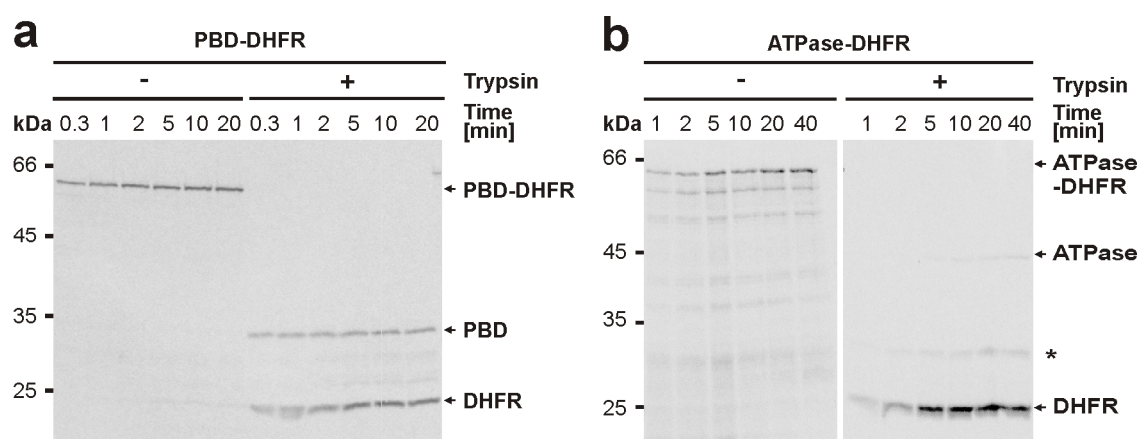


Figure 3.7. The PBD, but not the ATPase domain, folds in the context of the fusion protein

Radiolabelled precursors of PBD-DHFR (**a**) and ATPase-DHFR (**b**) were imported into wild-type mitochondria. To assess the import efficiency and folding state of the variants, samples were treated as described in Fig. 3.5 and analysed by SDS-PAGE and autoradiography. The asterisk indicates the folded DHFR domain with non-digested spacer.

Since the interdomain linker of Ssc1 interacts with the ATPase domain and induces conformational changes in this domain, we asked whether the interdomain linker has an effect on the folding of the ATPase domain. To this end, a radiolabelled ATPaseLinker-DHFR variant was synthesized and its folding upon import into mitochondria was followed. As a control, an ATPaseA4-DHFR variant in which the linker amino-acid residues VLLL were changed to four alanine residues, was tested (Fig. 3.6). Interestingly,

compared to the ATPase-DHFR variant, the trypsin digestion of the ATPaseLinker-DHFR led to the appearance of an additional protease-resistant fragment corresponding to the folded ATPase domain (Fig. 3.8a). In contrast, in the case of the ATPaseA4-DHFR, only the DHFR domain folded (Fig. 3.8b). This implies that the *de novo* folding of the ATPase domain depends on the presence of the interdomain linker.

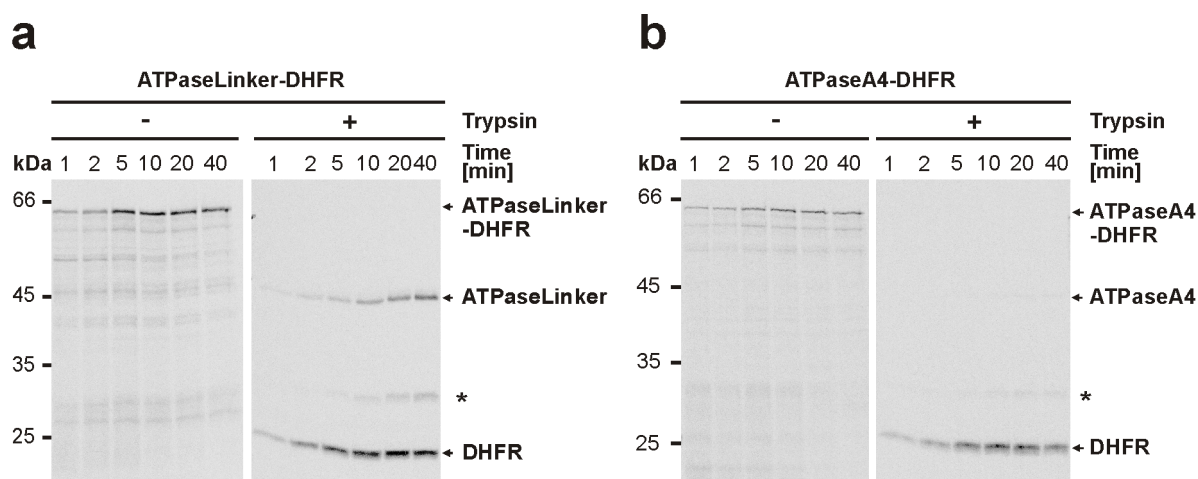


Figure 3.8. Folding of the ATPase domain relies on the interdomain linker

Radiolabelled precursors of ATPaseLinker-DHFR (**a**) and ATPaseA4-DHFR (**b**) were imported into wild-type mitochondria. When indicated, samples were treated with trypsin as described in Fig. 3.5 and analysed by SDS-PAGE and autoradiography. The asterisk indicates the folded DHFR domain with non-digested spacer.

To confirm the importance of the interdomain linker for the ATPase domain folding in the context of the full-length Ssc1 protein, the *de novo* folding of the Ssc1A4 mutant, in which the linker amino-acid residues were changed to four alanine residues, was tested. Trypsin treatment of the imported Ssc1A4 mutant generated a 35 kDa stable fragment, corresponding in size to the PBD (Fig. 3.9, left panel). An immunoprecipitation experiment with antibodies recognizing specifically either ATPase domain (α ATPase) or the C-terminus (α Cterm) of Ssc1, confirmed that the fragment was the PBD (Fig. 3.9, right panel). This means that upon the mutation of the interdomain linker, the ATPase domain cannot fold properly, in contrast to the PBD, which folds independently of the linker.

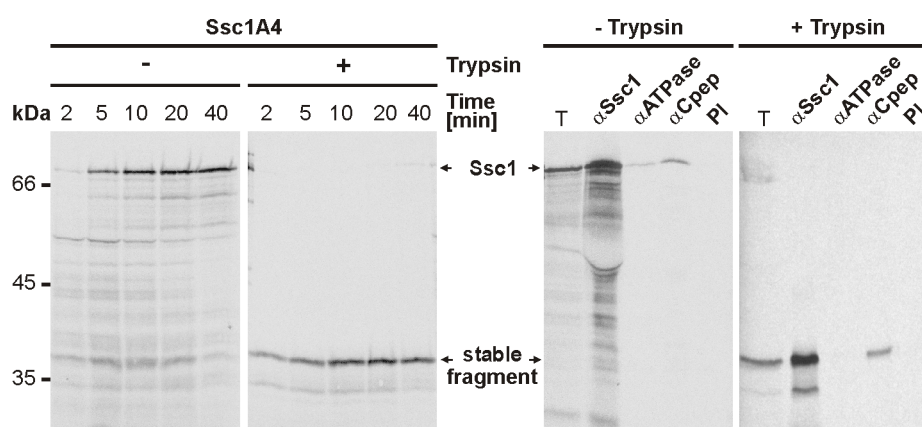


Figure 3.9. Folding of the ATPase domain of the Ssc1A4 linker mutant is impaired

Radiolabelled precursor of the Ssc1A4 linker mutant was imported into wild-type mitochondria for the indicated time periods. To assess the folding state of the variant, samples were treated as described in Fig. 3.5 and analysed by SDS-PAGE and autoradiography (left panel). Imported material as well as the stable trypsin-resistant fragment were subjected to immunoprecipitation with antibodies against full-length Ssc1 (α Ssc1), ATPase domain (α ATPase), C-terminus of Ssc1 (α Cpep) and with pre-immune IgGs (PI). Total material (T, 10 %) subjected to immunoprecipitation and the precipitated material were analysed by SDS-PAGE and autoradiography (right panel).

Taken together, the data indicate that both domains of Ssc1 are independent folding units. The ATPase domain requires the interdomain linker for the correct folding, whereas the PBD folds irrespectively of the presence of the interdomain linker.

3.2.3. Hep1 is required for the folding of the ATPase domain of Ssc1 *in organello*

In the section 3.1 it was shown that the chaperone Hep1 binds the aggregation-prone conformer of Ssc1 and thus prevents the formation of non-native Ssc1 oligomers. Is Hep1 also required to assist the *de novo* folding of Ssc1 upon import of Ssc1 into mitochondria? To answer this question, the folding of the radiolabelled Ssc1 in mitochondria lacking Hep1 was tested. In contrast to the wild-type mitochondria (wt), only a 35 kDa stable fragment, but no full-length protein was observed upon trypsin digestion (Fig. 3.10, left panel). This indicates that folding of Ssc1 is impaired in the absence of Hep1. The stable fragment could be immunoprecipitated with antibodies recognizing specifically the C-

terminus of Ssc1, demonstrating that it corresponds to the PBD (Fig. 3.10, right panel). This implies that in the absence of Hep1, the PBD of Ssc1 is able to fold, whereas the ATPase domain displays a folding defect.

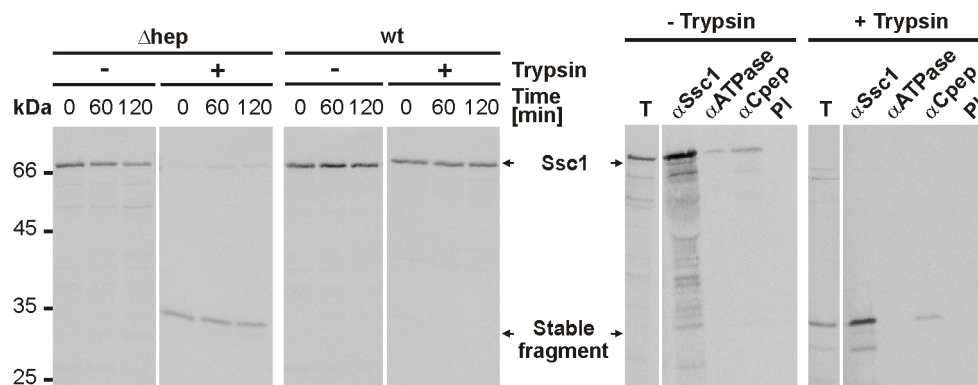


Figure 3.10. The folding of Ssc1 is impaired in the absence of Hep1 *in organello*

Radiolabelled Ssc1 precursor was imported into Δhep1 and wild-type (wt) mitochondria for 30 min. The import was stopped by addition of valinomycin, samples were further incubated for the indicated time periods and treated as described in Fig. 3.5 to assess the folding state of Ssc1 (left panel). Imported Ssc1, as well as the stable fragment present in the lysates of Δhep1 mitochondria was subjected to immunoprecipitation with antibodies against full-length Ssc1 (αSsc1), the ATPase domain (αATPase), the C-terminus of Ssc1 (αCpep) and with pre-immune IgGs (PI). Total material (T, 10 %) subjected to immunoprecipitation and the precipitated material were analysed by SDS-PAGE and autoradiography (right panel).

To confirm these findings, the folding of the individual Ssc1 domains in Δhep1 mitochondria using ATPaseLinker-DHFR and PBD-DHFR variants was assessed. Both variants were able to fold in the wild-type mitochondria. Moreover, folding of the DHFR in both variants was not affected by the lack of Hep1 (Fig. 3.11). However, in the absence of Hep1, only the PBD became trypsin-resistant (Fig. 3.11b), whereas almost all of the ATPase domain molecules were trypsin-sensitive (Fig. 3.11a). Thus, in the absence of Hep1, folding of the ATPase domain even in the context of the interdomain linker was impaired, in contrast to the folding of the PBD, which did not require Hep1.

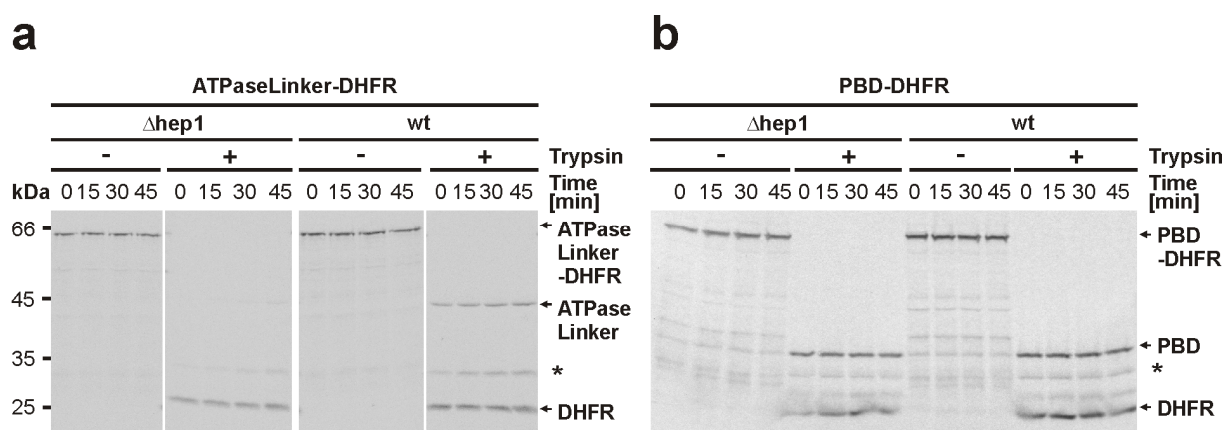


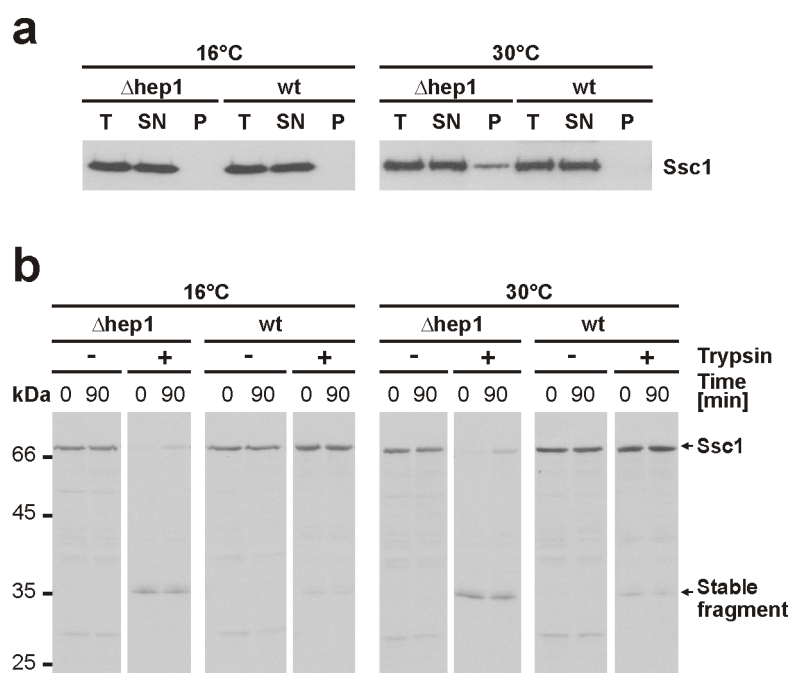
Figure 3.11. The ATPase domain requires Hep1 for folding, whereas the PBD folds without Hep1

Radiolabelled ATPaseLinker-DHFR (**a**) and PBD-DHFR (**b**) precursors were imported into $\Delta hep1$ and wild-type (wt) mitochondria for 15 min. Import was stopped by addition of valinomycin, samples were further incubated for the indicated time periods and treated as described in Fig. 3.5. Samples were analysed by SDS-PAGE and autoradiography. Asterisks indicate folded DHFR domain with non-digested spacer.

Taken together, these results clearly demonstrate that the chaperone Hep1 is crucial for the *de novo* folding of the Ssc1 *in organello*. Hep1 mediates the folding of the ATPase domain in the presence of the interdomain linker.

3.2.4. The folding defect of Ssc1 is a direct consequence of the absence of Hep1 *in organello*

Deletion of the *HEP1* gene has severe consequences for the correct functioning of yeast cells and it affects several cellular processes and features due to aggregation of mitochondrial Hsp70 proteins (Burri et al. 2004; Sanjuan Szklarz et al. 2005; Sichting et al. 2005; Yamamoto et al. 2005). To check whether the observed folding defect of newly imported Ssc1 in $\Delta hep1$ mitochondria is not a secondary effect of the accumulation of non-functional, aggregated Ssc1 in mitochondria, *de novo* folding of Ssc1 was tested in mitochondria from cells grown at different temperatures and displaying different phenotypic features. At low temperature (16 °C), a growth defect of $\Delta hep1$ cells and aggregation of Ssc1 was not observed (Fig. 3.12a, left panel), therefore mitochondria from those cells presumably did not accumulate strong secondary effects. On the other hand,



3.2.5. Folding of Ssc1 is independent of the Hsp60, the Hsp78 and the mtHsp70 chaperone system

There are several chaperone systems residing in the mitochondrial matrix, which are involved in protein folding. The mitochondrial chaperonin Hsp60 together with the chaperone Hsp10, as well as the mitochondrial Hsp70 chaperone system promotes the folding of various substrates *in vivo* and *in vitro*. The Hsp78 chaperone, a member of the Clp/Hsp100 family was reported to closely cooperate with the mtHsp70 chaperone system and play a role for protein reactivation upon heat stress. Do these mitochondrial chaperone systems assist in the *de novo* folding of Ssc1? To answer this question, the folding of Ssc1 in the mitochondria of the temperature-sensitive or deletion strains of these chaperones was tested. In the mitochondria of the *mif4* strain, containing a temperature-sensitive lethal mutation in the *HSP60* gene, Ssc1 folded with kinetics comparable to the folding in the wild-type mitochondria (Fig. 3.13a, left panel). In this strain, Hsp60 could not assemble into the functional homo-oligomeric complexes upon the shift to the non-permissive temperature and aggregated as previously shown (Fig. 3.13a, right panel) (Cheng et al. 1990). Moreover, Ssc1 folded properly with comparable kinetics in the presence and absence of Hsp78 (Fig. 3.13b). This indicates that folding of Ssc1 is independent of Hsp60 and Hsp78 chaperones. We also tested the mtHsp70 chaperone system itself, by using the strain lacking the J co-chaperone Mdj1. In the absence of Mdj1, the folding of Ssc1 was also not affected (Fig. 3.13c). This implies that the folding of Ssc1 does not require the mtHsp70 chaperone system.

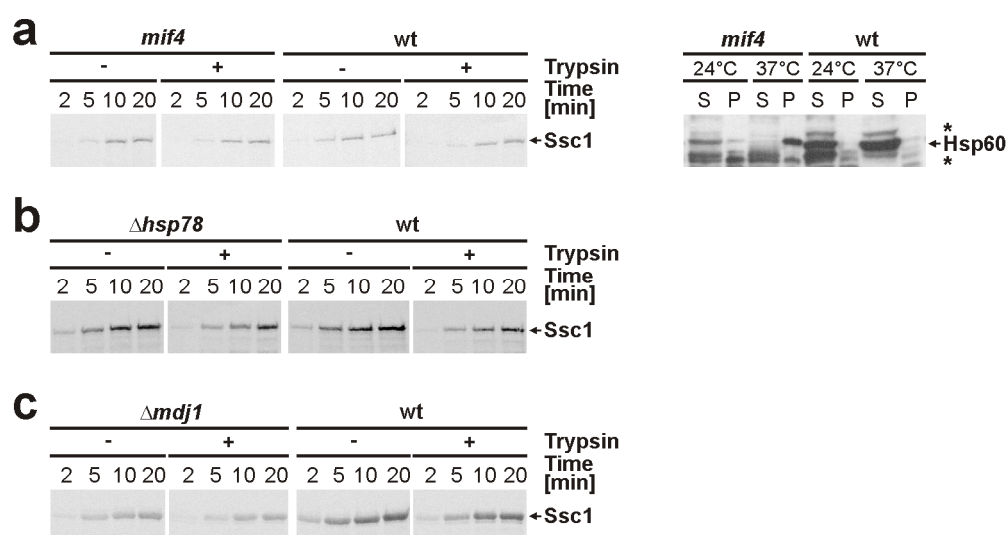


Figure 3.13. Defects in mitochondrial chaperone systems do not affect folding of Ssc1

Radiolabelled Ssc1 precursor was imported into mitochondria isolated from **(a, left panel)** *mif4*, **(b)** $\Delta hsp78$ and **(c)** $\Delta mdj1$ cells as well as from the corresponding wild-type (wt) cells for the indicated time periods. Samples were treated as described in Fig. 3.5 to assess the folding state of Ssc1 and analysed by SDS-PAGE and autoradiography. **(a, right panel)** Cells from *mif4* and the corresponding wild-type strain were grown at 24 °C and then shifted to 37 °C, when indicated, and grown further for 2.5 h. Then, cells were collected, disrupted with glass beads and solubilized with 1 % Triton X-100. Soluble (S) and aggregated (P) protein fractions were separated by centrifugation. These fractions were analysed by SDS-PAGE and immunodecoration with antibodies against bacterial GroEL, recognizing yeast Hsp60. Asterisks indicate cross-reaction of the antibodies.

Similarly, no folding defects were observed when the folding of the ATPaseLinker-DHFR variant was tested in all of the above-mentioned stains (Fig. 3.14). This implies that the folding of the ATPase domain is not affected in the absence of the functional chaperone systems of Hsp60, Hsp78 and mtHsp70.

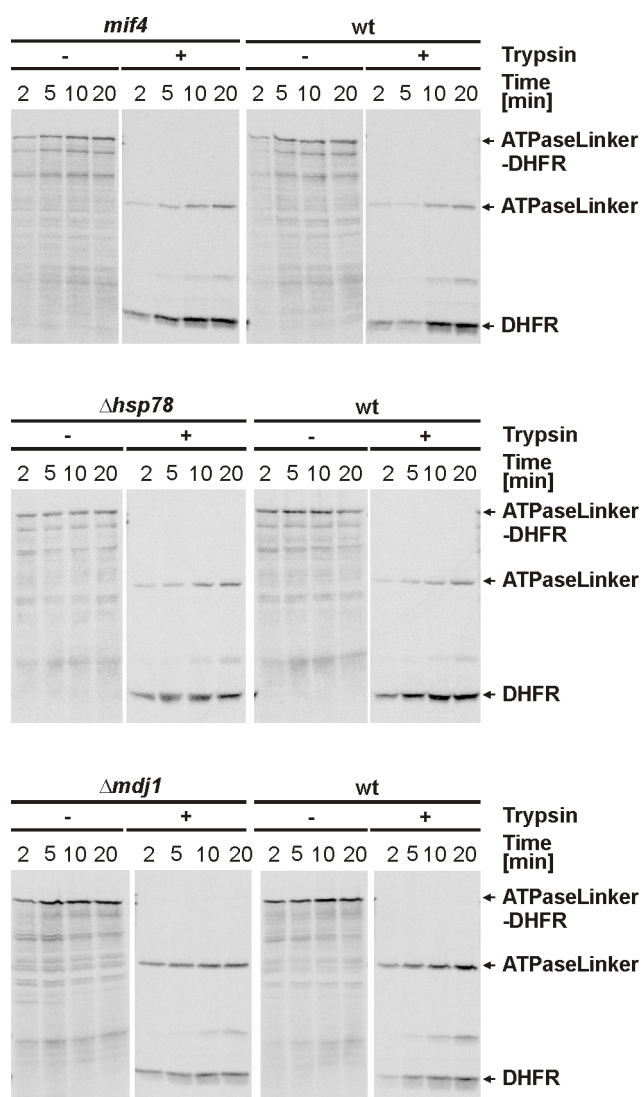


Figure 3.14. Defects in mitochondrial chaperone systems do not compromise folding of the ATPase domain with the interdomain linker

Radiolabelled ATPaseLinker-DHFR precursor was imported into mitochondria isolated from (a) *mif4*, (b) *Δhsp78* and (c) *Δmdj1* cells as well as from the corresponding wild-type (wt) cells for the indicated time periods. Samples were treated as described in Fig. 3.5 to assess the folding state of the imported variant and analysed by SDS-PAGE and autoradiography.

In summary, none of the tested mitochondrial chaperone systems seemed to be involved in the *de novo* folding of the Ssc1 chaperone *in organello*. This suggests that Hep1 is the main helper protein required for the biogenesis of Ssc1.

3.2.6. Hep1 is required for *de novo* folding of Ssc1 *in vivo*

To address, whether Ssc1 has a folding defect in the absence of Hep1 *in vivo*, an *in vivo* pulse-chase experiment, which allowed following the biogenesis of newly synthesized radiolabelled proteins in intact yeast cells, was performed. To this end, wild-type and $\Delta hep1$ cells were grown for 10 min in the presence of radioactive methionine to synthesize radiolabelled proteins (pulse). Next, protein synthesis was stopped, and an excess of cold methionine was added (chase). Mitochondria isolation, solubilization and trypsin digestion followed by an immunoprecipitation with anti-Ssc1 antibodies allowed the specific detection of the Ssc1 protein and its folding state. In $\Delta hep1$ and wild-type cells, the labelling efficiency and the mitochondrial import rates were comparable, since similar amounts of radiolabelled Ssc1 were precipitated from both mitochondrial extracts (Fig. 3.15, - Trypsin). However, in wild-type mitochondria the majority of Ssc1 was trypsin-resistant, thus folded. On the contrary, in $\Delta hep1$ mitochondria only a minor fraction of Ssc1 was trypsin-resistant and a stable fragment corresponding to the PBD was generated (Fig. 3.15). A chase of 20 min did not increase the amount of trypsin-resistant Ssc1 in mitochondria lacking Hep1. These results indicate a severe impairment of *de novo* folding of Ssc1 in the absence of Hep1 *in vivo*. Therefore, consistent with the findings *in organello*, the chaperone Hep1 mediates the *de novo* folding process of Ssc1 *in vivo*.

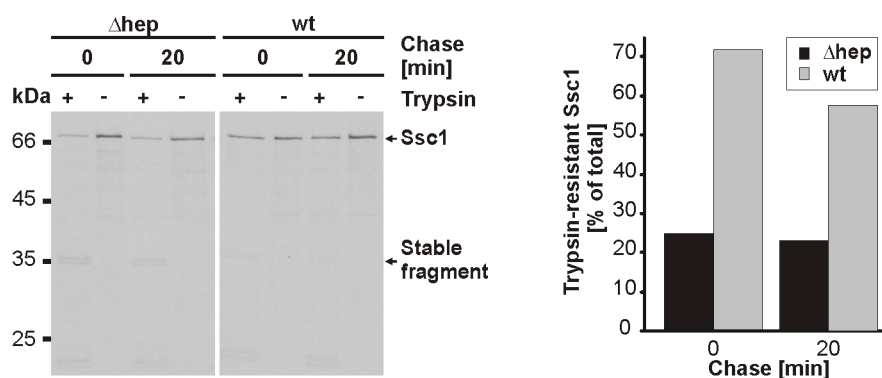


Figure 3.15. Folding of Ssc1 *in vivo* requires Hep1

Δhep1 and wild-type cells (wt) were radiolabelled with ^{35}S -methionine and chased with cold methionine for the indicated time periods after the protein synthesis was stopped. Mitochondria were then isolated, solubilized and mock-treated or treated with trypsin to assess the folding of proteins. Ssc1 was immunoprecipitated with antibodies against Ssc1 and samples were analysed by SDS-PAGE and autoradiography (left panel) and quantified by densitometry (right panel). Total material (- Trypsin) was set to 100 % for each time period, trypsin-resistant material is shown as percentage of total material.

3.3. Reconstitution of the *de novo* folding of the Ssc1 chaperone *in vitro*

3.3.1. Hep1 and ATP are crucial for the refolding of Ssc1 in the reconstituted *in vitro* system

To dissect the molecular mechanism of Ssc1 folding and characterise the function of Hep1 in this process, an attempt was made to reconstitute the *de novo* folding of Ssc1 using the purified components. To this end, mature Ssc1 and N-terminally his-tagged mature Hep1 were expressed as recombinant proteins and purified from bacterial cells (Fig 3.16).

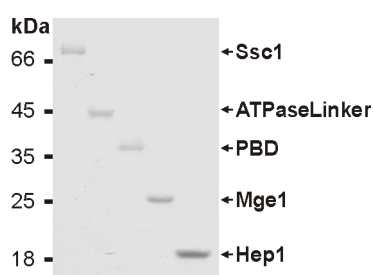


Figure 3.16. Purified recombinant proteins used in this study

Purified Ssc1, ATPaseLinker as well as his-tagged PBD, Mge1 and Hep1 (5 mg each) were analysed by SDS-PAGE and Coomassie staining.

The stability of the purified Ssc1 was tested by trypsin treatment using a broad range of trypsin concentrations. Purified Ssc1 was apparently not susceptible to trypsin, which implies that the purification yielded a stable, correctly folded protein (Fig. 3.17).

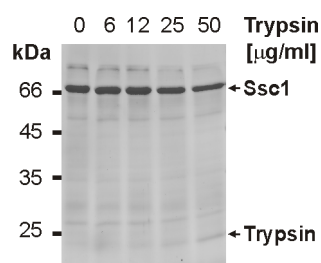


Figure 3.17. Purified recombinant Ssc1 is trypsin-resistant

Purified Ssc1 (6 mg) was treated with various concentrations of trypsin to assess the stability. Samples were analysed by SDS-PAGE and Coomassie staining.

To reconstitute the folding process of Ssc1, purified Ssc1 was denatured by precipitation and resuspension in buffer with 8 M urea and subsequently diluted in the

refolding buffer containing recombinant Hep1 and/or ATP. Refolding of Ssc1 was monitored by trypsin resistance. Only in the presence of ATP and equimolar amounts of Hep1, Ssc1 became resistant to trypsin. On the contrary, no or minor amounts of trypsin-resistant full-length Ssc1 were detected, when Hep1 or ATP were missing in the refolding buffer (Fig. 3.18). In summary, denatured Ssc1 can be efficiently refolded in an *in vitro* system. Hep1 and ATP are crucial for the refolding process.

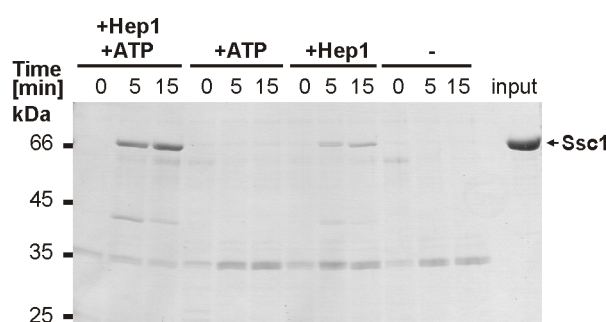


Figure 3.18. Ssc1 requires Hep1 and ATP for its refolding *in vitro*

Unfolded Ssc1 was diluted into refolding buffer with or without addition of recombinant Hep1 and/or ATP. At the indicated time points, samples were withdrawn and treated with trypsin. Samples were analysed by SDS-PAGE and Coomassie staining. Input, 100 % of protein added into the refolding reactions.

Next, the folding kinetics of Ssc1 in the presence of ATP was analysed in the refolding buffer with and without recombinant Hep1. In the presence of Hep1, Ssc1 became trypsin resistant in a few minutes (Fig. 3.19). Apart from full-length Ssc1, two fragments of 45 kDa and 35 kDa were observed upon trypsin treatment of the samples at the early time points of refolding (Fig. 3.18 and 3.19). These fragments presumably resulted from a digestion of a folding intermediate of Ssc1 and would correspond to the PBD and ATPase domain, respectively. This implies that both Ssc1 domains fold and become trypsin-resistant individually, before the full-length Ssc1 acquires the final, fully protease-resistant conformation. In the absence of Hep1, only a 35 kDa fragment corresponding to the PBD was formed, indicating that the PBD was able to fold, in contrast to the ATPase domain. This suggests that the PBD can fold independently of Hep1, whereas the ATPase domain needs Hep1 for folding *in vitro*.

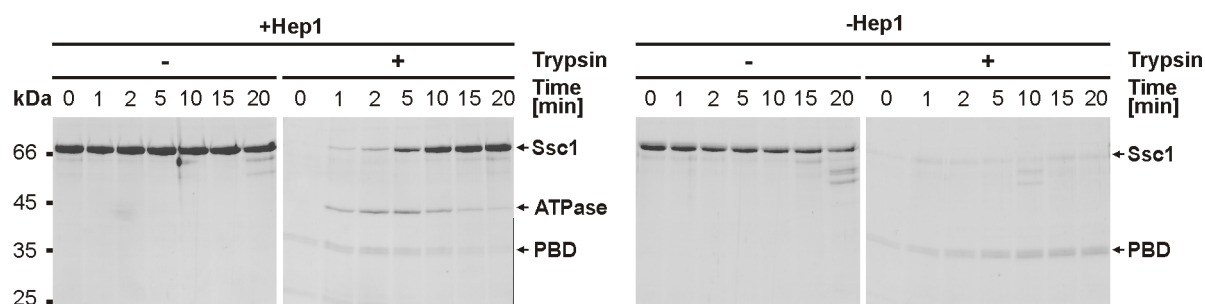


Figure 3.19. Kinetics of refolding of Ssc1 *in vitro*

Unfolded Ssc1 was diluted into refolding buffer containing ATP in the presence (left panel) or absence (right panel) of recombinant Hep1. At the indicated time points, samples were withdrawn and half of each sample was treated with trypsin to assess folding, the other half was mock-treated. Samples were analysed by SDS-PAGE and Coomassie staining.

3.3.2. Denatured Ssc1 does not aggregate and retains its folding competence in the absence of Hep1

In the absence of chaperones, unfolded or misfolded proteins have a tendency to acquire an off-pathway conformation and are not able to reach the native state. Such off-pathway conformers have a high propensity to form aggregates. It was tested whether denatured Ssc1 is able to fold when it is first diluted from urea and incubated in the refolding buffer in the absence of Hep1 and ATP. To this end, denatured Ssc1 was diluted in the refolding buffer and pre-incubated for 30 min at 37 °C. Then, ATP and recombinant Hep1 were added and the sample was further incubated at 25 °C to allow refolding. As a control, denatured Ssc1 was diluted into the refolding buffer with ATP and Hep1 added directly prior to Ssc1 addition. Samples were withdrawn at the indicated time points and half of each sample was treated with trypsin to assess the folding state of Ssc1. All samples were subjected to a clarifying spin and analysed by SDS-PAGE and Coomassie staining. For the mock-treated samples, no loss of protein upon pre-incubation in the absence of Hep1 and ATP was observed, implying that no large Ssc1 aggregates that could be removed by the clarifying spin were formed (Fig. 3.20, left panel). Moreover, upon addition of ATP and Hep1 to the pre-incubated Ssc1, the protein became trypsin-resistant, comparable to the control sample (Fig. 3.20, right panel). This suggests that denatured Ssc1, upon dilution in the refolding buffer, does not aggregate and remains in a folding-competent state in the absence of its folding helper.

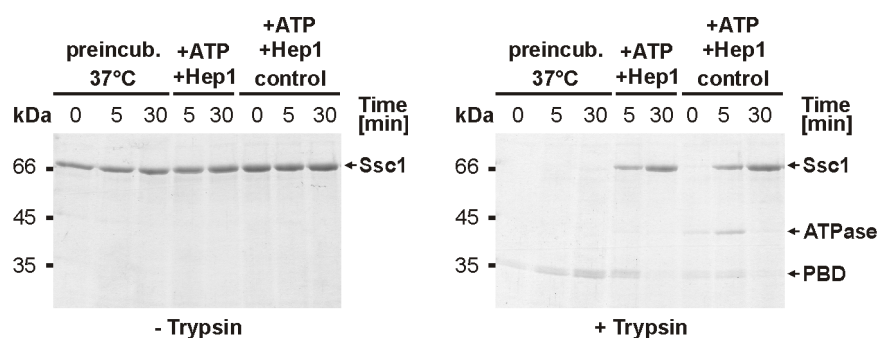


Figure 3.20. Denatured Ssc1 remains folding-competent upon pre-incubation in the absence of Hep1

Unfolded Ssc1 was diluted into refolding buffer and pre-incubated in the absence of recombinant Hep1 and ATP for 30 min at 37 °C. Then, ATP and Hep1 were added and the sample was further incubated at 25 °C to allow refolding. As a control, unfolded Ssc1 was directly incubated in the buffer containing ATP and Hep1 at 25 °C. Samples were withdrawn at the indicated time points and half of each sample was treated with trypsin (right panel) to assess the folding state of Ssc1. The other half was mock treated (left panel). Samples were analysed by SDS-PAGE and Coomassie staining.

3.3.3. Hep1 can be repeatedly used for refolding of Ssc1

Equimolar amounts of Hep1 efficiently promote refolding of Ssc1. Does the rate of the refolding reaction depend on the molar ratio between Hep1 and denatured Ssc1? To test this, the folding assay at the molar ratios of 1:1, 1:0.5 and 1:0.25 of Ssc1 to Hep1 was performed and the kinetics of refolding over a period of 120 min was measured. Although the initial rates of refolding of Ssc1 decreased with the decreasing concentration of Hep1, the amount of refolded Ssc1 at the end of the incubation reached 80–100 % in each case (Fig. 3.21). This suggests that each Hep1 molecule is able to mediate several rounds of Ssc1 refolding.

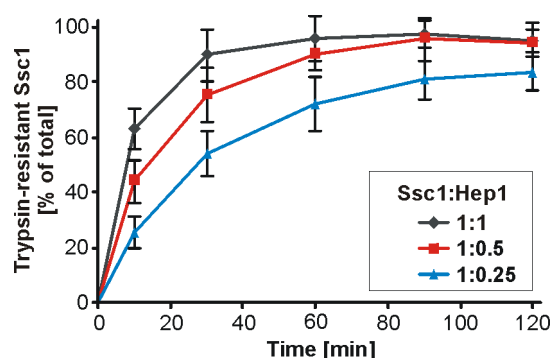


Figure 3.21. Hep1 mediates several rounds of refolding of Ssc1

Unfolded Ssc1 was diluted into refolding buffer containing ATP and recombinant Hep1 in molar ratios of 1:1, 1:0.5 and 1:0.25 of Ssc1 to Hep1. Samples were incubated at 25 °C to allow refolding, subsequently treated with trypsin and analysed by SDS-PAGE and Coomassie staining. The signals of trypsin protected full-length Ssc1 were quantified by densitometry. The signals of mock-treated samples of the longest time period (total) were set to 100 %. The graph shows mean values of 5 independent experiments +/- standard deviation.

3.3.4. Ssc1 refolded *in vitro* in the presence of Hep1 is enzymatically active

To prove that refolded Ssc1 is not only trypsin-resistant, but also enzymatically active, the ATPase activity of the refolded protein was measured. To this end, a coupled ATP-regenerating enzymatic assay was performed with the native Ssc1 and with the denatured Ssc1 subjected to the refolding procedure in the absence and presence of Hep1. Whereas the denatured Ssc1 remained inactive in the absence of Hep1 (Fig. 3.22a, Control), Ssc1 refolded in the presence of Hep1 regained almost 70 % of the activity as compared to the native protein (Fig. 3.22a). Similar results were obtained when a radioactive assay was used (Fig. 3.22b). Apparently, the protease-resistant conformation of Ssc1 that was adopted upon refolding in the presence of Hep1 represents the native and active form of Ssc1.

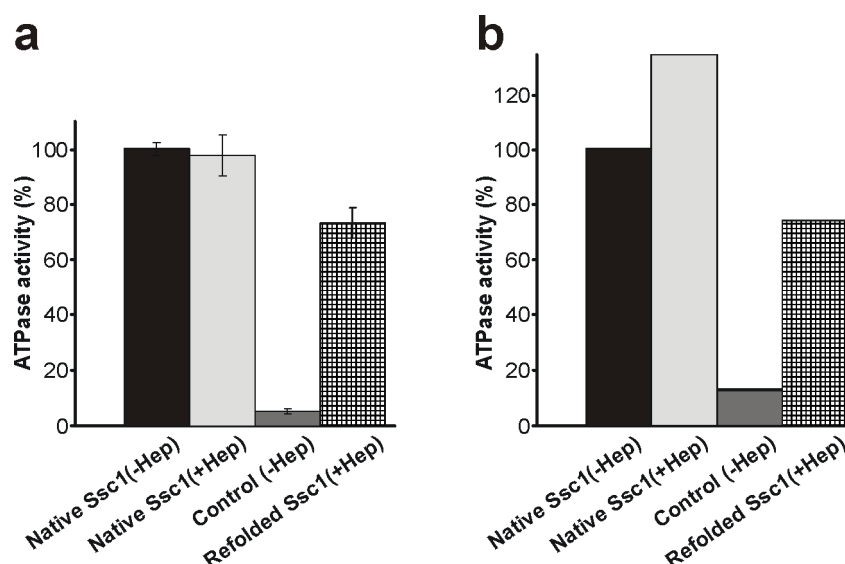


Figure 3.22. Refolded Ssc1 is enzymatically active

Native and unfolded Ssc1 were incubated in ATP-containing buffer in the presence or absence of recombinant Hep1. Then the ATPase activities of the samples in the presence of the co-chaperones Mge1 and DnaJ were measured using the spectrophotometric enzymatic assay (a) and the radioactive assay (b). The ATPase activity of native Ssc1 (-Hep1) was set to 100 %. Control, unfolded Ssc1 incubated in the absence of Hep1. Diagram (a) represents the mean values of three independent measurements +/- standard deviation.

3.3.5. Adenine nucleotides promote the folding of Ssc1 in the presence of Hep1

In section 3.3.1, it was demonstrated that ATP is required for the refolding of Ssc1 *in vitro* in the presence of Hep1 (Fig. 3.18). Do other adenine nucleotides promote the folding of Ssc1 as well? To address this, the refolding of Ssc1 in the presence of Hep1 with the addition of various adenine nucleotides, able to bind to the native Ssc1, was tested. Interestingly, both ATP and ADP promoted the folding of Ssc1 with a comparable efficiency. Addition of a non-hydrolyzable ATP analogue, ATP γ S, led to the folding of Ssc1 as well, albeit with slightly reduced efficiency. Virtually no trypsin-protected Ssc1 was formed in the presence of another non-hydrolyzable ATP analogue, AMP-PNP, or in the absence of any nucleotide (Fig. 3.23). This means, that the presence of nucleotide is necessary for the refolding of Ssc1 *in vitro*, and other adenine nucleotides, in addition to ATP, promote the folding of Ssc1 in the presence of Hep1. Apparently, the hydrolysis of ATP is not required for the folding of Ssc1, since ADP and ATP γ S can replace ATP in the refolding process. Instead, it is the binding of nucleotides that triggers the formation of a folded protein.

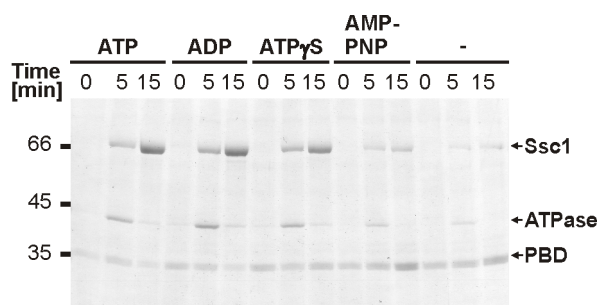


Figure 3.23. Adenine nucleotides are required for the folding of Ssc1 *in vitro*

Unfolded Ssc1 was incubated in refolding buffer containing recombinant Hep1 and the indicated nucleotides. After the indicated time periods, samples were withdrawn, treated with trypsin and analysed by SDS-PAGE and Coomassie staining.

3.3.6. Individual domains of Ssc1 fold *in vitro*

To analyse the requirements for the folding of the individual Ssc1 domains in the reconstituted system, mature non-tagged ATPaseLinker and C-terminally his-tagged PBD were expressed and purified from bacterial cells (Fig. 3.16) and subsequently denatured similarly to the purified Ssc1. Both proteins were diluted in the refolding buffer, incubated with and without Hep1 and ATP and their folding state was tested by trypsin digestion. The ATPaseLinker became trypsin-resistant only in the presence of both Hep1 and ATP (Fig. 3.24a). In contrast, the PBD was able to efficiently refold independently of Hep1 and ATP (Fig. 3.24b). These observations are consistent with the results obtained *in organello* and *in vivo*. It is apparently the ATPase domain in the full-length Ssc1 that requires Hep1 and an adenine nucleotide for the *de novo* folding. On the contrary, the PBD folds spontaneously in the absence of folding helpers.

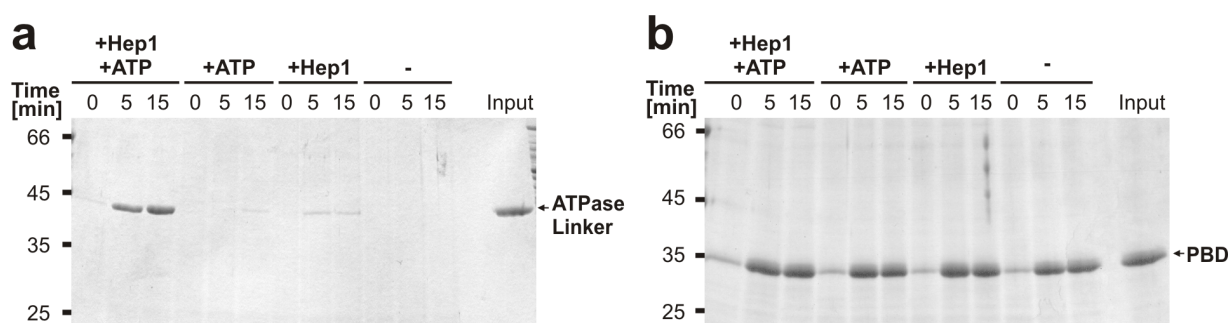


Figure 3.24. ATPaseLinker and PBD fold independently *in vitro*

Unfolded ATPaseLinker (**a**) or PBD (**b**) were incubated in refolding buffer containing ATP and/or recombinant Hep1. After the indicated time periods, samples were withdrawn, treated with trypsin and analysed by SDS-PAGE and Coomassie staining. Input, 100 % of protein added to the refolding reactions.

3.3.7. Mge1 does not promote the folding of Ssc1

Is the involvement in the refolding process of Ssc1 specific for Hep1? It was previously reported that another interaction partner of Ssc1 – the nucleotide exchange factor Mge1 – is able to keep Ssc1 soluble upon co-expression in *E.coli* (Momose et al. 2007). Therefore, the refolding of Ssc1 was tested in the presence of recombinant Mge1. Even a four-fold molar excess of Mge1 over Ssc1 did not promote the folding of the full-length Ssc1, nor of the ATPaseLinker (Fig. 3.25). Thus, the chaperone function in the folding of Ssc1 is most likely specific for Hep1 and cannot be taken over by other interaction partners of Ssc1 such as Mge1.

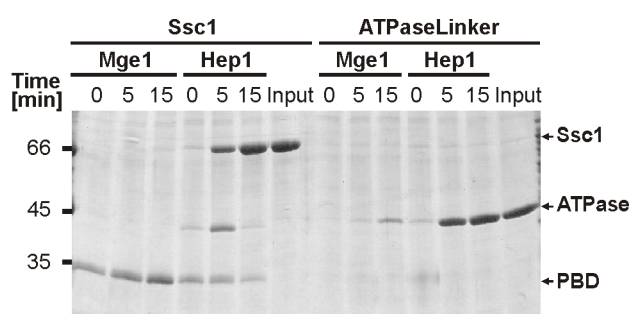


Figure 3.25. Mge1 does not mediate refolding of Ssc1 *in vitro*

Unfolded Ssc1 or ATPaseLinker were incubated in refolding buffer containing ATP and a four-fold molar excess of recombinant Mge1 or equimolar amount of recombinant Hep1 as a control. After the indicated time periods, samples were withdrawn, treated with trypsin and analysed by SDS-PAGE and Coomassie staining. Input, 100 % of protein added to the refolding reactions.

3.3.8. Hep1 interacts with a folding intermediate of Ssc1

Since Hep1 is required for the *de novo* folding of Ssc1 *in vitro*, it presumably transiently interacts with the folding Ssc1. To trap this interaction, a chemical crosslinking experiment with glutaraldehyde was performed. To this end, denatured Ssc1 was incubated with or without Hep1 and ATP and then the crosslinking reagent was added. All samples were analysed by SDS-PAGE and Coomassie staining, as well as western-blotting and immunodecoration against Hep1 and Ssc1. In the presence of Hep1, a crosslinked adduct containing Ssc1 and Hep1 was observed on the Coomassie-stained gel and on the immunodecoration (Fig. 3.26, lanes 8, 9, 13, 14, 18, 19). The crosslinked adduct was formed in the absence of ATP. Moreover, addition of ATP almost completely abolished the adduct formation (Fig. 3.26, lanes 9, 14, 19). This implies that in the absence of ATP the interaction between Hep1 and the folding Ssc1 was trapped, whereas in the presence of ATP the folding process of Ssc1 was completed and thus, Hep1 did not interact with Ssc1 anymore. In the presence of both Hep1 and ATP, a sharp band corresponding to the monomeric Ssc1 was observed on the Coomassie-stained gel and immunodecoration (Fig. 3.26, lanes 9, 19), reflecting that Ssc1 was able to adopt a folded structure, not accessible to the crosslinking reagent. In contrast, the signals in the other lanes were more diffuse due to the unfolded state of Ssc1, which allowed extensive modifications and the formation of intramolecular bridges by the crosslinking reagent. In conclusion, binding of Hep1 to Ssc1 is one of the early steps in the process of Ssc1 folding, and it precedes the nucleotide-dependent step.

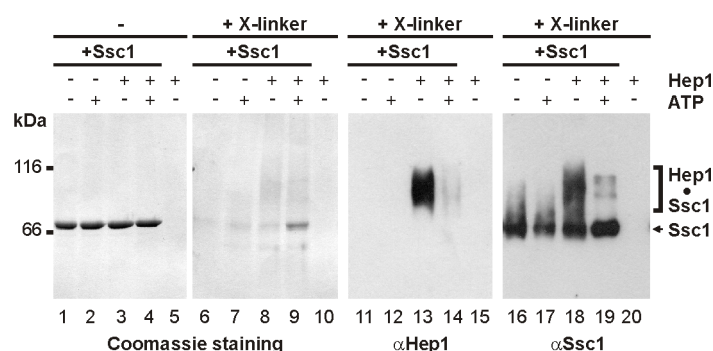


Figure 3.26. Hep1 interacts with folding Ssc1 in the absence of ATP

Unfolded Ssc1 was diluted into refolding buffer containing Hep1 and/or ATP, where indicated. Samples were incubated at 30°C for 20 min and then treated with or without the crosslinking reagent (glutaraldehyde). Samples were analysed by SDS-PAGE and Coomassie staining. Aliquots of the samples (15 %) were analysed by SDS-PAGE, western-blotting and immunodecoration with antibodies against Hep1 and Ssc1.

3.3.9. Incubation with ATP after removal of Hep1 does not promote folding of Ssc1

As shown in the previous section, Hep1 interacts with the folding intermediate of Ssc1 prior to the adenine nucleotide. Is Hep1 released from the partially folded intermediate of Ssc1 before the nucleotide binds and triggers further folding? To test this, denatured Ssc1 was incubated with Hep1 in the absence of nucleotides and subsequently a NiNTA pull-down was performed to deplete the sample of Hep1 containing a his-tag. As a control, the same procedure was carried out in the absence of Hep1. Non-bound material from the pull-down (FT) was then supplemented with ATP and, where indicated, freshly added Hep1, as a control, and the samples were further incubated to allow refolding of Ssc1. Half of each sample was treated with trypsin to assess the folding state of Ssc1 and all samples were analysed by SDS-PAGE and immunodecoration (Fig. 3.27a).

Following the incubation of Ssc1 with Hep1, Hep1 efficiently bound to the NiNTA beads and was fully depleted from the sample (Fig. 3.27b, upper panel). In that sample, upon the addition of ATP, Ssc1 remained trypsin-sensitive, thus, it did not fold (Fig. 3.27b, lower panel, lane 1). However, Ssc1 remained able to fold in the course of the procedure, since it became folded upon the addition of the fresh portion of Hep1 (Fig. 3.27, lower panel, lane 2). Similar results were obtained with Ssc1 incubated in the absence of Hep1 prior to the pull-down (Fig. 3.27b, lower panel, lanes 5 and 6). This means that Hep1

removal prior to the nucleotide addition, similarly to lack of Hep1, prevents folding of Ssc1. According to these findings, it is unlikely that Hep1 induces stable structural changes in the folding intermediate of Ssc1 and then dissociates from Ssc1 before the nucleotide-dependent step in the folding process takes place. It is more likely that the nucleotide binds to the folding intermediate of Ssc1 that is associated with Hep1 and triggers further folding.

Notably, only a small portion of Ssc1 was found in the fraction associated with the beads after pull-down of Hep1 (Fig. 3.27b, upper panel). This portion corresponded to the Ssc1 specifically bound to Hep1, since no Ssc1 was found in the bound fraction in the absence of Hep1. This suggests that under experimental conditions the interaction between the folding intermediate of Ssc1 and Hep1 is rather transient. Most probably, there is an equilibrium between free Ssc1 and Ssc1 transiently interacting with Hep1 in the absence of nucleotide. Removal of Hep1 shifts this equilibrium towards the folding intermediate of Ssc1 that is unable to fold further upon the addition of nucleotide.

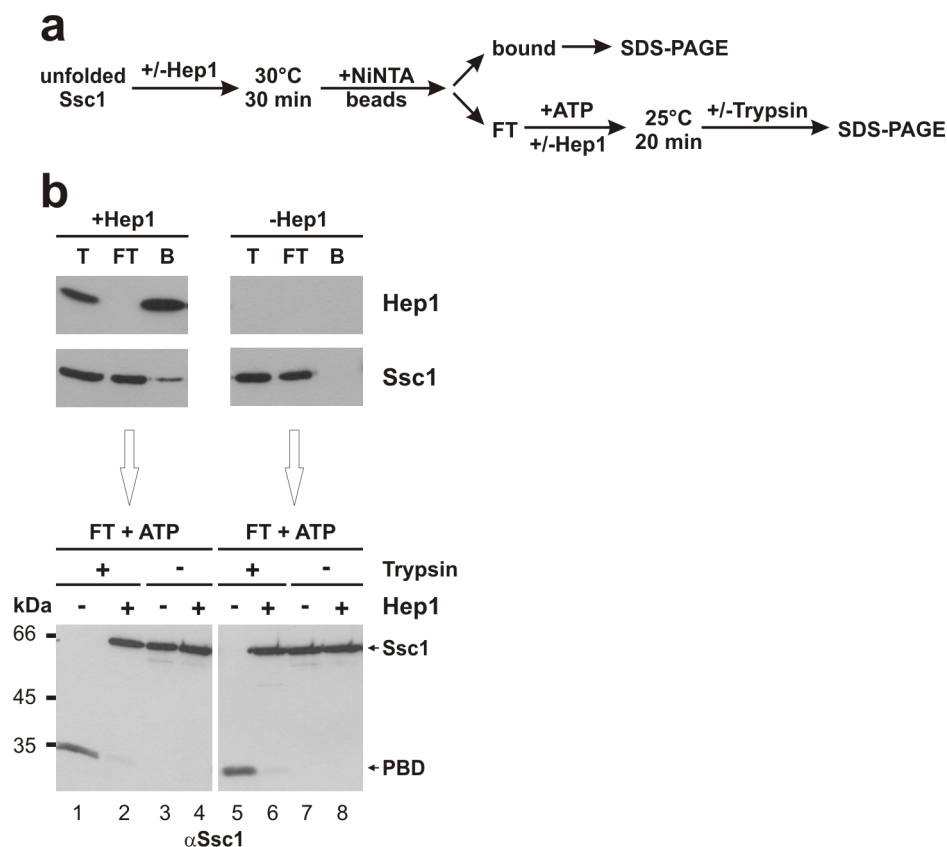


Figure 3.27. Removal of Hep1 prior to the nucleotide addition prevents the folding of Ssc1

(a) Scheme of the experimental procedure. **(b)** Unfolded Ssc1 was diluted into the buffer containing Hep1, where indicated. Samples were incubated at 30°C for 30 min and then Hep1 was removed by NiNTA beads. An aliquot of non-bound material (FT, 20 %) was withdrawn and analysed, together with total (T, 20 %) and bound fraction (B, 100 %) by SDS PAGE and immunodecoration against Ssc1 and Hep1 (upper panel). The remaining non-bound material was supplemented with ATP and, where indicated, Hep1, and further incubated at 25 °C for 20 min to allow refolding of Ssc1. Half of each sample was treated with trypsin to assess the folding state of Ssc1 (+ Trypsin); the other half was mock-treated

(- Trypsin) (lower panel). Samples were analysed by SDS-PAGE and immunodecoration with antibodies against Ssc1

3.3.10. Mge1 does not bind to the folding intermediate of Ssc1

In section 3.3.7 it was shown that nucleotide exchange factor Mge1 is not able to mediate the folding of Ssc1 (Fig. 3.25). To test whether Mge1 can at all interact with the folding intermediate of Ssc1, a chemical crosslinking in the absence of ATP was carried out. As a control, Hep1 instead of Mge1 was used. Samples were analysed by SDS-PAGE, Coomassie staining and immunodecoration with antibodies against Hep1 and Mge1. In contrast to Hep1, Mge1 did not form a crosslinked adduct with denatured Ssc1 (Fig. 3.28, lanes 3 and 11). It also did not influenced the adduct formation between denatured Ssc1 and Hep1, when all three proteins were incubated together (Fig. 3.28, lanes 4, 8, 12). This indicates that Mge1, unlike Hep1, is not able to recognize and bind to the folding intermediate of Ssc1, even though it interacts with the native Ssc1 under the same experimental conditions (data not shown). This lack of interaction between Mge1 and folding Ssc1 explains why Mge1 is not able to replace Hep1 during the folding process of Ssc1 *in vitro*.

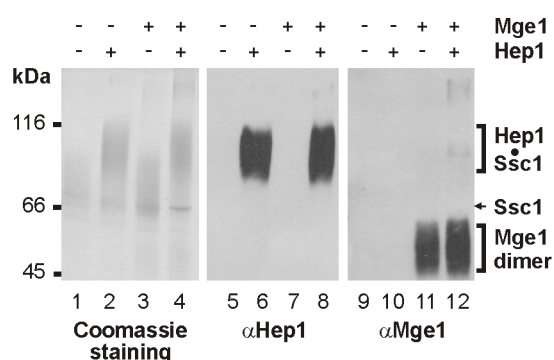


Figure 3.28. Mge1 does not interact with the folding Ssc1

Unfolded Ssc1 was diluted into the buffer containing, where indicated, Mge1 and/or Hep1. Samples were incubated at 30°C for 20 min and then treated with or without the crosslinking reagent (glutaraldehyde). Samples were analysed by SDS-PAGE and Coomassie staining. Aliquots of the samples (15 %) were analysed by SDS-PAGE and immunodecoration with antibodies against Hep1 and Mge1.

3.3.11. Adenine nucleotides trigger release of Hep1 from the complex with the folding intermediate of Ssc1

Since various adenine nucleotides affected the folding process differently (Fig. 3.23), the effects of those nucleotides on the formation of the crosslinked adduct between Ssc1 and Hep1 were analysed by SDS-PAGE, Coomassie staining and immunodecoration against Hep1. In the presence of ATP, as well as ADP, no Ssc1-Hep1 adduct was observed (Fig. 3.29a, lanes 7, 8, 12, 13) and a distinct band corresponding to the folded monomeric Ssc1 was present on the Coomassie-stained gel (Fig. 3.29a, lanes 7 and 8). Addition of ATP γ S led to low amounts of adduct (Fig. 3.29a, lanes 9 and 14), whereas in the presence of AMP-PMP, the adduct formed very efficiently (Fig. 3.29a, lanes 10 and 15). Similar results were obtained, when Ssc1 and Hep1 were pre-incubated together to allow complex formation prior to the nucleotide addition (chase). The pre-formed complex (Fig. 3.29b, no chase) dissociated upon incubation with ATP or ADP. Upon addition of ATP γ S, a reduction in the amount of the complex was observed. No effect on the complex was observed when AMP-PNP was added (Fig. 3.29b). These findings further support the results from section 3.3.8, demonstrating that Hep1 binds to the folding Ssc1 prior to the nucleotides. Binding of Hep1 induces structural changes in the folding intermediate of Ssc1 that trigger recognition of various nucleotides. Nucleotide binding promotes further folding of Ssc1 to the final native state and release of Hep1. Not every nucleotide tested, however, triggered dissociation of Hep1 with the same efficiency. The observed variability may reflect diverse affinities of the nucleotides to the Hep1-bound folding intermediate of Ssc1. On the other hand, binding of various nucleotides might induce different structural changes in Ssc1 that affect the stability of the complex with Hep1 differently.

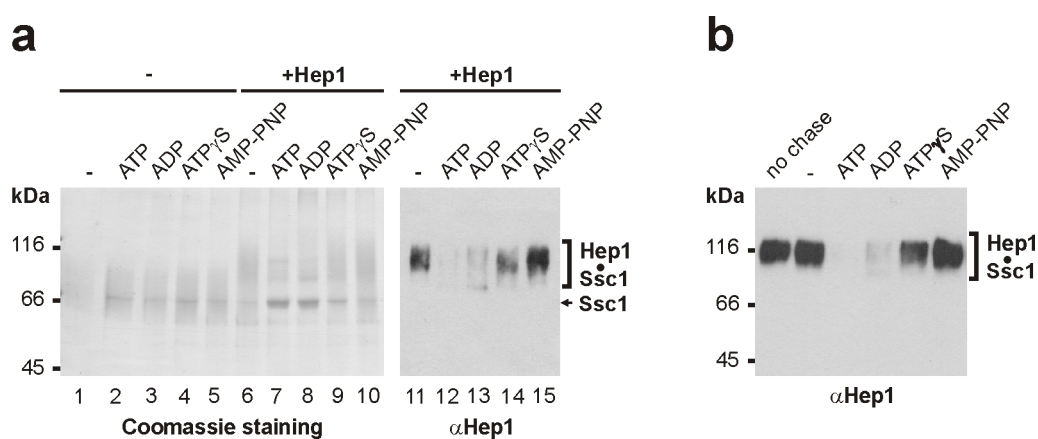


Figure 3.29. Nucleotides have various effects on the interaction of Hep1 with the folding intermediate of Ssc1

(a) Unfolded Ssc1 was incubated for 20 min at 30°C in the buffer containing, where indicated, Hep1 and different nucleotides. Then, samples were treated with the crosslinking reagent (glutaraldehyde) and analysed by SDS-PAGE and Coomassie staining. Aliquots of the samples (15 %) were analysed by SDS-PAGE, western-blotting and immunodecoration with antibodies against Hep1.

(b) Unfolded Ssc1 was incubated with Hep1 for 10 min at 30 °C (no chase). Then, nucleotides were added, where indicated, and samples were further incubated for 10 min. All samples were treated with glutaraldehyde and analysed by SDS-PAGE, western-blotting and immunodecoration with antibodies against Hep1.

4. Discussion

4.1. Aggregation of the Ssc1 chaperone

Yeast mitochondrial Hsp70 chaperone Ssc1 is an essential constituent of the import motor of the TIM23 translocase and one of the main folding helpers of the mitochondrial proteins, playing an indispensable role in the mitochondrial biogenesis. In order to fulfill its function, Ssc1 acquires a native structure that leads to the propensity of self-aggregation. This propensity is counteracted by the presence of the escort protein Hep1, which is required to keep Ssc1 in a soluble and active state.

The present study concentrated on the aggregation propensity of the Ssc1 chaperone in the absence of Hep1. It focused on the analysis of the nature of the Ssc1 aggregates as well as the structural determinants within Ssc1 that make it prone to aggregation. Moreover, this study characterised the smallest region within Ssc1 required for the interaction with Hep1. As shown, this region overlapped with the aggregation-prone region of Ssc1.

A propensity to self-oligomerize was previously observed for other Hsp70 proteins. Human cytosolic Hsp70 and Hsc70 proteins were reported to form oligomers in a temperature- and ATP-dependent manner (Angelidis et al. 1999). Moreover, a similar ATP-dependent behavior was observed for the ER Hsp70 chaperone Grp78 (Carlino et al. 1992) and for the bacterial DnaK (Thompson et al.; Schonfeld et al. 1995). Apart from ATP addition, the peptide substrates and, in the case of DnaK, co-chaperones were able to reverse the oligomerization process of those Hsp70 chaperones. This implies that the PBD was involved in the oligomerization process. In the case of Ssc1, the observed aggregation is irreversible, and it is not influenced by the addition of nucleotides and substrates (Sichting et al. 2005). Moreover, the Ssc1 aggregates formed *in vivo* in the absence of Hep1 were presumably much larger than those reported for the other Hsp70 chaperones, since a fraction of them was removed by low speed centrifugation. The remaining soluble Ssc1 species did not form any defined oligomers, but highly heterogeneous associates, corresponding most likely to intermediates in the aggregation process. In summary, a different mechanism seems to apply for the self-aggregation of Ssc1 than in the case of other Hsp70 proteins.

Why does the mitochondrial Hsp70 chaperone behave differently from other Hsp70 family members? Ssc1 is synthesized on cytosolic ribosomes and subsequently imported

into the mitochondrial matrix, where it has to fold *de novo* to acquire the native structure. Such a complicated biogenesis pathway might explain the aggregation propensity of this protein. However, bacterial DnaK, when expressed in yeast and directed to the mitochondria by a mitochondrial targeting signal, did not aggregate even in the absence of Hep1 (Blamowska et al. 2011). This implies that the import into mitochondria *per se* does not cause aggregation of mitochondrial Hsp70 proteins and that the aggregation propensity appears to be an intrinsic feature of the mitochondrial Hsp70 proteins. Mapa *et al.* (2010) showed that Ssc1 is much more flexible than bacterial DnaK. The binding of a substrate peptide induces additional conformational changes beside the changes induced by the nucleotide, particularly by the hydrolysis of ATP to ADP. Mapa *et al.* (2010) speculated that such behavior might reflect cross-talk between the Ssc1 and the translocase during the pre-protein import process. Therefore, such enhanced flexibility of Ssc1, required for its correct functioning in mitochondria, may result in the propensity to aggregate.

Another distinct feature of the aggregation process of Ssc1, in contrast to the oligomerization of other Hsp70 class members, is the involvement of the ATPase domain, not the PBD. However, the ATPase alone does not aggregate in the absence of Hep1. It is the interdomain linker segment that renders the ATPase domain prone to aggregation. The interdomain linker segment alone is, however, also not responsible for the aggregation propensity, since the linker fused to the PBD did not cause aggregation of the PBD. Moreover, DnaK contains the interdomain linker of the same sequence as Ssc1 and it was still soluble in the absence of Hep1 (Blamowska et al. 2011). Apparently, both ATPase domain and the adjacent linker contribute to the aggregation propensity of Ssc1. Since an interaction of the linker with the ATPase domain and a crucial role of the linker in interdomain communication and ATPase stimulation was reported for DnaK (Vogel et al. 2006; Swain et al. 2007), it is very likely that the linker binds to the ATPase domain and induces its aggregation-prone conformation in Ssc1.

In the presence of Hep1, however, no aggregation of Ssc1 was observed. Hep1 was able to bind to the ATPase in the context of the interdomain linker segment, preventing aggregation. Since Hep1 does not interact with the PBD in combination with the linker segment, nor with the DnaK protein, it is the ATPase domain of Ssc1 with the adjacent linker segment that is necessary and sufficient to bind Hep1. These findings are consistent with the study of Zhai *et al.* (2008), showing that the ATPase domain of the human mtHsp70 interacts with the human HEP protein. The ATPase construct used by Zhai *et al.* consists in fact of the ATPase domain and the interdomain linker. Since the ATPase

domain together with the interdomain linker acquires the aggregation-prone conformation within Ssc1, recognition of that aggregation-prone region by Hep1 reflects the anti-aggregation function of Hep1 on Ssc1.

Based on these findings, one can suggest a model of Hep1 action (Fig. 4.1). During the reaction cycle of the native Ssc1 in mitochondria, the ATPase domain in the context of the linker segment may adopt an aggregation-prone conformation. This conformation is likely to occur in the nucleotide-free state of Ssc1. In the absence of Hep1, the formation and accumulation of aggregation-prone conformers leads to their association in large non-functional aggregates. However, if Hep1 is present, it recognizes those conformers and binds to the ATPase domain and the linker, thus hindering aggregation and allowing Ssc1 to adopt the native conformation again.

The aggregation-prone conformer could also possibly be formed by Ssc1 during the *de novo* folding of newly synthesized Ssc1 imported into mitochondria. This possibility and therefore the involvement of Hep1 in the folding process of Ssc1 are discussed later.

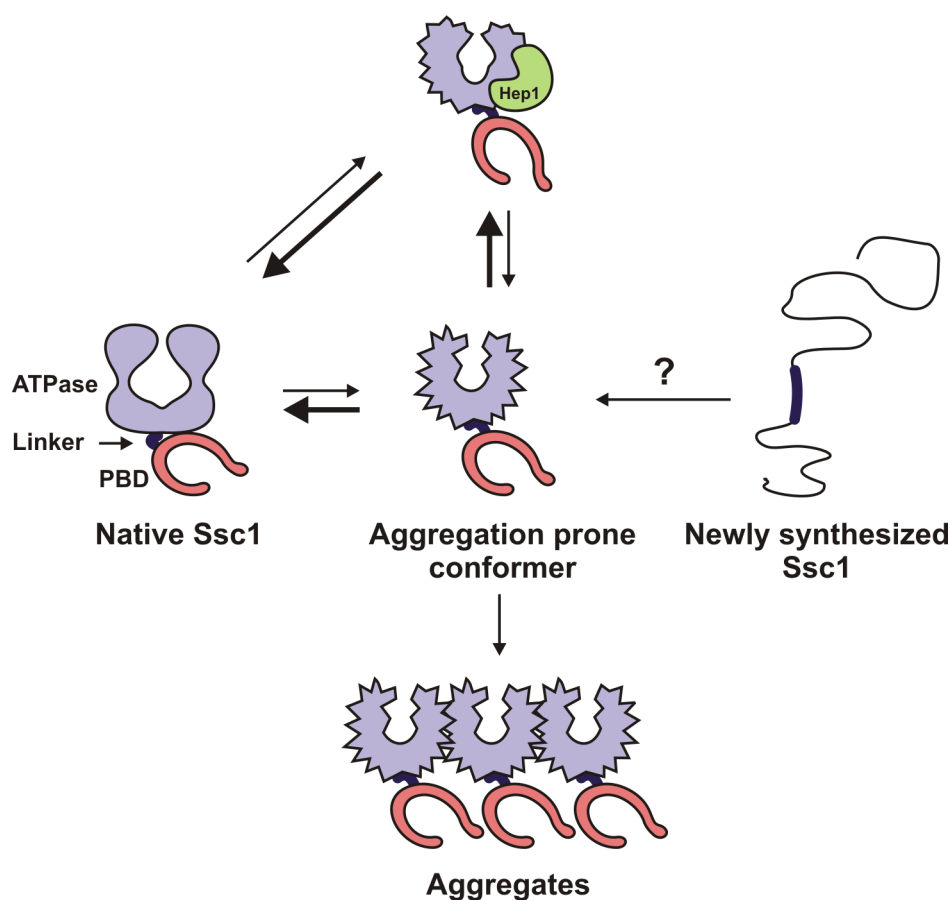


Figure 4.1. Model of anti-aggregating action of Hep1. See text for details.

The exact mechanism through which Hep1 escorts Ssc1 to the native state remains unclear. Presumably, Hep1 functions as a holdase and passively prevents Ssc1 aggregation by reducing the concentration of the free aggregation-prone species by kinetic partitioning. Alternatively, binding of Hep1 to the non-native conformers induces certain conformational changes within Ssc1 and stabilizes the native state of the chaperone. The release of Hep1 from Ssc1 may be achieved in a nucleotide-dependent manner, as it was reported that Ssc1 interacts with Hep1 upon ATP depletion (Sichting et al. 2005). Thus, binding of ATP to the ‘rescued’ Ssc1 molecule may result in dissociation of Hep1.

The formation of the aggregation-prone conformers by Ssc1 is likely not to occur too often, since the concentration of Hep1 molecules in mitochondria is much lower than the concentration of the very abundant Ssc1 chaperone (Sichting et al. 2005). Moreover, even minor amounts of Hep1 protein were sufficient to keep Ssc1 in a functional form, since no growth phenotype was observed upon down-regulation of Hep1 expression in yeast, in contrast to the *HEP1* deletion strain (unpublished results of M. Sichting, personal communication). In complete absence of Hep1, non-native aggregation-prone Ssc1 species accumulate with time.

Although the function of Hep1 in the prevention of aggregation of Ssc1 is well-documented, the mechanistic insights into this process are still missing. It would be interesting to study in detail the interaction between those two chaperones and unravel the mechanism through which this interaction is regulated under cellular conditions.

4.2. *De novo* folding of the Ssc1 chaperone

Due to their involvement in protein folding, assembly and degradation, molecular chaperones play an indispensable role in cellular proteostasis. Multiple functions of molecular chaperones in protein biogenesis have been extensively studied and characterised. In contrast, the biogenesis of chaperones has barely been described. Particularly, the folding of Hsp70 chaperones, the main protein folding helpers in the cell, has been poorly characterised to date.

The present study reports on the novel approaches to analyse the biogenesis of the yeast mitochondrial Hsp70 chaperone Ssc1 and on the exciting results obtained. First, the *de novo* folding of Ssc1 upon import into isolated mitochondria was followed and characterised. Second, the folding defect of Ssc1 in the absence of the helper protein Hep1

was described using *in organello* and *in vivo* studies. Hep1 and none of the general chaperone systems tested were found to be crucial for the correct folding of Ssc1, in particular for the ATPase domain of Ssc1. Third, the folding process of Ssc1 was reconstituted *in vitro* using purified components, and the main steps of the folding pathway of Ssc1 were dissected. Hep1 and ATP/ADP were found to be the only components, necessary and sufficient for the efficient folding of Ssc1 into the enzymatically active form.

In order to follow the folding process of Ssc1 and to distinguish between folded and unfolded Ssc1, a fast and precise assay based on trypsin resistance of the native Ssc1 was established. This assay was successfully used *in organello* and *in vivo* as well as in a modified form *in vitro*. To prove that the trypsin-resistant form of Ssc1 corresponds to the native and catalytically active conformation of the protein, the ATPase activity of the purified native and refolded Ssc1 was measured. Indeed, Ssc1 species that had refolded in a reconstituted system to the trypsin-resistant form regained their enzymatic activity, whereas the trypsin-sensitive Ssc1 species remained inactive. This implies that the trypsin-resistant Ssc1 species represent the native and folded protein, which validates the trypsin resistance-based approach used throughout the present study. The trypsin assay used *in organello* and *in vivo* allowed the identification of a severe folding defect of newly imported Ssc1 in *Δhep1* mitochondria. This finding was further corroborated in the reconstituted system, showing the crucial role of Hep1 in the folding process of Ssc1.

To date, the folding of chaperones was assumed to happen either spontaneously, or with the help of the cellular folding machinery, generally assisting in the folding of several proteins. For example, the folding of the Hsp60 chaperonin was reported to rely on native Hsp60 complexes (Cheng et al. 1990). Moreover, the bacterial Hsp70 counterpart, DnaK, could be efficiently refolded *in vitro* (Montgomery et al. 1993; Clerico et al. 2010) and its folding could be assisted by the large ribosomal RNA molecules that exhibit general folding activities (Ghosh et al. 2003; Kim et al. 2010). However, the present study describes for the first time a chaperone relying on a specialized proteinaceous factor for its folding. This specialized factor is Hep1 and it is crucial for the correct folding of the Ssc1 chaperone, both *in vivo* and *in vitro*.

A potential role of Hep1 in Ssc1 folding has previously been proposed. In addition to aggregation of native Ssc1, aggregated material could also be formed during the biogenesis of Ssc1 and a folding intermediate of Ssc1 could correspond to the aggregation-prone form of Ssc1. In such a case, the anti-aggregating function of Hep1 would be exhibited by its

assistance in the folding process of Ssc1. Previous reports support this hypothesis with some non-direct evidence. Sichting *et al.* (2005) showed that the co-expression of Ssc1 with the chaperone Hep1 in a bacterial system is essential to produce soluble and functional Ssc1. Moreover, Willmund *et al.* (2008) found that the plastidic counterpart of Hep1, HEP2, similarly rescues the cognate chaperone HSP70B from the algae *Chlamydomonas reinhardtii* upon co-expression in *E.coli*, and provided evidence for the involvement of HEP2 in the activation of HSP70B upon the cleavage of the transit peptide. These observations might reflect the assistance of Hep proteins in the *de novo* folding of cognate Hsp70 chaperones; a different mechanism, however, could not be excluded. The present work for the first time unambiguously demonstrates the key role of Hep1 in the folding of the Ssc1 chaperone, both *in vivo* and in a reconstituted system.

Using the *in organello* approach, as well as the reconstituted system, the key factors required for Ssc1 folding were identified and important mechanistic insights into this process were obtained.

In the case of multidomain proteins, single domains might first fold independently and then cooperate to adopt the overall native conformation in the final step of folding. This seems to be the case for Ssc1. The ATPase domain and the PBD acquire their native structure independently, both *in vivo* and *in vitro*. Whereas the ATPase domain requires the assistance of Hep1 and the presence of the nucleotides for folding, the PBD seems to fold spontaneously without any additional factors. These conclusions are supported by *in organello* and *in vitro* results using full-length Ssc1, as well as single domain constructs.

As shown in section 3.1.3, the interdomain linker segment is crucial for the interaction of Ssc1 with Hep1. Presumably, the ATPase domain of Ssc1 upon interaction with the linker acquires a conformation that is recognized by Hep1. At the same time, the linker seems to play an important role in the overall folding of the ATPase domain, since the DHFR fusion constructs consisting of ATPase alone or the ATPase with the mutated linker did not fold. Moreover, the ATPase domain in full-length Ssc1 with the mutated linker did not fold either. The lack of the correct interdomain linker, crucial for the interaction with Hep1, may hinder recognition of the folding intermediate of Ssc1 by Hep1. Therefore, Hep1 cannot assist in the folding process of Ssc1 and the process is stalled. It would be interesting to study the role of the interdomain linker for the folding of Ssc1 in the reconstituted system. Particularly, experiments addressing the interaction of a peptide resembling the linker peptide with Hep1 and the influence of this peptide added *in trans* on

the folding of the ATPase domain would help to understand the function of the linker for the folding process of Ssc1.

Besides Hep1, an adenine nucleotide was found to be crucial for the folding of Ssc1. Interestingly, since both ATP and ADP can be used in the folding process, it is not the hydrolysis of ATP but rather the binding of nucleotide to the ATPase domain that is required to trigger Ssc1 folding. Moreover, the nucleotide dependent step in the folding pathway of Ssc1 occurs apparently after binding of Hep1 to Ssc1. What is the function of the nucleotide in the folding process? The nucleotide binds to the Hep1-bound folding intermediate of Ssc1 and seems to be crucial for stabilizing a more compact conformation of the ATPase domain. The binding of the nucleotide to the nucleotide-binding pocket of Ssc1 stabilizes the pocket, and this presumably leads to the structural stabilization of the entire domain. Moreover, binding of the nucleotide apparently triggers dissociation of Hep1. This is consistent with the observation that Ssc1 interacts with Hep1 only upon depletion of ATP (Sichting et al. 2005). Ssc1 seems to have a distinct preference for nucleotides that promote its folding. Both ATP and ADP promote the folding of Ssc1 in the presence of Hep1, whereas an ATP analogue, AMP-PNP, despite binding to the native Ssc1, does not. The affinity of AMP-PNP to the unstructured folding intermediate of Ssc1 might be too low. It could also be that AMP-PNP does bind to the intermediate, but is not able to trigger release of Hep1, required for further folding of Ssc1. However, the second possibility is less likely, since the Ssc1-Hep1 complex is quite unstable and at least some of the Hep1 molecules would presumably spontaneously dissociate from Ssc1, allowing the formation of folded Ssc1. The third possibility is that AMP-PNP binding does not stabilize the ATPase domain similarly to ATP or ADP. Thus, Ssc1 is not able to acquire the native conformation.

The presented results allow the drawing of an initial model of the Ssc1 folding pathway (Fig. 4.2). As suggested above, both domains of Ssc1 start folding independently and simultaneously. The PBD folds on its own and rapidly acquires a trypsin-resistant, thus folded conformation. The ATPase domain together with the interdomain linker forms a folding intermediate, presumably partially folded, yet trypsin-sensitive, that cannot efficiently fold further without Hep1. This intermediate probably does not represent an off-pathway state, since Ssc1 remains folding-competent and does not aggregate upon a long incubation in higher temperatures in the absence of Hep1. Hep1 recognizes this folding intermediate and interacts with it, triggering the formation of the nucleotide binding pocket. This interaction is most probably transient, since the complex of Ssc1 intermediate

and Hep1 could not be observed without trapping it with a crosslinking reagent. In the next step, a nucleotide, ATP or ADP, binds to the folding intermediate and Hep1 is released from the complex. Binding of a nucleotide triggers further folding of the ATPase domain to the trypsin-resistant conformation. Finally, the folded ATPase domain and the PBD cooperate with each other to form the compact, native conformer of Ssc1, fully resistant against trypsin and catalytically active.

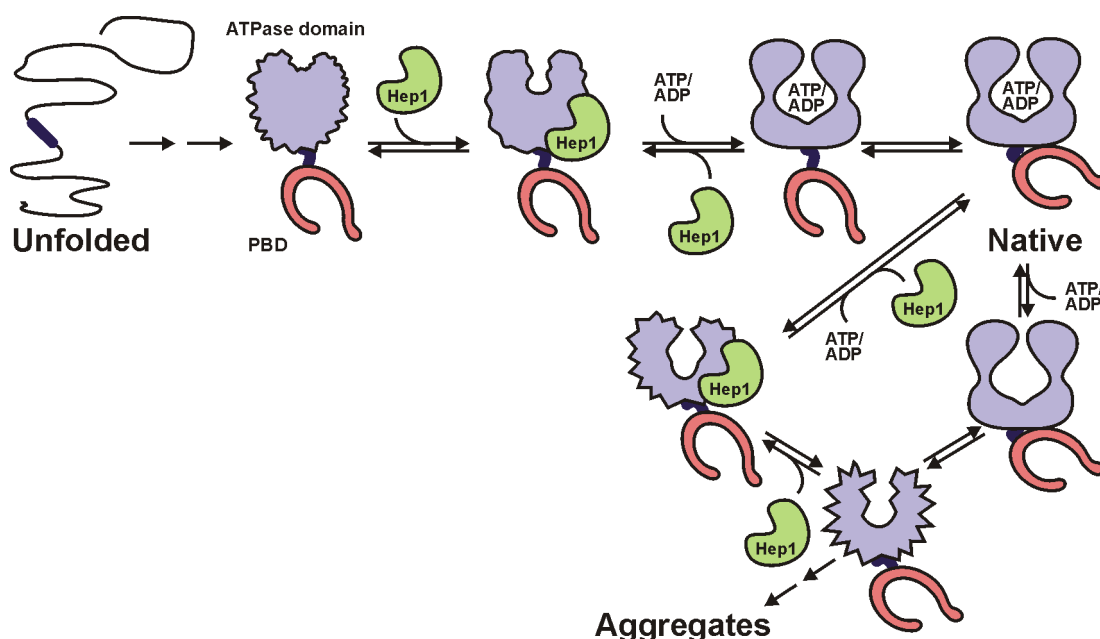


Figure 4.2. Model of the folding pathway of Ssc1.

The ATPase domain and the PBD of Ssc1 fold independently. The ATPase domain undergoes a Hep1-dependent and an ATP/ADP-dependent step in the folding process. The PBD domain folds spontaneously and, for simplicity, it is depicted in a folded state in all folding intermediates of Ssc1, although it presumably folds simultaneously with the ATPase domain. In the final folding step, both domains cooperate together to acquire the final structure of Ssc1. The presented model depicts additionally a transition of native Ssc1 to the aggregation-prone conformer and the anti-aggregation action of Hep1 presented in the previous model (see Fig. 4.1).

In the presented model, the folding of Ssc1 begins with the completely unfolded, mature protein. *In vivo*, newly synthesized Ssc1 is imported into mitochondria in an unfolded state as a pre-protein containing an N-terminal signal peptide. In the mitochondrial matrix, the newly imported Ssc1 has to be released from the native Ssc1 taking part in the translocation process, and the signal peptide has to be cleaved off. It is

not known whether those actions have been completed before Ssc1 starts folding. Moreover, one cannot exclude that the ATPase domain, which enters the mitochondria first, begins to fold while the rest of the protein is still being translocated. Definitely, *in vivo*, the folding process is very rapid, and it was not possible to separate it in time from the translocation process with the *in organello* approach. Although the situation seems to be more complex *in vivo* than in the reconstituted system, one can assume that the main steps on the folding pathway of Ssc1 *in vivo* and *in vitro* are similar, since all the results obtained *in vivo*, *in organello* and in the reconstituted system are consistent and lead to the same overall picture.

Does the involvement of Hep1 in the *de novo* folding of Ssc1 fit to the previous picture of Hep1 function in the prevention of Ssc1 aggregation? The presented model depicts additionally the formation of an aggregation-prone conformer of Ssc1 from the nucleotide-free form of Ssc1 during the ATPase cycle. Since denatured Ssc1 did not aggregate upon dilution in the refolding buffer in the absence of Hep1, it could mean that only folded Ssc1 can adopt an aggregation-prone conformation during the ATPase cycle. On the other hand, the presence of 400 mM urea in the refolding buffer upon dilution of denatured Ssc1 could prevent aggregation of unfolded Ssc1. Moreover, molecular crowding might contribute to the potential aggregation propensity of unfolded Ssc1 *in vivo*. Therefore, the transition of native Ssc1 through the nucleotide-free form to the aggregation-prone conformer, and Hep1 counteraction of aggregation is depicted in the model separately from the folding pathway of Ssc1. Whether the aggregation-prone conformer of Ssc1 is similar, at least partially, to the folding intermediate of Ssc1 and whether the Hep1 action in aggregation prevention and in the *de novo* folding of Ssc1 relies on a similar molecular mechanism, remain to be answered. In any case, the engagement of Hep1 in those two separate processes, protein folding and aggregation prevention, is a property typical of many chaperones.

Is the function in the *de novo* folding of Ssc1 specific for Hep1, or do other auxiliary interaction partners of Ssc1 also have a stabilizing effect on Ssc1? The co-chaperone Mge1, which binds to the ATPase domain of Ssc1, was reported to increase the solubility of Ssc1 upon co-expression in *E.coli*, similarly to Hep1 (Momose et al. 2007). However, our *in vitro* data show that Mge1 alone is not able to promote folding of Ssc1, since it fails to recognize and interact with a folding intermediate of Ssc1. This implies that the assistance in the *de novo* folding of Ssc1 is the primary function of Hep1, and not a

stabilizing effect that could be taken over by any interaction partner binding to the ATPase domain.

In organello and *in vitro*, Hep1 seems to be indispensable for the folding of the ATPase domain of Ssc1 at all temperatures tested. *In vivo*, however, deletion of the *HEP1* gene is lethal for yeast cells only at elevated temperatures and it seems to have a minor effect on the activity of Ssc1 at lower temperature. Therefore, it is likely that there is an additional folding helper for Ssc1 *in vivo*. In the mitochondrial matrix, several chaperone systems involved in protein folding and disaggregation have been described, including the Hsp60 chaperonin machinery, the mtHsp70 chaperone system as well as a member of the Clp/Hsp100 family, the Hsp78 chaperone (Voos 2009). Those three systems were tested for the involvement in the *de novo* folding of Ssc1 *in organello* and no significant effects of the mutation/deletion of those chaperone systems on the folding of Ssc1 were observed in the presence of Hep1. It is probable, however, that one of the aforementioned systems or/and an additional factor, yet to be identified, assists in the Ssc1 folding in the absence of Hep1 *in vivo*, and in the presence of Hep1 it plays only a minor role. One cannot exclude that a pool of newly imported Ssc1 is able to fold spontaneously. Nevertheless, due to the effect of strong molecular crowding *in vivo*, it is more likely that Ssc1, as a rather large, two-domain protein is assisted in folding by additional factors, which are working efficiently in the absence of Hep1 at lower temperatures, but are not sufficient *in organello*.

Interestingly, Hep1 homologues were identified only in mitochondria and chloroplasts of eukaryotes, and no Hep-related proteins were found in bacteria (Sichting et al. 2005; Willmund et al. 2008). Despite some evidence for additional functions of Hep1 homologues (Burri et al. 2004; Zhai et al. 2008; Goswami et al. 2010), this study shows that the assistance in the *de novo* folding of the cognate Hsp70 chaperones is the primary and major function of Hep proteins. This suggests that the folding pathway of mitochondrial and plastidic Hsp70 chaperones is more complex and their free-energy folding landscapes are much more rugged than those of the other Hsp70 family members. Thus, they may require the specialized folding helpers to overcome the energetic barriers and to rapidly reach the native state. Why do the folding pathways of mitochondrial and plastidic Hsp70 chaperones differ from the other ones? As discussed before, those chaperones, due to their involvement in the complicated processes of protein translocation in parallel to protein folding-related functions, need to acquire highly flexible and dynamic structures. Reaching these structures during *de novo* folding of those chaperones may thus

lead to the formation of kinetically trapped intermediates and only binding of the Hep-related protein catalyzes further folding into the final conformation. On the other hand, Hep dependence might be a cellular way to prevent non-physiological folding of the chaperone in the cytosol, before it is translocated into the target organelle.

The present study focused on the biogenesis of the yeast mitochondrial Hsp70 Ssc1 and the function of yeast Hep1. The human Hep1 homologue, HEP, called also DNLZ, was shown to interact with the ATPase domain in combination with the interdomain linker of the human mtHsp70, mortalin, and to increase the solubility of mortalin upon co-expression in bacteria, similarly to the yeast counterparts (Zhai et al. 2008). In contrast to Hep1, however, several studies demonstrated that human HEP stimulates the ATPase activity of mortalin (Zhai et al. 2008; Goswami et al. 2010; Zhai et al. 2011; Vu et al. 2012). Moreover, HEP was shown to have an anti-aggregation effect on the model substrate rhodanese *in vitro* (Goswami et al. 2010). Since the human HEP could partially complement the deletion of Hep1 in yeast (Vu et al. 2012), one could expect that its primary function is conserved. It would be, therefore, interesting to study the role of human HEP for the *de novo* folding of mortalin as well as to investigate in detail the new functions that the human HEP seemed to have acquired during the evolution. Furthermore, accumulating evidence points towards the involvement of mortalin in the regulation of cellular senescence and immortalization. This links mortalin to carcinogenesis and aging processes. Mortalin was shown to be highly upregulated in many tumor tissues including breast, brain and colon carcinoma and has already been suggested to be a biomarker for the prediction of recurrence in hepatocellular carcinoma (Wadhwa et al. 2006; Yi et al. 2008; Chen et al. 2011). Moreover, it was reported to be involved in the pathogenesis of Parkinson's disease (Burbulla et al. 2010). Multiple functions of mortalin and their complicated regulation make the human mtHsp70 system more complex than the yeast one. It would be, therefore, highly exciting to study the biogenesis of mortalin, using the well-defined tools developed for the study of the yeast Ssc1 chaperone.

5. Summary

Molecular chaperones of the Hsp70 class are essential for a number of cellular processes. The yeast mitochondrial Hsp70 chaperone Ssc1 plays an indispensable role for the mitochondrial biogenesis. As an essential component of the import motor of the TIM23 translocase, Ssc1 drives the ATP-dependent translocation of proteins into the mitochondrial matrix. Moreover, it mediates the *de novo* folding and the assembly of several proteins in the mitochondrial matrix and prevents the formation of protein aggregates. Surprisingly, Ssc1 itself has a propensity to self-aggregate. Thus, it requires a helper protein, the chaperone Hep1 that prevents Ssc1 aggregation and maintains its structure and function. The mechanism of the protective function of Hep1 on Ssc1, however, is not understood.

In the present study, the structural determinants of Ssc1 that make it prone to aggregation and the structural requirements of Ssc1 for its interaction with Hep1 were analysed and provided insights into the mechanism of prevention of Ssc1 aggregation by Hep1. The aggregation studies demonstrate that a variant of Ssc1 consisting of the ATPase domain and the subsequent interdomain linker aggregates in absence of Hep1. In contrast, the PBD and the ATPase domain alone are not prone to aggregation. Moreover, the interaction studies reveal that the aggregation-prone region seems to be the smallest entity within Ssc1 required for the interaction with Hep1. Taken together, the native Ssc1 adopts an aggregation-prone conformation, in which the ATPase domain with the interdomain linker has the propensity to aggregate. Hep1 binds to this aggregation-prone region and thereby counteracts the aggregation process and keeps the native Ssc1 in a functional and active state.

Although Hsp70 chaperones are important for the biogenesis of a multitude of proteins, little is known about the biogenesis of these chaperones themselves. The present study reports on the analysis of the folding process of the mitochondrial Hsp70 chaperone Ssc1. *In organello*, *in vivo* and *in vitro* assays were established and then employed to study the *de novo* folding of Ssc1. Upon import into mitochondria, Ssc1 folds rapidly with the ATPase domain and the PBD adopting their structures independently of each other. Notably, the ATPase domain requires the presence of the interdomain linker for its folding, whereas the PBD folds without the linker. Moreover, in the absence of Hep1, the ATPase domain with the interdomain linker displays a severe folding defect, which indicates a role

of Hep1 in the folding process of Ssc1. Apart from Hep1, none of the general mitochondrial chaperone systems seem to be important for the folding of Ssc1. Furthermore, the folding process of Ssc1 was reconstituted *in vitro* and the main steps of the folding pathway of Ssc1 were characterised. Hep1 and ATP/ADP are required and sufficient for the folding of Ssc1 into the native, catalytically active form. In an early step of folding, Hep1 interacts with the folding intermediate of Ssc1. This interaction induces conformational changes which allow binding of ATP/ADP. The binding of a nucleotide triggers Hep1 release and further folding of the intermediate into a native Ssc1.

The present study provides the first direct evidence for the requirement of Hep1 for the folding of the Ssc1 chaperone. Thus, it demonstrates for the first time that the *de novo* folding of an Hsp70 chaperone depends on a specialized proteinaceous factor.

In conclusion, Hep1 fulfils a dual chaperone function in the cell. It mediates the *de novo* folding of Ssc1 and maintains folded Ssc1 in a functional state during the ATPase cycle. Therefore, the Hep1 chaperone plays a crucial role for the protein biogenesis and homeostasis in mitochondria.

6. Literature

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7. Abbreviations

AAA	ATPase Associated with various cellular Activities
ADP	adenosine diphosphate
Amp	ampicillin
AMP-PNP	adenylyl imidodiphosphate
APS	ammonium persulfate
Arg	arginine
Asp	aspartic acid
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
ATP γ S	Adenosine-5'-(γ -thio)-triphosphate
BSA	Bovine Serum Albumin
C-terminus	carboxy-terminus
CCT	chaperonin containing TCP-1
cDNA	complementary DNA
CNBr	cyanogen bromide
CTP	cytidine triphosphate
cyt	cytochrome
DHFR	dihydrofolate reductase
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DSG	disuccinimidyl glutarate
DTT	dithiotreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylendiamine tetraacetate
ER	endoplasmic reticulum
FT	flow-through
g	acceleration of gravity
GTP	guanosine triphosphate
h	hour
HCl	hydrochloric acid
HEPES	N-2 hydroxyl piperazine-N'-2-ethane sulphonic acid
His	histidine
His-tag	hexahistidine tag
HRP	horseradish peroxidase
Hsc	heat shock cognate
Hsp	heat shock protein

IgG	immunoglobulin G
IM	inner membrane
IMS	intermembrane space
IP	immunoprecipitation
IPTG	isopropyl- β ,D-thiogalactopyranoside
kb	kilobase
kDa	kiloDalton
kV	kilovolt
LB	Luria Bertani
MDa	megaDalton
Mdj	mitochondrial DnaJ
Mia	Mitochondrial intermembrane space Import and Assembly
MOPS	N-morpholinopropane sulphonic acid
mRNA	messenger ribonucleic acid
mt	mitochondrial
MTS	matrix targeting signal
N-terminus	amino-terminus
NAC	Nascent chain-Associated Complex
NADH	nicotine amide adenine dinucleotide
NEF	nucleotide exchange factor
NiNTA	nickel-nitrilo triacetic acid
NMR	nuclear magnetic resonance
OD ₆₀₀	optical density at 600 nm
OM	outer membrane
O/N	overnight
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBD	peptide binding domain
PCR	polymerase chain reaction
PEG	polyethylene glycol
PI	pre-immune serum
PK	proteinase K
PMSF	phenylmethylsulfonyl fluoride
RAC	Ribosome-Associated Complex
RNA	ribonucleic acid
RNasin	ribonuclease inhibitor
rpm	rounds per minute
RT	room temperature
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SAM	Sorting and Assembly Machinery
SD	selective dextrose
SDS	sodium dodecyl sulfate

SH	sorbitol, HEPES
SOC	Super Optimal broth with Catabolite repression
STI	soybean trypsin inhibitor
Su9	subunit 9
TAE	TRIS, acetic acid, EDTA
TBS	TRIS buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylene diamine
TIM	translocase of inner mitochondrial membrane
TOB	Topogenesis of mitochondrial Outer-membrane β -Barrel proteins
TOM	translocase of outer mitochondrial membrane
TRiC	TCP-1 Ring Complex
TRIS	tris-(hydroxymethyl)-aminomethane
U	unit
UTP	uridine triphosphate
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
WT	wild-type
YPD	yeast extract, peptone, dextrose
YPG	yeast extract, peptone, glycerol
YPGal	yeast extract, peptone, galactose
$\Delta\Psi$	membrane potential

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