Chapter 2
Mortalin's Machinery

Custer C. Deocaris, Sunil C. Kaul and Renu Wadhwa

Abstract Mortalin/mtHsp70 performs a wide array of cellular functions and has been implicated in aging, cancer and neurodegenerative diseases. Similar to other Hsp70s, its ability to chaperone misfolded proteins and bind to a myriad of clients is derived from its N-terminal nucleotide-binding domain (NBD) regulating substrate affinity of the C-terminal substrate-binding domain (SBD) in a nucleotide- and co-chaperone-dependent mechanism. To understand the structural dynamics of its allostery making this relevant to mortalin's cellular function, this chapter describes key structural features of these two domains as well as provide an appreciation as to possibly how a single amino acid change, Gly to Arg in the SBD that can be viewed so minor, is able to metamorphose from a life-extending species of mortalin (mot-2) into one that induces senescence and even inhibits tumor growth (mot-1).

Keywords Mortalin · Chaperone · Structure · Binding domains · Functions

2.1 Introduction

Having around 40–60% identity between its eukaryotic and prokaryotic members, the Hsp70 superfamily comprises one of the most highly conserved proteins in biology (Hunt and Morimoto 1985; Karlin and Brocchieri 1998). These chaperones are also strictly required for cellular viability in every organism. Despite their diversity, it has been observed that each type does not have overlapping or redundant functions. In yeast, among its highly identical Hsp70s (amino acid identity: 60–80%), none of the members could be functionally interchanged for another.

Within the mitochondrion, a key component of the organellar import machinery and protein quality control is mortalin (mtHsp70). With a molecular weight of 73,913 Da, this mitochondrial chaperone is composed of 679 amino acids and shares

C. C. Deocaris (✉)
Department of Food Science and Technology, College of Home Economics, University of the Philippines, Diliman, Quezon City, Philippines
e-mail: cdeocaris@gmail.com

S. C. Kaul · R. Wadhwa
National Institute of Advanced Industrial Science and Technology (AIST), Central 4, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8562, Japan

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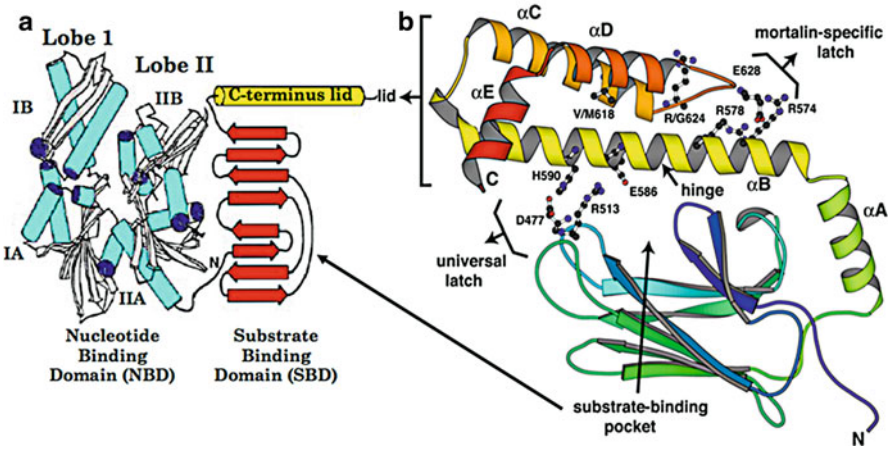


Fig. 2.1 Ribbon diagrams of mortalin domains. **a** N-terminal Nucleotide Binding Domain (NBD) shows the 2 sub-domains: IA and IB (Lobe 1), and IIA, and IIB (Lobe 2). The folding of these lobes, makes up for a deep pocket that binds to ATP (Deocaris et al. 2007b). Substrate-binding domain (SBD) is shown here as two sets of four-stranded anti-parallel β-sheets (*red*) resembling a twisted sandwich that forms the substrate-binding site. **b** The "substrate lid" is composed of alpha-helices A–C. Electrostatic interactions among Arg574, Arg578, and Asp628 constitute the "mortalin-specific latch", and among Asp477, Arg513, Glu586, and His590 that forms the "common latch" which has been found to be evolutionarily-conserved among all Hsp70s. (Modified from Deocaris et al. 2006a)

high degree of identity with other Hsp70s, including *Escherichia coli* DnaK (51%), *Saccharomyces cerevisiae* SSC1p (65%), the constitutive cytosolic Hsp70 from rat, Hsc70 (46%), and the rat endoplasmic reticulum isoform, BiP (49%) (Webster et al. 1994). The molecular structure of mortalin/mtHsp70 has not yet been elucidated, as previous attempts to crystallize it had been unsuccessful. Given the strict evolutionary conservation among Hsp70s, the "mortalin machinery" can readily be modeled after the well-known allosteric dynamics of two canonical inter-connected modules: the N-terminal Nucleotide Binding Domain (also called the ATPase domain) and C-terminal substrate-binding domain (SBD) (Fig. 2.1a, b). The interaction of a substrate with the SBD, the affinity and kinetics of substrate binding are being controlled by the activity of NBD. Exchange of ADP for ATP in the NBD causes significant local conformational changes that ultimately lead to the opening of the lid sub-domain. This action is concomitant to the decrease in binding affinity for substrates. Upon ATP hydrolysis, the protein is returned to the ADP-bound configuration ready for another round of substrate binding and release. With the interplay between these domains which constitute a basis for the chaperoning activity of mortalin, we offer a hypothesis on how its natural allelic forms in mouse may possess contradictory biological functions: one which is associated with aging (mortalin 1, or mot-1) and the other which confers lifespan-extension (mortalin 2, or mot-2) (Deocaris et al. 2006b).

2.2 N-terminal Nucleotide Binding Domain (NBD)

The NBD is the chaperone's control unit. To appreciate the function of this domain, we made a model through a homology-based method using the crystal structure of ATP-bound human Hsp70 (1S3X) (Deocaris et al. 2007b) as this region contains a high degree of sequential (>80%) and structural conservation (Mayer and Bukau 1998). Mortalin's NBD is similar to the ATPases of several other functionally diverse proteins such actin, hexokinase, etc. (Bork et al. 1992).

As shown in Fig. 2.1a, the 44-kDa structure folds into a pair of lobes each with 2 sub-domains: subdomains IA and IB (in lobe I), and, IIA and IIB (in lobe II). The folding of these lobes, stabilized by metal-binding, creates a deep pocket for the catalysis of ATP (Sriram et al. 1997). As ATP lodges into this central pocket, particularly at the interface between sub-domains IIA and IIB, the geometric and energetic effects of its binding and hydrolysis is rapidly communicated throughout the entire NBD (Smock et al. 2010). NMR readout of *E. coli* DnaK showed that in the ADP-bound state, NDB and SBD are both dissociated and largely independent (Bertelsen et al. 2009). In contrast, upon ATP binding, conformational rearrangement in the NBD weakens binding of substrates within the C-terminal SBD and promotes release of the substrate. Of note, the inter-domain linker connecting the NBD and SBD is highly conserved underscoring the importance of this short segment in transmitting the allosteric changes (Bertelsen et al. 2009; Bhattacharya et al. 2009; Mayer 2011; Mayer and Bukau 2005).

The turnover rate of Hsp70 ATPase is rather slow (0.02–0.2 min^{-1}) and is insufficient to assist chaperoning during cellular stress. Therefore, it is essential that co-factors modulate mortalin's ATPase to fine-tune the regulation between its ATP- and ADP-bound states (Ha and McKay 1994, 1995). It has been established that the precise functioning of the NBD critically involves its interaction with two co-chaperone families: the J-domain proteins that catalyze ATP hydrolysis (Craig et al. 2006) and the nucleotide exchange factors (NEFs) that help in the replacement of ADP with ATP (Kabani 2009; Xu et al. 2008). Studies on *E. coli* Hsp70, DnaK, first suggested that the ATPase activity of the protein could be cyclically stimulated by co-chaperones DnaJ and GrpE. DnaJ permits the hydrolysis of Hsp70-bound ATP allowing the ADP-bound Hsp70 to interact more strongly with unfolded proteins. The nucleotide exchange factor GrpE enables the recycling of Hsp70 back intoan ATP-bound state permitting the efficient release of its substrate. Multiple GrpE-like proteins and a unique human GrpE homologue, HMGE, have been reported to be restricted to the mitochondria, and form chaperone-pairing with mortalin/mtHsp70 (Deocaris et al. 2006a). Catalysis of ADP-ATP exchange, and hydrolysis by J-domain proteins and NEFs, i.e. the GrpEs, has to be balanced to facilitate equilibrium between substrate binding and release.

a) *J-domain proteins.* DnaJ proteins are 80-amino acid small proteins containing the N-terminal J domain essential for stimulating ATPase activity. Transport of preproteins into the mitochondrial matrix is mediated by the presequence translocase–associated motor (PAM) that has four essential subunits: (1) mortalin,

(2) the peripheral membrane protein, Tim44, (3) the nucleotide exchange factor, Mge1 and (4) J-domain protein PAM18 that stimulates the ATPase activity of mortalin. PAM18 is required for the interaction of mortalin with Tim44 and protein translocation into the matrix, see review by Deocaris et al. (2006a). The function of other co-chaperones, such as mtDnaJ (Zhao et al. 2002), dj2 and dj3 in regulating mortalin is still obscure although it is interesting to note that these cytosolic DnaJs are also translocated in the mitochondria (Goswami et al. 2010; Terada et al. 1997; Terada and Mori 2000). Another co-chaperone that stimulates ATPase activity of mortalin is the Hsp70 escort protein (Hep). Previously, it has been shown that Hep is critical to the solubility of mortalin and inhibits self-association of the chaperone (Zhai et al. 2008). The binding sites of Mge1 and Tim44 to mortalin have been assigned at the variable region along C-terminal alpha-helical lid (Strub et al. 2003).

b) *NEFs.* Nucleotide exchange factor Mge1 and multiple GrpE-like proteins are restricted to the mitochondria and form chaperone pairing with mortalin (Choglay et al. 2001; Naylor et al. 1998). Association of GrpE with ADP-bound Hsp70 hastens nucleotide exchange by as much as 5000-fold and reduces affinity for ADP by 200-fold (Packschies et al. 1997). Essential during the translocation of precursor proteins into the mitochondrion, Mge1 has been demonstrated to enhance the otherwise low intrinsic ATPase activity of mortalin *via* the release of ADP and Pi (Dekker and Pfanner 1997). This results in the dissociation of Tim44-mortalin complex and the enhanced binding of mortalin to the transiting pre-protein (Schneider et al. 1996). Subdomain IA, which contains both N- and C-terminus and links to the remaining part of mortalin, is a target of the J-domain proteins (Jiang et al. 2007; Xu et al. 2008). This subdomain is highly conserved but more rigid than the other subdomains (Flaherty et al. 1990). NEFs target, in most cases, subdomain IIB. Subdomain IIB is characterized by its high mobility, especially at the β-sheet E and the exposed loop connecting the two strands of this sheet. Upon binding by GrpE, there is suppression in its mobility.

MKT-077, mitochondriotopic drug that binds to and abrogates mortalin function, has been deduced to target the alpha-helix within sub-domain IIB of the catalytic cleft based on immunoaffinity studies with truncated versions of mortalin (Wadhwa et al. 2000). Using various spectrophotometric tools to analyze protein structure, i.e. circular dichroism polarimetry, ANS and tryptophan fluorescence, we have deduced that binding of MKT-077 induces not just (local) conformational changes, but also global tertiary conformational shifts in mortalin. As reported, the presence of MKT-077 ablated mortalin chaperone function based on *in vitro* (insulin aggregation assay) and *in vivo* (luciferase test) assays (Deocaris et al. 2007b).

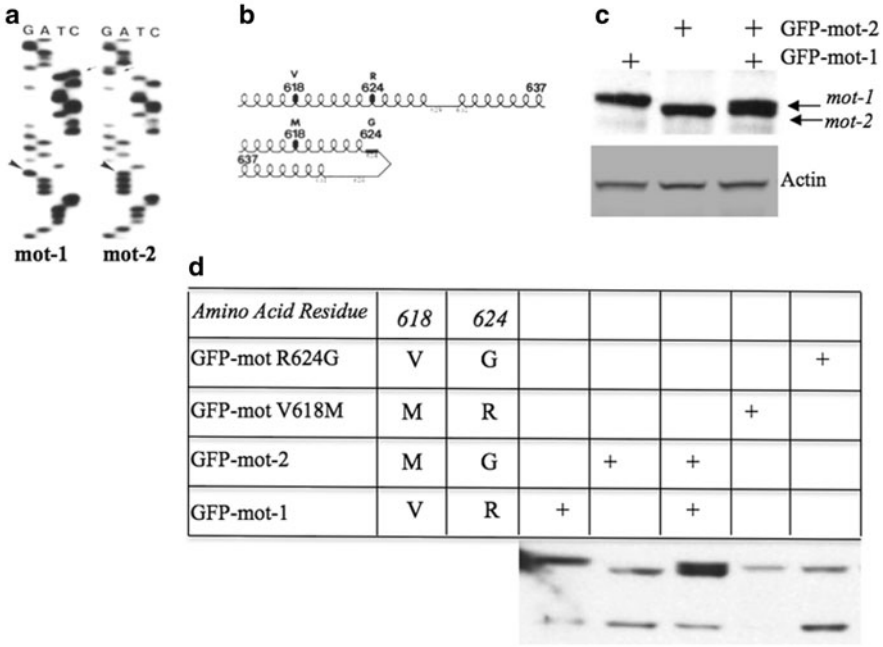
2.3 C-terminal Substrate Binding Domain (SBD)

There is a wider sequence variation in the SBD, compared to the ATPase domain signifying the diversification of Hsp70 substrates and clients (Rudiger et al. 1997, 2000). Comprising of five distinct helical domains, mortalin's SBD is 18-kDa in weight.

The SBD is composed of two sets of four-stranded anti-parallel β-sheets forming a twisted sandwich. The most predominant feature of SBD is its "substrate lid" that "swallows and seals" substrates during an ADP-bound state (Zhu et al. 1996). The 10-kDa substrate "lid" is composed of a helix kinked in the middle and bent upwards. This is a common structural feature among Hsp70s allowing it to flip-flop with the substrates ingress and egress (Fig. 2.1b). A consensus motif recognized by DnaK has been identified by screening a phage-display library. Consistent with the observed features of signal sequences of proteins destined to the mitochondrial compartment, Gragerov and Gottesman (1994) found that peptides containing internal alipathic residues, such as NRLLTG, and those that contain basic (not acidic) residues favor binding to this prokaryotic Hsp70. Additionally, these sequences are found buried deep in the α-strands of folded peptides and are generally exposed only when misfolded. Based on various protein folding algorithms employed, the distal end of the predominantly alpha-helical lid that is followed by an aperiodic glycine/proline rich region may adopt disorder in conformation. While the function of this variable C-terminal domain remains unclear, a disordered secondary structural state may permit the binding to co-factors specific to a type of Hsp70 to expand its bioactivity (Horton et al. 2001; Stahl et al. 1999) and also elicit immunomodulatory functions (Deocaris et al. 2005; Pockley et al. 2008).

2.4 Mortalin-1 *Versus* Mortalin-2: A Case of a Substrate Lid "Overbite"

Discussion on the structure-function relationship of mortalin is not complete without mentioning the differences between the 2 species of mortalin in mice: the senescence-inducing mouse mot-1, an allelic variant that differs by only two amino acids in the SBD from mot-2, the functional equivalent of human mortalin (Kaul et al. 1998, 2003, 2005, 2007). Structurally, the two proteins and their single amino acid mutants displayed different mobilities on SDS-polyacrylamide gel presenting an evidence for their different secondary structures (Fig. 2.2a–d). Based on the interaction, immunohistochemical and reporter assays, it was deduced that only mot-2 interacts with and inactivates p53 function (Kaul et al. 2000). However, primary structural difference between mot-1 and mot-2 at amino acid residues 618 or 624 did not concern the interaction of mortalin with p53. Later, it was determined that the interaction of mortalin and p53 involved N-terminal region of the mortalin protein (Kaul et al. 2001). However, interestingly, when assessed for chaperoning function of different mortalin constructs, we found that mot-2 served as a better chaperone compared to mot-1 (Deocaris et al. 2008; unpublished). In addition to the roles of ADP-ATP exchange in propagating allosteric changes in the SBD, another major feature is the presence of "locking" mechanisms that help facilitate the entry/exit of protein substrates. These so-called electrostatic "latches" between the lid and substrate-binding region are important in holding protein clients clasped into the SBD. Previous studies identified evolutionary conserved "latches" (common to all Hsp70s) consisting of



Amino Acid Residue	*618*	*624*					
GFP-mot R624G	V	G					+
GFP-mot V618M	M	R				+	
GFP-mot-2	M	G		+	+		
GFP-mot-1	V	R	+		+		

Fig. 2.2 Structural difference between mouse mot-1 and mot-2. Nucleotide changes G to A and C to G **a** resulting in the amino acid changes V618 M and R624G **b** are shown. Mobility of GFP tagged mot-1 and mot-2 proteins **c** and of the single amino acid mutants **d** are shown Order of mobility (slow to fast) was: mot-1 (V618, R624), mutant V618 M (M618, R624), mutant R624G (V618, G624) and mot-2 (M618, G624). (Modified from Wadhwa et al. 1993b; Kaul et al. 2000)

Asp477, Arg513, Glu586 and His590 (Mayer et al. 2000). In our homology work, we discovered an additional "latch" on the opposite end, consisting of Arg574, Arg578 and Glu628 that are mortalin-specific (see Fig. 2.1b). A replacement of Gly624 (in mot-2), located at the C-terminus of α-helix C, by Arg (in mot-1) is likely to extend the α-helix C. In contrast, Gly, a strong helix breaker, shortens the L3 (C-D) loop (Fig. 2.1b). The latter should perturb the structure of the "mortalin-specific latch", presumably pulling apart the electrostatic attraction and in effect weakening the attractive forces as residues fall beyond the scope of the electrostatic field, a case of a chaperone "overbite". From these, we view that the single amino acid change could therefore influence acquisition of substrates consistent with the ageing-inducing property of murine mot-1 (Nakabayashi et al. 1997; Wadhwa et al. 1993b) vis-à-vis the known life-span extending and immortalization features of mouse and human mot-2 chaperones (Kaul et al. 1998, 2003; Yokoyama et al. 2002). Using mot-1 and mot-2 recombinant proteins and the antibodies that react specifically to either of them, we isolated mot-1 and mot-2 binding proteins from human cell lysates. Analysis of these complexes by 2-D gel electrophoresis and mass spectrometry has revealed some proteins that bind preferentially either to mot-1 or to mot-2 (Wadhwa

et al.; unpublished). The unique biology of the different mortalin species demonstrates the flexibility of cellular function vis-à-vis the minor structural changes in the protein. In the various systems of natural aging and escaped aging (with immortal and tumor cells), this phenomenon can also be demonstrated. As an example, when mortalin becomes oxidized, it may be transformed into a "sick chaperone" that can even induce the building up a catastrophe of misfolded proteins. Our data indicated that H_2O_2-oxidized mortalin, more than just remaining benign and non-functional, promotes aggregation of misfolded proteins at concentrations in excess relative to mortalin. Consistent to this, the oxidized form of mortalin has been reported abundant in tissues from Alzhemier's and Parkinson's Diseases (Choi et al. 2004).

The basic paradigm of molecular chaperones is that they recognize and selectively bind non-native, but not native proteins (or even themselves) to form relatively stable complexes. It is interesting to note that the trajectory of our understanding on the cellular roles of mortalin is quite unlike the way other Hsp70s have gone (Kaul et al. 2007). Other chaperones was seen as only helpers in maintaining proteome integrity, however, mortalin was initially discovered as a "mortality" associated gene (Wadhwa et al. 1993a), a stress-associated protein targeted by toxicants (Bruschi et al. 1993), then as an antigen-processing protein (Domanico et al. 1993) and finally as the main motor component for mitochondrial biogenesis (Webster et al. 1994). Unexpectedly, mortalin has been shown participate in regulating the dynamic organization of the cytoskeleton (Cicchillitti et al. 2009) and in cellular uptake mechanisms via the lipid rafts (Wittrup et al. 2010). It, thus, appears that chaperonization may be just one mechanism on how this molecule executes its diverse functions within the mitochondria and beyond (Gupta et al. 2008). In addition to several specialized review articles on the structure-function relationship of chaperones (Baker et al. 2007; Genevaux et al. 2007; Hohfeld et al. 2001; Kim et al. 2006; Liberek et al. 2008; Mogk and Bukau 2004; Mayer and Bukau 2005; Saibil 2008; Slepenkov and Witt 2002; Tomkiewicz et al. 2007; Young et al. 2003), there have been few recent reviews published specifically on mortalin (Deocaris et al. 2006a, 2007a, 2009; Kaul et al. 2007). The present review provides just a basic appreciation of the pro-aging or anti-aging properties vis-à-vis to mortalin's amazing molecular architecture.

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