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Kinetic and Structural Characterization of Human Mortalin

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Abstract

Human mortalin is an Hsp70 chaperone that has been implicated in cancer, Alzheimer's and Parkinson's disease, and involvement has been suggested in cellular iron-sulfur cluster biosynthesis. However, study of this important human chaperone has been hampered by a lack of active material sufficient for biochemical characterization. Herein, we report the successful purification and characterization of recombinant human mortalin in *Escherichia coli*. The recombinant protein was expressed in the form of inclusion bodies and purified by Ni-NTA affinity chromatography. The subsequently refolded protein was confirmed to be active by its ATPase activity, a characteristic blue-shift in the fluorescence emission maximum following the addition of ATP, and its ability to bind to a likely physiological substrate. Single turnover kinetic experiments of mortalin were performed and compared with another Hsp70 chaperone, *Thermotoga maritima* DnaK; with each exhibiting slow ATP turnover rates. Secondary structures for both chaperones were similar by circular dichroism criteria. This work describes an approach to functional expression of human mortalin that provides sufficient material for detailed structure-function studies of this important Hsp70 chaperone.

Keywords

mortalin; Hsp70; chaperone; iron-sulfur cluster biosynthesis

Introduction

Human mortalin (also termed PBP74, mtHSP70 and GRP75) is a member of the Hsp70 family of chaperones, and has been implicated with several major human diseases: including cancer, Alzheimer's and Parkinson's disease [1,2]. Mortalin is overexpressed in several cancer cell lines [3–6]; although in Parkinson's disease the protein is downregulated [7,8]. The direct link between mortalin and these disease states remains uncertain, however, it has been identified as a potential therapeutic target or biomarker. Mortalin resides in multiple cellular locations that include the endoplasmic reticulum, cytoplasmic vesicles, and the cytosol, but primarily is found in mitochondria. Moreover, mortalin has been associated with multiple functions, including intracellular trafficking, antigen processing, regulation of cell proliferation, aging,

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differentiation and tumorigenesis [1,2,9], while also interacting with many other partner proteins, including Tim44 in the mitochondrial protein import machinery [10,11].

As with all Hsp70 family chaperones, mortalin is composed of two domains: an N-terminal nucleotide binding domain (NBD) and C-terminal substrate binding domain (SBD). The NBD sequence is highly conserved in all Hsp70 family members. By contrast the SBD demonstrates significant variation among members, while substrate specificity is also found to vary. It is believed that for all Hsp70 chaperones, these two domains can interact with each other in an allosteric regulatory fashion [12,13]. Nucleotide (ATP) binding to the NBD can induce a conformational change in the SBD that results in further release of the substrate. Conversely, substrate binding to the SBD can promote ATP hydrolysis in the NBD [14-17]. Structural details of the mechanism of allosteric regulation are unclear, although several important residues involved in allosteric regulation have recently been identified [18-20]. Studies of interdomain communication have been hampered by the lack of structural information on intact Hsp70 chaperones; however, the recently resolved crystal structures for truncated bovine Hsc70 and *Geobacillus kaustophilus* DnaK as well as the NMR structure for the full-length *E. coli* DnaK, obtained as a co-complex with both its substrate and ADP, have provided useful information on possible structural mechanisms [21-23].

Human mortalin has also been suggested to play a role in iron-sulfur (Fe-S) cluster biosynthesis [24]. In yeast, an Hsp70 family member, Ssq1, was found to interact with the Fe-S cluster scaffold protein ISU1, while certain mutations in Ssq1 were found to result in the accumulation of mitochondrial iron [25,26]. Interestingly, in yeast *Saccharomyces cerevisiae*, there exist three subsets of mitochondrial Hsp70 chaperone, Ssq1, Ssc1 and Ecm10; while most eukaryotic systems have only one subset, namely Ssc1. The yeast homologue of mortalin, Ssc1, was found to play the main role in the mitochondrial protein import machinery, although it has the ability to functionally substitute for Ssq1 in *S. cerevisiae* when it is present in 2,000-fold excess over Ssq1 [27,28].

Despite the importance of human mortalin in cellular chemistry, biochemical studies of this protein are scarce and are mostly limited to immunodetection [8,29-31]. The lack of sufficient quantities of full-length protein has been a major stumbling block to progress. Herein we report the overexpression of human mortalin in *E. coli*, and the isolation of active protein in sufficient yield for detailed biochemical and spectroscopic characterization. Single turnover kinetic and structural characteristics of mortalin are also compared with another Hsp70 protein, *Thermotoga maritima* DnaK (*Tm* DnaK).

The fact that no Ssq1 homolog exists in human cells suggests mortalin to be a strong candidate for involvement as an Hsp70 chaperone in Fe-S cluster biosynthesis, and it is shown that the refolded active human mortalin can interact with the proposed binding motif of human ISU1, LPPVK [24]. These facts suggest that mortalin, which displays broad substrate selectivity, is a multi-functional protein that serves as a chaperone in human cellular Fe-S cluster biosynthesis, in addition to other roles that are not normally demonstrated by the yeast Ssq1 protein.

Materials and Methods

General materials

Unless otherwise specified, all chemical reagents were purchased from Sigma. The plasmid DNA purification kit and Ni²⁺-NTA poly-His tag purification resin were purchased from Qiagen. *E. coli* strain DH5 α was used as a carrier for the plasmid constructs and was purchased from Invitrogen. *E. coli* strain BL21-CodonPlus(DE3)-RIL was used for expression of plasmid constructs and was purchased from Stratagene. Primers were synthesized by Integrated DNA

Technologies. DNA sequencing was performed by Genewiz. The vector pET-28b(+) was purchased from Novagen. Restriction enzymes were purchased from New England BioLabs. Talon poly-His tag purification resin was purchased from Clontech. The fluorescein-labeled peptide, LSLPPVKLHK-fluorescein was synthesized by Genemed Synthesis, Inc.

Construction of plasmids

Recombinant *Tm* DnaK (NCBI reference sequence, protein accession number: **NP_228184.1**) with an N-terminal His₆-tag was prepared as previously described [32]. Human mortalin (GenBank nucleotide accession number: **L15189**), lacking the probable mitochondrial targeting sequence (residues 1-51), was amplified by the polymerase chain reaction (PCR) from the Marathon-Ready™ Human Heart cDNA library (Clontech, CA). PCR amplification of the construct was achieved by use of the following primers: 5'-GGA AGG CCA TAT GAT CAA GGG AGC AGT TGT TGG TAT TGA TTT-3' as the forward primer, and 5'-ACC GCT CGA GTG TCC TTC TGG CTT CAA AAT TTC TGC TA-3' as the reverse primer. The underlined motifs correspond to *Nde*I and *Xho*I restriction sites, respectively. The amplified human mortalin construct was digested with restriction enzymes and ligated to the double-digested vector pET-28b(+) with T4 DNA ligase, which introduces an additional His₆-tag at the N-terminus of the construct. The resulting plasmid was sequenced to confirm its integrity and then transformed into *E. coli* DH5α competent cells. To express human mortalin, the resulting construct was transformed into *E. coli* BL21-CodonPlus(DE3)-RIL competent cells.

Overexpression of proteins

E. coli strain BL21-CodonPlus(DE3)-RIL was transformed either with *Tm* DnaK miniprep DNA, or with human mortalin miniprep DNA. The strain was grown overnight at 37 °C with aeration in Luria-Bertani (LB) medium containing 50 mg/ml kanamycin (about 15 h). The overnight culture was then diluted 100-fold into fresh LB medium with kanamycin and grown at 37 °C until an A₆₀₀ ~ 0.6 was obtained. Protein expression was then induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside, and the strain was grown for 4 h at 30 °C following induction. Cells were harvested by centrifugation at 2,600×g for 15 min at 4 °C. The resulting cell pellet was stored at -80 °C if not used immediately.

Purification of proteins

The purification of N-His₆-*Tm* DnaK (termed *Tm* DnaK in the text that follows) was described previously [32]. In brief, the cell pellet was resuspended homogeneously in 8 volumes of lysis buffer, 40 mM Tris-HCl, and 500 mM NaCl, pH 8.0, supplemented with 75 ng/mL lysozyme and 1 mM phenylmethanesulfonylfluoride (PMSF). To remove DNA from the protein sample, 10 mg/ml protamine sulfate was added to the resuspended cell solution, which was placed on ice and then disrupted by sonication (10 s pulse every 2 min for 30 min), and the lysate then centrifuged at 27,000×g for 30 min. After centrifugation, the supernatant was incubated at 65 °C for 15 min to remove protein impurities, and the resulting solution was centrifuged at 27,000×g for 30 min. After the second centrifugation, the supernatant was loaded onto a Talon column which was pre-equilibrated with lysis buffer. The resin was subsequently washed with 20 bed volumes of washing buffer, 40 mM Tris-HCl, 500 mM NaCl, and 10 mM imidazole, pH 8.0, and the protein was eluted using elution buffer, 40 mM Tris-HCl, 500 mM NaCl, and 200 mM imidazole, pH 8.0. The fractions containing N-His₆-*Tm* DnaK were pooled. The purity of the protein (>90%) was assessed by 10% SDS-PAGE and the protein concentration was determined by a Bradford assay (Bio-Rad). The protein solution was then concentrated, and the buffer system was exchanged according to the requirements of subsequent experiments by use of Amicon YM-30 ultrafiltration. The resulting protein band on Coomassie Brilliant Blue

stained gel was excised and peptide mass fingerprinting was performed using MALDI-MS/MS by Applied Biomics (Hayward, CA) to confirm the integrity of the protein.

For purification of human N-His₆-mortalin ($\Delta 1-51$, corresponding to a plausible mitochondrial targeting sequence), which will be termed human mortalin in the text that follows, the cell pellet was resuspended homogeneously in 8 volumes of lysis buffer, 40 mM Tris-HCl and 500 mM NaCl, pH 8.5, supplemented with 75 ng/mL lysozyme and 1 mM PMSF. The resuspended cell solution was disrupted through sonication (10 s pulse every 2 min for 30 min) on ice, and the lysate was centrifuged at $27,000\times g$ for 30 min. Human mortalin resides in the pellet, and so, following centrifugation, the pellet was solubilized in 40 mM Tris-HCl, 500 mM NaCl, and 2 M GdmHCl, pH 8.5, supplemented with 1 mM PMSF, and then sonicated. The lysate was then centrifuged at $27,000\times g$ for 30 min. After the second centrifugation, the supernatant (termed S2 in Fig. 1) was loaded onto a Ni²⁺-NTA column which was pre-equilibrated with 40 mM Tris-HCl, 500 mM NaCl, and 2 M GdmHCl, pH 8.5. The resin was then washed with 20 bed volumes of washing buffer, 40 mM Tris-HCl, 500 mM NaCl, 2 M GdmHCl, and 20 mM imidazole, pH 8.5. After the washing step, the protein was eluted using elution buffer, 40 mM Tris-HCl, 500 mM NaCl, 2 M GdmHCl, and 400 mM imidazole, pH 8.5. The fractions containing N-His₆-mortalin ($\Delta 1-51$) were pooled. The purity of the protein (>90%) was examined by 10% SDS-PAGE. The pooled protein fractions were concentrated to about 10 ml by ultrafiltration through an Amicon YM-30 membrane. The concentrated protein was refolded by dropwise dilution using a 25-fold excess of 100 mM HEPES, 100 mM KCl, pH 7.6 into the protein solution with rapid stirring. The refolded protein solution was concentrated again to about 10 ml. The buffer was exchanged by multiple ultrafiltrations, depending on the requirements for subsequent experiments, but with 10% glycerol in most cases to maintain the stability of the refolded protein. Although the protein solution can be kept at 4 °C for 2-3 days without loss of ATPase activity (described later), freshly prepared and filtered protein solution was used in all experiments, and the concentration was determined prior to each experiment by measuring the UV absorption at 280 nm. The calculated extinction coefficient of human mortalin at 280 nm is $21,025\text{ M}^{-1}\text{cm}^{-1}$ [33]. To further ensure the purified protein is human mortalin, the resulting protein band on Coomassie Brilliant Blue stained gel was excised and peptide mass fingerprinting was performed using MALDI-MS/MS by Applied Biomics (Hayward, CA) to confirm the integrity of the protein.

UV-Visible and fluorescence spectroscopy

UV-visible spectra were recorded on a Hewlett-Packard 8425A diode array spectrophotometer at 25 °C. A 1.0 cm pathlength quartz cell was used for all measurements. Fluorescence spectra were obtained on a Perkin-Elmer LS50B luminescence spectrometer, and a 3 mm pathlength quartz cell was used in all measurements. Intrinsic fluorescence excitation and emission spectra of human mortalin were recorded using 20 μM of mortalin in 100 mM HEPES, 100 mM KCl, and 10% glycerol, pH 7.6, at 25 °C. The ATP-induced blue-shift of tryptophan fluorescence was monitored at 25 °C by following the excitation at 295 nm with 9 nm slit widths.

ATPase activity assay and single turnover kinetics studies

ATPase activity was determined by monitoring the formation of inorganic phosphate with the EnzChek phosphate assay kit (Invitrogen, Molecular Probes) [34]. The formation of 2-amino-6-mercapto-7-methylpurine resulting from inorganic phosphate coupling with the enzyme purine nucleoside phosphorylase (PNP) and its substrate 2-amino-6-mercapto-7-methylpurine riboside (MESG), was monitored over time at 360 nm in a 1 mL sample volume. For assay reactions the final concentrations of PNP and MESG were 1 unit/mL and 0.2 mM (following dilution from a 1 mM stock), respectively. All assays were performed at 37 °C following the instructions from the manufacturer on a Hewlett-Packard 8425A diode array spectrophotometer.

For single turnover kinetics experiments, in the case of *Tm* DnaK, 15 μ M of ATP was used in all assays. 150 μ M of *Tm* DnaK in 100 mM HEPES, 100 mM KCl, and 1 mM MgCl₂, pH 7.6 was used in all measurements. Samples were pre-incubated at 37 °C for 5 min prior to the addition of ATP; total volumes were 1.5 ml. Reactions were quenched at various time points with 10 μ l 50% trichloroacetic acid to a 100 μ l aliquot of sample solution. The quenched samples were then centrifuged at 15,000 $\times$ g for 3 min to remove precipitated protein, and 50 μ l of solution was taken to determine the amount of phosphate by use of the EnzChek assay kit. In the case of human mortalin, 10 μ M of ATP was used in all assays. 100 μ M of human mortalin in 100 mM HEPES, 100 mM KCl, 1 mM MgCl₂ and 10% glycerol, pH 7.6, was used in all measurements.

Fluorescence anisotropy

Steady-state fluorescence anisotropy experiments were performed using a Varian Cary Eclipse fluorescence spectrophotometer equipped with a manual polarizer at 37 °C. Samples of mortalin solution in 100 mM HEPES, 100 mM KCl, and 10% glycerol contained 10 nM of a peptide corresponding to the plausible binding motif of human ISU1 with fluorescein attached to the C-terminus (LSLPPVKLHK-fluorescein). The solution was consecutively diluted with 10 nM of the fluorescein labeled peptide in the same buffer. The background control (samples without mortalin) was set as the zero point of the fluorescence anisotropy. The excitation and emission wavelength were respectively fixed at 495 and 525 nm with 10 nm slit widths. Each anisotropy value resulted from the average of 4 measurements, and the whole set of experiment was repeated for three times.

Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded using an AVIV Model 202 Circular Dichroism Spectrometer. Spectra were acquired in the wavelength range of 190–400 nm using a 0.1 cm quartz cell at 25 °C. The signal at each wavelength was recorded with an average time of 5 s and a bandwidth of 2.0 nm. A constant nitrogen flow was used to flush the CD chamber during the experiment. Spectra for *Tm* DnaK were obtained using 1.08 μ M of sample in 10 mM potassium phosphate buffer, pH 7.4. The spectra of human mortalin were obtained using 4.15 μ M of sample in 10 mM potassium phosphate buffer, pH 7.0. All spectra represent the average of 3 scans following subtraction of background values obtained with buffer. Secondary structures were estimated by use of the CD deconvolution program K2d (<http://www.embl.de/~andrade/k2d/>) [35]. The secondary structures of *Tm* DnaK and human mortalin were also predicted from the primary sequences by using the programs nnpredict (University of California, San Francisco, <http://www.cmpchem.ucsf.edu/~nomi/nnpredict.html>) [36] and Porter (University College Dublin, Ireland, <http://distill.ucd.ie/porter/>).

Results and Discussion

Purification of human mortalin

Recombinant human mortalin was substantially overexpressed in *E. coli* but was found to form inclusion bodies (Fig. 1, lane 2–4). Purification of the recombinant protein required consideration of several issues, including solubilization of protein aggregates, refolding of the target protein, and recovery of its bioactivity [37–39]. Solubilization of the protein aggregates was achieved by use of the chaotropic agent GdmHCl at 2 M concentration, where the rather mild solubilization conditions proved beneficial in obtaining active protein [38,39]. Expressed human mortalin included an N-terminal His₆-tag that facilitated purification by Ni²⁺-NTA affinity chromatography.

Purification of solubilized human mortalin through a Ni^{2+} -NTA column yielded an average of 3.0 mg/ml total protein after elution, and at least 30 mg of human mortalin per liter of cell culture was obtained, yielding a single band by SDS-PAGE (Fig. 1, lane 8). To verify expression of the correct protein, the band from the SDS-PAGE gel of the eluted peak was excised, subjected to in-gel tryptic digestion, and the extracted peptides were analyzed by MALDI-MS/MS. The result from peptide mass fingerprinting verified that the protein with the highest score was the 73681.9 Da human mortalin (GenBank protein accession number, [AAA67526.1](#)) (Fig. S1).

The refolding of human mortalin was achieved through slow dropwise addition of a 25-fold excess of buffer into the protein solution with rapid stirring. This step was important for practical reasons, since protein aggregation might otherwise occur during the process. Other approaches were also attempted, but often resulted in problems. For example, efforts to refold the protein during dialysis resulted in serious protein aggregation. Following successful refolding, the buffer system was exchanged by ultrafiltration. Glycerol was required in the new buffer system to maintain the stability of human mortalin. Removal of glycerol was attempted; however, precipitation of the protein frequently occurred unless the concentration of the protein was reduced to the sub- μM range. The activity of the refolded protein was examined by use of an ATPase activity assay (described later). Size exclusion chromatography was performed following the buffer exchange; however, precipitation compromised the yield of the protein (data not shown). Therefore, this step was not applied to assure sufficient amount of protein sample can be obtained for the subsequent experiments.

While the expressed protein resides in inclusion bodies, this fact was beneficial with regard to the yield of isolable protein and protection of the protein from proteases in *E. coli* [37-39]. Indeed, a considerable amount of human mortalin was obtained (more than 30 mg protein per liter of cell culture). Prior studies have shown that expressed proteins in inclusion bodies might retain partial secondary structure, and the preservation of that structure can facilitate the proper refolding of the protein. The application of a modest level of a chaotropic reagent (2M GdmHCl) for solubilization of the inclusion bodies resulted in fewer problems in subsequent steps. The rate of buffer addition and careful mixing during dropwise dilution have proven to be important for the successful refolding without protein aggregation.

Spectroscopic characterization of human mortalin

Human mortalin was characterized by both UV-visible and fluorescence spectroscopies. The UV-visible spectrum was determined in the range of 240-800 nm and a peak absorbance was observed at 280 nm (Fig. S2). This result is not surprising since human mortalin contains a large number of aromatic residues (1 tryptophan, 10 tyrosine and 18 phenylalanine residues). The spectrum also indicated that no additional nucleotide chromophores were present. The intrinsic excitation and emission fluorescence spectra were monitored, and the results are shown in Fig. S3. The excitation wavelengths corresponding to tyrosine and tryptophan residues were used, and the emission spectra were obtained. Excitation at 278 nm, which results in excitation of both tyrosine and tryptophan residues, yields the most intense emission spectrum [40]. Nevertheless, with the intention to monitor the emission resulting from the single tryptophan residue, emission spectra from higher excitation wavelengths were also acquired. Excitation at both 290 and 295 nm provided an acceptable emission response for this purpose with a peak maximum near 340 nm. Conversely, excitation at 305 nm barely generated sufficient signal, and so 295 nm excitation was chosen to monitor tryptophan fluorescence and minimize the influence from tyrosine residues. Since the fluorescence spectrum of tryptophan is sensitive to its microenvironment, the wavelength of the peak maximum suggests that the single tryptophan in human mortalin is exposed to a polar environment [40,41]. The fact that human mortalin contains only one tryptophan residue simplified the fluorescence experiments,

and will enable future studies of protein-protein interactions and functional studies of this chaperone.

A common hallmark of all Hsp70 family proteins is a shift (red or blue) of the emission maximum of the tryptophan fluorescence that is generally observed upon ATP addition [42-44]. The intrinsic fluorescence of 15 μ M human mortalin was acquired both in the absence and in the presence of 45 μ M ATP, and resulted in a blue-shift of ~ 3 nm following nucleotide addition (Fig. 2). This suggests that the blue-shift originates from ATP binding and that this binding induces a conformational change that causes the solvent exposed tryptophan to become buried in a more hydrophobic environment [40,41], consistent with prior observations for Hsp70-type chaperones.

Single-Turnover ATPase Activity

To better demonstrate the complete and uniform folding of human mortalin, kinetic parameters for single turnover ATP hydrolysis were obtained for human mortalin and compared with data for *Tm* DnaK. The concentration of either human mortalin or *Tm* DnaK used in single turnover experiments was 10-fold in excess relative to the ATP substrate, and the rate profile for inorganic phosphate formation is illustrated in Fig. 3 a. The rate of ATP hydrolysis can be described by equation 1 [45-49]:

$$[\text{ADP}]_t = [\text{ADP}]_{\infty} \cdot [1 - [k_{\text{hyd}}/(k_{\text{hyd}} - k_{\text{syn}})] \cdot \exp[-(k_{\text{hyd}} - k_{\text{syn}}) \cdot t]] \quad (1)$$

where k_{hyd} is the rate constant for ATP hydrolysis, k_{syn} is the rate constant for the reverse reaction and the concentration of ADP formed is the same as the concentration of inorganic phosphate generated from the reaction. Assuming that the rate of ATP hydrolysis is much larger than the rate of the reverse reaction, equation 1 can be simplified to equation 2.

$$[\text{ADP}]_t = [\text{ADP}]_{\infty} \cdot (1 - \exp[-k_{\text{hyd}} \cdot t]) \quad (2)$$

The time course for the formation of inorganic phosphate was fit to a first-order exponential function, as in the above equation, and yielded an observed first-order rate constant for ATP hydrolysis, k_{hyd} , of $6.0 \times 10^{-4} (\pm 0.7 \times 10^{-4}) \text{ s}^{-1}$.

For comparison, the single turnover kinetics for ATP hydrolysis by *Tm* DnaK was studied in a similar fashion (Fig. 3 b) and yielded an observed first-order rate constant for hydrolysis, k_{hyd} , of $3.7 \times 10^{-4} \text{ s}^{-1} (\pm 0.3 \times 10^{-4}) \text{ s}^{-1}$, which is in the same range as that obtained for human mortalin. The rate constants for ATP hydrolysis, k_{hyd} , for both human mortalin and *Tm* DnaK are consistent with the data obtained from *E. coli* DnaK, which is determined to be $6.0 \times 10^{-4} \text{ s}^{-1}$ [50]. These results suggest that both human mortalin and *Tm* DnaK exhibit slow basal turnover. While we have not yet determined the kinetic constants for each step in the ATPase cycle, previous studies of the Hsp70 family of proteins indicate that ATP hydrolysis is the rate limiting step, and the values obtained for k_{hyd} are consistent with k_{cat} values previously reported for steady state turnover for such ATPase activities ($\sim 1.4 \times 10^{-3} \text{ s}^{-1}$ for *E. coli* Hsc66 [49] and $6.3 \times 10^{-4} \text{ s}^{-1}$ for *E. coli* DnaK [50]).

Substrate binding

To address the potential involvement of mortalin in Fe-S cluster biogenesis, steady-state fluorescence anisotropy experiments were conducted to evaluate the binding affinity of a putative peptide binding motif, LPPVK, from the human Fe-S cluster scaffold protein, ISU1,

to mortalin. Fluorescence anisotropy reflects the rate of rotational diffusion, which depends on the size and the shape of the molecule or complex. This technique can be applied to the quantification of protein-protein and/or protein-peptide interaction [51]. The affinity of mortalin for a C-terminal fluorescein-labeled LSLPPVKLHK peptide was monitored (Fig. 4) and fitting to a one-site binding equation yielded a dissociation constant, K_d , of $1.2 \pm 0.1 \mu\text{M}$; in good agreement with prior estimates from studies of yeast Ssq1 (K_d of $2.8 \pm 0.4 \mu\text{M}$) [26]. This result is consistent with mortalin serving as a chaperone with direct involvement in human mitochondrial Fe-S cluster biogenesis through interaction with the ISU1 scaffold protein via the LPPVK binding motif.

Secondary structure

Circular dichroism spectroscopy was utilized to probe the secondary structure of human mortalin (Fig. 5). Analysis of the data through the K2d program revealed a secondary structure of 24% alpha-helix, 19% beta-strand and 57% random coil, with a square distance parameter of 71.65 and maximum error of 0.2. The neural networking method (K2d program) assesses the variation of the fitted and actual spectra, and data that yield a square distance under 227 are viewed as acceptable [35].

For comparison, the secondary structure of *Tm* DnaK, another Hsp70 family chaperone, was also determined (Fig. S4) and yielded a secondary structure of 27% alpha-helix, 15% beta-strand and 58% random coil at pH 7.4, with a square distance parameter of 33.89 and maximum error of 0.08. The secondary structures of both human mortalin and *Tm* DnaK were also predicted through their primary sequences by use of the programs nnpredict and Porter (Fig. S5 a and S5 b). For human mortalin, nnpredict yielded a secondary structure of 30.6% alpha-helix, and 13.6% beta-strand, while a secondary structure of 33.6% alpha-helix and 14.3% beta-strand was determined for *Tm* DnaK (Table 1).

The results from the primary sequence analysis are consistent with the observations from CD spectroscopy, suggesting that both human mortalin and *Tm* DnaK contain approximately 30% alpha-helix and 15% beta-strand. Alignment of the amino acid residues from these two Hsp70 family members shows that they share 50% sequence identity and 69% sequence similarity, further supporting the resemblance between their secondary structures (Fig. S6). Both results from circular dichroism spectroscopy and primary sequence analysis suggest that the refolded human mortalin adopts a folded structure, which was confirmed by the fact that the refolded human mortalin demonstrated both ATPase activity and a blue-shift in the emission maximum of tryptophan fluorescence following ATP binding. Both observations demonstrate that the purified refolded human mortalin is active and amenable to meaningful spectroscopic characterization.

Role for mortalin in Fe-S cluster biosynthesis

Comparison of human mortalin with *Tm* DnaK shows that both Hsp70 proteins share similar secondary structures and parallel kinetic behavior. The striking similarities in secondary structures of *Tm* DnaK and human mortalin lead to more interesting questions regarding common roles in Fe-S cluster biogenesis. For example, while human ISU1 possesses the plausible substrate binding LPPVK motif, *Tm* IscU possesses a related but non-identical NYPAR motif. The binding motif of *Tm* IscU that can be recognized by chaperones is still unraveled, and can be interesting to probe, considering that it might serve as the future drug target in other parallel pathogens. Furthermore, in some bacteria, a dedicated Hsc66 or HscA chaperone has been implicated in Fe-S cluster biosynthesis [52,53], while the DnaK type chaperone is usually implicated in general cellular roles requiring chaperone assistance [54]. However, in bacteria lacking an HscA-type chaperone, DnaK appears to fill the chaperone role for cluster biosynthesis [32]. In yeast, Ssq1 is the principal chaperone implicated in Fe-S cluster

biosynthesis [25], while in human cellular system only the multifunctional Ssc1-type chaperone mortalin has been implicated in Fe-S cluster biosynthesis. Interestingly, the Ssc1- and Ssq1-type chaperones are phylogenetically related, but are discrete from the HscA-type orthologs that are common to bacteria [24,55].

Conclusions

In this work, we have successfully purified and characterized human mortalin. Human mortalin has been implicated in several serious human diseases. While the underlying role in these disease states remains unclear, it is likely that the involvement of human mortalin in iron homeostasis is at least partly responsible for the occurrence of several of these disease states. In summary, this work provides a basic biochemical characterization of human mortalin and offers a foundation for future detailed biophysical and kinetic studies of its function and its relationship to several human disease states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation used

ATP	Adenosine-5'-triphosphate
NTA	nitrilotriacetic acid
<i>Tm</i> DnaK	<i>Thermotoga maritima</i> DnaK
Tris	tris(hydroxymethyl)aminomethane
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
GdmHCl	guanidinium chloride
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
Fe-S	iron-sulfur

Fig. 1.

SDS-polyacrylamide gel (10%) results for the purification of human mortalin. *Lane 1*, molecular weight markers (94, 67, 43, 30, 20.1 kDa from the top); *lane 2*, total protein from the supernatant after lysozyme treatment, sonication and centrifugation (S1); *lane 3*, total soluble protein from inclusion bodies after lysozyme treatment, sonication, centrifugation, solubilization with 2M GdmHCl and the second centrifugation (S2); *lane 4*, total insoluble protein from inclusion bodies after lysozyme treatment, sonication, centrifugation, solubilization with 2 M GdmHCl and the second centrifugation (P2); *lane 5*, flow-through of unbound proteins from a Ni^{2+} -NTA column; *lanes 6 and 7*, buffer wash; *lane 8*, elution portion, eluted with 400 mM imidazole in the elution buffer. GdmHCl is present in the gel sample for *lanes 3-8*. *Lane 8* shows a single band at approximately 70 kDa. The single band from *lane 8* was excised and used for peptide mass fingerprinting.

Fig. 2.

ATP-induced blue-shift of the emission maximum in the tryptophan fluorescence spectrum. The filled circle line represents the intrinsic fluorescence spectrum of 15 μM human mortalin in 100 mM HEPES, 100 mM KCl, 1 mM MgCl_2 and 10% glycerol, pH 7.6. The open circle line represents the fluorescence spectrum of 15 μM human mortalin in 100 mM HEPES, 100 mM KCl, 1 mM MgCl_2 , 45 μM ATP, and 10% glycerol, pH 7.6.

Figure 3 a

Fig. 3. Single turnover kinetic profile of human mortalin and *Tm* DnaK at 37 °C (n = 3). (a) ATP hydrolysis by 100 μM human mortalin in 100 mM HEPES, 100 mM KCl, 10 μM ATP, 1 mM MgCl₂, and 10% glycerol, pH 7.6. (b) ATP hydrolysis by 150 μM *Tm* DnaK in 100 mM HEPES, 100 mM KCl, 15 μM ATP, 1 mM MgCl₂, and 10% glycerol, pH 7.6. The solid curve is the best fit to the first order exponential function.

Fig. 4. Steady-state fluorescence anisotropy measurements for the binding of LSLPPVKLHK-fluorescein to human mortalin at 37 °C (n=4). Both background control data obtained in the absence of mortalin (open circles) and binding data obtained with mortalin (filled circles) were obtained in 100 mM HEPES, 100 mM KCl, and 10% glycerol, pH 7.6, with 10 nM of LSLPPVKLHK-fluorescein. The solid curves represent the best fit to the one-site binding function, which yielded K_d of 1.2 ± 0.1 μ M for the binding of mortalin to LSLPPVKLHK-fluorescein.

Fig. 5. Circular dichroism (CD) spectra of a 4.15 μ M solution of human mortalin in 10 mM potassium phosphate buffer, pH 7.0 at 25 $^{\circ}$ C.

Table 1

Quantitation of secondary structure elements determined by CD and primary sequence analysis.

	Human mortalin		<i>Tm</i> DnaK	
	α -helix	β -strand	α -helix	β -strand
CD Spectroscopy	24.0	19.0	27.0	15.0
Primary Sequence Analysis	30.6	13.6	33.6	14.3