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Biochemical and Biophysical Research Communications 333 (2005) 1160–1170
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# Differential degradation of variant medium-chain acyl-CoA dehydrogenase by the protein quality control proteases Lon and ClpXP ☆

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Received 1 June 2005
Available online 15 June 2005

**Abstract**

The coordinated activities of chaperones and proteases that supervise protein folding and degradation are important factors for deciding the fate of proteins whose folding is impaired by missense variations. We have studied the role of Lon and ClpXP proteases in handling of wild-type and a folding-impaired disease-associated variant (R28C) of the mitochondrial enzyme medium-chain acyl-CoA dehydrogenase (MCAD). Using an *Escherichia coli* model system, we co-overexpressed the MCAD variants and the respective proteases at two conditions: at 31 °C where R28C MCAD protein folds partially and at 37 °C where it misfolds and aggregates. Co-overexpression of Lon protease considerably accelerated the degradation rate of a pool of R28C variant MCAD synthesised during a 30 min pulse and counteracted accumulation of aggregates at 37 °C, whereas increasing the amounts of ClpXP protease had no clear effect. Co-overexpression of either Lon or ClpXP protease markedly decreased the steady state levels of both wild-type and R28C mutant MCAD at 37 °C but not at 31 °C. Our results suggest that Lon is more efficient than ClpXP in elimination of non-native MCAD protein conformations, and accordingly, that Lon can recognise a broader spectrum of MCAD protein conformations.


*Keywords:* Mitochondrial; Variant protein; Misfolding; Degradation; Protein quality control; Proteases; Chaperones

Most recessively inherited enzyme deficiencies are caused by loss-of-function of the gene product. In a majority of cases, loss-of-function is due to mutations that result in failure to express the functional enzyme either by jeopardising pre-mRNA processing or mRNA stability [1] or it is due to missense mutations that compromise folding of the translated variant protein to the native conformation [2,3]. The residual function displayed by a variant protein impaired in folding is determined by its ability to fold correctly versus its propensity to form misfolded conformations with increased sensitivity to premature degradation or aggregation [4]. Molecular chaperones that monitor the folding status of proteins play an important role in promoting correct folding and avoiding protein misfolding and aggregation. Proteins that fail to fold may, if they are not recognised and degraded by cellular proteases, form inter-molecular interactions with other partially folded or misfolded polypeptides leading to aggregates [5]. The combined action of chaperones and proteases in these processes has been designated protein quality control [6]. The quality control mechanisms that decide if the fate of a protein will be correct folding, degradation,

☆ *Abbreviations:* Hsp, heat shock protein; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MCAD, medium-chain acyl-CoA dehydrogenase; PAGE, polyacrylamide gel electrophoresis; SCAD, short-chain acyl-CoA dehydrogenase.

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doi:10.1016/j.bbrc.2005.06.024

or aggregation have not completely been elucidated. It has been proposed that the decision or triage mechanisms are based on competition between chaperones on one side and proteases on the other [6,7]. As chaperones and proteases both can bind hydrophobic patches exposed on non-native protein conformations their individual affinity for these conformations and the stoichiometric balance between the involved chaperone and protease components may be important for triage mechanisms in a competition model.

Medium-chain acyl-CoA dehydrogenase (MCAD) is an enzyme localised in the mitochondrial matrix. The enzymatically active MCAD tetramer catalyses an essential step in the mitochondrial β-oxidation of fatty acids. Deficiency of this enzyme is potentially fatal and is the most frequently observed defect in mitochondrial β-oxidation of lipids [8,9]. The MCAD enzyme deficiency is most often caused by missense mutations that compromise the folding of the variant protein into its native conformation but do not affect the thermal stability of the correctly folded tetramer [3]. More than 20 different disease-associated missense variations in the MCAD gene have been reported [10–14]. Several other diseases where missense variations cause a loss-of-function of a variant protein by impairing its folding to the native conformation and increasing its susceptibility to proteolytic breakdown have been characterised [15–17].

An organelle-specific portfolio of proteases and chaperones handles proteins localised in mitochondria [18–20]. Most nuclear encoded proteins that reside in the mitochondrial matrix become associated with Hsp70 molecules as an integrated part of the mitochondrial import process. A subset of the imported proteins, including the MCAD protein, subsequently requires binding to Hsp60/10 chaperone complexes in order to fold into their native conformation [21,22]. Several folding-impaired MCAD and short-chain acyl-CoA dehydrogenase (SCAD) (a mitochondrial paralogue to MCAD) variant proteins have been shown to be retained in Hsp60 bound complexes for an extended period of time when compared to the corresponding wild-type proteins [22,23]. Furthermore, despite prolonged interaction between the variant proteins and Hsp60, the formation of tetrameric conformations was severely inhibited. A group of variant SCAD proteins, that displays extensive binding to Hsp60 complexes has been shown to be more susceptible to intra-mitochondrial degradation than wild-type SCAD [23]. Rescue of the folding and tetramerisation of MCAD proteins expressed in *Escherichia coli* has been shown for several variants by co-overexpression of the bacterial Hsp60/10 chaperone system GroEL/ES and/or by lowering of the growth temperature [13,24,25]. These folding-optimising conditions increased the fraction of correctly folded variant MCAD protein considerably compared to standard conditions and resulted in build-up of enzymatically active variant protein equivalent to that of wild-type MCAD. In contrast to the substantial knowledge on chaperone-assisted biogenesis, the degradation mechanisms of variant MCAD proteins and the role of specific mitochondrial matrix protein quality control proteases herein are not well understood.

In the mammalian mitochondrial matrix two ATP-dependent proteolytic systems are known, namely the Lon and ClpXP proteases [26–28]. Molecular investigations of these proteases and identification of their substrates have mostly been performed for orthologs in bacteria and yeast. For the *E. coli* ClpXP, cellular substrates have been characterised and it appears that these are recognised on the basis of sequence motifs either in the N- or C-terminus [29]. For Lon-type proteases in bacteria, yeast, and mammals no sequence motifs that trigger degradation have been identified. Rather, a general specificity towards non-native conformations and proteins damaged by oxidation has been suggested [30–34].

To study degradation mechanisms of the MCAD protein we have utilised an *E. coli* model system based on independently replicating vectors that enables controllable and timed overexpression of several genes simultaneously. Since a great overlap between the functionality of the orthologous chaperones and proteases from the mammalian mitochondrial matrix and from *E. coli* exists, the use of this simplified model is appropriate as a first approach to gain a better understanding of degradation mechanisms of variant MCAD proteins.

We hypothesise that the decision of degradation is based on the conformational state of MCAD protein variants, and that Lon and/or ClpXP proteases play a role in MCAD degradation. To test our hypotheses, we have used two model proteins that differ in their folding propensity, namely wild-type MCAD and a disease-causing missense variant that has an amino acid substitution of arginine with cysteine in position 28 of the mature MCAD protein (R28C) [10]. Both wild-type and R28C MCAD proteins fold to the native state and display comparable specific enzyme activities and thermal stabilities under permissive growth conditions in *E. coli* but the variant protein misfolds under non-permissive conditions [10,13].

We have in the present study co-overexpressed the ClpXP and Lon-type proteases in *E. coli* to regulate the balance between chaperones and proteases, and monitored the degradation of co-overexpressed wild-type and R28C MCAD proteins. Specifically, we wanted to investigate: (1) whether Lon and/or ClpXP degrade MCAD protein variants, (2) whether R28C MCAD protein is degraded faster than wild-type MCAD due to the increased propensity of R28C MCAD to misfold, and