Misfolding, Degradation, and Aggregation of Variant Proteins

THE MOLECULAR PATHOGENESIS OF SHORT CHAIN ACYL-CoA DEHYDROGENASE (SCAD) DEFICIENCY*

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Short chain acyl-CoA dehydrogenase (SCAD) deficiency is an inborn error of the mitochondrial fatty acid metabolism caused by rare variations as well as common susceptibility variations in the SCAD gene. Earlier studies have shown that a common variant SCAD protein (R147W) was impaired in folding, and preliminary experiments suggested that the variant protein displayed prolonged association with chaperonins and delayed formation of active enzyme. Accordingly, the molecular pathogenesis of SCAD deficiency may rely on intramitochondrial protein quality control mechanisms, including degradation and aggregation of variant SCAD proteins. In this study we investigated the processing of a set of disease-causing variant SCAD proteins (R22W, G68C, W153R, R359C, and Q341H) and two common variant proteins (R147W and G185S) that lead to reduced SCAD activity. All SCAD proteins, including the wild type, associate with mitochondrial hsp60 chaperones; however, the variant SCAD proteins remained associated with hsp60 for prolonged periods of time. Biogenesis experiments at two temperatures revealed that some of the variant proteins (R22W, G68C, W153R, and R359C) caused severe misfolding, whereas others (R147W, G185S, and Q341H) exhibited a less severe temperature-sensitive folding defect. Based on the magnitude of in vitro defects, these SCAD proteins are characterized as folding-defective variants and mild folding variants, respectively. Pulse-chase experiments demonstrated that the variant SCAD proteins either triggered proteolytic degradation by mitochondrial proteases or, especially at elevated temperature, aggregation of non-native conformers. The latter finding may indicate that accumulation of aggregated SCAD proteins may play a role in the pathogenesis of SCAD deficiency.

Short chain acyl-CoA dehydrogenase (SCAD) is one of the four acyl-CoA dehydrogenases that catalyze the initial step in the mitochondrial β-oxidation of straight-chain fatty acids (1). Subunits of the SCAD enzyme are nuclear encoded and synthesized in the cytosol as precursor proteins that are imported into the mitochondrial matrix. Newly imported SCAD proteins are proteolytically processed, folded, and assembled into the biologically active homotetrameric form (168 kDa) containing one molecule of FAD per subunit (2). Hereditary deficiency of SCAD has been reported in 20 patients worldwide with a variety of clinical symptoms (3–13). The most frequent symptoms are chronic and episodic neuromuscular disabilities, including cognitive and muscular developmental delay, hypotonia, and seizures. All patients reported with documented SCAD deficiency excrete increased levels of ethylmalonic acid in the urine, originating from the accumulation of butyryl-CoA, the substrate for SCAD (14). SCAD deficiency is inherited in an autosomal recessive fashion due to variations in both alleles of the SCAD gene. At present, 14 disease-causing variations have been reported in the SCAD gene, all of which are of the missense type except one (a 3-bp deletion) (4, 12, 15–17). Besides the rare pathogenic variations in the SCAD gene leading to a complete deficiency of SCAD activity, two common SCAD variations have been identified in Western European populations that encode proteins with reduced catalytic activity and/or thermostability: 625G>A (G185S) and 511C>T (R147W) (12, 17, 18). The 625A and 511T alleles are overrepresented in homozygous or in compound heterozygous form in patients with elevated ethylmalonic acid levels (>18 mmol/mol creatinine) in the urine (69 versus 14% in the general population) (17, 18). The majority of patients reported with apparent SCAD deficiency carry only the common variant genotypes (625A/625A, 511T/511T, and 625A/511T) or a genotype defined by common variations in one allele and rare variations in the other (12, 17). This agrees with the mutation spectrum observed in a larger group of SCAD patients. Because the common SCAD variants are detected in 14% of the general population, they cannot be sufficient to cause SCAD deficiency. The role of 625G>A and 511C>T in the pathogenesis of SCAD deficiency, therefore, is not clear. We have hypothesized that these variations represent susceptibility alleles that, in combination with other genetic and/or environmental factors, can trigger disease in some individuals (12).

To elucidate the pathogenesis of SCAD deficiency, the molecular properties of some of the identified variant SCAD enzymes, including the R147W and G185S susceptibility variants, have been investigated in a variety of in vitro systems.

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‡‡ The abbreviations used are: SCAD, short chain acyl-CoA dehydrogenase; MCAD, medium chain acyl-CoA dehydrogenase; hsp, heat-shock protein; OTC, ornithine transcarbamylase; SOD-1, Cu/Zn-superoxide dismutase.

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The R147W and G185S variant proteins were produced at lower levels in *Escherichia coli*, but could be partly rescued and subsequently purified by co-expression of the bacterial GroEL/ES chaperonin-system (bacterial homologue to hsp60/hsp10) (18, 19). Purified G185S SCAD showed impaired catalytic efficiency with butyryl-CoA as substrate (10 μM⁻¹s⁻¹ versus 33 μM⁻¹s⁻¹), whereas the catalytic efficiency (33 μM⁻¹s⁻¹) and thermostability of the R147W variant enzyme were similar to that of wild type SCAD (33 μM⁻¹s⁻¹) (19). This is in accordance with previous observations in extracts of transfected COS-7 cells (20, 21). Furthermore, the levels of active R147W and G185S SCAD enzymes produced in COS-7 cells were shown to be temperature-dependent (17). The amount of soluble variant protein at high temperature, however, was not decreased proportionally to the enzyme activity, but paralleled wild type levels. The low levels of inactive soluble R22W, G68C, W153R, and R359C protein produced in COS-7 cells suggested that these variations are disease-causing (17, 20, 21). The R22W SCAD variant has further shown to be imported normally into mitochondria, but partially folded or misfolded forms of the variant protein were eliminated from the soluble fraction at a much faster rate compared with the wild type enzyme, which was interpreted to be caused by degradation (20).

Partially folded subunits of medium chain acyl-CoA dehydrogenase (MCAD) have been shown to transiently interact with the mitochondrial chaperones hsp70 and hsp60 following import and then subsequently fold into the native conformation. Experiments with wild type MCAD and the K304E variant enzyme (the most common variation found in MCAD-deficient patients) have shown that the variant polypeptide was retained for longer time periods in high-molecular weight complexes with hsp60 following mitochondrial import compared with wild type (22, 23). Similar to the results obtained with variant SCAD proteins, the yield of overexpressed K304E MCAD in *E. coli* was increased when it was co-expressed with GroEL/ES chaperonins (24).

The aim of the present study was to compare the processing mechanism, including folding, degradation, and aggregation, of a series of rare variant SCAD enzymes, which exhibit a low thermostability of the wild type enzyme (the most common variation found in MCAD-deficient patients) have shown that the variant polypeptide was retained for longer time periods in high-molecular weight complexes with hsp60 following mitochondrial import compared with wild type (22, 23). Similar to the results obtained with variant SCAD proteins, the yield of overexpressed K304E MCAD in *E. coli* was increased when it was co-expressed with GroEL/ES chaperonins (24).

### EXPERIMENTAL PROCEDURES

**Plasmid Construction**—The coding region from position +17 to +1270 of human wild type SCAD cDNA was cloned into the pcDNA3.1 (+) expression vector (Invitrogen). The 274G>T (G68C), 511C>T (R147W), 529C>T (W153R), 625G>A (G185S), and 1095G>T (Q341H) variations were each introduced into the pcDNA3.1-SCAD wild type plasmid using PCR-based mutagenesis. Positions of these mutations are shown together with the common SCAD variations (17) including the wild type enzyme (Q341H). All variations are located distant from the active center.

**In Vitro Transcription/Translation of Precur sor SCAD cDNA**—In *in vitro* transcription and translation of wild type and variant precursor SCAD cDNAs in pcDNA3.1 (+) were performed in the presence of [35S]methionine (20 μCi/50 μl reaction, 10 μCi/μl, Amersham Biosciences PhosphoImager (STORM 840) and quantified using the ImageQuant® software.

**Effects of Apyrase and Oligomycin on Tetramer Assembly and hsp60 Complexes**—One volume of freshly prepared BALB/cByJ (SCAD−−) mouse liver mitochondria was mixed with two volumes of *in vitro*
Fig. 2. Analysis of in vitro synthesized SCAD enzymes in isolated mitochondria. In vitro-translated SCAD precursor proteins were incubated with isolated mitochondria from SCAD-deficient BALB/cByJ (+/−) and from SCAD normal BALB/cA (+/+) mouse liver for 15 min at 26 °C. Mitochondrial lysates were analyzed by native PAGE followed by Western blotting. The blotted membrane was cut into “lanes” and exposed to phosphorimaging (lanes 1 and 2) or treated separately with specific antibodies recognizing SCAD (lanes 3, 4, and 5) or hsp60 (lane 6). The bands marked by * in lanes 3–6 represent unspecific binding of the antibodies.

RESULTS

SCAD-deficient Mitochondria as in Vitro Model System—To investigate the folding of in vitro synthesized SCAD proteins we have utilized a model system in which SCAD proteins of interest are synthesized in vitro in the presence of [35S]methionine and imported into isolated mitochondria from SCAD-deficient BALB/cByJ (SCAD−/−) mouse liver. Wild type precursor protein incubated with SCAD−/− mitochondria at 26 °C for 15 min were analyzed by native (non-denaturing) PAGE followed by Western blotting (Fig. 2). The phosphorimaging analysis of imported radiolabeled SCAD (Fig. 2, lanes 1 and 2) shows two bands with apparent molecular masses of 450 and 170 kDa, respectively. The 170-kDa band most likely represents the mature, tetrameric form of SCAD (predicted molecular mass, 168 kDa). To confirm this, Western blotting was performed with an antibody specifically recognizing SCAD. Endogenous SCAD protein in mitochondria isolated from a normal BALB/cA (SCAD+/+) mouse strain (lane 3) co-migrated with the 170-kDa band from the import assay (lane 2), confirming that the latter band represents SCAD tetramer. SCAD protein imported in vitro into SCAD−/− mitochondria was present at only a small fraction of the level of endogenous SCAD in normal (SCAD+/+) mice (Fig. 2, lanes 3 and 4). SCAD was absent in purified mitochondria from the BALB/cByJ (SCAD−/−) mouse strain (lane 5 in Fig. 2).

To investigate the nature of the SCAD protein migrating at 450 kDa, Western blotting was performed with specific antibodies against the mitochondrial hsp60 chaperonin. Fig. 2 shows co-migration of hsp60 (lane 6) with the 450-kDa SCAD band (lane 2), suggesting that this band constitutes SCAD protein associated with the mature form of hsp60. The smear observed in the middle of lane 6 may represent monomers or other oligomeric forms of hsp60.

Identification of the “450 kDa Form” of SCAD as a Complex between SCAD and hsp60—Mitochondrial hsp60 chaperonins are involved in folding and assembly of newly imported proteins within the mitochondria (30–34). To support the identity of the 450-kDa SCAD band as a complex between newly imported SCAD protein and hsp60 chaperonins, isolated BALB/cByJ (SCAD−/−) mitochondria were incubated with in vitro synthesized radiolabeled hsp60 protein for 15 min at 26 °C, and the soluble matrix fraction was analyzed by native PAGE (Fig. 3). Fig. 3A shows that in vitro imported hsp60 protein co-migrated with the 450-kDa SCAD band. Because the release of proteins from hsp60 is known to be ATP-dependent (30, 34), we examined the effect of ATP depletion on hsp60/SCAD-complex stability in BALB/cByJ (SCAD−/−) mitochondria. After 5 min import at 26 °C mitochondria were incubated with or without oligomycin and apyrase for another 30 min. Oligomycin specifically inhibits the mitochondrial ATP-synthetase thereby preventing the intramitochondrial synthesis of ATP, whereas apyrase catalyzes the breakdown of ATP outside the mitochondria. The import mixtures were then analyzed by native PAGE (Fig. 3B). When oligomycin and apyrase were added to the import reaction, 70 and 30% of SCAD were found associated in 450 kDa complex and tetramer, respectively. In contrast, without oligomycin and apyrase only 10% of the SCAD protein was retained in the 450-kDa complex, whereas the remaining 90% was found in the tetrameric form. These findings are compatible with the interpretation that the 450-kDa band represents synthesized SCAD wild type translation product at 26 °C. After 5 min import the mixture was treated with or without oligomycin and apyrase to final concentrations of 20 μg and 20 units/ml, respectively, and the mixture was incubated at 26 °C for 30 min. Mitochondrial lysates were analyzed by native PAGE as described above.

Western Blotting—One volume of fresh purified BALB/cByJ (SCAD−/−) or BALB/cA (SCAD+/+) mouse liver mitochondria was incubated with two volumes of in vitro synthesized SCAD wild type protein for 15 min at 26 °C. Mitochondrial lysates were subjected to electrophoresis on native 4–15% Tris-HCl Criterion polyacrylamide gels (Bio-Rad). Blotting was performed as described previously (29). The blots were incubated with polyclonal anti-SCAD (1) or monoclonal anti-hsp60 antibodies (H-3524, Sigma), and bound antibodies were visualized by chemiluminescence using the ECL Plus™ Western blotting detection reagent (Amersham Biosciences). An Amersham Biosciences Phosphor-Imager (STORM 840) was used for direct image analysis.

Detergent Treatment of Insoluble Proteins—One volume of freshly isolated BALB/cByJ (SCAD−/−) mitochondria was incubated with two volumes of in vitro synthesized SCAD or OTC protein for 30 min at 28 °C. The reaction mixtures were then incubated at 41 °C for a further 160 min. The mitochondria were treated and sonicated twice as described above, and the mitochondrial pellet was then sonicated in buffer with or without 1% Triton X-100. The soluble and insoluble lysate fractions were analyzed by SDS-PAGE as described above.
Biogenesis of Wild Type and Variant SCAD Proteins in SCAD-deficient Mitochondria—We next compared the biogenesis of wild type SCAD with a set of variant SCAD enzymes (R22W, G68C, R147W, W153R, G185S, Q341H, and R359C; for positions in SCAD protein structure see Fig. 1). In vitro translated precursor proteins were incubated with isolated BALB/cByJ mitochondria for up to 60 min at 26 °C, and the mitochondrial lysates were examined by native PAGE analysis. Imported SCAD and hsp60 proteins were quantified by phosphorimaging. B, in vitro translation product containing SCAD wild type precursors was incubated with purified BALB/cByJ (SCAD−/−) mitochondria at 26 °C. After 5 min of incubation the mixture was supplemented with (+) or without (−) oligomycin (20 μM, see Ref. 47) and apyrase (20 units/ml, see Ref. 22), and further incubated at 26 °C for 30 min. The mitochondrial lysates were analyzed by native PAGE and radiolabeled SCAD proteins were detected by phosphorimaging (right panel). Amounts of SCAD bound to hsp60 were quantitatively determined and compared with tetrameric protein in the presence or absence of oligomycin and apyrase (left panel).

Fig. 3. The 450-kDa form of SCAD is a complex between SCAD and hsp60. A, isolated BALB/cByJ (SCAD−/−) mouse liver mitochondria were incubated with in vitro translated radiolabeled SCAD wild type (lane 1) or hsp60 wild type protein (lane 2) for 15 min at 26 °C, and the mitochondrial lysates were examined by native PAGE analysis. Imported SCAD and hsp60 proteins were quantified by phosphorimaging. B, in vitro translation product containing SCAD wild type precursors was incubated with purified BALB/cByJ (SCAD−/−) mitochondria at 26 °C. After 5 min of incubation the mixture was supplemented with (+) or without (−) oligomycin (20 μM, see Ref. 47) and apyrase (20 units/ml, see Ref. 22), and further incubated at 26 °C for 30 min. The mitochondrial lysates were analyzed by native PAGE and radiolabeled SCAD proteins were detected by phosphorimaging (right panel). Amounts of SCAD bound to hsp60 were quantitatively determined and compared with tetrameric protein in the presence or absence of oligomycin and apyrase (left panel).
The within batch coefficients of variation ($C_V$) of the incubation reaction and of the gel-loading in this study were estimated from an experimental approach involving five unrelated translation reactions. We found that $C_V$ of the incubation reaction was 8–9%, whereas $C_V$ of the gel loading was 6–7% (data not shown).

Fig. 4A shows native PAGE analysis of appearance of tetramers of wild type and variant SCAD enzymes following in vitro import into mitochondria at 26 and 37 °C, respectively. Tetramers were formed at both temperatures for wild type SCAD as well as for the R147W, G185S, and Q341H variants. Tetrameric variant enzymes accumulate to higher levels at 26 versus 37 °C, suggesting that folding was impaired at 37 °C and could be partially rescued at lower incubation temperature. The slightly faster migrating band just below the SCAD tetramer band in these gels disappeared after treatment of the
sample with alkaline phosphatase, indicating that it represents a phosphorylated form of the SCAD tetramer (results not shown). This is compatible with previous reports of phosphorylation of the MCAD enzyme (35). In the upper section of the native gels in Fig. 4A significant amount of radiolabeled SCAD protein was detectable as a smear. This SCAD protein species was neither tetrameric nor hsp60 complexed, but might contain soluble folding intermediates and/or oligomeric forms of SCAD complexed with other mitochondrial chaperones, possibly hsp70. No tetrameric forms were seen at either temperature upon mitochondrial import of the R22W, G68C, W153R, and R359C SCAD variants. In contrast, these variant SCAD proteins remained associated with the hsp60 chaperonins forming high molecular weight complexes. Because the R22W and R359C variations were originally identified along with the common G185S variation in the same allele in patients, we used expression plasmids containing the G185S variation (i.e. SCAD proteins R22W/G185S and R359C/G185S). We have repeated the import experiments with expression plasmids lacking the G185S variation, and obtained identical results (not shown), suggesting that G185S variation has no significant additional effect.

To examine the degree of defective folding, the levels of tetrameric SCAD enzymes were related to the amounts of SCAD protein bound to hsp60 chaperonins after 60 min incubation. This relation is shown in Fig. 4B. The levels of tetramer and hsp60-complexed SCAD were normalized with respect to the total amount of soluble radiolabeled SCAD protein in the entire lane of the native gel (36, 37). Taken as a whole, these results suggested that biogenesis of all the SCAD enzymes depends on interaction with mitochondrial hsp60 chaperonins to fold correctly. However, the ratios between tetramer and hsp60 demonstrated a prolonged association of all variant SCAD proteins with the hsp60 chaperonin system compared with wild type.

**Fig. 5. Formation of heterotetrameric SCAD enzymes in mitochondria containing endogenous SCAD protein.** In vitro translation product containing SCAD R147W precursor proteins was incubated with SCAD normal BALB/cA (+/+; lane 1) or SCAD-deficient BALB/cByJ (SCAD−/−; lane 2) mouse liver mitochondria at 37 °C for 60 min. Mitochondrial lysates were analyzed by native PAGE, and phosphorimaging was used for quantifying the radiolabeled SCAD R147W proteins.
Detergent-soluble and Detergent-insoluble SCAD Species—To study the nature of the insoluble SCAD species in more detail, we have examined their solubility in Triton X-100 detergent. The orthologous very long chain acyl-CoA dehydrogenase enzyme, which is bound to the inner mitochondrial membrane, could be solubilized in Triton X-100 detergent (not shown).
shown). However, lysis of mitochondria with buffer containing Triton X-100 did not lead to an increased yield of soluble SCAD protein (Fig. 7A), nor could Triton X-100 solubilize the insoluble SCAD species following lysis in buffer without detergent (Fig. 7B). This is similar in behavior to an ornithine transcarbamylase mutant protein (OTC-Δ) previously shown to produce detergent insoluble aggregates within the mitochondrial matrix (25). Import of *in vitro* synthesized wild type and mutant OTC proteins into isolated BALB/cByJ (SCAD−/−) mitochondria demonstrated that the mutant form of OTC was completely insoluble in Triton X-100 (Fig. 7B), whereas wild type OTC was presented mainly in the soluble fraction (70–80%; Fig. 7A). Accordingly, the insoluble accumulation of non-tetrameric SCAD intermediates may represent aggregates and not membrane-associated soluble species.

**DISCUSSION**

A variety of rare and common missense variations have been identified in patients with SCAD deficiency. Functional studies to date have suggested that although the variant enzymes would have catalytic impairment ranging from mild to complete, many of them are actually unstable in cells, potentially exacerbating concurrent functional defects. We have previously shown that an R147W variant SCAD fails to fold properly upon import into mitochondria, and preliminary experiments suggested that the abnormal protein was retained in the mitochondrial matrix in a soluble form associated with chaperonins, which was slowly converted into active enzyme compared with wild type SCAD (21). In the current study, we have investigated the folding of a variety of SCAD proteins harboring missense variations localized in different regions of the SCAD monomer (Fig. 1). These included five variations reported to cause complete SCAD deficiency (R22W, G68C, W153R, Q341H, and R359C) along with two common SCAD variations (R147W and G185S) thought to predispose to neuromuscular disease. Newly synthesized orthologous MCAD protein has been shown to interact with mitochondrial hsp70 chaperones (mhs70) for a short period of time following import into isolated mitochondria and is then quickly transferred to the hsp60/hsp10 chaperonin complex, which completes folding into the native structure (22). In contrast, a K304E variant MCAD remained complexed with hsp60 for longer periods of time (22, 23). We therefore hypothesized that derangements in interactions with the hsp60/hsp10 chaperonin system would play a crucial role in the abnormal folding of variant SCAD proteins. To eliminate the confounding problems of interaction of variant SCAD proteins with endogenous wild type protein from the mitochondria, we use mitochondria isolated from SCAD-deficient mouse liver cells for import experiments.

As anticipated, all of the SCAD proteins were found to interact with the mitochondrial chaperonin system upon import into mitochondria. As with other mitochondrial proteins, assumption of the proper final conformation of the SCAD upon import is likely to be accomplished through repeating rounds of folding in the cavity of the hsp60/hsp10 chaperonin ring complex with ATP-driven release of the folding intermediate. Variations that impair the ability of SCAD to fold properly would be expected to require additional rounds of hsp60/hsp10 mediated folding before either reaching the native conformation or being targeted to the mitochondrial protein degradation system. Folding of the R147W, G185S, and Q341H variant proteins was clearly temperature-sensitive, with higher levels of tetrameric SCAD enzyme being formed at a lower incubation temperature (Fig. 4). In contrast, the R22W, G68C, W153R, and R359C variants showed severely impaired folding irrespective of incubation temperature. These enzymes displayed an increased dependence on the hsp60 machinery and remained associated in hsp60 complexes or as other soluble folding intermediates of SCAD for a prolonged time, particularly the W153R variant (Fig. 4B). We have designated the former group of variant enzymes as mild folding variants, and the latter group folding-defective, and propose a model for the chaperone-assisted folding pathway of SCAD in the mitochondrial matrix (Fig. 8).

To further characterize these groups of variants, the turnover of the various SCAD proteins was studied with *in vitro* mitochondrial pulse-chase experiments, and comparison of the levels of soluble and insoluble SCAD protein following import (Fig. 6). The common R147W and G185S variants and the rare Q341H variant enzyme accumulated almost the same amount of SCAD protein as the wild type enzyme after 260 min chase at 37 °C; however, the ratios of tetrameric to non-tetrameric soluble protein were different. The G185S and Q341H proteins were both retained in non-tetrameric forms either associated with hsp60 or other soluble SCAD species longer than the wild type, again suggesting a decreased folding ability. At 41 °C, the

![Image](https://example.com/image.png)
Q341H variant showed increased intramitochondrial accumulations of insoluble protein compared with wild type SCAD; however, G185S was handled similar to the wild type. Turnover of R147W SCAD at 37°C was similar to wild type enzyme; however, as seen in Fig. 4 folding of the R147W variant was more efficient at lower temperatures. These observations are consistent with previous studies in COS-7 cells (17). Increasing the incubation temperature following import to 41°C resulted in elevated amounts of insoluble R147W SCAD protein (Fig. 6B). Classification of the R147W, G185S, and Q341H variant enzymes as mild folding variants appears to have some physiological relevance as some individuals with these genotypes are only occasionally symptomatic. Thus, they can be viewed as being conditionally related to SCAD deficiency.

The folding-defective variants (R22W, G68C, W153R, and R359C) demonstrated higher degradation rates than the wild type enzyme at 37°C, and the small amount of soluble SCAD protein present was all in a non-tetrameric form, indicating that they never acquire the native conformation. Increasing the incubation temperature after import to 41°C resulted in elevated amounts of insoluble R147W SCAD protein. These results suggest that the folding-defective SCAD enzymes are degraded by mitochondrial proteases at 37°C but form insoluble inclusions at higher temperatures. A similar phenomenon has been observed in a familial form of amyotrophic lateral sclerosis, an adult-onset neurodegenerative disease, caused by mutations in the Cu/Zn-superoxide dismutase (SOD-1) gene (38). Most mutant SOD-1 proteins were degraded by the proteasome pathway involved in the proteolysis of denatured or misfolded proteins; however, several of the mutant SOD-1 proteins had an increased tendency to precipitate in solution relative to wild type SOD-1, resulting in the formation of cytoplasmic aggregates. Moreover, the mutant SOD-1 proteins in the cytoplasmic aggregates were associated with cytoplasmic hsp70 chaperones. Similarly, it is possible that the insoluble variant SCAD proteins observed in our study may be associated with chaperones, which were unable to fold the variant proteins and/or to facilitate their degradation. The insoluble SCAD proteins could form aggregates, which may be of pathological interest in SCAD deficiency. Accumulation of unfolded mutant OTC protein within the mitochondrial matrix has been shown to result in a specific transcriptional up-regulation of nuclear genes encoding mitochondrial stress proteins, including hsp60 and hsp10, and ClpP proteases (25). As our experiments showed a similarity between the insoluble aggregates of SCAD and OTC-Δ proteins at 41°C, accumulations of unfolded variant SCAD protein may also induce a mitochondrial stress response. Studies are in progress to investigate whether cells from SCAD patients display increased expression levels of mitochondrial stress genes.

Milewski et al. (39) have suggested that the intracellular accumulation of misfolded proteins is a selective process determined by the sequences of the aggregating polypeptides, and demonstrated that substitution of two amino acids could eliminate the aggregation of a cystic fibrosis transmembrane conductance regulator-derived polypeptide. A theoretical study based on the acylphosphatase protein proposed that variations that specifically perturb the rate of aggregation are located in certain regions of the protein sequence, each of which has a relatively high hydrophobicity and propensity to form β-sheet structures (40). These properties are not significantly changed in the variant SCAD proteins (PepPlot; Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI), thus we would not predict that the insoluble SCAD inclusions are ordered β-sheet aggregates as seen in Alzheimer’s disease and in other neurodegenerative disorders (41–44). Instead precipitation of the variant SCAD proteins may arise from hydrophobic interactions of incompletely folded, misfolded, or even partially folded variant SCAD proteins that are augmented during thermal stress and other environmental conditions affecting the degree and rate of intermolecular association. Such disorganized assemblies have been defined by Dobson as “amorphous” structures (45).

In summary, the results from the present study indicate that impaired folding of the variant SCAD enzymes play an important role in the molecular pathogenesis related to these variations, and that the mitochondrial protein quality control system seems unable to handle some of these variant proteins appropriately. The intramitochondrial processing partitioned between chaperone-assisted folding, degradation by intracellular proteases and aggregation, and the competition of chaperones and proteases for non-native conformations determines whether or not the variant SCAD polypeptide will be degraded before it is properly folded. If correct folding fails and degradation is not initiated rapidly, non-functional conformations may form insoluble aggregates (Fig. 8). Additionally studies should provide important insights into the role of aberrant interactions with mitochondrial chaperonins, aggregation of variant proteins, and the effect of physiological stress on the development of disease related to SCAD variations.

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**Fig. 8. Proposed model for the SCAD folding pathway inside the mitochondrial matrix.** Newly imported SCAD polypeptides interact briefly with mhs70 chaperones followed by transferring to the mitochondrial hsp60/hsp10 chaperonin system. Depending on the nature of the SCAD mutation/variation, the unfolded SCAD polypeptides will achieve the native conformation after a number of cycles in the hsp60/hsp10 folding machinery. If the polypeptides are released from the chaperonin system without being converted into the native structure they remain in a pool of non-native intermediates. These SCAD intermediates may rebind the chaperone machinery for another attempt to fold or encounter a mitochondrial protease, thus being degraded and thereby removed from the intramitochondrial pool of non-native intermediates. Alternatively, if correct folding is unachievable and degradation is not initiated rapidly, the SCAD polypeptide may interact with other unfolded or partially folded SCAD species, leading to the formation of aggregates.
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REFERENCES
1. Ikeda, Y., Okamura-Ikeda, K., and Tanaka, K. (1985) J. Biol. Chem. 260, 1311–1329
2. Naito, E., Otsuka, H., Ikeda, Y., and Tanaka, K. (1989) J. Clin. Invest. 83, 1605–1612
3. Amendt, B. A., Greene, C., Sweetman, L., Cloherty, J., Shih, V., Moon, A., Teel, L., and Rhein, J. W. (1987) J. Clin. Invest. 79, 1303–1309
4. Seidel, J., Streek, S., Bollstedt, K., Vianey-Saban, C., Pedersen, C. B., Vockley, J., Korall, H., Roskos, M., Deufel, T., Trefz, K. F., Sewell, A. C., Kauf, E., Zinnl, F., Lehner, W., and Gregersen, N. (2003) J. Inherit. Metab. Dis. 26, 37–42
5. Coates, P. M., Hale, D. E., Finocchiaro, G., Tanaka, K., and Winter, S. C. (1988) J. Clin. Invest. 81, 171–175
6. Sewell, A. C., Herwig, J., Bolehs, H., Rinaldo, P., Bhala, A., and Hale, D. E. (1993) Eur. J. Pediatr. 152, 923–924
7. Bhala, A., Willi, S. M., Rinaldo, P., Bennett, M. J., Schmidt-Sommerfeld, E., Briones, P., Lamantea, E., Sentis, M., Barcelo, A., and Roig, M. (1998) Eur. J. Pediatr. 157, 317–329
8. Tres, F. A. (1999) Neurology 52, 366–372
9. Corydon, M. J., Vockley, J., Rinaldo, P., Rhein, W. J., Kjeldsen, M., Winter, V., Riggs, C., Babovic-Vukosavic, D., Stavaint, J. de Jong, J., Levy, H., Sewell, A. C., Roe, C., Matern, D., Haslam, R. H., Bennett, M. J., and Vockley, J. (2002) J. Clin. Invest. 109, 11126–11133
10. Corydon, T. J., Bruss, P., Jensen, T. G., Corydon, M. J., Lund, T. B., Jensen, U. B., Kim, J. J., Gregersen, N., and Bolund, L. (1998) J. Biol. Chem. 273, 13063–13071
11. Gregersen, N., Boris, A., Pedersen, C. B., Corydon, T. J., and Bolund, L. (2001) J. Inherit. Metab. Dis. 24, 189–212
12. Saio, T., Welch, W. J., and Tanaka, R. (1994) J. Biol. Chem. 269, 4401–4408
13. Yokota, I., Saio, T., Vockley, J., and Tanaka, K. (1992) J. Biol. Chem. 267, 26004–26010
14. Bross, P., Jespersen, C., Jensen, T. G., Andersen, B. S., Kristensen, M. J., Winter, V., Nandy, A., Krauie, F., Ghisla, S., and Bolundi, L. (1995) J. Biol. Chem. 270, 10284–10290
15. Zhao, Q., Wang, J., Levichkin, I. V., Stasinopoulos, S., Ryan, M. T., and Hoogenraad, N. (2002) EMBO J. 21, 4411–4419
16. Wood, P. A., Amendt, B. A., Rhein, W. J., Millington, D. S., Inoue, P., and Armstrong, D. (1989) Pediatr. Res. 25, 38–43
17. Bross, P., Winter, V., Pedersen, C. B., and Gregersen, N. (2003) in Protein Misfolding and Disease: Principles and Protocols (Bross, P., and Gregersen, N., eds) pp. 285–293, Humana Press Inc., Totowa, New Jersey
18. Gregersen, N. (1979) Pediatr. Res. 13, 1227–1230
19. Jorgensen, M. M., Jensen, O. N., Holst, H. U., Hansen, J. J., Corydon, T. J., Bross, P., Bolund, L., and Gregersen, N. (2000) J. Biol. Chem. 275, 33861–33868
20. Hartl, F. U. (1996) Nature 381, 571–579
21. Martin, J. (1997) J. Bioenerg. Biomembr. 29, 35–43
22. Ryan, M. T., Naylor, D. J., Hoj, P. B., Clark, M. S., and Hoogenraad, N. J. (1997) Int. Rev. Cytol. 174, 127–193
23. Fink, A. L. (1999) Physiol. Rev. 79, 425–449
24. Ostermann, J., Horwich, A. L., Neupert, W., and Hartl, F. U. (1989) Nature 341, 125–130
25. Maceroux, P., Sanner, C., Buttnar, H., Kieweg, V., Ruterjans, H., and Ghisla, S. (1997) Biochim. Biophys. Acta 1318, 1381–1385
26. Riet-Vosnisci, M., and Wilisson, K. R. (2000) J. Mol. Biol. 304, 81–98
27. McCormack, E. A., Rohman, M. J., and Wilisson, K. R. (2001) J. Struct. Biol. 135, 185–197
28. Shindler, G. A., Lacourse, M. C., Minotti, S., and Durand, H. D. (2001) J. Biol. Chem. 276, 12791–12796
29. Mlewecki, M. I., Mickle, J. E., Forrest, J. K., Stanton, B. A., and Cutting, G. R. (2002) J. Biol. Chem. 277, 34462–34470
30. Chiti, F., Taddei, N., Baroni, F., Capanni, C., Stefani, M., Ramponi, G., and Dobson, C. M. (2001) Nature 410, 137–143
31. Dobson, C. M. (2003) Philos. Trans. R. Soc. Lond. Biol. Sci. 358, 133–145
32. Martin, J. B. (1999) Nat. Engl. J. Med. 340, 1970–1980
33. Huang, T. H., Yang, D. S., Fraser, P. E., and Chakrabartty, A. (2000) J. Biol. Chem. 275, 36436–36447
34. Shastry, B. S. (2003) Neurochem. Int. 43, 1–7
35. Dobson, C. M. (2003) Nat. Rev. Drug Discov. 2, 154–160
36. Battaile, K. P., Molin-Case, J., Paschke, R., Wang, M., Bennett, D., Vockley, J., and Kim, J. J. (2002) J. Biol. Chem. 277, 12200–12207
37. Stuart, R. A., Grubber, A., van, d. K., I, Guiard, B., Koll, H., and Neupert, W. (1994) Eur. J. Biochem. 220, 9–18