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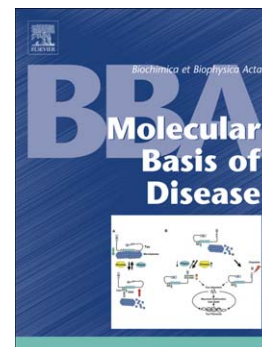
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Clinical and molecular characterization of five patients with Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency

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Running title: Five patients with SCOT deficiency

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Research Highlights

- Five patients with SCOT deficiency were reported.

- All developed multiple severe ketoacidosis but their outcome was good.

- Six missense mutations were analyzed by expression analysis of mutant cDNAs.

- Their effects were viewed from the tertiary structure of SCOT molecule.

- Main effects were destabilization of SCOT molecules; some also affected catalytic activity.

Abstract

Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency is an inborn error of ketone body metabolism and causes episodic ketoacidosis. We report clinical and molecular analyses of 5 patients with SCOT deficiency. Patients GS07, GS13, and GS14 are homozygotes of S405P, L327P, R468C, respectively. GS17 and GS18 are compound heterozygotes for S226N and A215V, and V404F and E273X, respectively. These mutations have not been reported previously. Missense mutations were further characterized by transient expression analysis of mutant cDNAs. Among 6 missense mutations, mutants L327P, R468C, and A215V retained some residual activities and their mutant proteins were detected in immunoblot analysis following expression at 37 °C. They were more stable at 30 °C than 37 °C indicating their temperature sensitive character. The R468C mutant is a distinct temperature sensitive mutant which retained 12% and 51% of wild-type residual activities at 37 °C and 30 °C, respectively. The S226N mutant protein was detected but retained no residual activity. Effects of missense mutations were predicted from the tertiary structure of the SCOT molecule. Main effects of these mutations were destabilization of SCOT molecules, and some of them also affected catalytic activity. Among 5 patients, GS07 and GS18 had null mutations in both alleles and the other three patients retained some residual SCOT activities. All 5 developed a first severe ketoacidotic crisis with blood gas pH <7.1, and experienced multiple ketoacidotic decompensations (two of them had seven such episodes). In general, the outcome was good even following multiple ketoacidotic events. Permanent ketosis or ketonuria is considered a pathognomonic feature of SCOT deficiency. However, this condition depends not only on residual activity but also on environmental factors.

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Key words: ketoacidosis, OXCT, mutation, SCOT, succinyl-CoA:3-ketoacid CoA transferase, structure-function analysis

1. **Introduction**

Ketone bodies, produced mainly in the liver, are an important source of energy for extrahepatic tissues [1]. Succinyl-CoA: 3-ketoacid CoA transferase (SCOT; EC 2.8.3.5, gene symbol OXCT1), is a mitochondrial homodimer essential for ketone body utilization. SCOT catalyzes acetoacetate activation to acetoacetyl-CoA in mitochondria. The human OXCT1 gene is mapped to 5p13 and consists of 17 exons [2,3]. Human SCOT cDNA encodes a precursor subunit of 520 amino acids.

Patients with SCOT deficiency (OMIM 245050) experience episodic ketoacidosis and are usually asymptomatic between episodes. Fewer than 30 affected individuals are known [2-22]. Urinary organic acid analysis and acylcarnitine analysis show non-specific profiles in this disorder. Hence, in vitro methods of diagnosis, such as enzyme assay and mutation analysis are essential for the definite diagnosis. Permanent ketosis or ketonuria is a pathognomonic feature of this disorder but is not always present [17, 20, 22]. We previously identified 11 mutations of the OXCT1 gene [6, 8-14] in 12 SCOT-deficient families.

Recently, the human SCOT tertiary structure has become available (PDB entry 3DLX). This has enabled us to evaluate effects of missense mutations more precisely than homology modeling using porcine SCOT

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structure [20] .

We herein describe 5 SCOT-deficient patients and characterize their mutations by transient expression analysis of mutant cDNAs and discuss the mutation sites on the tertiary structure of human SCOT.

2. Patients, materials and methods.

2.1 Patients

GS07: Clinical findings of GS07 have been reported previously [11]. Briefly, he presented with two episodes of ketoacidosis during infections at 17 and 25 months of age.   He was followed until age 6 years without any further episodes.

GS13: Some aspects of GS13 have already been published [14]. Although parents are not known to be related, the family originates from a small socially isolated area, making consanguinity not unlikely. After an initial severe crisis at age 6 months, the girl had three additional, but milder crises during infancy. After six hours of fasting during a test performed after the first crisis, her blood pH was 7.42, HCO_3 12.5 mmol/L and BE -9.1 mmol/L, with high serum ketones 3.55 mmol/L and low FFA of 0.29 mmol/L. At the age of 12.5 years she is now doing well, attending a regular school. She usually takes her last meal at about 10 p.m. and takes first morning meal in school at about 9 to 10 a.m. without any signs or symptoms. Acid-base balance was checked three times at about 8 a.m. and was always normal.

GS14: This female patient of 21 years of age was born to consanguineous parents in October 1988. She had many hospitalizations in the pediatric ward for episodes of hypoglycemia. Her first hospitalization was at 19

months for seizures and coma. On physical exam she had deep respiration. Her blood glucose was 1.3 mmol/L, She had severe metabolic acidosis with pH 6.93, HCO_3 6 mmol/L and ketonuria 3+. She had six similar episodes of hypoglycemia with ketoacidosis without seizures, usually after episodes of infections. Her neuro-developmental status was normal. She had persistent ketonuria between episodes of decompensation.

GS17: She is a Caucasian girl born to non consanguineous parents at full-term with a birth weight of 2230 g. She had low blood glucose levels of 1.6mmol/L on the first day of life and received intravenous glucose for 1-2 days. She was discharged on day five of life. She was well until 3 years of age when she developed tachypnea and lethargy following gastroenteritis. Her blood pH was 6.99 with BE -25 mmol/L and her blood glucose was 7.5mmol/L. Urine analysis showed massive ketones. She was intubated and admitted to the intensive care unit. She responded to intravenous glucose and bicarbonate and was discharged after 7 days. Six months later she was readmitted to a local hospital with mild lethargy, tachypnea and ketoacidosis which developed during a febrile upper respiratory infection. The patient's mother reported that the patient has had trace to moderate ketonuria, even when she was in good health. SCOT deficiency was confirmed by an enzyme assay using fibroblasts. The patient has not had any more episodes of ketoacidosis for 6 years. She is doing well and receives a mildly protein-restricted diet (2.0 g/kg/day), avoids prolonged fasting and adheres to "sick day" precautions such as increasing calories from carbohydrates in her diet and intravenous glucose as needed.

GS18: The male patient, first child of healthy non-consanguineous parents from Vietnam, was born at 39 weeks with a birth weight of 3390 g. After

a normal clinical presentation during the first days of life he was readmitted to hospital at the age of 3 days with polypnea. Biochemically he presented with severe metabolic acidosis (pH 7.08, pCO_2 25 mmHg, BE –22.6 mmol/l) and pronounced ketonuria. Beside ketones, metabolic screening revealed unremarkable urinary organic acids. With intravenous fluid with sodium bicarbonate the patient recovered within hours. During the first year of life the patient was hospitalized three times because of episodes of severe ketoacidosis (minimal pH 6.98, pCO_2 15 mmHg, BE -28mmol/l). At the age of 1 year, SCOT deficiency was confirmed by an enzyme assay using his lymphocytes and platelets. He was on treatment that consisted of avoidance of prolonged fasting and moderate protein restriction (1.5 g/kg/d). Subsequently the patient has had three more severe episodes of ketoacidosis in the course of intercurrent diseases. Otherwise, he has permanent mild ketonuria. At his present age of 10 years psychomotor and physical development are normal.

Table I summarizes the clinical presentations and laboratory data of these 5 SCOT deficient patients. This study has been approved by The Ethical Committee of Graduate School of Medicine, Gifu University.

2.2 Enzyme assay and immunoblot analysis

Assays for acetoacetyl-CoA thiolase and for SCOT were performed as described [7, 23], using acetoacetyl-CoA as a substrate and measuring its disappearance spectrophotometrically.

2.3 Mutation analysis

Total RNA was purified from peripheral blood mononuclear cells with an

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ISOGEN kit (Nippon Gene, Tokyo, Japan). RT-PCR was as described [2]. Mutations were detected by amplifying cDNA spanning the full-length coding sequence, and sequencing more than 5 clones.

Genomic DNA was purified with a Sepa Gene kit (Sanko Junyaku, Tokyo, Japan). Mutation analysis at the genomic level was done by PCR for each exon and its intron boundaries (at least 20 bases from the exon/intron boundaries for both directions) followed by direct sequencing [3].

2.4 Construction of eukaryote transient expression vectors

Wild-type full-length SCOT cDNAs [3] were subcloned into the pTZ18U and pCAGGS eukaryote expression vectors [24] and designated the pTscotWild-type and pCAGGSscotWild-type, respectively. Mutations were introduced into the pTscotWild-type using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla CA), confirmed by sequencing, and then transfered into pCAGGS.

2.5 Transient expression analysis

Wild-type and mutant SCOT expression vectors (4 μg) were first transfected using Lipofectamine 2000 (GIBCO BRL Invitrogen Inc., Carlsbad, CA) in ~10^5 SV40-transformed SCOT-deficient fibroblasts of GS01[2]. One μg of the cytosolic acetoacetyl-CoA thiolase (CT)-expressing vector, pCAGGSct [25], was cotransfected to monitor transfection efficiency. Transfection was done at 37 °C for 24 hours was followed by a further 48-hour incubation at 37 °C or 30 °C. The cells were harvested and stored at -80 °C until SCOT and CT activities were assayed.   Immunoblot was done using a mixture of anti-[human SCOT]

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antibody and anti-[human CT] antibody as the first antibody [26]. The quantity of mutant protein was estimated densitometrically, and was compared to the signal intensities of serially diluted samples of the wild-type SCOT protein .

2.6 Tertiary structural model of human SCOT

To analyze the putative structural implications of the SCOT mutations, the recently determined crystal structure from the Structural Genomics Consortium (PDB entry 3DLX) of human SCOT was taken as a starting point. Prior to the analysis, the structure was subjected to further refinement in PHENIX [27] and COOT [28], including the addition of missing side chains and rebuilding of the solvent network. The figures describing the structural details were prepared with PyMOL.

3. Results and Discussion

3.1 Enzyme assay.

Enzyme assay data for 4 patients are shown in Table 2. All four patients' fibroblasts presented with decreased SCOT activity, whereas they had a potassium-ion activated acetoacetyl-CoA thiolase activity which was a specific character of mitochondrial acetoacetyl-CoA thiolase (T2). In immunoblot analysis, SCOT protein was scarcely detected in these patients' cells whereas T2 protein was clearly detected (data not shown). Lymphocytes and platelets from GS18 had no apparent SCOT activity (data not shown). These results confirmed the diagnosis of SCOT deficiency in the 5 patients.

3.2 Mutation analysis

Both genomic mutation analysis and cDNA analysis were done in all the cases except for GS18 of whom RNA was not available. The results of mutation analyses are shown in Table 1. Three patients with definite or possible consanguinity had homozygous mutations (c.1213T>C (S406P) in GS07; c.980T>C (L327P) in GS13; c.1402C>T (R468C) in GS14). GS17 is a compound heterozygote of c.644C>T (A215V) from the father and c.677G>A (S266N) from the mother. GS18 is also a compound heterozygote of c.817G>T (E273X) from the father and c.1210G>T (V404F) from the mother. We also detected three single nucleotide polymorphisms. Among them, c.173C>T (T58M) (rs75134564) was previously identified in a Japanese patient (GS02) and demonstrated not to reduce enzyme activity [13].

3.3 Transient expression analysis of mutant cDNAs

We performed transient expression analysis of wild-type and mutant cDNAs in SCOT-deficient SV40-transformed fibroblasts. Following expression of SCOT cDNAs for 48h at either 37 °C or 30 °C, an enzyme assay and immunoblots were performed (Fig. 2). The transfection of wild-type SCOT cDNA produced high SCOT activity, whereas that of mock cDNA produced no demonstrable enzyme activity at any temperature. Among 6 missense mutations, S226N, V404F, and S405P did not retain   residual SCOT activity. The A215V, L327P and R468C mutants retained detectable residual activities, 3.5, 4.7 and 12% of the wild-type value, respectively, in expression at 37 °C. Their relative residual SCOT activities to wild-type in expression at 30 °C were 3-4 fold higher than those in expression at 37 °C. In particular, R468C mutants retained a 51%

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activity of wild-type value in the expression at 30 °C. In immunoblot analysis, V404F and S405P protein was not detected in expression at 30°C and 37°C. S226N protein was clearly detected in the expression at 30 °C without any detectable residual activity, indicating that S226N protein was an inactive protein. The relative amount of the A215V, L327P, and R468C mutant proteins, as compared to the wild-type, was estimated to be 30%, 30%, and 50%, respectively, in expression at 30 °C. These proteins were more stable at 30°C than at 37°C. Specific activities (activity/protein) of A215V, L327P, and R468C mutants could be calculated to about 50%, 50%, and 100% that of wild-type, respectively.

3.4 Tertiary structural model of human SCOT and mutations

A number of mutations have been characterized for SCOT deficiency, and several of them have been structurally analyzed before [3, 20, 22], based on homology models of human SCOT, made with the help of the pig SCOT crystal structure [20]. Figure 2A shows a dimer of human SCOT, with presently and previously identified mutations highlighted on the SCOT monomer in Figure 2B. It is noteworthy that most of the mutations are located around two 'hot-spots' in 3D space; these areas correspond to a small beta sandwich domain in the N-terminal lobe, and a larger beta sandwich structure close to the C terminus. Sporadic mutations are also seen closer to the active site cavity.

The mutation A215V involves the residue A215, which in the wild-type protein is in the middle of a beta sheet, pointing inwards into the protein. The terminal carbon atom of A215 is only 3.6 Å away from the terminal methyl group carbons of L269 in an opposing beta sheet. Thus, even a small valine residue

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cannot be incorporated into this position without structural strain and changes. The position is located at a small beta sandwich domain involved in SCOT dimer formation. A215 is, furthermore, in the very close vicinity of the previously characterized SCOT mutations G219E and V221M [3]. These observations on the tertiary structure are in accord with the results that the main mutant effect of A215V is the instability of the mutant protein since the mutant protein amount was 12.5% and 30% relative to wild-type at 37°C and 30°C, respectively.

S226 is located close to the dimerization interface, although not being directly involved in it. The side chain is hydrogen-bonded to the backbone carbonyl of D362 and *via* a buried water molecule to N345; N345 is vicinal to the crucial catalytic residue E344 (Figure 2D). Thus, the S226N mutation is likely to disturb the structure at least locally, and could also affect the properties of the catalytic site. As expected from the view of tertiary structure, the S226N mutant protein was revealed to be unstable and non functional protein was detected in transient expression analysis

L327 locates to an alpha helix on the SCOT surface, close to the active site entrance (Figure 2D). The side chain of L327 is solvent-exposed and disordered in the crystal. This helix could form part of the CoA substrate-binding site, and a proline mutation in the central part of this helix may both perturb the helical structure and affect the functional mobility of this region, especially since the neighbouring residue (326) is also a proline. A main mutant effect of L327P is the instability of the mutant protein since the mutant protein amount was 12.5% and 30% relative to wild-type at 37°C and 30°C, respectively.

V404 locates to the C-terminal beta sandwich domain of SCOT (Figure 2E). The side chain points inwards, into a tightly packed hydrophobic core

between two beta sheets. There clearly is no room for a large Phe residue, as there are several short distances from the side chain of V404 to residues from the opposing beta sheet. S405 is neighboring V404, at the end of a beta strand, preceding a short tight beta turn (Figure 2E). In light of this, the side chain hydroxyl group is within hydrogen bonding distance from three main-chain NH groups from residues 407-409. Such an arrangement would be completely destroyed upon mutation of residue 405 to proline. As expected from the tertiary structure, these mutant proteins were too unstable to detect in either the 37 °C or 30 °C expression.

R468 is an exposed residue, present in the beta sheet opposite to that harboring V404 and S405. Its side chain is a central residue in a salt bridge network with E488, R308, E312, K436, and D477 (Figure 2F). Mutation of the R468 residue will be detrimental to this large hydrophilic region on the surface of the C-terminal domain of SCOT. Since its specific activity is similar to wild-type, R468C does not affect catalytic activity; the active site residues are far from this mutation. The main mutational effect of R468C is also instability of the molecule.

3.5 Clinical phenotypes and genotypes.

We reported herein 5 SCOT deficient patients and their clinical and molecular aspects are summarized in Table 1. They developed the first ketoacidotic episodes from 3 days of age to 3 years of age. The episodes were associated with very severe metabolic acidosis with blood pH ranging from 6.90 to 7.08. They all recovered from the first ketoacidotic crises and were well managed after the diagnosis of SCOT deficiency was made. We previously reported clinical and molecular characters for 12 SCOT deficient families and

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now have added to those 5 more

Permanent ketosis or persistent ketonuria is pathognomonic feature of SCOT deficiency. We, however, previously showed that SCOT-deficient patients with "mild" mutations may have no permanent ketosis. V221M, R268H, or T435N homozygotes and T435N/null mutation compound heterozygotes did not show permanent ketosis or permanent ketonuria [3, 17, 20, 22]. V221M, R268H and T435N mutations retained 10%, 34% and 25%, respectively, relative activity to wild-type in 37 °C expression using the same expression system. In the present study, L327P and R468H mutations retained 4.7% and 12%, respectively, relative activity in 37 °C expression. A L327P homozygote, GS13, did not have permanent ketonuria but a R468H homozygote, GS14, did. To our knowledge, patients with null mutations all showed permanent ketonemia or ketonuria. Hence "mild" mutation with residual activity may be a necessary condition but not a sufficient condition for the absence of permanent ketonemia or ketonuria. Environmental factors may also affect a clinical phenotype of persistent ketonuria.

Acknowledgment

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Figure legends

Figure 1.   Transient expression results for wild-type and mutant SCOT cDNAs. Wild-type and mutant SCOT expression vectors (4 µg) were transfected together with 1 µg of the cytosolic acetoacetyl-CoA thiolase (CT)-expressing vector, pCAGGSct, to SV40-transformed fibroblasts of GS01 of which the mutation is S283X/S283X. Transient expression was done 37 °C and 30 °C . Mock, transfection of 1 µg of pCAGGSct and 4 µg of pCAGGS vectors without insert. A) SCOT activities relative to those in wild-type transfection are shown. The mean values are displayed together with the SD of three independent experiments. B) Immunoblots for SCOT and CT are shown. The protein amounts applied are shown above the lanes. The first antibody was a mixture of an anti-CT (cytosolic thiolase) antibody and anti-SCOT antibody. The positions of the bands for CT and SCOT are indicated by arrows.

Figure 2.   Mutation sites on the tertiary structure of SCOT monomer

A) An overall structure of human SCOT dimmer (PDB entry 3DLX). B) A monomer of human SCOT. The N and C termini are labelled. The two clusters of mutations are indicated by ellipsoids. The positions of the mutations identified in this study are in red, and the ones previously identified are shown in yellow. The position of the catalytically active glutamate residue 344 is marked with a blue sphere.

C) The environment of the A215V mutation in the tightly packed hydrophobic core of the small beta sandwich. The N terminus of the crystal structure is at the bottom front.

D) S226 lies close to the active site, and interacts with N345 via a water-mediated hydrogen bond (green) and by van der Waals interactions. The catalytic residue is E344. L327 also lies close to the entrance of the catalytic cavity and could be involved in substrate binding.

E) V404 and S405 are next to each other, V404 being buried at the hydrophobic core of a beta sandwich unit. S405 interacts with the backbone amides of a tight turn (blue).

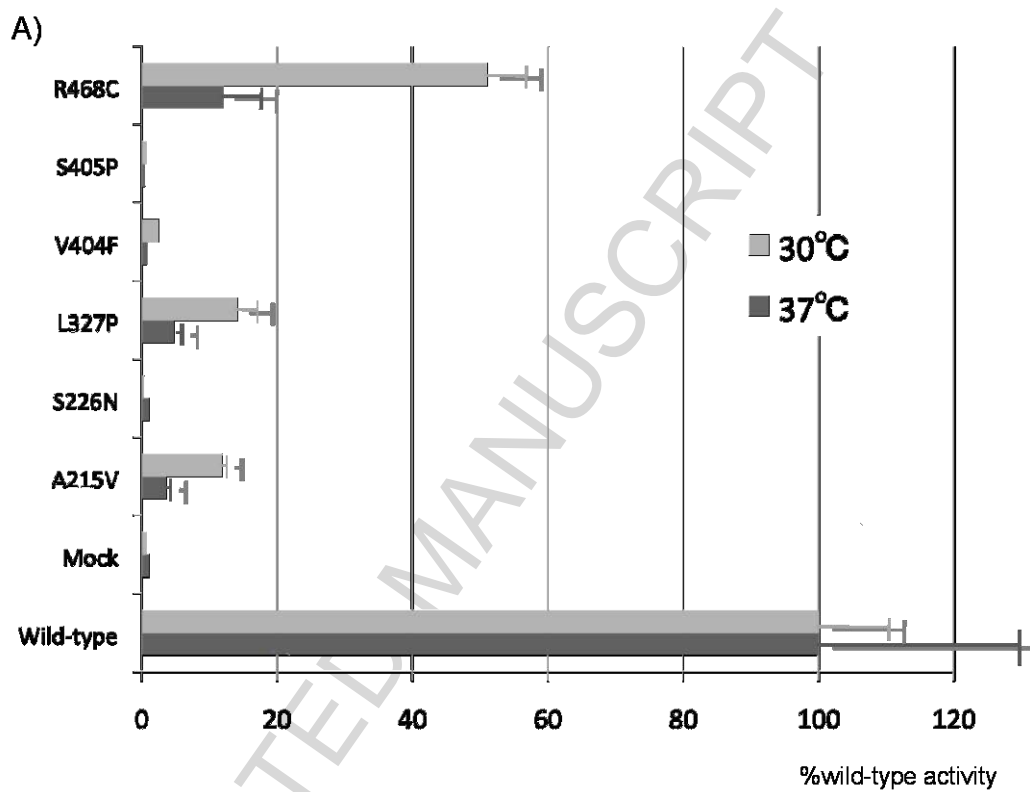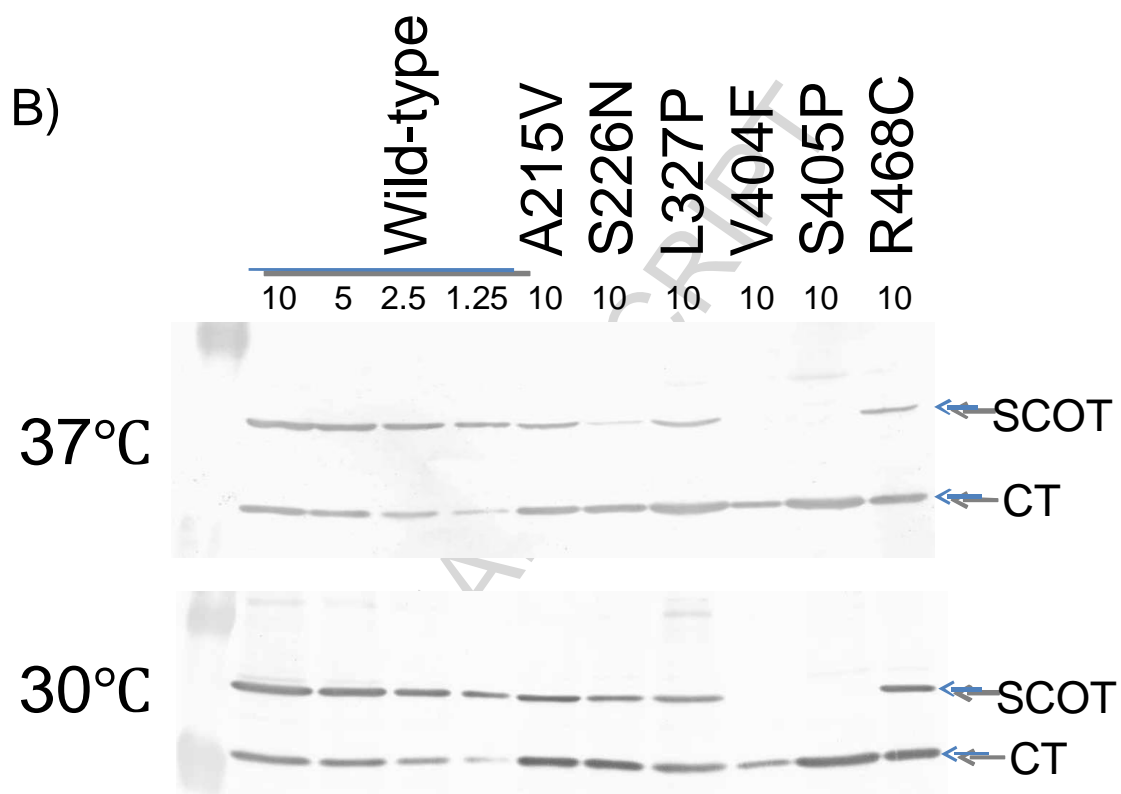F) R468 plays a central role in a salt bridge network linking a beta sheet and a helix.
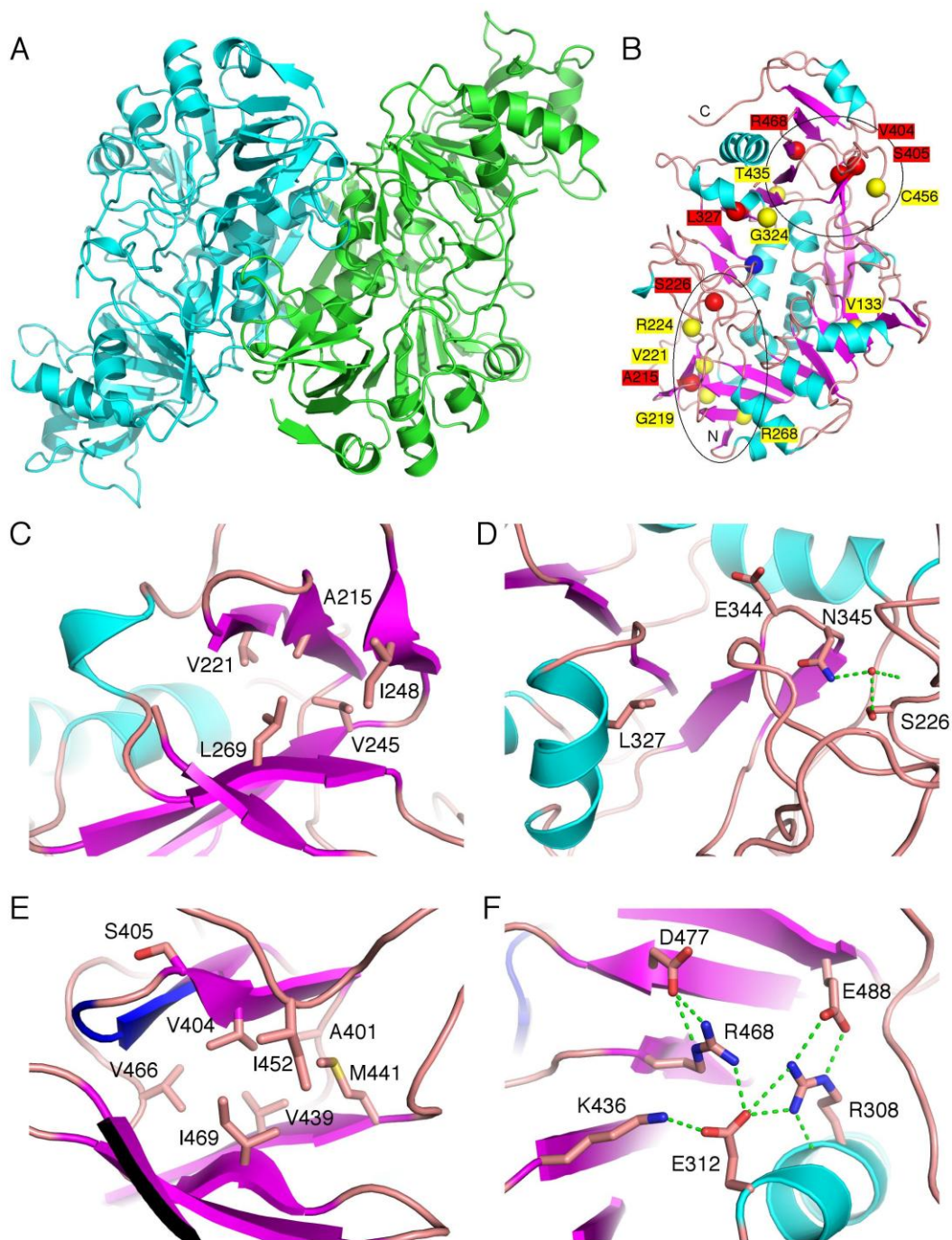
Fig. 1a

Fig.1b

Fig. 2

Table 1. Clinical presentation and mutation of SCOT deficient cases

NP: nothing particular
+ possible consanguinity
***GS18 is Vietnamese*

Table 2. Enzyme assay using fibroblasts

	acetoacetyl-CoA thiolase			SCOT	SCOT/+K⁺
	-K⁺	+K⁺	+K⁺/-K⁺		
GS 0 7	5.9	12.9	2.2	1.9	0.2
GS 1 3	4.3	9.1	2.1	0.8	0.1
GS 1 4	3.6	7.3	2.0	1.2	0.2
GS 1 7	7.0	14.9	2.1	1.2	0.1
Controls (n=5)	5.0±0.7	10.8±0.9	2.2±0.3	6.7±2.1	0.6±0.2

Enzyme activity is expressed as nmol/min/mg protein

Fibroblasts from GS18 were not available.

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