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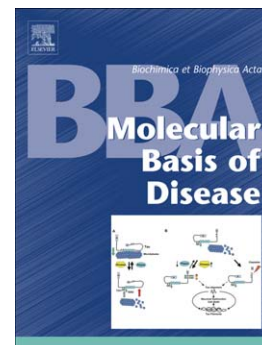
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**The Molecular Basis of Aminoacylase 1 Deficiency**

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**Abstract**

Aminoacylase 1 (ACY1) is a zinc-binding enzyme which hydrolyzes *N*-acetyl amino acids into the free amino acid and acetic acid. Deficiency of ACY1 due to mutations in the *ACY1* gene follows an autosomal-recessive trait of inheritance and is characterized by accumulation of *N*-acetyl amino acids in the urine. In affected individuals neurological findings such as febrile seizures, delay of psychomotor development and moderate mental retardation have been reported. Except for one missense mutation which has been studied in *Escherichia coli*, mutations underlying ACY1 deficiency have not been characterized so far. This has prompted us to approach expression studies of all mutations known to occur in ACY1 deficient individuals in a human cell line (HEK293), thus providing the authentic human machinery for posttranslational modifications. Mutations were inserted using site directed mutagenesis and ACY1 enzyme activity was assessed in cells overexpressing ACY1, using mainly the natural high affinity substrate *N*-acetyl methionine. Overexpression of the wild type enzyme in HEK293 cells resulted in an approximately 50-fold increase of the ACY1 activity of homogenized cells. Most mutations resulted in a nearly complete loss of enzyme function. Notably, the two newly discovered mutations p.Arg378Trp, p.Arg378Gln and the mutation p.Arg393His yielded considerable residual activity of the enzyme, which is tentatively explained by their intramolecular localization and molecular characteristics. In contrast to ACY1 variants which showed no detectable ACY1 activity, ACY1 proteins with the mutations p.Arg378Trp, p.Arg378Gln and p.Arg393His were also detected in Western blot analysis. Investigations of the molecular bases of additional cases of ACY1 deficiency contribute to a better understanding of this inborn error of metabolism whose clinical significance and long-term consequences remain to be elucidated.

### Research Highlights

- Clinical and biochemical data on aminoacylase1 (ACY1) deficiency are expanded.
- Transient overexpression of all known mutations underlying ACY1 deficiency.
- Most mutations result in nearly complete loss of enzyme function.
- Newly discovered mutations yield considerable residual activity of the enzyme.
- Intramolecular localizations and molecular characteristics can explain residual activities.

**Keywords:** aminoacylase, amidohydrolase, amino acid, organic acid, inborn error of metabolism, *N*-acetyl amino acid, *N*-acetyl methionine, *N*-acetyl glutamic acid

## 1. Introduction

*N*-acylation of a protein usually leads to extension of its half life. 50 % to 80 % of all cellular proteins show formylated or acetylated *N*-termini [1]. Following the degradation of proteins, free amino acids can be recycled by enzymatic hydrolysis of *N*-acylated amino acids catalyzed by aminoacylases [2;3]. Aminoacylases are evolutionary conserved cytosolic zinc-binding metalloenzymes which belong to the M20 family [4;5].

Aminoacylase 2, also known as aspartoacylase (ASPA; EC 3.5.1.15) hydrolyzes specifically *N*-acetyl-L-aspartate. ASPA deficiency causes spongy degeneration of the brain known as Canavan disease [6]. Canavan disease is a leukodystrophy leading to severe impairment and, reportedly, often to death in early childhood [7]. Patients show increased urinary levels of *N*-acetylaspartic acid.

In contrast to aminoacylase 2, aminoacylase 1 (ACY1; EC 3.5.1.14) (encoded by the *ACY1* gene) has a wide substrate specificity, comprising many *N*-acylated amino acids [8;9]. In agreement with this, genetic deficiency of aminoacylase 1 has recently been discovered in several children with accumulation of *N*-acetylated amino acids in urine. They were identified by organic acid analysis in urine, which is part of the diagnostic procedure if an inborn error of metabolism is suspected [10;11]. *ACY1* is highly expressed in human kidney and brain. The majority of patients reported so far as being affected by ACY1 deficiency, presented with neurological features like psychomotor delay or febrile seizures [10;11]. ACY1 deficiency has also been described in a patient with autistic behaviour [12]. All patients with a characteristic pattern of urinary metabolites showed strongly decreased activity of ACY1 in lymphocytes/fibroblasts and presented with biallelic mutations in the *ACY1* gene. Five different mutations in the coding region of the *ACY1* gene could be identified by mutation analysis [10,11;13]. However, expression analyses of mutated ACY1 enzyme have not been performed so far with the exception of a single missense mutation (c.1057 C>T, p.Arg353Cys), which has been studied in *Escherichia coli* [11].

Pittelkow et al have suggested that porcine ACY1 (pACY1) protein has two glycosylation sites, although the presence of carbohydrates in the purified enzyme has not been demonstrated yet [14]. pACY1 presents with acetylation as a posttranslational modification of the alanine residue following the starter methionine [15]. The protein sequences of human ACY1 and pACY1 show 85 % homology [5], but posttranslational glycosylation patterns have not been investigated for human ACY1 so far. The human ACY1 protein shows one possible glycosylation site in the peptide dimerization domain (Asn-Lys-Thr) and two glycosylation sites in the C-terminal peptidase domain (Asn-Lys-Thr; Asn-Arg-Thr), respectively [16]. Since human ACY1 reference protein has not been crystallized yet, assumptions on its structure are largely based on information on the porcine enzyme, on a crystallized

human ACY1 T347G mutant and on the high percentage of homology of human and pig ACY1 [5;17;18;].

In view of possible posttranslational modifications of ACY1, which could be lost in a different expression system, characterization of the previously reported mutations was now approached using a mammalian system. Here, we report expression studies in a human cell line (HEK293) of all five known mutations identified in ACY1 deficient individuals, thus providing the authentic machinery for posttranslational modifications. In addition, three newly diagnosed patients with ACY1 deficiency are described, whose *ACY1* mutations are included in the expression studies.

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## 2. Patients, Material and Methods

### 2.1 Patients

For most mutations studied here, patients with ACY1 deficiency have been reported previously [10-13]. However, case descriptions of three newly identified individuals expand both the clinical picture and the information on mutations in *ACY1*.

AC007 II-1 is the first child of reportedly non-consanguineous parents originating from Liberia. Following several episodes of febrile seizures the boy presented at 4.5 years with severe mental retardation, autistic features and lack of speech development. When he was 6 years and 9 months old, cerebral imaging revealed bihemispheric, frontally accentuated, cortical dysplasia with gray matter heterotopia.

AC014 II-1 is the fifth child of consanguineous parents originating from the Lebanon. The girl presented with psychomotor retardation (started walking at 2.5 years, only few words of active speech at 3 years). Her siblings are healthy.

AC013 II-1: The seven-year-old daughter of consanguineous Turkish patients presented with mental retardation, short stature (1<sup>st</sup> percentile) and adiposity (body-mass index 93<sup>rd</sup> percentile). Metabolic studies were initiated after the patient had complained because of pain in her left hip.

All three individuals were identified by the assessment of urinary organic acids which is routinely performed as part of a metabolic work-up if an inborn error of metabolism is considered.

### 2.2 Mutation analysis

Genomic DNA was extracted directly from peripheral blood or from Epstein-Barr virus (EBV) transformed lymphocytes using by standard methods. Mutation analysis followed essentially the description by Sass et al. 2006 [10].

Some primers were redesigned using *Primer3* software (<http://frodo.wi.mit.edu/primer3>) and were purchased from *Eurofins MWG/OPERON*. All exons and exon-intron boundaries of the *ACY1* gene from patient DNA samples were amplified by PCR and prepared for bidirectional sequencing by a commercial provider (*LGC Genomics*, Berlin, Germany). DNA sequences were analyzed using *BioEdit* software (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>).

### **2.3 Restriction fragment length analysis**

In order to screen for the mutation c.1156C>T (p.Arg386Cys) in exon 15 of the *ACY1* gene, a 600 bp PCR product was amplified using primers Ex15\_RV\_F (5'-CTGCCACTGACAACCGCTAT-3') and Ex15\_RV\_R (5'-GGTGGCCACTCAAATACCAA-3'), followed by a restriction digest using BtgI (*New England Biolabs*) at 37 °C, 20 h. DNA fragment size was verified by agarose gel electrophoresis. For comparison, in addition to samples of *ACY1* deficient patients and their parents, samples of 105 control individuals were studied who usually originate from Southwestern Germany.

### **2.4 Cloning and site-directed Mutagenesis**

A pOTB7 *ACY1* cDNA clone (MGC-2251) carrying chloramphenicol resistance and the gene for human *ACY1* was purchased from the *American Type Culture collection* (ATCC). The human *ACY1* cDNA consists of 1415 base pairs (bp) with 61 bp and 188 bp untranslated sequence at the 5' and the 3' end, respectively. The *ACY1* cDNA sequence was cloned from the pOTB7 *ACY1* cDNA clone into the mammalian expression vector pcDNA3.1/+ (*Invitrogen*) by using the restriction sites EcoRI and XhoI. The pcDNA3.1/+ plasmid provides ampicillin resistance for selection. The restriction digest with EcoRI and XhoI was carried out at 37 °C, 2 h. Subsequently, the endonucleases were inactivated by heating the reaction mix at 80 °C, 20 min, followed by gel extraction using *QIAquick Gel Extraction Kit* (*Qiagen*). Successful cloning was verified by colony PCR using the primers pcDNA3.1+\_F 5'-CGGTGGGAGGTCTATATAAGCA-3' and pcDNA3.1+\_R 5'-TTAGGAAAGGACAGTGGGAGTG-3'.

All six mutations in the *ACY1* gene which have been reported previously [10; 11; 13] as well as two novel mutations were inserted into the pcDNA3.1/+ *ACY1* cDNA clone using the *Stratagene QuickChange Site-Directed Mutagenesis Kit*. Mutagenesis primers were designed using *QuickChange T<sub>m</sub> calculator* (<https://www.genomics.agilent.com/>). Plasmid DNA was prepared using *Qiagen kits*.

### **2.5 Cell culture and transfection**

Expression studies for ACY1 were performed in HEK293 cells, transformed human embryonic kidney cells. HEK293 cells were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) and subcultured in DMEM (Dulbecco's Modified Eagle's Medium) from Gibco supplemented with 10 % heat-inactivated fetal bovine serum, 100 units/ml penicillin G (sodium salt), 100 µg/ml streptomycin sulfate and 25 µg/ml amphotericin B (Gibco). Transfections of 10 µg plasmid DNA (including mutants and controls) into HEK293 cells were carried out using Xfect transfection reagent (Clontech) according to manufacturer's instructions.

## 2.6 ACY1 enzyme activity

ACY1 enzyme activity was assessed in homogenized EBV-transformed lymphocytes and HEK293 cells using on a ninhydrin-based photometric assay described by Mitz and Schlueter et al. 1958, which was adapted to cell homogenate probes and to a microscale reaction [19]. Substrate *N*-acetyl-L-methionine was used at a final concentration of 2 mM, substrate *N*-acetyl-L-glutamate was used at 100 mM. The absorption of the reaction products of released amino acids was determined at 570 nm with the Synergy HT photometer (BIOTEK). Gen5 1.6 software was used for data evaluation.

## 2.7 SDS-PAGE and Western Blot analysis

ACY1 expression was studied on the protein level by SDS-PAGE followed by Western blot analysis. The protein concentrations of the samples were determined by the Lowry method using bovine serum albumin as the standard [20]. The Precision Plus Protein™ standard (BIORAD) was used to estimate the protein sizes. Proteins were transferred onto a polyvinylidene fluoride membrane using a Trans-Blot® semi dry blot system (BIORAD). Anti-ACY1 antibody produced with a peptide consisting of amino acids 89-336 of human ACY1 was used at a dilution of 1:1,000 (GeneTex, Inc.; GTX110348). The secondary antibody (goat anti-rabbit IgM + IgG) was obtained from Southern Biotech (4010-05) and used at a dilution of 1:8,000. The comparison of the intensities of the different ACY1 wild type (WT) and ACY1 mutant protein bands was performed using the ImageJ (<http://rsbweb.nih.gov/ij/>) software program. The intensity of the ACY1 WT protein band was set to 100 %.

### 3. Results

#### 3.1 Patients

All patients studied presented with abnormal patterns of urinary organic acids as assessed by gas chromatography-mass spectrometry.

Notably, in the three newly identified patients abnormalities in the metabolite pattern (accumulation of *N*-acetylated amino acids) were much less pronounced than in previously reported patients. While *N*-acetyl-L-glutamate remained detectable in the urine (except for one organic acid pattern of patient AC014 II-1, which was considered completely normal), *N*-acetyl-L-methionine, previously considered another sensitive marker for ACY1 deficiency, was not detectable in the urine samples of these children.

#### 3.2 Mutation analysis

Two novel point mutations (c.1132C>T [p.Arg378Trp]; c.1133G>A [p.Arg378Gln]) were identified in exon 15 of the *ACY1* gene of patients AC013 II-1 and AC014 II-1, respectively. Patient AC007 II-1 showed a single point mutation at the nucleotide position 1156 of the *ACY1* gene (c.1156C>T [p.Arg386Cys]) (Table 1). Our results suggest homozygosity for the specific mutations in all three cases. Full sequencing of the coding region of *ACY1* revealed no further deviation from the reference sequence. For patient AC014 II-1, the assumption of homozygosity was supported by the detection of heterozygosity for the specific *ACY1* mutation c.1132C>T (p.Arg378Trp) in the DNA of the parents. No DNA of the parents of patients AC007 II-1 and AC013 II-1 was available for analysis, but in view of known consanguinity or at least a common geographical background of the parents homozygosity appears plausible in those cases as well, although it was not formally proven.

#### 3.3 Expression studies and Western blot analysis

The ACY1 wild type (WT) protein was expressed in HEK293 cells and yielded approximately 50-fold higher ACY1 enzyme activity in cell homogenates than cells transfected with the pcDNA3.1/+ mock vector.

In contrast, the mutants ACY1 Arg197Trp, ACY1 Glu233Asp, ACY1 Arg353Cys, ACY1 Pro369fsX46, ACY1 Arg386Cys displayed a loss in enzyme activity compared to overexpressed ACY1 WT enzyme. Recombinants ACY1 Arg378Trp, ACY1 Arg378Gln and ACY1 Arg393His showed major residual enzyme activity (Fig. 1), whereas the ACY1 Arg378Gln and ACY1 Arg393His mutants yielded the same enzyme activity level as the ACY1 WT enzyme. ACY1 Arg378Trp showed 50 % residual enzyme activity of ACY1 WT. ACY1 protein expression in HEK293 cells was analyzed using Western blot analysis. The housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a positive loading control in Western blot analysis (Fig. 2). Enzyme activity data are well in agreement with the results of Western blot analysis using an antibody against human ACY1. No protein band corresponding to ACY1 was detected in transfected cells carrying ACY1 mutants, which resulted in no measurable enzyme activity. In contrast, strong ACY1 signals were obtained in the Western blot analysis not only for the ACY1 WT enzyme, but also for ACY1 Arg393His. A considerable amount of ACY1 protein, although less than for ACY1 WT, was also detected for ACY1 Arg378Trp and ACY1 Arg378Gln.

### ***3.4 Restriction fragment length analysis of c.1156C>T (p.Arg386Cys) in ACY1***

Biallelic loss of the BtgI restriction site, comprising nucleotide 1156 of the ACY1 coding sequence, results in a single DNA band of 606 bp. Heterozygosity for the mutation c.1156C>T (p.Arg386Cys) or another sequence variation in the *ACY1* gene, which disrupts the BtgI restriction site, results in three bands of 206 bp, 400 bp and 606 bp. 105 reference samples corresponding to 210 chromosomes, all yielded bands of 206 and 400 bp only, revealing a functional BtgI restriction site and therefore no evidence for the mutation c.1156C>T (Fig. 3).

#### 4. Discussion

Transient overexpression of ACY1 WT protein in HEK293 cells resulted not only in strong protein bands reacting with the anti-ACY1 antibody in Western blot analysis, but also in high enzyme activity. This suggests that the human cell expression system chosen here fulfills the requirements for the synthesis of a correctly folded, active protein.

In contrast to the ACY1 WT protein, overexpression of mutants ACY1 Arg197Trp and ACY1 Glu233Asp yielded no detectable ACY1 activity. This is in agreement with the results of enzyme activity tests in corresponding patient cells and has been ascribed to the localization of the mutations in the highly evolutionary conserved and functionally relevant ACY1 protein dimerization domain [10;13]. The zinc-binding site is situated in the centre of the ACY1 dimerization domain (Fig.4) [18]. Even though both amino acid residues are localized contrary to the zinc binding dimerization and catalytic site, mutations p.Arg197Trp and p.Gln233Asp result in non-functional ACY1 protein. Moieties of both the amino acids 197 and 233 are oriented towards the second monomer and thus may play a role in stabilization of the homodimeric structure (Fig.4).

In contrast, the exchange of Arg353 with Cys (p.Arg353Cys) probably disrupts the globular catalytic subunit [11]. It is likely that impaired interactions of the His373 residue with the Zn<sup>2+</sup> ion of the catalytic site result not only directly in loss of enzyme activity, but yields also in an unstable dimeric protein structure prone to degradation (Fig.4) [18]. This mutation has been found several times in control populations, both by Van Coster et al. 2005 and by Sass et al 2006 [10;11]. Although not indicated as a single nucleotide polymorphism (SNP) in the SNP database (NCBI SNP data base; <http://www.ncbi.nlm.nih.gov/projects/SNP>), it is likely that it represents a polymorphism affecting ACY1 activity.

The frame shift mutation c.1105\_1106insAC (p.Pro369fsX46) even results in loss of the catalytically important amino acid His373 and hence results in loss of ACY1 enzyme functions. The insertion suggests an altered DNA sequence resulting in loss of the original stop codon and major elongation of the translation product making it prone to early degradation.

While some residual activity was still observed in lymphocyte homogenate of patient AC007 II-1, who is homozygous for the mutation p.Arg386Cys, transient overexpression of the mutation in HEK293 cells resulted in complete loss of ACY1 activity. The latter can be explained by perturbation of an alpha-helix structure in the C-terminal peptidase domain (Fig.4). This sequence variation has been reported as a SNP in the NCBI SNP database, occurring, e.g., in individuals from Nigeria. However, we did not observe p.Arg386Cys in any of 105 control samples collected in Southwestern Germany.

It is in agreement with a profound loss of protein stability/ early protein degradation that all five mutations listed above did not only result in completely abolished enzyme activity, but also in loss of immunoreactive ACY1 protein.

This is in sharp contrast to both studied mutations affecting Arg378. Mutations p.Arg378Trp and p.Arg378Gln resulted not only in ACY1 protein detectable by Western blot analysis, but also in major residual enzyme activity. Arg378 is present in the C-terminal peptidase domain but is oriented away from the catalytic site and its zinc-binding centre. It is expected that the replacement of arginine with tryptophan has a higher impact on the protein secondary structure compared to an exchange with glutamine because tryptophan is an aromatic amino acid. Tryptophan would occupy more space in the structure and its side chain may interact with the proline369 in an adjacent loop structure (Fig.4) which could result in a change of catalysis. Arginine and glutamine are both basic amino acids and their side chains are structurally alike, whereas tryptophan is a lipophilic, aromatic amino acid and may change the characteristics of this protein region much more than an exchange with glutamine. This is well reflected by the lower enzyme activity of ACY1 Arg378Trp compared to ACY1 Arg378Gln. High residual ACY1 activities of lymphocytes of patients AC013 II-1 and AC014 II-1, which are homozygous for the mutations p.Arg378Trp and p.Arg378Gln in the *ACY1* gene, corroborate the results obtained with the overexpressed mutated protein. Although abnormalities in the pattern of urinary organic acids were less pronounced for these two patients than is usually observed in ACY1 deficiency, it still pointed to this diagnosis. Since there was almost no increase in urinary *N*-acetyl-L-methionine, it was speculated that the mutations p.Arg378Trp and p.Arg378Gln may have modified substrate specificity, keeping hydrolysis of *N*-acetyl-L-methionine possible. Therefore, the enzyme activity assay was also performed with *N*-acetyl-L-glutamate, one of the alternative substrates indeed identified in these patients' urine samples. However, this yielded no preference of the enzyme variants of patients AC013 II-1 and AC014 II-1, if compared to the use of the standard substrate *N*-acetyl-L-methionine and to reference samples.

The mutation p.Arg393His affects an amino acid residue beyond the C-terminal peptidase domain of ACY1, which most likely does not interact with the catalytic center and amino acid His373 (Fig.4). This is compatible with the unaffected enzyme activity of overexpressed ACY1 Arg393His and with the strong signal of the mutated protein in Western blot analysis, which resembles that of the WT protein.

However, preservation of ACY1 activity does not necessarily mean that a mutation such as p.Arg393His plays no biological role. Maceyka et al. (2004) have shown that ACY1 protein can interact with the sphingosine kinase type 1 (SphK1). This suggests functions of ACY1 in regulating other proteins such as SphK1, which, for instance, is known to promote cell growth and inhibit apoptosis of tumor cells [21;22].

Notably, seven of the mutations which we have identified in the *ACY1* gene affect primarily the peptidase dimerization domain, the C-terminal peptidase domain or an even subsequent part of the protein. No patient has been identified so far with mutations affecting the *N*-terminus of the ACY1 protein. It is remarkable that Maceyka et al. have demonstrated that a recombinant ACY1 variant lacking the first 231 amino acids can also bind SphK1 [21]. However, this variant protein has distinct consequences if compared with interactions of full-lengths ACY1 with SphK1. The shorter variant inactivates SphK1 thus resulting in decreased proliferation and enhanced apoptosis in tumor cells [21]. Hence, one may speculate that the *N*-terminal region of the *ACY1* gene is rather conserved since its integrity may be crucial for the quality of the effects of ACY1 on SphK1 or other regulatory mechanisms. As mentioned before [13], a role of ACY1 as a potential modifier could explain the phenotypic variability observed in ACY1-deficient individuals.

Our data show that the HEK293 cell system is suitable for expressing human ACY1 proteins. Expression analysis confirmed the causal relation between mutations in the *ACY1* gene, ACY1 activity and metabolite patterns. Ongoing research in our laboratory is aiming at a better understanding of possible regulatory functions of ACY1.

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Table 1: Aminoacylase 1 (ACY1) activities in homogenates prepared from EBV-transformed

	<b>Mutation (DNA)</b>	<b>Mutation (Protein)</b>	<b>Mean ACY1 enzyme activity (in lymphocytes, substrate: <i>N</i>-Acetyl-L-methionine) [<math>\mu\text{mol} * \text{g}^{-1} * \text{min}^{-1}</math>]</b>	<b>Standard deviation</b>	<b>Mean ACY1 enzyme activity (in lymphocytes, substrate: <i>N</i>-Acetyl-L-glutamate) [<math>\mu\text{mol} * \text{g}^{-1} * \text{min}^{-1}</math>]</b>	<b>Standard deviation</b>
positive control	-	-	0.00	0.01	0.02	0.03
AC007 II-1	homozygous c.1156C>T	p.Arg386Cys	0.22	0.14	n.d.	n.d.
AC013 II-1	homozygous c.1133G>A	p.Arg378Gln	0.99	0.41	0.69	0.13
AC014 II-1	homozygous c.1132C>T	p.Arg378Trp	0.32	0.01	0.08	0.09
negative controls:						
A	-	-	1.85	0.87	0.67	0.26
B	-	-	1.40	0.53	0.97	0.22
C	-	-	2.16	0.85	0.79	0.32

patient lymphocytes. Mean values are calculated from at least n=3 experiments. n.d. = not determined

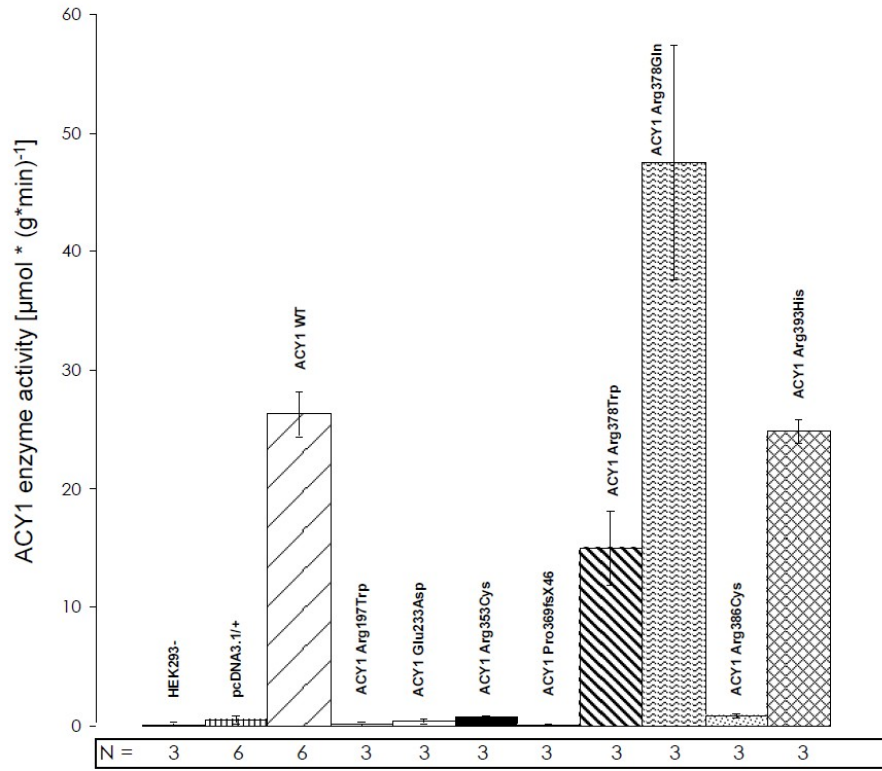
Fig. 1: Assessment of ACY1 activities in HEK293 cells revealed loss of enzyme activity with missense mutations ACY1 Arg197Trp, ACY1 Glu233Asp, ACY1 Arg353Cys, ACY1 Arg386Cys and with frame shift mutation ACY1 Pro369fsX46. Mutants ACY1 Arg378Trp, ACY1 Arg378Gln and ACY1 Arg393His showed not more than a slight decrease in ACY1 activity, if compared to HEK293 cells expressing ACY1 WT. (HEK293-: non-transfected cells; pcDNA3.1/+: mock control, HEK293 cells transfected with the empty vector).

Fig. 2: Western blot analysis of ACY1 protein (molecular weight of the monomer = 46 kDa). Use of GAPDH as the control demonstrates comparable loading of all lanes. Based on 100% intensity for ACY1 WT protein overexpressed in HEK293 cells, relative intensities were determined for variants ACY1 Arg378Trp (40 % intensity), ACY1 Arg378Gln (66 % intensity) and ACY1 Arg393His (243 % intensity). No ACY1 protein was detected with mutants ACY1 Arg353Cys, ACY1 Arg197Trp, ACY1 Glu233Asp, ACY1 Arg386Cys and ACY1 Pro369fsX46.

Fig. 3: Map of the restriction digest using BtgI (Primer F - forward primer; Primer R - reverse Primer) Point mutation c.1156 C>T (p.Arg386Cys) results in loss of the BtgI restriction site. DNA samples of heterozygous individuals yielded three bands of 206 bp, 400bp and 606 bp on a 1% agarose gel. Samples of patients displayed only one band of 606 bp. The negative control lacks the c.1156C>T mutation, thus yielding a double-band pattern (400 bp and 206 bp).

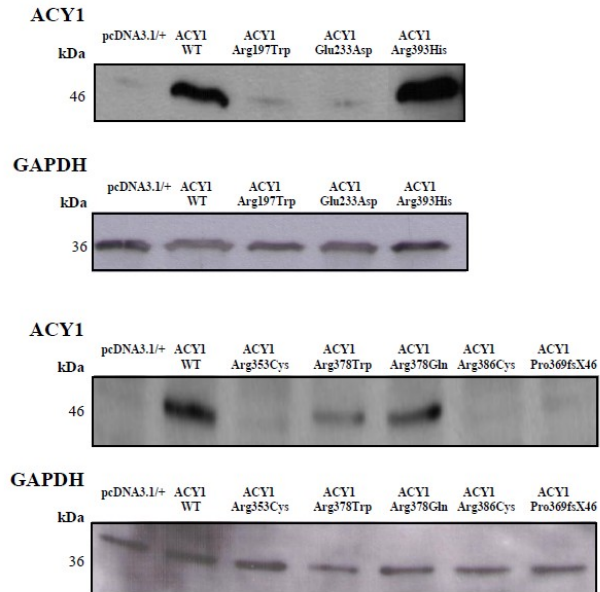
Fig. 4: ACY1 T347G mutant homodimeric protein structure (structure is available at: <http://www.rcsb.org/pdb/explore/explore.do?structureId=1Q7L>) [21]: One monomer of the structure is coloured in grey and the other one in black. The zinc ions are labelled blue and their interacting amino acid residues are marked in orange. Amino acids (Arg197, Arg353, Pro369, Arg378, Arg386 and Arg393) which were alternatively replaced by our approach using site-directed mutagenesis in the *ACY1* gene are coloured in purple and labelled in one of the monomers.

Figure 1



ACCEPT

Figure 2



ACCEPTED

Figure 3

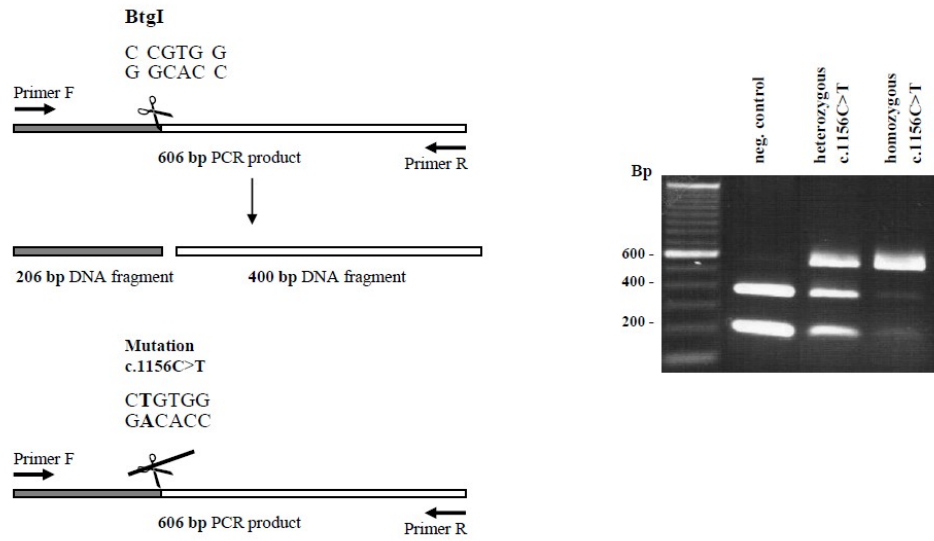
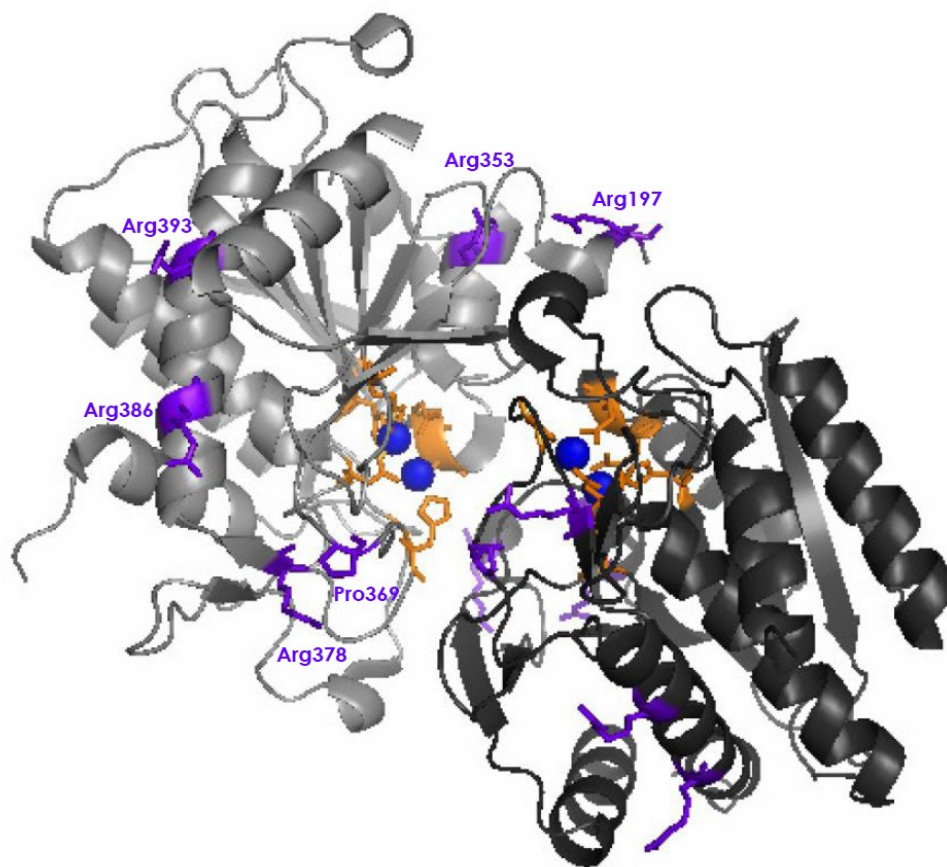


Figure 4



ACCEPT