Aminoacylase I deficiency: A novel inborn error of metabolism

R.N. Van Coster a,*, E.A. Gerlo b, T.G. Giardina c, U.F. Engelke d, J.E. Smet a, C.M. De Praeter e, V.A. Meersschaut f, L.J. De Meirleir g, S.H. Seneca g, B. Devreese h, J.G. Leroy a, S. Herga c, J.P. Perrier c, R.A. Wevers d, W. Lissens g

a Department of Pediatrics, Division of Neurology and Metabolism, University Hospital, Ghent, Belgium
b Department of Clinical Chemistry, University Hospital, Free University, Brussels, Belgium
c Institut Méditerranéen de Recherche en Nutrition, INRA-UMR 1111 Service 342, Université Paul Cézanne—Aix-Marseille III, Faculté des Sciences et Techniques, 13397 Marseille Cedex 20, France
d Laboratory of Pediatrics and Neurology, University Medical Center Nijmegen, Nijmegen, The Netherlands
e Department of Neonatal Intensive Care, University Hospital, Ghent, Belgium
f Department of Radiology, Division of Pediatric Radiology, University Hospital, Ghent, Belgium
g Department of Medical Genetics, University Hospital, Free University, Brussels, Belgium
h Laboratory of Protein Biochemistry and Protein Engineering, Ghent University, Ghent, Belgium

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Abstract

This is the first report of a patient with aminoacylase I deficiency. High amounts of N-acetylated amino acids were detected by gas chromatography–mass spectrometry in the urine, including the derivatives of serine, glutamic acid, alanine, methionine, glycine, and smaller amounts of threonine, leucine, valine, and isoleucine. NMR spectroscopy confirmed these findings and, in addition, showed the presence of N-acetylglutamine and N-acetylasparagine. In EBV transformed lymphoblasts, aminoacylase I activity was deficient. Loss of activity was due to decreased amounts of aminoacylase I protein. The amount of mRNA for the aminoacylase I was decreased. DNA sequencing of the encoding ACY1 gene showed a homozygous c.1057 C > T transition, predicting a p.Arg353Cys substitution. Both parents were heterozygous for the mutation. The mutation was also detected in 5/161 controls. To exclude the possibility of a genetic polymorphism, protein expression studies were performed showing that the mutant protein had lost catalytic activity.

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Aminoacylase I (EC 3.5.1.14) is a homodimeric zinc-binding metalloenzyme located in the cytosol [1]. It catalyzes the hydrolysis of N-linked acyl groups in L-amino acids, including the N-acetylated derivatives of serine, glutamic acid, alanine, methionine, glycine, leucine, and valine.

Among the proteolytic systems known to be involved in intracellular catabolism of intrinsic proteins, the intracellular catabolism of N-acetylated proteins is mediated by the ATP-ubiquitin-dependent proteasome [2]. This large complex degrades proteins into peptides 5–30 amino residues long. N-acetylated peptides are then cleaved by acylpeptidase hydrolyase (EC 3.4.19.1) with the release of the N-acetylated amino acid and a shorter free peptide. In the next step, aminoacylase I hydrolyzes the N-acetylated amino acid to acetate and its free amino acid.

The detection of several N-acetylated amino acids in the urine of a young infant with an encephalopathy to be described in this report has led to the identification of a deficiency of aminoacylase I.

Materials and methods

Patient description. The propositus was born at 39 weeks gestation after an uneventful pregnancy as the first child of non-consanguineous parents. He had a birth weight of 2.565 g. Length and head circumference...
were 48 and 33 cm, respectively. Delivery was by elective caesarian section due to breech presentation. On the third day of life, the newborn no longer drank effectively, started vomiting and became cyanotic. Seizures and apnoeic spells were noticed. In addition to daily bouts of convulsions, generalized hypotonia was most prominent. During the following days, episodes of bradycardia compounded the hypotonia. Cerebral MRI showed abnormal signals in the cortico-subcortical zones of the fronto-parietal and tempo-parietal areas consistent with cortical laminar necrosis. A sensorineural hearing deficit (left 70 dB, right 60 dB) was detected by auditory brain stem response. The convulsions ceased around the age of two weeks, when also the feeding problems started improving. At six weeks, the feeding difficulties had subsided and convulsions had not recurred. Clinical examination revealed no abnormalities. However, auditory brain stem response confirmed significant hearing deficit (left 65 dB, right 100 dB). The MRI revealed mild signs of cerebral atrophy. Clinical examination at six months of age was normal. At nine months, he had reached normal milestones and abnormal clinical neurological signs were not detected.

**Organic acids.** Organic acids were extracted by a standard ethyl acetate-diethyl ether procedure, converted to their trimethylsilyl (TMS) derivatives, and analyzed on a Hewlett Packard 5973 gas chromatography-mass spectrometry (GC-MS) system [3]. For a detailed description, see Gerlo et al. (in preparation).

**Proton NMR spectroscopy.** Proton NMR spectroscopy of urine (1-dimensional and COSY) was performed on a Bruker 500 MHz spectrometer as described before [4].

**Enzyme activities.** Cultured lymphoblast cell lines were washed in cold phosphate-buffered saline, pelleted, and resuspended in 2 mL of 10 mM Hepes buffer at pH 7.2, containing 5 mM glucose. After cells were lysed with an Ultraturax for 10 s in ice-cold Hepes buffer, the resulting homogenate was centrifuged at 100,000g for 30 min. The supernatant was either directly used for measurement of acylcarnitine activity, or for acylase I activity, as previously described [5]. Protein concentrations were determined using Bradford's method [6].

**Immunoblotting.** Cultured lymphoblast cell lines were washed with PBS buffer. The resulting pellets were homogenized in buffer containing 10 mM sodium phosphate, 150 mM sodium chloride, and 2 mM EDTA (pH 7.4), and centrifuged at 16,000g. Protein concentration in the supernatant was determined according to Lowry et al. [7]. Sixty micrograms of protein was loaded per lane. Proteins were separated in a 16.5% tricine SDS-PAGE gel as described by Laemmli [8]. Electrobolting was performed according to Towbin [9]. As primary antibody an anti-aminocyclase 1 polyclonal serum was used (Abnova, Taiwan). To demonstrate equal loading, the membrane was reprobed with a β-actin antibody (Abcam, UK).

**Gene analysis.** PCR primers were manually designed to cover the 14 exons and parts of the flanking intronic regions of the ACY1 gene. Genomic and cDNA sequences of the gene were from GenBank (Accession Nos. AC115284 and L07548, respectively). The 14 exons were PCR amplified from genomic DNA using 1 U Platinum Taq polymerase One-step RT-PCR System (Invitrogen Life Technologies) at 45°C for 20 min, and terminated by heating at 95°C for 5 min, for RT enzyme inactivation. PCR primers used in this study were designed based on the EMBL/GenBank (Accession #: Rattus β actin, NM_031144; ACY1, NM_001005383), and the β-actin and ACY1 PCR primer sequences were 5'-ATCCTGGGACC ACACCTTCTACATGAGCTGCG-3' and 5'-GTGTTACATTCTCTGAGGCG-3', respectively. The expected sizes of RT-PCR products were 838 bp for the β-actin and 747 bp for the ACY1. Each cycle of PCR included 30 s of denaturation at 94°C. The annealing step was carried out at appropriate temperature using 0.4 μmol of each couple of primers for 2 min, whereas the extension step was performed at 72°C for 3 min using an overall volume of 50 μl and a total number of 40 cycles for PCR in a Mastercycler gradient 5331 thermocycler (Eppendorf). After appropriate cycles, 10 μl of the RT-PCR products was separated on a 1.2% TAE (Tris acetate EDTA)-agarose gel, stained with ethidium bromide, and photographed under UV light.

For detection of the ACACCTTCTACATGAGCTGCG primers, RT-PCR products were separated by electrophoresis, the gel was denatured (0.4 M NaOH, 1.5 M NaCl) for 30 min, rinsed twice with distilled water, neutralized with 0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5 buffer, and transferred onto a nylon membrane (Hybond, Amersham Pharma Biocience). After blotting, the membranes were dried and cross-linked by ultraviolet light.

**Oligonucleotides with internal sequences to our ACY1 sequence were used for synthesis of the labelled-PCR probe (5'-GCCAGGACAGAAGAGGTTGGAGC-3' and 5'-CCCGCTCTCAGG-3'). The ACY1-PCR DNA probe was random primed by using the DIG-high prime DNA labelling kit according to the manufacturer's instructions (Roche Diagnostics Corporation). Finally, the RT-PCR products were probed at 45°C in the manufactured hybridization conditions and detected by using the DIG detection system kit II (Roche).

**Cloning of human aminocyclase I.** The clone (#IRIAup969D1218D) containing the complete open reading frame of hACY1 was obtained from RZPD German Resource Center for Genome Research (Berlin, Germany). hACY1 nucleotide sequence, fused to an upstream reading frame, was cloned using the Gateway system (Invitrogen). hACY1 cDNA was first synthesized, in a PCR mixture, using the primers, 5'-GGG ACA AGT TGT TAC AAA AAA GCA GCC TTG GTT CCG CTT GGA TTC CAG GGC TCA GC-3', and 5'-GCCGAGCTCTCGACG-3'. The 14-fragment DNA probe was then probed by using the DIG-high prime DNA labelling kit according to the manufacturer’s instructions (Roche Diagnostics Corporation). Finally, the RT-PCR products were probed at 45°C in the manufactured hybridization conditions and detected by using the DIG detection system kit II (Roche).

**Site-directed mutagenesis kit.** Cloning of human aminocyclase I. The clone (#IRIAup969D1218D) containing the complete open reading frame of hACY1 was obtained from RZPD German Resource Center for Genome Research (Berlin, Germany). hACY1 nucleotide sequence, fused to an upstream reading frame, was cloned using the Gateway system (Invitrogen). hACY1 cDNA was first synthesized, in a PCR mixture, using the primers, 5'-GGG ACA AGT TGT TAC AAA AAA GCA GCC TTG GTT CCG CTT GGA TTC CAG GGC TCA GC-3', and 5'-GCCGAGCTCTCGACG-3'. The 14-fragment DNA probe was then probed by using the DIG-high prime DNA labelling kit according to the manufacturer’s instructions (Roche Diagnostics Corporation). Finally, the RT-PCR products were probed at 45°C in the manufactured hybridization conditions and detected by using the DIG detection system kit II (Roche).

**Expression and purification.** Expression and purification of the Site-directed mutagenesis kit (Strategene Europe, Amsterdam Zuidoost, The Netherlands) with two overlapping complementary oligonucleotides containing the corresponding nucleotide changes 5'-AACCCGATATATCTGCGGCTGGGGTGCCTCCCA-3' and 5'-GGGACCCACCCGCGCAGAATAGGCGTT-3'. Mutations were confirmed by DNA sequencing which was carried out using the dyeoxychian-termination procedure by Genome Express (Grenoble, France).

**Site-directed mutagenesis kit.** The mutation was introduced into the pGEX1/T-ACY1 plasmid using the QuickChange XL site-directed mutagenesis kit (Strategene Europe, Amsterdam Zuidoost, The Netherlands) with two overlapping complementary oligonucleotides containing the corresponding nucleotide changes 5'-AACCCGATATATCTGCGGCTGGGGTGCCTCCCA-3' and 5'-GGGACCCACCCGCGCAGAATAGGCGTT-3'. Mutations were confirmed by DNA sequencing which was carried out using the dyeoxychian-termination procedure by Genome Express (Grenoble, France). The plasmids were inserted into E. coli BL21 DE3 competent cells to induce the expression of the proteins required.

**Protein expression and purification.** Expression and purification of the wild-type and mutant proteins were performed as follows. Bacterial lysis was carried out in a 10 mM Tris-HCl, pH 8.0, buffer containing 1 mM
EDTA, 150 mM NaCl, and 10 mg/ml lysozyme (STE buffer). The digestion was carried out at 4 °C for 15 min. Cells were then sonicated for 1 min in STE containing 1 M α-dithiothreitol and 10% Sarkosyl. After a 16,000 rpm centrifugation run, the supernatant was supplemented with 10% Triton X-100 and incubated for 30 min at room temperature. The resulting soluble fraction was mixed with GST-agarose beads pre-equilibrated with PBS. The fusion protein was first cleaved from GST by incubating the gel overnight with thrombin (20 U/ml gel bed volume) at room temperature, and the resulting beads were subsequently washed with PBS buffer. The soluble fraction was used for characterization, while the remaining bound GST was released from the column into a 20 mM reduced glutathione solution containing 50 mM Tris–HCl buffer, pH 8.0.

Polyacrylamide gel electrophoresis and protein determination. SDS–PAGE was performed in 12% (w/v) polyacrylamide gel as described by Laemmli[8] to check the protein purity and determine the molecular mass. Molecular-mass markers and ACY1 samples were heated to 100 °C in the presence of 2% SDS under reducing conditions prior to performing electrophoresis and Coomassie brilliant blue staining. Protein concentration was determined using Bradford’s method with bovine serum albumin as the standard [6].

Results

Metabolites in urine

GC–MS analysis of organic acids extracted from the patient’s urine was performed. The profile was characterized by multiple unusual peaks identified as the TMS-derivatives of N-acetylated amino acids by matching the retention times and the mass spectra of TMS-derivatives prepared from model compounds. N-acetylated derivatives of alanine, glutamic acid, glycine, methionine, and, serine, and smaller amounts of isoleucine, leucine, threonine, and valine were consistently found in four urine samples collected over a two-month period (Table 1). Other organic acids including N-acetylaspartic acid were within reference limits. Proton nuclear magnetic resonance spectroscopy of a patient’s urine sample confirmed the presence of these compounds and also revealed the presence of N-acetylated glutamine, asparagine, and an unidentified N-acetylated derivative (Table 1). N-Acetylaspartic acid was present in the patient’s urine but was not significantly increased. The same held for the dipeptide N-acetylaspartylglutamate. N-acetylated forms of the aromatic amino acids tyrosine, phenylalanine, and tryptophan were not found, and N-acetyllysine was also undetectable.

Enzyme activities

Aminoacylase I activity was significantly decreased in the patient derived cell strains (Table 2). The activity of acylpeptide hydrolase was not significantly different in EBV transformed patient and control lymphoblast cell strains. The acylpeptide hydrolase/aminoacylase I activity ratio was 272 in the patient versus 23 in control cells.

Immunoblotting

Western blotting showed that aminoacylase I CRM was significantly decreased in the lymphoblast cells of the patient (Fig. 1). Immunoblotting with β-actin antibody

Table 1
Urinary excretion (mmol/mol creatinine) of N-acetylated amino acids, as determined by gas chromatography mass–spectrometry (GC–MS) and proton magnetic resonance spectrometry (H1 NMR)

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Identifications in different (#) samples</th>
<th>GC–MS</th>
<th>H1 NMR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>#1 D21ª</td>
<td>#2 D51ª</td>
</tr>
<tr>
<td>N-acetyl-serine</td>
<td>230</td>
<td>240</td>
<td>330</td>
</tr>
<tr>
<td>N-acetyl-glutamic acid</td>
<td>310</td>
<td>390</td>
<td>560</td>
</tr>
<tr>
<td>N-acetyl-glutamine</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>N-acetyl-alanine</td>
<td>200</td>
<td>210</td>
<td>250</td>
</tr>
<tr>
<td>N-acetyl-methionine</td>
<td>110</td>
<td>30</td>
<td>80</td>
</tr>
<tr>
<td>N-acetyl-glycine</td>
<td>160</td>
<td>180</td>
<td>190</td>
</tr>
<tr>
<td>N-acetyl-aspartic acid</td>
<td>16</td>
<td>17</td>
<td>16</td>
</tr>
<tr>
<td>N-acetyl-asparagine</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>N-acetyl-leucine</td>
<td>18</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>N-acetyl-isoleucine</td>
<td>5</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>N-acetyl-valine</td>
<td>11</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>N-acetyl-threonine</td>
<td>60</td>
<td>50</td>
<td>80</td>
</tr>
<tr>
<td>N-acetyl-X</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detectable.
ª Age of the patient (days).

Table 2
Specific activities of aminoacylase I (ACY1) and acylpeptide hydrolase (APH) in lymphoblasts from the patient and controls (U/mg protein)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Specific activity APH</th>
<th>Specific activity ACY1</th>
<th>Ratio specific activity APH/ACY1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean controls (n = 3)</td>
<td>82 ± 22</td>
<td>3.6 ± 0.23</td>
<td>23 ± 6</td>
</tr>
<tr>
<td>Patient</td>
<td>49</td>
<td>0.18</td>
<td>272</td>
</tr>
</tbody>
</table>
yielded comparable results in the patient and two control cell lines.

**RT-PCR and Southern blot analyses**

RT-PCR product analyses were performed to compare the expression of aminoacylase I mRNA between the patient and two controls. A strong band for aminoacylase I mRNA was detected in the controls and only a weak band in the patient (Fig. 2A). The β-actin cDNA amplified as internal marker was present in all control and patient cells at a similar level (Fig. 2A). Using the RT-PCR products, Southern blotting was performed with a DIG-ACY1 PCR fragment confirming the above results (Fig. 2B). Moreover, a nested PCR with an aliquot of the RT-PCR from the patient was performed which allowed to conclude that the weak band observed was indeed aminoacylase I mRNA (data not shown).

**Genomic analysis**

Genomic sequencing of all 14 exons/introns in the patient and his parents showed that the patient was homozygous for a c.1057C > T change in exon 13, while both of his parents were heterozygous for this mutation. This nucleotide transition predicts the p.Arg353Cys. Five out of 161 controls were also heterozygous for this transition.

**Site-directed mutagenesis and purification of wild-type and mutants ACY1**

The wild-type and mutated ACY1 genes were expressed and purified to homogeneity by affinity chromatography. The ACY1 protein size was checked by SDS–PAGE after removal of the GST tag by thrombin proteolysis. Each enzyme produced a single band of an appropriate size (45 kDa) (data not shown).

**Role of R353C mutation in ACY1**

To investigate the possible importance of the arginine residue at position 353 for human ACY1 activity, arginine was mutated into a cysteine. The R353C mutant resulted in complete absence of enzyme activity.

**Discussion**

Acetylation of the N-terminal amino acid (α-NH₂ acetylation) is a common protein modification in eukaryotes but is rarely encountered in prokaryotes. In mammalians, 80–90% of the cytosolic proteins are subjected to an irreversible, cotranslational amino acid acetylation at their N-terminus [10]. Many structural proteins such as actin and tropomyosin are N-acetylated. This holds as well for several cytosolic enzymes, calcium and metal-binding proteins, transfer proteins, and peptide hormones [2]. N-acetylated proteins are catabolized in the cytosol by the ATP-ubiquitin-dependent proteasome pathway [2]. Functional aminoacylase I is crucial in the last step in this degradation as it catalyzes the hydrolysis of N-acetylated amino acids into acetate and the free amino acid. In this paper, a patient is described with a severe deficiency of the aminoacylase I. The propositus presented neonatally with an acute encephalopathy with onset on the third day of life and duration of about two weeks. This is the first report of a patient with a congenital defect in the cytoplasmic protein degradation pathway of N-acetylated proteins.

Detection of several N-acetylated amino acids in the patient’s urine pointed to an as yet undescribed inborn error. The pattern of N-acetylated derivatives in the urine of the patient matched the substrate specificity of aminoacylase I. The enzyme is known to have a preference for neutral and aliphatic N-acetyl-amino acids [11]. The patient’s urine did not contain N-acetylated forms of the basic and aromatic amino acids. N-acetylaspartic acid was also not increased. These findings were consistent with the normal function of separate
Aminoacylases deacytating N-acetylaspartic acid (aspartoacylase) and N-acetylated aromatic amino acids (aryl acylamidase) [11]. Aspartoacylase, deficient in Canavan disease [12], is specific for N-acetylaspartic acid. The clinical and neuroradiologic features in our patient were different from the ones characterizing Canavan disease.

The suspected deficiency of aminoacylase I was confirmed enzymatically in the EBV transformed lymphocytes of the propositus. SDS-PAGE and immunoblotting showed that the loss of catalytic activity was due to severely decreased amounts of aminoacylase I protein. The amount of aminoacylase I mRNA was also significantly decreased, probably due to inefficient transcriptional processing or to mRNA instability. In the DNA extracted from the peripheral leucocytes, the homozygous point mutation (R353C) due to the c.1057 C > T transition was detected in exon 13 of the ACY1 gene. Both parents, who denied consanguinity, were heterozygous for this mutation. Based on a porcine ACY1 model [13] each ACY1 monomer consists of two domains: a globular catalytic subunit (residues 1–188 and 311–399) consisting of a β-sheet sandwiched between α-helices and a second, β-sheet located on the surface, and the dimerization domain (residues 189–310) folding into a β-sheet flanked on one side by two α-helices. Therefore, the replacement of R353 by a C residue could create a perturbation in the vicinity of the active site.

Also, 5 out of 161 controls were found to be carrier of this missense mutation. To exclude the possibility of a genetic polymorphism, protein expression studies were performed which showed that the mutant protein had lost all catalytic activity. Therefore, the metabolic condition described might not be exceptionally rare. From the results of the study presented it is expected that the abnormal compounds would be detected in 1/4,147 individuals. However, as organic acid analysis has become part of routine testing for any encephalopathic patient at most major medical centers, one would predict that other patients with this unusual organic acid profile would have been identified in the past. N-Acetylated amino acids have poor extraction efficiency which can vary greatly from laboratory to laboratory. Therefore, it is not excluded that the abnormal profile has been missed in other patients. Another theoretical possibility is that not all patients with this enzyme deficiency excrete the abnormal compounds in the urine because of the existence of an alternative pathway for degradation of N-acetylated amino acids. Until now, however, such an alternative pathway has not been demonstrated.

As this is a report of a single patient, there is no direct evidence that aminoacylase I deficiency has caused the clinical findings observed. The clinical phenotype associated with aminoacylase I deficiency and the long-term clinical implications of this hitherto unreported inborn error of metabolism must await further follow-up of the propositus presented and the reporting of more patients with the same enzyme defect.

References