Molecular Genetics and Metabolism 113 (2014) 267–273

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Recurrence of carbamoyl phosphate synthetase 1 (CPS1) deficiency in Turkish patients: Characterization of a founder mutation by use of recombinant CPS1 from insect cells expression

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ARTICLE INFO

Article history:
Received 9 September 2014
Received in revised form 30 September 2014
Accepted 30 September 2014
Available online 7 October 2014

Keywords:
Urea cycle disorder
Carbamoyl phosphate synthetase 1 (CPS1) deficiency
Founder mutation
Baculovirus/insect cell expression system
Enzyme activity
Thermostability

ABSTRACT

Carbamoyl phosphate synthetase 1 (CPS1) deficiency due to *CPS1* mutations is a rare autosomal-recessive urea cycle disorder causing hyperammonemia that can lead to death or severe neurological impairment. CPS1 catalyzes carbamoyl phosphate formation from ammonia, bicarbonate and two molecules of ATP, and requires the allosteric activator N-acetyl-L-glutamate. Clinical mutations occur in the entire *CPS1* coding region, but mainly in single families, with little recurrence. We characterized here the only currently known recurrent *CPS1* mutation, p.Val1013del, found in eleven unrelated patients of Turkish descent using recombinant His-tagged wild type or mutant CPS1 expressed in baculovirus/insect cell system. The global CPS1 reaction and the ATPase and ATP synthesis partial reactions that reflect, respectively, the bicarbonate and the carbamate phosphorylation steps, were assayed. We found that CPS1 wild type and V1013del mutant showed comparable expression levels and purity but the mutant CPS1 exhibited no significant residual activities. In the CPS1 structural model, V1013 belongs to a highly hydrophobic β-strand at the middle of the central β-sheet of the A subdomain of the carbamate phosphorylation domain and is close to the predicted carbamate tunnel that links both phosphorylation sites. Haplotype studies suggested that p.Val1013del is a founder mutation. In conclusion, the mutation p.V1013del inactivates CPS1 but does not render the enzyme grossly unstable or insoluble. Recurrence of this particular mutation in Turkish patients is likely due to a founder effect, which is consistent with the frequent consanguinity observed in the affected population.

1. Introduction

Carbamoyl phosphate synthetase 1 deficiency (CPS1D; OMIM #237300) caused by mutations in the human *Carbamoyl phosphate synthetase 1* (*CPS1*, MIM #608307) gene is a rare autosomal-recessive urea cycle disorder (UCD) with an estimated incidence of 1:50,000–1:300,000 based on the reports of the Japanese and American cohorts

Abbreviations: hCPS1, human carbamoyl phosphate synthetase 1; CPS1D, carbamoyl phosphate synthetase 1 deficiency; DSF, differential scanning fluorimetry; V1013del, CPS1-valine 1013 deletion; CP, carbamoyl phosphate; NAG, N-acetyl-L-glutamate.

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http://dx.doi.org/10.1016/j.ymgme.2014.09.014
1096-7192/

[1,2]. Like in other urea cycle disorders, CPS1D patients can present hyperammonemia at any age leading to death or to severe neurological impairments. In plasma, CPS1D is biochemically characterized by high ammonia and glutamine and low citrulline levels with no increase in urine orotic acid (which is the marker for another mitochondrial UCD, ornithine transcarbamylase (OTC) deficiency). CPS1D can be identified as lethal neonatal onset within the first days of life but also less severe late onset is described in children or adults [3,4]. The severity of the clinical presentation varies depending on the time of onset and the level of residual enzyme activity.

Human *CPS1* is located on chromosome 2q34–35 [5] and is comprised of 38 exons with 4500 nucleotides coding sequence [6–8]. To date, more than 220 mutations have been detected over the entire *hCPS1* coding region [2–4,9–15]. Different types of mutations were described, including single-nucleotide substitutions (~77%) with preference of missense changes (~61%), deletions (~14%), small insertions or duplications (~7%), indels (~2%), and nonsense mutations (~7%) [2]. Most missense mutations are non-recurrent [2,6,7,10,12,14–16]. Only very few of the missense mutations (<10%) were found in more than one unrelated family and true recurrence was not yet found [2].

CPS1 (EC 6.3.4.16) is the first and rate-limiting urea cycle enzyme which catalyzes the synthesis of carbamoyl phosphate (CP) from ammonia, bicarbonate and two molecules of ATP [2,8,17–24]. The complex reaction requires the cofactor N-acetyl-L-glutamate [25,26] and takes place in three sequential steps, namely, bicarbonate phosphorylation, carbamate formation from carboxyphosphate and ammonia, and carbamate phosphorylation [27,28]. The bicarbonate-dependent ATPase partial reaction and the partial reaction of ATP synthesis from ADP and carbamoyl phosphate reflect the steps of bicarbonate and carbamate phosphorylation, respectively [20,27–29]. *CPS1* is highly expressed in liver [30,31] and can also be detected in small intestine [32] as well as in pancreas [30].

Expression systems for investigations of the functional relevance of missense mutations associated with CPS1D were developed using bacterial CPS [29] or, with the mammalian enzyme, using insect cells [33,34], or, in the case of a common single nucleotide polymorphism (p.Thr1406Asn), yeast (*Schizosaccharomyces pombe*) [35]. Hereby, the role of specific residues and amino acid changes and their functional consequences for CPS1 function were defined. Importantly, at least the recombinant human CPS1 expressed in the baculovirus/insect system showed essentially the same kinetic and molecular properties as the natural human enzyme allowing reliable characterization of disease-causing mutations [34].

In this study, we expressed the recombinant human CPS1 in insect Sf9 cells to characterize the molecular basis of the only currently known recurrent mutation, the deletion of a valine 1013 residue (p.Val1013del) in the carbamate phosphorylation domain of the *CPS1* gene. This mutation, that is located in a region which is not a “hot spot” for mutations, was found in eleven Turkish CPS1D patients from apparently unrelated families. We found that CPS1 WT and p.Val1013 deleted mutant (CPS1-V1013del) were produced at comparable level and in similarly high purity as judged by SDS-PAGE. However, the CPS1-V1013del mutant yielded no significant residual activity in the global as well as the partial reactions and, judged from differential scanning fluorimetry (thermofluor assays), exhibited slightly decreased thermal stability of uncertain functional relevance.

2. Materials and methods

2.1. Mutation analysis in CPS1 deficiency

In the period from September 2008 until May 2014, we made in our laboratory the diagnosis of CPS1D in a total of 78 patients. For clinical genetic testing, we used cDNA derived from cultured fibroblasts or stimulated lymphocytes as described [9,10]. Within this cohort of 78 patients, 18 had a Turkish background and eleven of them were found to carry the mutation c.3037_3039del (r.3037_3039del; p.Val1013del; according to reference sequences for CPS1: GenBank NG_008285.1 and NM_001875.4 and NP_001866.2) in a homozygous state. All eleven patients (6 female, 5 male) had a neonatal onset of disease between day 1 and 6 and died in the neonatal period or infancy. Although some of the families originated from Eastern regions in Turkey, there was no apparent relationship between the families. All parents of whom DNA was available (which was the case in five of the eleven families) were carriers of the respective mutation confirming segregation on each one parental allele. Clinical information was available for all eleven patients confirming the severity of CPS1D in all cases (e.g. max. ammonia levels were between 970 and 2957 μmol/l). Mutation analysis was done after parental written informed consent (obtained according to local regulations). More details to patients and their families can be found in Supplementary Table 1.

2.2. Generation of wild type and mutant CPS1 constructs

The recombinant mature human CPS1 WT construct carrying a His6-tag at the N-terminal position hereby replacing the mitochondrial targeting sequence (pFastBac-CPS1-WT) was generated as described previously [34]. Based on the pFastBac-CPS1-WT, site-directed mutagenesis was performed by the overlapping extension method (Quickchange kit from Stratagene) using the forward V1013delF′ (5′ GACGGTGGTGAATTGCAATCCTGAGAC3′) and reverse V1013delR′ (5′ GTCTCAGGATTGCAATTCACCACCGTC3′) primers yielding the mutant pFastBac-CPS1-V1013del. The established CPS1 constructs were verified by sequencing using the BigDye Terminator cycle sequencing kit version 1.1 (ABI sequence, Applied Biosystems).

2.3. Recombinant human CPS1 protein expression, purification and Western blot analysis

The CPS1 constructs were expressed by using the Bac-to-Bac® Baculovirus Expression System (Invitrogen) as described before [34]. In brief, the plasmids pFastBac-CPS1-WT and pFastBac-CPS1-V1013del were transformed into *Escherichia coli* DH10Bac competent cells by heat shock. The individual bacmids containing the recombinant CPS1 constructs were extracted using the blue white screen and further verified by PCR screening. The Sf9 insect cells were grown in a shaker flask with serum-free SF 900 II SFM cell culture medium (Invitrogen) containing 0.1% Pluronic F-68 (Invitrogen) and 0.5% penicillin/streptomycin solution (Sigma) and incubated at 27 °C with orbital shaking at 125 rpm in a humidified atmosphere. Cells were transfected with bacmids carrying CPS1 WT or mutant (CPS1 c.3037_3039delGTG) cDNA using Cellfectin II Reagent/Grace medium in a 6-well plate for 5 h according to the manufacturer's instructions (Invitrogen) followed by 3-day culture in growth medium without shaking. The produced baculovirus was enriched for 2 days prior to infecting the Sf9 cells for CPS1 expression. The infected cells were harvested after 3 days by centrifugation at 300 *g* for 10 min.

The cell pellets containing recombinant His-tagged human CPS1 WT or mutant proteins were lysed followed by purification using a HisTrap HP 1-ml column fitted in an AKTA FPLC system (GE Healthcare). The CPS1-containing fractions, monitored by SDS-PAGE (8% polyacrylamide gel) with Coomassie staining, were pooled, concentrated to >1 mg protein/ml using centrifugal ultrafiltration (100-kDa cutoff membrane, Amicon Ultra, Millipore), enriched with extra 10% glycerol and 1 mM dithiothreitol (DTT), and snap frozen at −80 °C. The concentrations of purified CPS1 proteins were determined by the method of Bradford [36] using bovine serum albumin as a standard.

Western blotting was performed as described by Laemmli [37]. Cell extracts (~20 μg of total protein) were separated by 8% denaturing SDS-PAGE, and subsequently transferred to nitrocellulose transfer membranes (Whatman GmbH, Dassel, Germany). The primary monoclonal antibody anti-6xHis (Clontech, CA) was used at a dilution of