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Understanding carbamoyl-phosphate synthetase I (CPS1) deficiency by using expression studies and structure-based analysis

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ABSTRACT

Carbamoyl-phosphate synthetase I (CPS1) deficiency (CPS1D), a recessively inherited urea cycle error due to *CPS1* gene mutations, causes life-threatening hyperammonemia. The disease-causing potential of missense mutations in CPS1 deficiency can be ascertained with the recombinant CPS1 expression and purification system reported here, which uses baculovirus and insect cells. We study with this system the effects of nine clinical mutations and one polymorphism on CPS1 solubility, stability, activity and kinetic parameters for NAG. Five of the mutations (p.T471N, p.Q678P, p.P774L, p.R1453Q and p.R1453W) are first reported here, in three severe CPS1D patients. p.P774L, p.R1453Q and p.R1453W inactivate CPS1, p.T471N and p.Y1491H greatly decrease the apparent affinity for NAG, p.Q678P hampers correct enzyme folding, and p.S123F, p.H337R and p.P1411L modestly decrease activity. p.G1376S is confirmed a trivial polymorphism. The effects of the C-terminal domain mutations are rationalized in the light of this domain crystal structure, including the NAG site structure [Pekkala et al. Biochem J 424:211-220]. The agreement of clinical observations and *in vitro* findings, and the possibility to identify CPS1D patients which might benefit from specific treatment with NAG analogues because they exhibit reduced affinity for NAG highlight the value of this novel CPS1 expression/purification system.

Keywords: CPS1 deficiency; hyperammonemia; urea cycle diseases; inborn errors; baculovirus expression system; CPS1 structure; CPS1 site-directed mutagenesis.

INTRODUCTION

Carbamoyl-phosphate synthetase I (CPS1; EC 6.3.4.16) deficiency (CPS1D; MIM# 237300), a rare autosomal (2q35 [Summar et al., 1995]) recessive inborn error of the urea cycle, causes severe neonatal hyperammonemia when there is little or no CPS1 activity in the liver, [Brusilow and Horwich, 2001], or less severe late onset forms believed to be associated with substantial residual enzyme activity [Shi, 1976]. CPS1, a 1463-residue polypeptide (1500 before cleavage of its N-terminal mitochondrial targeting sequence) [Haraguchi et al., 1991; Nyunoya et al., 1985] is found exclusively in hepatocytes and enterocytes. Similarly to *Escherichia coli* CPS (EcCPS) [Thoden et al., 1997], it appears to be folded into six major domains (Figs. 1A,B) [Rubio, 1993; Rubio, 1994; Rubio and Cervera, 1995]. The three-step (Fig. 1C) irreversible reaction catalyzed by CPS1 ($2\text{ATP} + \text{HCO}_3^- + \text{NH}_3 \rightarrow 2\text{ADP} + \text{P}_i + \text{carbamoyl-phosphate}$) [Metzenberg et al., 1958] yields the carbamoyl-phosphate used to make the arginine precursor citrulline. In contrast to other types of CPS, including bacterial CPSs [Meister, 1989], CPS1 uses ammonia with high affinity and cannot use glutamine, and it requires N-acetyl-L-glutamate (NAG), an allosteric activator without which CPS1 is inactive [Lusty, 1983; Rubio et al. 1981]. Perhaps because of the rarity of CPS1D and of the difficulties of its genetic analysis (*CPS1* gene size, 38 exons and 37 introns) [Funghini et al., 2003; Häberle et al., 2003; Summar et al., 2003], relatively small numbers of mutations have been reported in CPS1D, with a large fraction of them being missense changes scattered throughout the protein [Eeds et al., 2006; Kurokawa et al., 2007; Summar, 1998], for which there is little information concerning whether they are truly disease-causing or trivial rare polymorphisms.

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3 Confirmation of the disease-causing nature of *CPS1* missense mutations by site-directed
4 mutagenesis experiments was not possible because of the lack of suitable *CPS1* expression
5 systems, although recombinant EcCPS has been used as a model system for mutations affecting
6 the two CPS catalytic domains [Yefimenko et al., 2005]. However, mutations falling in the 150-
7 residue C-terminal domain could not be studied in this system because of large sequence
8 differences therein and also because this domain is involved in allosteric regulation, and both the
9 effectors and the resulting effects are widely different for EcCPS and *CPS1* (reviewed in [Lusty,
10 1983; Meister, 1989; Pekkala et al., 2009]. Similarly, mutations mapping in the N-terminal 400
11 residues of *CPS1* could not be meaningfully studied in EcCPS because the corresponding region
12 in this enzyme is a separate subunit which binds and cleaves glutamine, yielding the ammonia
13 used in the reaction [Meister, 1989], whereas in *CPS1* this region role is unclear (as already
14 indicated, *CPS1* uses no glutamine and utilizes ammonia with high affinity).

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17 We have developed [Pekkala et al., 2009] and we perfect now a baculovirus/insect cell
18 recombinant *CPS1* expression and purification system that allows rapid production of milligram
19 amounts of pure *CPS1*. We use this system here to study the effects of nine missense *CPS1*
20 mutations found in *CPS1D* patients and one *CPS1* amino acid-changing polymorphism (Fig.
21 1A,B; Table 1). *CPS1* polymorphisms may be important, since they have been associated to
22 vascular pathology and necrotizing enterocolitis [Moonen et al., 2007; Pearson et al., 2001;
23 Summar et al., 2004]. Seven of these changes fall in the two *CPS1* regions that are
24 misrepresented in the EcCPS model, whereas three changes affect the bicarbonate-
25 phosphorylation domain (Fig. 1A). Five mutations are reported here for the first time in three
26 patients with severe *CPS1D*. Our results demonstrate the disease-causing nature of the studied
27 clinical mutations, clarifying their effects, and revealing for two mutations decreased affinity for
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3 NAG, a finding that raises the possibility of pharmacologic therapy. We also exploit our
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5 identification of the NAG site [Pekkala et al., 2009] in the recently determined structure of the
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7 CPS1 C-terminal domain [Xie et al., 2008] to rationalize the effects of mutations affecting this
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9 domain (Fig. 1D).
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12 13 14 15 **PATIENTS AND METHODS**

16 17 **Patients and CPS1 mutations**

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20 Table 1 lists the ten amino acid substitutions studied here, giving some details on the type
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22 of clinical presentation in the patients carrying these mutations. Five mutations were reported
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24 already, whereas five are novel changes identified by one of us (UF) in three neonatal CPS1D
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26 patients (patients 3, 4 and 5, Table 1) presenting negligible liver or small intestine CPS1 activity.
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28 Two of these patients died at an early age and one (patient 3) survives after liver transplantation.
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30 Mutations in these patients were identified in the cDNA prepared from peripheral blood, using
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32 exonic primers for amplification of the cDNA into several overlapping fragments as reported
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34 [Finckh, et al., 1998]. Genomic DNA from patients 4 and 5 was also amplified using intronic
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36 primers deduced from the genomic sequence (GenBank entry NG_008285.1; primers will be
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38 provided upon request). PCR products were sequenced using dye terminator chemistry (Applied
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40 Biosystems). All mutations were confirmed by duplicate analysis or, when possible, by
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42 restriction fragment analysis. Compound heterozygosity for two mutants *CPS1* alleles in the
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44 three new patients (Table 1) was confirmed by segregation analysis using parental genomic
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46 DNA. Samples were obtained with full informed consent of the patients parents in order to
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48 perform molecular genetic diagnostics for clinical purposes.
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Baculovirus-insect cell rat liver CPS1 production and purification

Production of recombinant rat liver CPS1 in Sf9 insect cells using a baculovirus expression system was reported recently [Pekkala et al., 2009]. For enzyme purification, the pellet from a 50-ml 65-hour culture at 27°C of Sf9 cells infected at a virus-to-cell ratio of two, was suspended in 3 ml of a solution containing 20 mM Na phosphate, pH 7, 20 mM KCl, 1 mM dithiothreitol, 10% (v/v) glycerol, 0.1% Triton X-100, 0.4 mM phenylmethylsulphonyl fluoride, 0.1% (v/v) histidine-target specific protease inhibitor mixture, and 5 µM cysteine protease irreversible inhibitor E-64 (the last two components were from Sigma). After 30 min at 0°C, the suspension was twice frozen in liquid nitrogen and thawed in ice, centrifuged (15 min, 16000xg, 4 °C), and the supernatant was applied to a HisTrap 1-ml column in a FPLC AKTA system (GE Healthcare) which was equilibrated with buffer A (20 mM Na phosphate, pH 7/0.5 M NaCl/1 mM dithiothreitol/20 mM imidazole/10% glycerol/) at 4°C. After 10-ml washing with buffer A, a 10-ml linear gradient of 20-500 mM imidazole in buffer A was applied. The fractions containing pure CPS1 (monitored by SDS-PAGE) were pooled, concentrated to >1 mg protein ml⁻¹ by centrifugal ultrafiltration (100 kDa cutoff Amicon Ultra, from Millipore), enriched with 10% extra glycerol, and either used immediately or frozen at -80°C.

Site directed mutagenesis and production of mutant enzyme forms

A pFastBac-HTA (from Invitrogen)-derived plasmid that harbors the cDNA encoding the mature form of CPS1 [Pekkala et al., 2009] was mutated by the overlapping extension method using a commercial kit (Quickchange, from Stratagene), a high fidelity thermostable DNA polymerase (Pfu Turbo from Stratagene) and the appropriate mutagenic forward and reverse oligonucleotides (Supp. Table S1). Mutant plasmids were sequenced to corroborate the presence

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3 of the desired mutation and the absence of unwanted mutations. Production of the mutant
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5 proteins by the insect cells and enzyme purification were as for the wild type enzyme.
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8 9 10 **Enzyme activity assay**

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12 CPS1 activity was assayed by measuring colorimetrically citrulline [Nuzum and
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14 Snodgrass, 1976] in an ornithine transcarbamylase-coupled 10-min assay at 37°C [Pekkala et al.,
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16 2009] containing 50 mM glycyl-glycine pH 7.4, 0.1 M KCl, 0.1 M NH₄Cl, 5 mM ATP, 7 mM
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18 MgSO₄, 20 mM KHCO₃, 10 mM NAG, 5 mM L-ornithine, 1 mM dithiothreitol, 10 U/ml
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20 ornithine transcarbamoylase, and 5-200 µg/mL of CPS1 (wild-type or mutant). In some cases
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22 ADP was measured also at the end of the 10-min incubation, after terminating the reaction with
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24 10% trichloroacetic acid [Fresquet et al., 2000]. When indicated, to approach the conditions
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26 prevailing in the mitochondrial matrix of the hepatocyte [Garcia-España et al., 1991; Siess et al.,
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28 1982], we lowered the NH₄Cl and NAG concentrations to 1 and 0.1 mM, respectively. Because
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30 of the low activity of the p.T471N mutant, a coupled assay was utilized for determination of the
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32 K_a^{NAG} of this mutant, in which ADP production was monitored continuously at 340 nm
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34 [Guthohrlein and Knappe, 1968] in the standard assay mixture supplemented with 2.5 mM
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36 phosphoenol pyruvate, 0.25 mM NADH, 0.04 mg/ml pyruvate kinase and 0.025 mg/ml lactate
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38 dehydrogenase. Kinetic constants were determined in the standard assay by varying the
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40 concentration of the desired substrate or of NAG, using program GraphPad Prism (GraphPad
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42 Software, San Diego, California) for fitting the data to hyperbolae.
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51 52 **Other assays**

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3 CD spectra (10 scans per spectrum) were taken in the far-UV region (195–250 nm; 4°C;
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5 0.1 cm-pathlength cell) with a Jasco 810 spectropolarimeter, on 1-2 μM solutions of wild type or
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7 mutant proteins in 20 mM Tris-HCl pH 7.4, 20 mM KCl, 20% glycerol and 0.5 mM
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9 dithiothreitol.
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12 Thermal stability was monitored by 15-min heating at the indicated temperatures of 1 mg
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14 ml^{-1} enzyme solutions, in 50 mM glycyl-glycine pH 7.2, 20 mM KCl an 20% glycerol, followed
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16 by flash-cooling to 0°C, taking sample for immediate enzyme activity assay at 37°C.
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19 Protein was determined according to [Bradford, 1976] utilizing bovine serum albumin as
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21 standard.
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24 25 26 27 **RESULTS AND DISCUSSION**

28 29 **Mutations studied**

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31 p.S123F and p.H337R, which map, respectively, in the N-terminal and the glutaminase-
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33 like domains of the N-terminal region of CPS1 (Fig. 1, A,B), supposedly do not completely
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35 inactivate CPS1, since they were found in late onset patients which carried either p.S123F as the
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37 only mutation detected in cDNA studies (patient 1, Table 1) [Summar, 1998]), or p.H337R in
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39 heterozygosis with an inactivating deletion/frameshift (patient 2, Table 1) [Aoshima et al., 2001].
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43 Of the five C-terminal amino acid substitutions studied here (Fig. 1, A,B,D; Table 1),
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45 p.R1453W and p.R1453Q are novel mutations affecting the same residue. They were found,
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47 respectively, in patients 3 and 5 (Table 1), in heterozygosis with the novel bicarbonate
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49 phosphorylation domain mutations p.T471N (patient 3) and p.P774L (patient 5, Table 1), two
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51 mutations that are also expressed and analyzed functionally here. Patients 3 and 5 presented very
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53 severe CPS1D with negligible liver CPS1 activity, and thus, the four mutations found in these
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3 patients, p.T471N, p.P774L, p.R1453W and p.R1453Q, should inactivate or decrease very
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5 drastically the activity of CPS1. In contrast, p.P1411L and p.Y1491H (Table 1, patients 6 and
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7 7), were reported in late onset CPS1D [Summar, 1998] and are unlikely to be totally inactivating,
8
9 since p.P1411L was found in heterozygosis with the nonsense change p.Q478X and p.Y1491H
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11 was the only *CPS1* allele detected (Table 1). The amino acid substitution p.G1376S was found
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13 by one of us (UF) in a severely deficient patient (patient 4, Table 1) before it was recognized as a
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15 polymorphism [Summar et al, 2003], along with a missense change for p.Q678P on the paternal
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17 allele and a 486 bp deletion causing exon 8 skipping (revealed by cDNA analysis) on the
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19 maternal allele (Table 1), illustrating the difficulties in ascertaining the disease-causing nature of
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21 missense changes when they coexist with unrecognized polymorphisms.
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29 **Expression, isolation and stability of recombinant wild type CPS1 and of its clinical** 30 **mutants** 31 32

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34 Our recently reported [Pekkala et al., 2009] baculovirus-insect cell system for CPS1
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36 expression, slightly modified to improve enzyme purification (see Methods section), was used
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38 for production of the wild type rat liver enzyme and the corresponding mutant enzymes. Rat and
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40 human CPS1 can be used interchangeably because they share 96% sequence identity [van den
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42 Hoff et al., 1995] and have virtually identical properties [Rubio et al., 1981]. The expressed
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44 recombinant rat liver enzyme, lacking its N-terminal mitochondrial targeting sequence (residues
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46 1-37) and having an N-terminal 6×His-tag for facilitating purification, represented ~5% of the
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48 soluble protein (judged by SDS-PAGE) in cell extracts when using an optimized virus-to-cell
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50 ratio and postinfection incubation period, yielding typically, per 200-ml culture, 3-5 mg pure
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52 CPS1 (Fig. 2A) having normal activity ($1.31 \pm 0.03 \mu\text{mol citrulline min}^{-1} \text{mg}^{-1}$) and substrate and
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3 activation kinetics (respective K_m values for ATP, HCO_3^- and NH_4^+ , 1.06 ± 0.11 mM,
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5 6.43 ± 0.60 mM and 1.07 ± 0.1 mM; K_a^{NAG} , 0.13 ± 0.01 mM) [Pekkala et al., 2009].
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8 The mutations studied here did not hamper CPS1 expression, solubility or enzyme
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10 purification (Fig. 2A), except in the case of the p.Q678P substitution, which resulted in poor
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12 CPS1 expression and solubility. Nevertheless, although with low yield, it was still possible to
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14 purify this mutant. UV-CD spectra of the mutant and wild-type enzymes were essentially
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16 identical, excluding gross misfolding of the mutants. In those mutants that were active (all but
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18 p.P774L, p.R1453Q and p.R1453W, see below) the reaction stoichiometry was normal (2 ADP
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20 molecules made per carbamoyl-phosphate molecule synthesized, data not shown), and the
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22 stability to heating (p.T471N was also excluded from this study because of its very low activity)
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24 was not changed significantly, excluding lack of stability as a main reason for the clinical
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26 deficiency (but see below).
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34 **The two mutations affecting the N-terminal region decrease but do not abolish enzyme** 35 **activity** 36 37

38 As expected (see above), the recombinant p.S123F and p.H337R mutant forms were
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40 active. However, their activities were reduced by ~60% and ~70%, respectively, relative to wild
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42 type CPS1 (Table 2), apparently because of reduced rates of catalysis (k_{cat}), since apparent K_m
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44 values for the three substrates (not shown) and K_a^{NAG} were not substantially changed by these
45
46 mutations (Table 2). The presence of substantial enzyme activity with the two mutations agrees
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48 with the late onset presentation. This reduction in the activity suggest that the N-terminal region
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50 of CPS1 partially activates the catalytic moiety of the enzyme, an activation that is reminiscent
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52 of that of EcCPS by glycine (an inert glutamine analog; glutamine binds to the N-terminal
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3 region of EcCPS [Meister, 1989]). Perhaps the main role of the CPS1 N-terminal region is
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5 activation of the enzyme catalytic power. In fact, removal of the entire N-terminal region
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8 dramatically reduced the activity of CPS1 [Ahuja and Powers-Lee, 2008].
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10 11 12 **p.G1376S is a polymorphism with no functional consequences**

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15 The frequent p.G1376S polymorphism [Summar et al., 2003] located in the C-terminal,
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17 regulatory domain of CPS1 has no substantial effect, within experimental error, on CPS1 activity
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19 at both saturating and subsaturating ammonia/NAG concentrations (Table 2), on the K_a^{NAG}
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21 (Table 2), or on the thermal stability of the enzyme (Fig. 2B). This lack of effect can be
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23 rationalized in terms of the known structure of the CPS1 regulatory domain, given the small size
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25 of both Gly and Ser, precluding large structural effects of their mutual substitution, particularly
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27 since the torsion angles of the enzyme main chain at the site of G1376 are permissive for the
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29 occurrence of serine at this site, and also because, in the CPS1 C-terminal domain, G1376 looks
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31 towards the aqueous solvent (Fig. 1D), an excellent environment for a serine side-chain.
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39 **The regulatory domain mutations p.R1453Q and p.R1453W and the bicarbonate** 40 41 **phosphorylation domain mutation p.P774L inactivate the enzyme**

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43 Three mutations, p.P774L, p.R1453Q and p.R1453W, rendered CPS1 inactive (Table 2),
44
45 confirming their disease-causing nature (Table 1). R1453 is located in the middle of the allosteric
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47 domain helix 4 (Fig. 1D), a helix that shapes the site for the essential allosteric activator NAG
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49 and which provides NAG site residues (I1452, N1449 and F1445). Therefore, the p.R1453Q/W
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51 mutations may distort the NAG site, in addition to hampering the interactions made by the
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53 R1453 side chain with the key NAG binding residue N1437, which thus could be misplaced for
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3 proper NAG binding. In summary, these mutations may inactivate CPS1 by rendering it
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5 insensitive to its essential activator NAG. Furthermore, transmission of the NAG signal to the
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7 catalytic machinery may also be hampered, given the important contacts of the residue
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9 corresponding in EcCPS to R1453 of CPS1, R1030, with the carbamate phosphorylation domain
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11 [Thoden et al., 1997]. The ion pair formed between R1030 and D902 (corresponding to R1453
12
13 and D1322 in human CPS1) should be abolished by the mutation of the arginine to glutamine or
14
15 to tryptophan. D902 is in one possible path for NAG signal transmission to both phosphorylation
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17 domains, since it belongs to the carbamate phosphorylation domain loop 900-914, which is
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19 sandwiched between the C-terminal domain and the bicarbonate phosphorylation domain loop
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21 367-372 (Fig. 1D). In any case, mutations affecting this invariant arginine (R1453 in human)
22
23 should be inactivating only in CPS1, since other CPSs are active in the absence of any effectors.
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25 Indeed, mutation to alanine of the corresponding arginine of EcCPS and of yeast CPS (R1030A
26
27 and R1060A mutations, respectively) fails to inactivate these CPSs [Czerwinski et al., 1995; Lim
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29 and Powers-Lee, 1997].
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36 The inactivation of the enzyme by the p.P774L mutation agrees with the previous
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38 observations that the corresponding mutation in EcCPS, P360L [Delannay, et al., 1999;
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40 Yefimenko, et al., 2005] drastically decreased enzyme activity and strongly shifted the allosteric
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42 equilibrium towards the inactive enzyme conformation, an equilibrium that is very displaced
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44 already towards the inactive form in wild type CPS1 (as shown by the lack of activity in the
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46 absence of NAG).
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53 **A large decrease in the apparent affinity for NAG is triggered by the p.Y1491H and**
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55 **p.T471N mutations**
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3 The p.Y1491H mutation decreased ~4-fold CPS1 activity at saturation of the substrates
4 and NAG, and even more at subsaturating ammonia and NAG concentrations (Table 2),
5 reflecting ~70-fold increased K_a^{NAG} and a more modest decrease of V_{max} (Table 2). The
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10 substantial residual activity of the mutant enzyme possibly accounts for the late onset
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12 presentation in patient 7, who appeared to be homozygous for this mutation (Table 1). Similarly,
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14 the p.T471N mutation, affecting a residue of the bicarbonate phosphorylation domain (Fig. 1A),
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16 importantly decreased the apparent affinity for NAG, but it decreased also ~13-fold enzyme
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18 activity at NAG saturation, considerably more than the p.Y1491H mutation (Table 2). These
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20 effects, combined with the inactivating nature of the p.R1453W mutation found also in the same
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22 patient (patient 3 was a compound heterozygote for the p.T471N and p.R1453W mutations),
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24 explain the very severe deficiency observed in this patient. These mutations at Y1491 and T471
25
26 may hamper the cross-talk between the ATP and NAG sites that is manifested in the fact that
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28 NAG induces a dramatic decrease in the K_m^{ATP} [Rubio, et al., 1983] and that ATP increases very
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30 importantly the affinity of CPS1 for NAG [Alonso and Rubio, 1983]. Cleavage of the C-terminal
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32 20 residues of rat liver CPS1, including among them Y1491 (the 10th residue from the C-
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34 terminus), increased >30-fold K_a^{NAG} [Marshall and Fahien, 1988], and the deletion of the 14
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36 residues at the C-terminus of EcCPS abolished any actions of the allosteric effectors of the
37
38 enzyme and dramatically decreased and increased, respectively, V_{max} and the K_m for ATP
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40 [Czerwinski, et al., 1995]. Interestingly, in the EcCPS structure (the only known structure for a
41
42 complete CPS; PDB file 1CE8 [Thoden et al., 1997]), the 14 C-terminal residues are projected
43
44 far away from the C-terminal domain as a loop and a terminal α -helix, contacting extensively
45
46 both phosphorylation domains (Fig. 1E) through a hydrophobic cluster that includes V1065 and
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48 T56, the residues corresponding to Y1491 and T471 of CPS1 (Fig. 1E). In this context, the
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3 p.Y1491H and p.T471N mutations, by introducing more polar residues, would disturb this
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5 hydrophobic cluster, hampering the cross-talk between the ATP and the NAG sites.
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8 9 10 **p.P1411L is a mild mutation**

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12 The decrease of the order of 50% in enzyme activity even at suboptimal ammonia and
13 NAG concentrations triggered by the p.P1411L mutation (Table 2) would be expected to yield in
14 patient 6, who hosts this mutation and a second inactivating *CPS1* allele (a nonsense change), a
15 liver CPS1 activity of the order of 25% of normal, possibly in the limit for substantial deficiency
16 [Eather et al., 2006], agreeing with the late onset presentation observed in this patient. The
17 P1411L mutation does not increase the K_a^{NAG} despite the fact that P1411 is next to the NAG site
18 lid residue W1410, an important residue which when mutated to lysine triggered ~60-fold
19 increase in the K_a^{NAG} in addition to decreasing V_{max} to a similarly extent than p.P1411L [Pekkala
20 et al., 2009]. The lack of NAG site distortion by the P1411L mutation that is revealed by the
21 failure of the mutation to increase the K_a^{NAG} may result from the specific amino acid substitution
22 in this mutation, since in the structure of the CPS1 C-terminal domain P1411 faces I1452, a
23 residue of the hydrophobic cavity that surrounds the end-methyl of the acetamide group of NAG
24 [Pekkala et al., 2009]. Therefore, the P1411L replacement would not appear ill-suited for
25 interaction with I1452 or for providing a hydrophobic environment to the NAG methyl group.
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48 **Low efficiency of correct folding appears the major reason for the pathogenic role of the** 49 **p.Q678P mutation**

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51 As already mentioned, this mutation greatly decreases the yield of soluble recombinant
52 enzyme (Fig. 2A), with most of the expressed protein being recovered in the pellet. This may be
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3 the main reason for the drastic deficiency observed clinically in patient 4, a heterozygote having
4 as the second allele a necessarily inactivating exon 8-skipping mutation (Table 1). Because of an
5 unfavourable partition between well-folded and misfolded enzyme, only a low level of soluble
6 functional enzyme may exist *in vivo*. Nevertheless, the small fraction that is well folded and
7 soluble is active, although it exhibits ~6-fold decreased activity (per mg protein) in the standard
8 assay, again supporting the disease-causing nature of this mutation. Previous studies with the
9 corresponding EcCPS mutant, Q262P, yielded similar results: poor folding and ~80% decrease in
10 the velocity of the enzyme [Yefimenko, et al., 2005]. These effects were attributed to the helix-
11 breaking effect of proline (Q262 sits in an α helix), predicting a partial block in the tunnel (Fig.
12 1B) that brings the ammonia to the bicarbonate phosphorylation site for catalysis of reaction step
13 2 (Fig. 1C). Such disturbances would not be expected if the mutation does not break the α helix.
14 In agreement with this prediction, the mutation Q262A of EcCPS, which does not have the helix-
15 breaking potential of Q262P, failed to trigger enzyme precipitation or decreased activity [Kim
16 and Raushel, 2004].
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39 Final remarks

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41 The present work supports the value of a commercial kit-based baculovirus-insect cell
42 expression system used here for testing the disease-causing potential of missense mutations
43 identified in CPS1D patients. There is essentially full agreement between the results obtained by
44 this approach and the expectations derived from genotype and clinical observations in the
45 patients. The system has been proven here to be highly valuable for determining the effects of
46 mutations affecting the N-region and the C-terminal domain, two regions of CPS1 for which the
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3 EcCPS model used previously for catalytic domain mutations [Yefimenko et al., 2005] does not
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5 appear appropriate.
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8 While the present work was being performed, another system has been reported [Ahuja
9
10 and Powers-Lee, 2008] for human CPS1 expression, which is based on the use of
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12 *Schizosaccharomyces pombe* as expression host. This system has not been used for the
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14 expression of clinical mutations found in CPS1D patients. However, its utilization for testing the
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16 effects of a common polymorphism [Ahuja and Powers-Lee, 2008] which has been associated
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18 with vascular pathology and with necrotizing enterocolitis, suggests that this system can also be
19
20 valuable for testing the disease-causing nature of clinical CPS1D mutations. Nevertheless, more
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22 details on this system should be provided to compare it in terms of convenience of use with the
23
24 present expression/purification system.
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29 An interesting result of the present studies is the identification of one live patient with a
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31 clinical mutation (p.Y1491H) having as the dominant disease-causing trait a large decrease in the
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33 affinity of CPS1 for NAG. It is conceivable that the partial deficiency observed in this patient
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35 could be overcome by greatly increasing the concentration of NAG in the liver. Perhaps the
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37 administration of large arginine doses could effect such increase [Tatibana and Shigesada, 1976],
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39 since arginine is an activator of NAG synthase [Tatibana et al., 1976], the enzyme that makes the
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41 NAG used for CPS1 activation. Alternatively, large doses of the deacylase-resistant NAG
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43 analogue N-carbamoyl-L-glutamate [Caldovic et al., 2004; Rubio and Grisolia, 1981] could be
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45 administered to this patient to try to increase CPS1 activation. The latter treatment appears
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47 particularly feasible since N-carbamoyl-L-glutamate is little toxic
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51 (<http://www.emea.europa.eu/humandocs/PDFs/EPAR/carbaglu/534602en6.pdf>) and is already
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3 marketed in Europe under the name Carbaglu[®] (Orphan Europe) as an orphan drug for treating
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5 secondary CPS1 deficiency due to primary deficiency of NAG synthase.
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11
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FIGURE LEGENDS

Figure 1. Structural bases of CPS1 function and deficiency. (A) Bar scheme of the domain composition of the CPS1 polypeptide, color-coding the different domains, mapping with arrows above the bar the position of the different amino acid substitutions studied here, and giving below the bar the number for the first and last residue of each domain. The same color code for the different domains is used in other panels. The mass of the different domains, in kDa, is given in black within the bar. The question sign denotes the so-called oligomerization domain of EcCPS. (B) Structure of EcCPS1 (as a coil representing the main chain; PDB file, 1BXR), localizing with numbered spheres the amino acids corresponding to the mutated residues studied here. Residue numbering is that for human CPS1. The CPS1/EcCPS residue correspondence is as follows: S123/S72_{ss} (the subindex denotes the small subunit of EcCPS; when not using subindex, reference is made to the large subunit); H337/H312_{ss}; T471/T56; Q678/Q262; P774/P360; G1376/ D959; P1411/V994; R1453/1030; Y1491/V1065. Pink shadowing highlights the intramolecular tunnel found in the structure of EcCPS. The two bound nucleotide molecules are shown in sticks representation, in black. (C) Steps of the reaction catalyzed by CPS1. Each step is shown under the domain catalyzing it, and it is colored as the corresponding domain. The empty arrow in black denotes the migration of carbamate from the bicarbonate phosphorylation domain to the carbamate phosphorylation domain. (D) Stereo view of the crystal structure of the allosteric domain of human CPS1 (C^α trace in black; PDB file 2YVQ), fitting in it the bound NAG molecule (in sticks and colored) [Pekkala et al., 2009]. The green and brown loops belong, respectively, to the bicarbonate and carbamate phosphorylation domains of the superimposed

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3 structure of EcCPS. Small spheres mark every tenth residue (numbering in small type). Large
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5 blue spheres correspond to the indicated allosteric domain residues, which are those at which
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7 amino acid substitutions are studied here (except Y1491, affecting a residue not included in the
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9 experimental crystal structure). (E) Cartoon representation of the C-terminal helix (in reddish) of
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11 EcCPS, viewed to show its relations with the bicarbonate (in green) and the carbamate (in
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13 brownish) phosphorylation domains. The positions of V1065 and T56 (corresponding to the
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15 CPS1 residues Y1491 and T471) are illustrate with spheres.
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22 **Figure 2.** Purified wild-type and mutant forms of CPS1, and thermal stability of these forms. (A)
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24 SDS-PAGE (8% polyacrylamide) [Laemmli, 1970] of the different purified enzyme forms, after
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26 Coomassie staining. (B) Thermal stability of the enzyme activity of the different CPS1 forms
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Table 1. *CPS1* mutations found in CPSD patients tested here in recombinant CPS1

CPS1 domain	Mutation ^a	Residue conservation	Patient	Second <i>CPS1</i> allele	Clinical severity	Report
N-terminal ₁	p.S123F	Invariant in CPS1 and CPSIII. Largely conserved in other CPSs	1	No second <i>CPS1</i> allele found in cDNA study	Late onset	Summar, 1998
N-terminal ₂ (glutaminase-like)	p.H337R	Invariant in all CPSs	2	Deletion and frameshift	Late onset	Aoshima <i>et al.</i> , 2001
Bicarbonate-phosphorylation	p.T471N c.1412C>A	Invariant in all CPSs	3	p.R1453W	Neonatal. No liver CPS1 activity. Survives at 3.5 years thanks to emergency liver transplantation	Present work
	p.Q678P^b c.2033A>C	Conserved in CPS1	4^b	486 bp deletion leading to exon 8 skipping ^c	Neonatal. Death at 2 months. Intestinal CPS1 activity, <2% of control	Present work
	p.P774L c.2321C>T	Invariant in all CPSs	5	p.R1453Q	Neonatal. Death at 3.5 months. Liver CPS1 activity <5% of control	Present work
C-terminal (NAG-binding, regulatory)	p.G1376S ^b	Polymorphism	4^b		See p.Q678P	
	p.P1411L	Invariant in CPS1/CPSIII. V in bacterial CPS	6	p.Q478X	Late onset	Summar, 1998

p.R1453Q c.4358G>A	Invariant in all CPSs	5	p.P774L	See p.P774L	Present work
p.R1453W c.4357C>T		3	p.T471N	See p.T471N	Present work
p.Y1491H	Invariant in CPS1/CPSIII	7	No second <i>CPS1</i> allele found in cDNA study	Late onset	Summar, 1998

Novel mutations and patients are highlighted in boldface. When the mutation is novel, the cDNA change is given also.

^aReference sequence: GenBank entry: NG_008285.1. For cDNA numbering +1 corresponds to the A of the ATG translation initiation codon in the reference sequence according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon must be codon 1.

^bThe mutation p.Q678P and the polymorphism p.G1376S coexist in the same CPS1 allele.

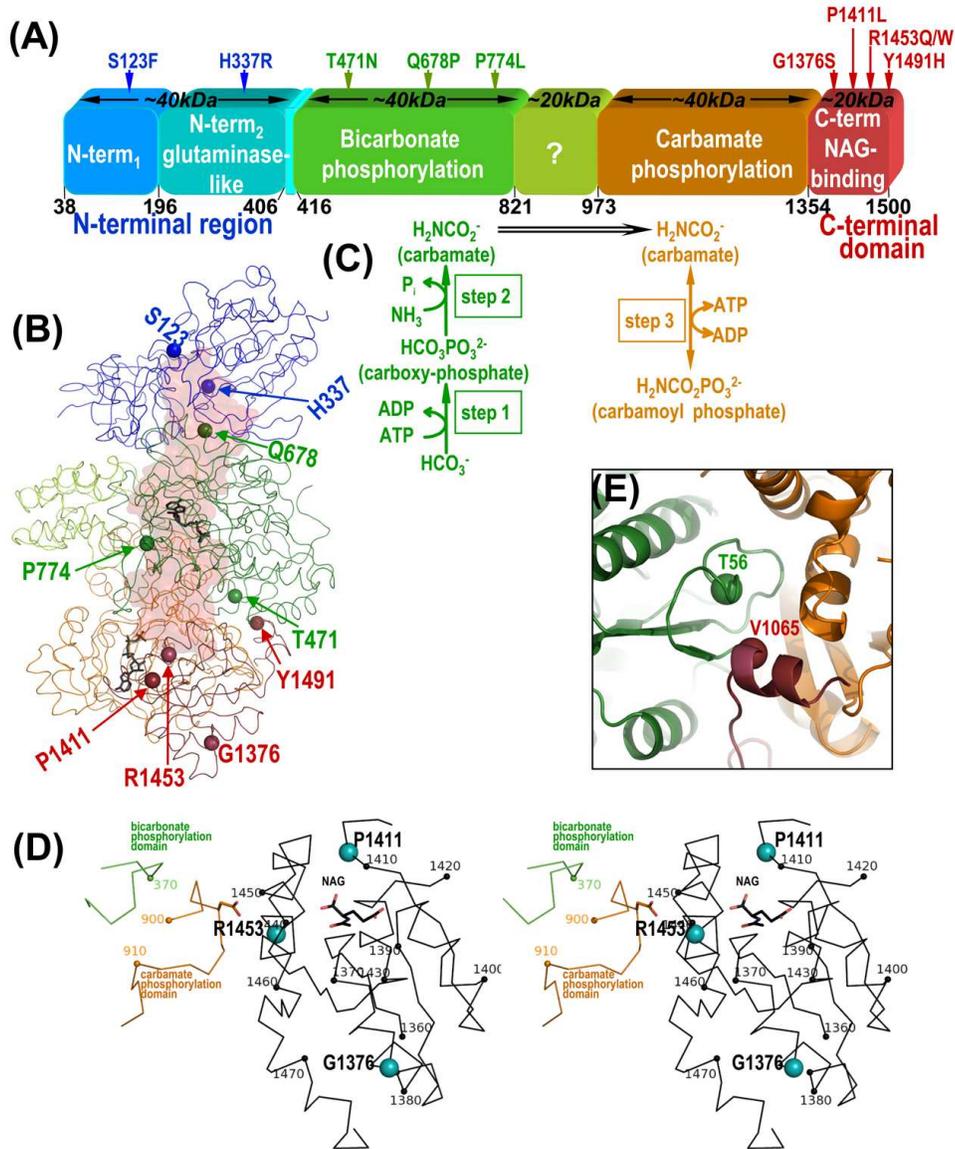
^cDNA change, 116624(±1)_116139(±1)del (del 486bp) including 56-58 bp of exon 8, with exon 8 skipped in-frame. Redundancy of 3 bp at the deletion breakpoints precluded unambiguous determination of the deletion endpoints. The 486 bp deletion encompasses 428-430 bp of the 3'-end of intron 7, and 56-58 bp of the 5' end of exon 8, leading to exon 8 skipping (confirmed by PCR using exonic primers flanking exon 8 and the mutant cDNA as template).

Table 2. Influence of clinical CPS1 mutations on enzyme activity and on enzyme activation by NAG*

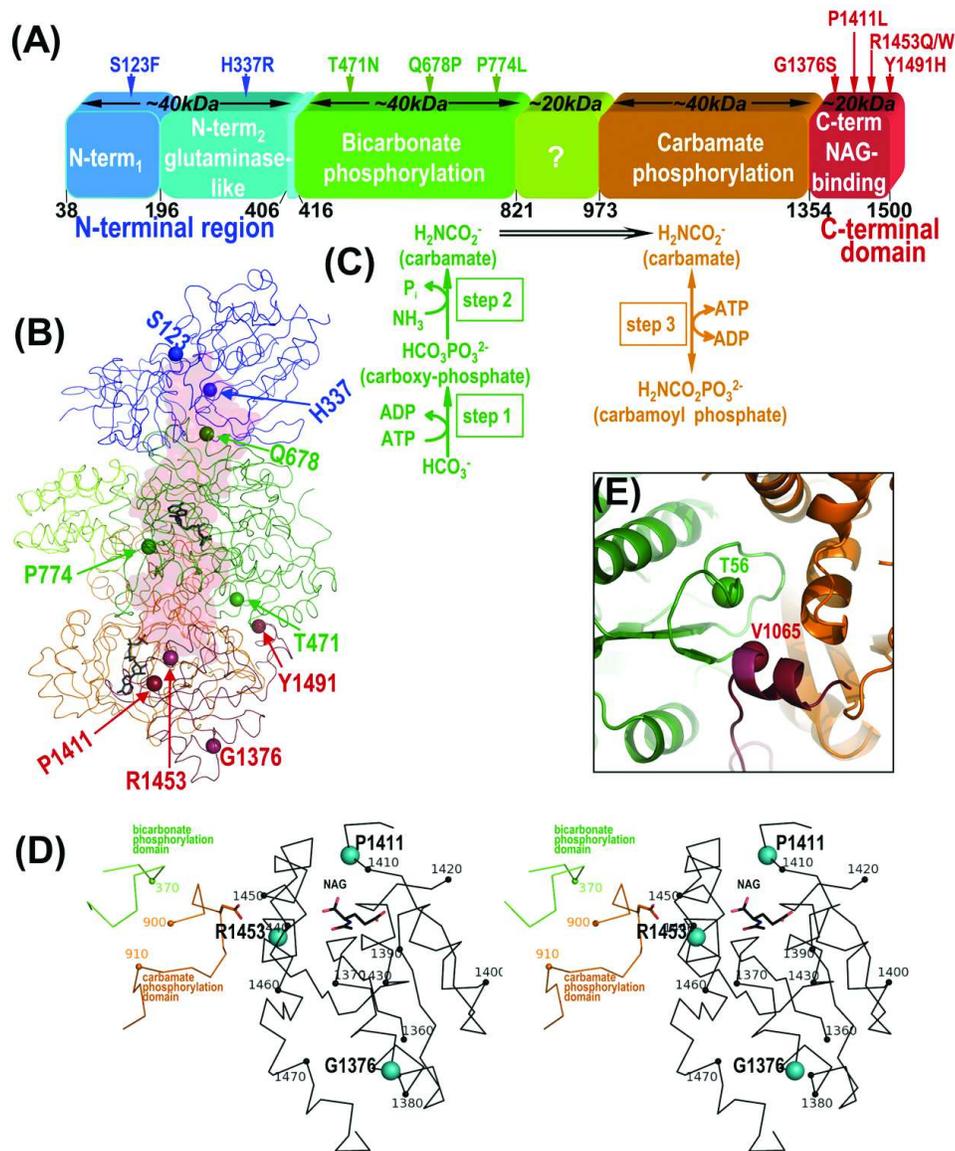
CPSI form	CPSI activity assayed at		[NAG] dependence of CPS1 activity	
	10 mM NAG 100 mM NH ₄ ⁺	0.1 mM NAG 1 mM NH ₄ ⁺	V ^{[NAG]=∞}	K _a
		μmol min ⁻¹ mg ⁻¹		mM
WT	1.31 ± 0.03	0.31 ± 0.04	1.34 ± 0.02	0.13 ± 0.01
p.S123F	0.51 ± 0.01	0.098 ± 0.004	0.52 ± 0.01	0.20 ± 0.02
p.H337R	0.38 ± 0.04	0.077 ± 0.002	0.38 ± 0.03	0.16 ± 0.04
p.T471N	0.10 ± 0.02	<0.01 ^a	0.18±0.01	4.9 ± 0.3
p.Q678P	0.22 ± 0.02	0.042 ± 0.001	0.22 ± 0.01	0.21 ± 0.04
p.P774L	<0.01 ^a	<0.01 ^a	-----	-----
p.G1376S	1.32 ± 0.06	0.27 ± 0.01	1.35 ± 0.04	0.19 ± 0.03
p.P1411L	0.83 ± 0.03	0.18 ± 0.01	0.81 ± 0.02	0.15 ± 0.02
p.R1453Q	<0.01 ^a	<0.01 ^a	-----	-----
p.R1453W	<0.01 ^a	<0.01 ^a	-----	-----
p.Y1491H	0.31 ± 0.01	<0.01 ^a	0.51 ± 0.01	6.9 ± 0.5

*For details, see Methods

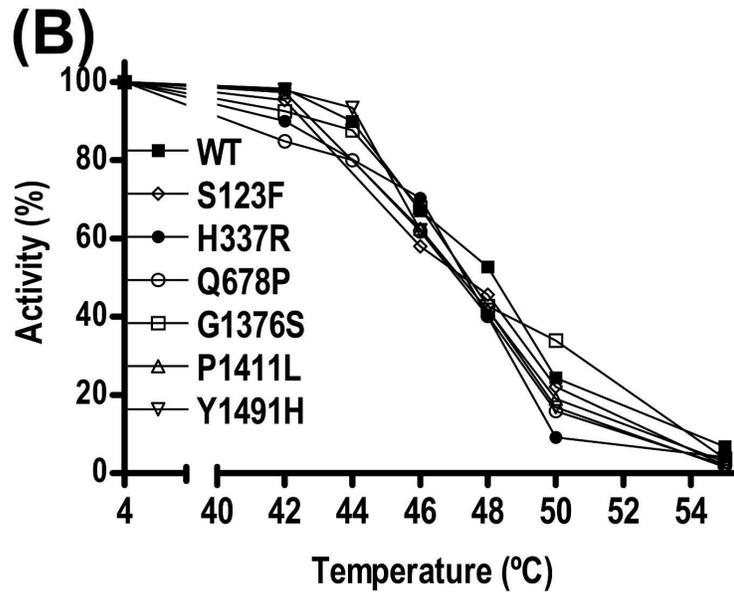
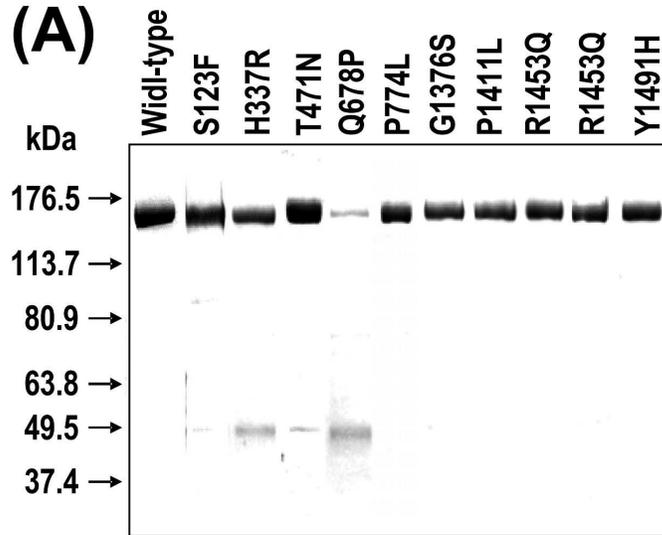
^aDetection limit



167x194mm (200 x 200 DPI)



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122x188mm (600 x 600 DPI)

Supp. Table S1. Synthetic oligonucleotides used in site-directed mutagenesis

Mutation	Direction	Sequence
S123F	Forward	5'G AAT AAG TAC ATG GAG TTT GAT GGA ATC AAG G3'
S123F	Reverse	5'C CAC CTT GAT TCC ATC AAA CTC CAT GTA C3'
H337R	Forward	5'C ATA ACT GCT CAG AAT CGT GGC TAT GCT CTG G3'
H337R	Reverse	5'GGT GTT GTC CAG AGC ATA GCC ACG ATT CTG AGC A G3'
T471N	Forward	5'GCA TCC GTG CAG AAC AAC GAG GTG3'
T471N	Reverse	5'CAA TCC CAC CTC GTT GTT CTG CAC GGA TG3'
Q678P	Forward	5'C AAT GCA GAG TTC CCG ATG TTG AGA CG3'
Q678P	Reverse	5'GCG TCT CAA CAT CGG GAA CTC TGC ATT GG3'
P774L	Forward	5'C ATG GTG ACC AAG ATT CTC CGC TGG G3'
P774L	Reverse	5'G GTC CCA GCG GAG AAT CTT GGT CAC C3'
G1376S	Forward	5'CGT CCA AGA TTC CTT AGT GTT GCT GAG C3'
G1376S	Reverse	5'G TAA CTG CTC AGC AAC ACT AAG GAA TCT T3'
P1411L	Forward	5'C CCA GTG GCT TGG CTA TCT CAG GAA GG3'
P1411L	Reverse	5'CTG TCC TTC CTG AGA TAG CCA AGC CAC3'
R1453Q	Forward	5'GAT AAT TAT GTG ATT CAG AGG ACA GCT GTG G3'
R1453Q	Reverse	5'C CAC AGC TGT CCT CTG AAT CAC ATA ATT ATC3'
R1453W	Forward	5'GAT AAT TAT GTG ATT TGG AGG ACA GCT GTG3'
R1453W	Reverse	5'C AGC TGT CCT CCA AAT CAC ATA ATT ATC ATG G3'
Y1491H	Forward	5'CC AAG AGT CTG TTC CAC CAC AGG CAG TAC AGC3'
Y1491H	Reverse	5'CC AGC GCT GTA CTG CCT GTG GTG GAA CAG AC3'

The triplets introducing the desired mutations are indicated in bold.