Review

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Glutamine synthetase as a central element in hepatic glutamine and ammonia metabolism: novel aspects

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Abstract: Glutamine synthetase (GS) in the liver is expressed in a small perivenous, highly specialized hepatocyte population and is essential for the maintenance of low, non-toxic ammonia levels in the organism. However, GS activity can be impaired by tyrosine nitration of the enzyme in response to oxidative/nitrosative stress in a pH-sensitive way. The underlying molecular mechanism as investigated by combined molecular simulations and *in vitro* experiments indicates that tyrosine nitration can lead to a fully reversible and pH-sensitive regulation of protein function. This approach was also used to understand the functional consequences of several recently described point mutations of human GS with clinical relevance and to suggest an approach to restore impaired GS activity.

Keywords: ammonia; glutaminase; glutamine synthetase; hyperammonemia; molecular dynamics simulations; protein tyrosine nitration.

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Introduction

There is a sophisticated structural and functional organization of ammonia and glutamine metabolizing pathways in the liver acinus (Häussinger 1983) (for review see Häussinger 1990). Glutamine synthetase (GS) is restricted to a small perivenous hepatocyte population surrounding the hepatic venules, whereas periportal hepatocytes contain liver-type glutaminase (GLS2) and urea cycle enzymes (Gaasbeek Janzen et al. 1984; Gebhardt and Mecke 1983; Häussinger 1983) (for a review see Häussinger 1990; Häussinger and Schliess 2007). GLS2 is activated by its product ammonia and acts as an intramitochondrial pH-modulated ammonia amplifier. This amplification step is required for urea synthesis in view of the high K_m (ammonia) of carbamoylphosphate synthetase I, which exceeds by far the physiological ammonia concentration in portal venous blood. Ammonia amplification by GLS2 is very pH sensitive, which provides one basis for adjusting flux through the bicarbonate-consuming urea cycle to the needs of acid-base balance (for review see Häussinger 1990). Ammonia that escaped periportal urea synthesis is eliminated with high affinity by GS-containing hepatocytes at the acinar outflow. Thus, in the liver acinus, glutamine is hydrolyzed in periportal hepatocytes, whereas it is resynthesized by perivenous hepatocytes from the ammonia left over by periportal urea synthesis (Figure 1). This is the so-called intercellular glutamine cycle, whose regulation is essential for the maintenance of bicarbonate and ammonia homeostasis in the organism. Depending on the acid-base status, that way, the liver can switch ammonia elimination from urea to glutamine synthesis.

Characteristics of perivenous GS-expressing hepatocytes

The perivenous GS-containing hepatocytes have also been termed scavenger cells because they eliminate not only ammonia with high affinity but also at least some

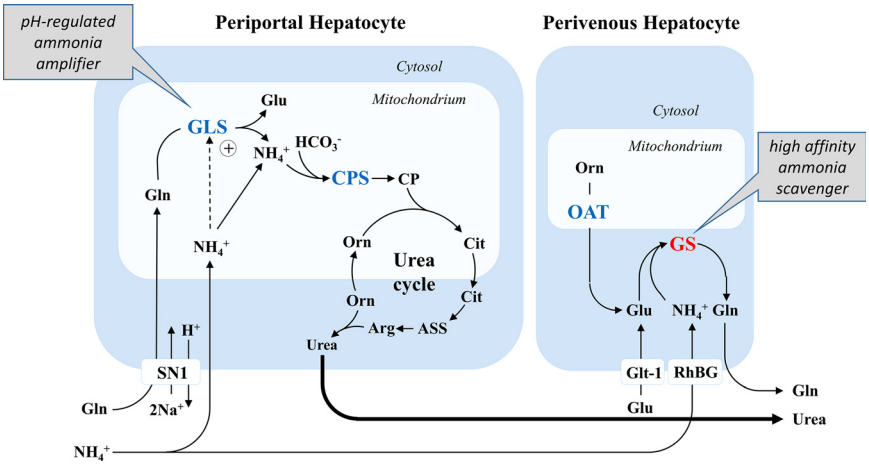

Figure 1: Structural–functional organization of hepatic glutamine and ammonia metabolism.
Following the bloodstream, ammonia removal by urea and glutamine synthesis are organized in sequence. Periportal urea synthesis is a high-capacity, but low-affinity system for ammonia removal, whereas downstream glutamine synthetase corresponds to a high-affinity system for ammonia removal. Liver glutaminase (GLS2) acts as a pH-modulated ammonia amplifier and adjusts bicarbonate-consuming urea synthesis to the needs of acid-base homeostasis. Perivenous glutamine synthetase expressing hepatocytes (so-called "perivenous scavenger cells") also express Glt1, RhBG, and OAT to allow for high-affinity ammonia removal via glutamine synthesis. Adapted from Häussinger (1990).

signal molecules before the acinar blood enters the systemic circulation (Häussinger and Stehle 1988). Perivenous scavenger cells are well equipped for their task to eliminate ammonia with high affinity through glutamine synthesis. They are the only hepatocytes also expressing the ammonium transporter RhBG, the glutamate/aspartate transporter Glt1, ornithine aminotransferase (OAT), and specifically take up glutamate and related dicarboxylates (Cadoret et al. 2002; Ginguay et al. 2017; Kuo et al. 1991; Stoll and Häussinger 1991; Weiner et al. 2003) (Figure 1). The ß-catenin pathway critically controls the zonal distribution of GS, OAT, RhBG, and axin2 (Leibing et al. 2018; Merhi et al. 2015; Sekine et al. 2006, 2007; Yang et al. 2014). Axin2 is a universal transcriptional target of β-catenin-dependent Wnt signaling, and axin2- and GS-positive cells surrounding the central vein have been implicated in the homeostatic renewal of the liver (Wang et al. 2015). In line with this, a recent study on proteome profiling of separated GS-expressing hepatocytes identified several proteins being highly enriched in perivenous GS-expressing hepatocytes compared to GS-negative hepatocytes (Paluschinski et al. 2021). Among these proteins, heat shock protein 25 and basic transcription factor 3 (BTF3), which triggers undifferentiated, stem cell-like properties in prostate tumor cells (Hu et al. 2019), were identified (Paluschinski et al. 2021). This study also suggested that GS-positive hepatocytes may not be uniform, but may comprise subpopulations, because immunohistochemistry showed that only 50–70% of the GS-expressing hepatocytes also expressed Hsp25 and BTF3 (Paluschinski et al. 2021).

Liver glutamine synthesis and ammonium homeostasis

Destruction of the perivenous area in rat liver by applying appropriate doses of CCl_4 impaired ammonia removal in perfused rat liver by the abolition of glutamine release, whereas urea synthesis remained unaffected. This finding suggested an important role of perivenous scavenger cells in maintaining ammonia homeostasis. The suggestion was confirmed by the finding that liver-specific deletion of GS in mice, without affecting other scavenger cell markers, such as Glt-1, OAT, and RhBG, triggered systemic hyperammonemia *in vivo* with corresponding sequelae such as cerebral protein tyrosine nitration and RNA oxidation (Qvartskhava et al. 2015). Downregulation of liver GS is also observed in human liver cirrhosis (for review see Häussinger 1990), which may contribute to the development of hyperammonemia in cirrhosis. Interestingly, hyperammonemia was also observed in taurine transporter (TauT) knockout mice (Qvartskhava et al. 2019). In young (three months old) TauT k.o. animals, this was due to a downregulation of RhBG-mediated ammonia uptake into perivenous scavenger cells. By contrast, in older animals (12 months old), hyperammonemia was due to an inactivating protein tyrosine nitration of liver GS (Qvartskhava et al. 2019).

GS and protein tyrosine nitration

Protein tyrosine nitration (PTN) of liver GS not only occurs in old TauT-knockout mice, but also after exposure to lipopolysaccharide (LPS) (Görg et al. 2005). PTN of GS in the human brain was observed in epilepsy (Bidmon et al. 2008), after ammonia exposure of rat astrocytes and portocavally shunted rats (Schliess et al. 2002), in hypoosmotically or benzodiazepine-treated astrocytes (Häussinger and Görg 2010), as a response to genetic deletion of GS in mice (Qvartzkava et al. 2015), and in the brain from humans with liver cirrhosis and hepatic encephalopathy (Görg et al. 2010).

Previous analyses of sequences and structural and functional aspects revealed three classes of GS. Of these, GS class II enzymes occur in eukaryotes and a few bacteria families (Darrow and Knotts 1977; Edmands et al. 1987; Kumada et al. 1990), and human GS belongs to this class (Liaw and Eisenberg 1994). Its 10 identical subunits form a homodecamer in which two pentameric rings stack against each other (Krajewski et al. 2008) (Figure 2A). The β-barrel-shaped catalytic sites are harbored in the interfaces between two neighboring subunits, resulting in 10 catalytic sites. Computational (Issoglio et al. 2016; Moreira et al. 2017) and *in vitro* experiments (Eisenberg et al. 2000; Liaw and Eisenberg 1994; Wedler and Boyer 1972; Wedler and Horn 1976) on GS-catalyzed glutamine synthesis and ammonia detoxification suggest a two-step catalytic mechanism (Figure 2B). First, adenosine triphosphate (ATP) binds to the catalytic site and induces conformational changes necessary for glutamate binding. After transfer of the terminal phosphate group of ATP to the γ-carboxylate group of glutamate yielding adenosine diphosphate (ADP) and γ-glutamyl phosphate (GGP), an ammonium ion binds to a negatively charged site (Moreira et al. 2017) and is deprotonated to ammonia as the nucleophile (Krajewski et al. 2005; Moreira et al. 2016, 2017). Ammonia then attacks GGP, and glutamine, ADP, and inorganic phosphate are released (Moreira et al. 2017).

Mass spectrometry of peroxynitrite-exposed sheep GS showed that PTN occurred in the highly conserved YFEDR motif of GS, likely targeting Y336 (Figures 2C and 3A), and resulted in inactivation of GS (Görg et al. 2005, 2007). PTN modifies key properties of a tyrosine residue, including the phenol group pK_a, redox potential, hydrophobicity, and volume (Batthyany et al. 2017; Radi 2013). Free energy computations predicted that the binding affinity of ATP towards Y336-nitrated GS is significantly reduced relative to non-nitrated GS, but only in the presence of the deprotonated and negatively charged 3′-nitro tyrosinate (Frieg et al. 2020). By contrast, in the presence of the neutral 3′-nitro tyrosine, the computations suggested a more favorable binding affinity of ATP (Frieg et al. 2020). This observation could be explained by an electron-withdrawing effect of the nitro group that likely reduces repulsive forces between the phenyl ring and the electron-rich purine ring system of ATP (Martinez and Iverson 2012), promoting favorable stacking interactions (Frieg et al. 2020). The negatively charged 3′-nitro tyrosinate not only reversed this effect but introduced increased repulsive forces, explaining the decreased affinity towards ATP (Frieg et al. 2020). By contrast, configurational free energy computations indicated that Y336 nitration only weakly influences the kinetics of ATP binding (Frieg et al. 2020), which is at variance with the prediction for tyrosine nitration in human manganese superoxide dismutase, according to which a drastically increased energetic barrier for ligand entry results (Demicheli et al. 2016; Moreno et al. 2011).

The pK_a value of the phenolic hydroxyl group of free 3′-nitrotyrosine is ~7.3 (Radi 2013) and was calculated to decrease to ~5.3 in the case of nitrated Y336 within human GS (Frieg et al. 2020) (Figure 3B). Hence, under experimental conditions previously chosen (Görg et al. 2005, 2006, 2007) and at physiological pH of 7.4, >99% of nitrated Y336 exist as 3′-nitro tyrosinate according to the computed pK_a. By contrast, at pH 4, ~95% of the nitrated Y336 exist as 3′-nitro tyrosine. Indeed, the catalytic activity of Y336-nitrated GS could be restored at pH 4 *in vitro*, whereas it was reduced at pH 6 and 7 (Figure 3C). These results indicate a fully reversible and pH-sensitive mechanism for regulating protein function by tyrosine nitration (Frieg et al. 2020).

Congenital GS deficiency

Although defects of urea cycle enzymes in humans have been known for decades, it was in 2005 when the first cases of human glutamine synthetase mutations were described (Häberle et al. 2005, 2006) (for review see Spodenkiewicz et al. 2016). Two mutations have been described initially, R324C and R341C, but the list of mutations is growing (Bennett et al. 2020; Spodenkiewicz et al. 2016). In addition, also a homozygous deletion of the Glul gene has been reported (Roifman et al. 2020). The R324 and R341C mutations result in early neonatal death accompanied by multiple organ failure, severe cerebral malformations, and skin abnormalities (Häberle et al. 2005, 2006). By contrast, a patient with a homozygous R324S mutant (Häberle et al. 2011) showed developmental delay and neurological impairment, but survived six years (Spodenkiewicz et al. 2016). Here, glutamine supplementation improved the clinical condition (Häberle et al. 2012).

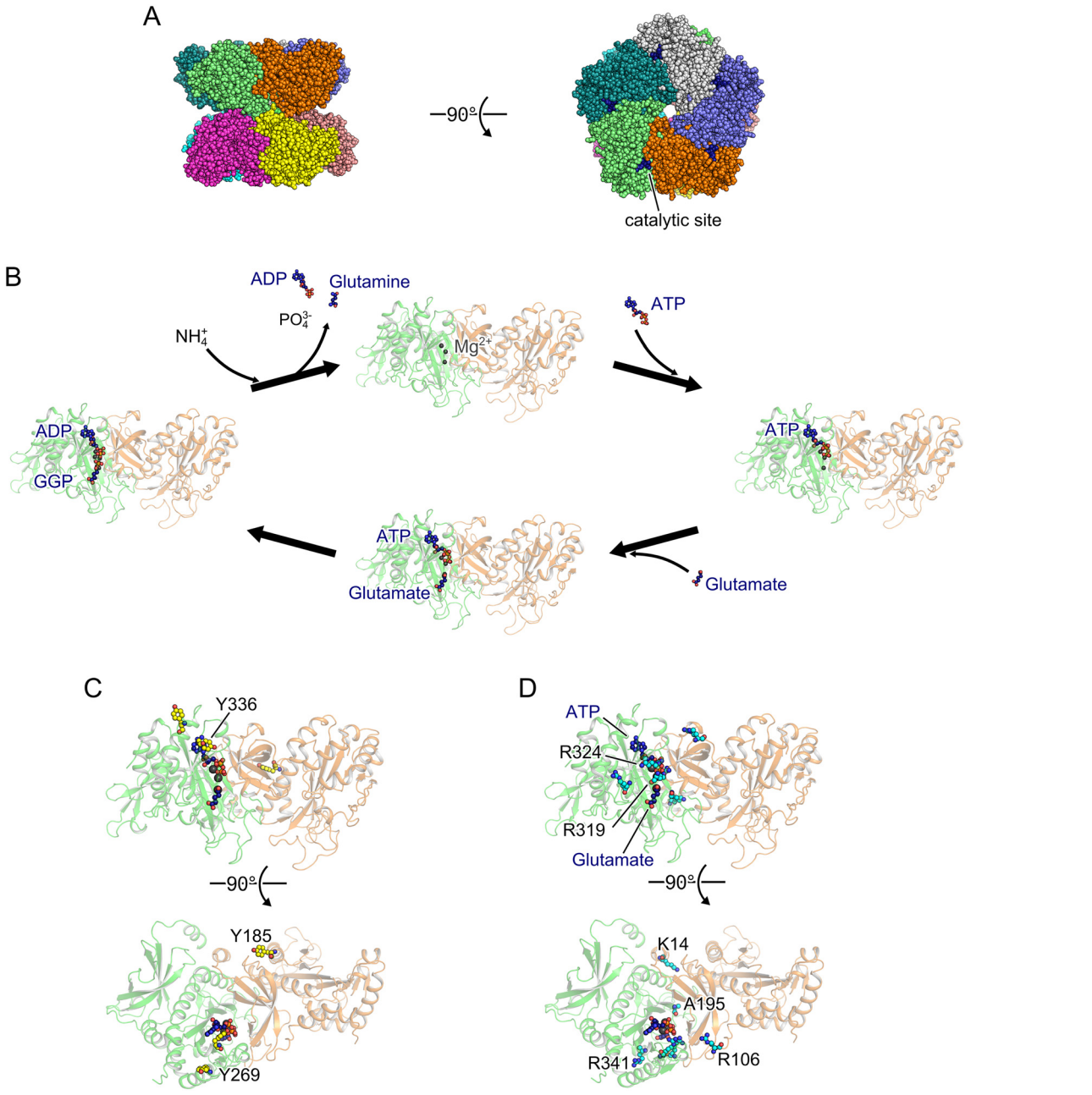

Figure 2: Structure of the human glutamine synthetase.
A: 3D structure of human glutamine synthetase (GS) (PDB-ID 2QC8 [Krajewski et al. 2008]). The 10 individual subunits are colored differently, with atoms depicted as sphere-model. The bound ADP (dark blue sphere-model) is in the catalytic site in the interface of two adjacent subunits. B: Schematic visualization of glutamine synthesis catalyzed by GS (Eisenberg et al. 2000). The structural models of apo GS, GS bound to ATP, ATP and glutamate, and ADP and γ-glutamyl phosphate (GGP) were taken from (Frieg et al. 2016a). C, D: Dimeric GS model, in which two neighboring subunits form a single catalytic site, complexing the substrates ATP, glutamate, and magnesium ions. Amino acids identified as a target for tyrosine nitration (Bartesaghi et al. 2016; Görg et al. 2005) (B) or as clinically relevant mutation sites (Bennett et al. 2020; Häberle et al. 2005, 2011; Spodenkiewicz et al. 2016) (C) are shown as yellow or cyan sphere-models, respectively. In panels B–D, ATP, ADP, glutamate, GGP, and magnesium ions are shown as dark blue or gray sphere-models, respectively.

R324 is part of the catalytic site (Krajewski et al. 2008), and we showed that it is directly involved in ATP binding (Frieg et al. 2016a) (Figure 4A). Molecular simulations revealed that the direct interaction is lost in both the R324S and R324C variants (Frieg et al. 2016a). However, this loss is partially compensated by indirect, water-mediated interactions between the sidechains of S324 or C324 and the β-phosphate group of ATP (Figure 4B) (Frieg et al. 2016a).

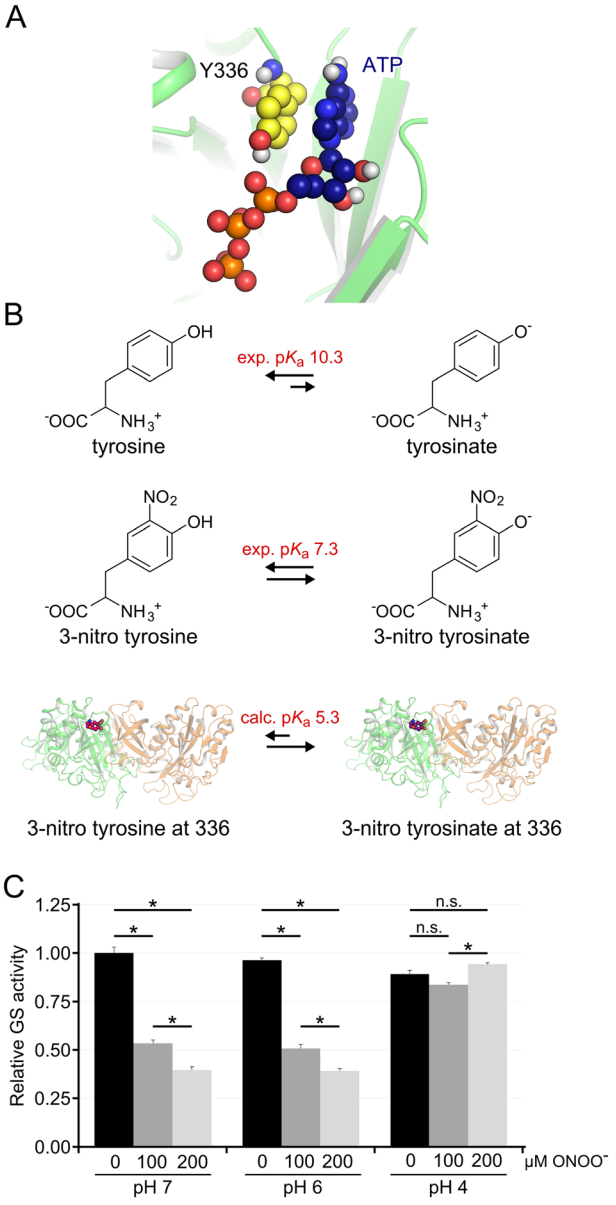

Figure 3: pH-sensitive inhibition and activation of human GS. A: ATP (blue) and tyrosine 336 (Y336, yellow) are depicted as a sphere-model in our ATP-bound model of GS (Frieg et al. 2020). B: Schematic of the effect of tyrosine nitration. Nitration of free tyrosine decreases the pK_a of the phenolic hydroxyl group by three log units (Radi 2013), leading to an equilibrium between 3-nitro tyrosine and 3-nitro tyrosinate at physiological pH (adapted from ref. Radi 2013). In GS, the calculated phenolic pK_a decreases by two additional log units (Frieg et al. 2020), such that the deprotonated state is preferred at physiological pH. C: pH-dependent and $ONOO^-$-mediated inhibition of GS activity. Purified human GS was exposed to $ONOO^-$ at concentrations of 0, 100, or 200 μM, and aliquots were taken for measuring GS activity. GS activity in vehicle-treated control at pH 7 was set to 1, and activities measured under the other experimental conditions are given relative to it. *: statistically significantly different. n.s.: not statistically significantly different. Taken from ref. Frieg et al. (2020).

The indirect interactions were significantly more frequent in the case of R324S than R341C, explaining why the R324S variant likely conserved a higher level of residual activity (Figure 4C) (Frieg et al. 2016a).

No cure is currently available for targeted treatment of inborn GS deficiency (Häberle et al. 2012). We hypothesized that molecules bridging the S324/ATP interaction better than water result in tighter ATP binding, that way (partially) restoring („repairing“) GS activity. We focused on trimethylglycine (betaine) as one such molecule (Frieg et al. 2016b) since it spontaneously bound to the correct epitope in the vicinity of S324 and weakly stabilized ATP in molecular simulations. Furthermore, it is a safe, well-tolerated, and inexpensive substance and has been used to improve serum levels of liver enzymes in the context of fatty liver diseases (NASH) (Abdelmalek et al. 2001; Barak et al. 1996; Craig 2004). Betaine and structural analogs are currently being investigated concerning their *in vitro* potency to restore the R324S GS activity.

In the R341C GS, a long-range interaction that causes catalytic inhibition of GS was identified (Frieg et al. 2016a). In wild type GS, R341 is pointing away from the catalytic site and not directly involved in substrate binding (Frieg et al. 2016a). Instead, it interacts with amino acids harbored on the solvent-exposed helix H8, particularly H281, H284, and Y288 (Figure 4D). Molecular simulations suggested that R341C significantly reduces the mechanical stability around helix H8 (Frieg et al. 2016a). For glutamate to bind to GS, ATP needs to induce a structural rearrangement of helix H8 (Krajewski et al. 2008). Consequently, glutamate binding was predicted to be disfavored in the R341C variant relative to wild type GS, and functional *in vitro* experiments corroborated the prediction (Frieg et al. 2016a).

Recently, several suspected cases of patients carrying novel variants of the GS were reported (Bennett et al. 2020; Spodenkiewicz et al. 2016) (Figure 2D). As we previously investigated all relevant stages of the GS catalytic cycle towards glutamine (Frieg et al. 2016a), we use these results to suggest explanations at the structural level for impaired GS activity.

A case report of a five-year-old boy with severe epileptic encephalopathy was associated with two probably damaging mutations, A195D and R319H (Spodenkiewicz et al. 2016). A195 forms a hydrophobic pocket with C163 and W202 but is not directly involved in substrate binding (Figure 4E). Interestingly, the neighboring E196 is relevant for glutamate binding and Mg^{2+} coordination according to our structural models (Frieg et al. 2016a) (Figure 4E). The introduction of a negatively charged aspartate in the case of the A195D GS likely weakens the

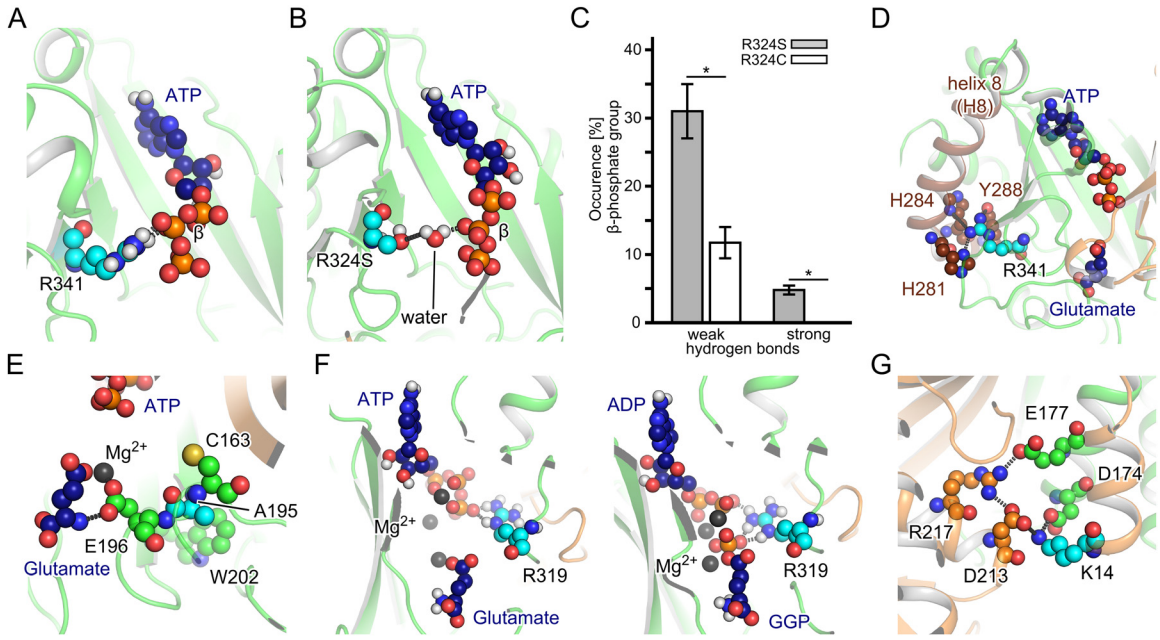

Figure 4: Structural interpretation of clinically relevant GS mutations.
The structural model of the human GS bound to ATP, glutamate, and Mg^{2+} ions (Frieg et al. 2016a) allows for the structural interpretation of GS mutations. The enzyme structure is shown as cartoon model with subunits colored green and orange. ATP, glutamate, Mg^{2+} ions, and relevant amino acids are depicted as sphere-model. Amino acids targeted by mutagenesis are colored cyan. A, B: Close up view of the catalytic site. The wild type R324 interacts directly with the β-phosphate group of ATP (A). Instead, R324S uses an indirect, water-mediated interaction to stabilize the substrate (B). C: Mean relative occurrence (error bars denote the standard error of the mean) of water-mediated hydrogen bonds that connect the β-phosphate group of ATP and residues S324 or C324 in the GS variants (adapted from Frieg et al. 2016a). Strong and weak hydrogen bonds were defined by distance cutoffs of 2.8 and 3.2 Å (Desiraju and Steiner 2001). The stars indicate a significant difference ($p < 0.05$) D: Interactions between R341 and H281, H284, and Y288 on helix H8 (colored brown). These interactions are lost in R341C GS, leading to destabilization of H8, which, in turn, hampers structural adaptation mechanisms required for glutamate binding (Frieg et al. 2016a). E: A195 forms a hydrophobic pocket with C163 and W202 and is in the immediate neighborhood of E196, which binds to glutamate and Mg^{2+}. F: R319 is part of the catalytic site and complexing the phosphate groups of ATP (left) or ADP and the reactive intermediate γ-glutamyl phosphate (GGP) (right). G: K14 contributes to a hydrogen-bond network involving amino acids from two adjacent subunits, likely contributing to the inter-subunit stability.

hydrophobic contacts, which may displace E196 and, thereby, hamper glutamine synthesis. R319 is highly conserved in prokaryotes and eukaryotes (Eisenberg et al. 2000), suggesting an essential catalytic function. R319 binds to the terminal phosphate group of ATP during the first steps of glutamine synthesis and the phosphate groups of ADP and GGP during the later catalytic stages (Figure 4F), suggesting that R319 is essential for the phosphate transfer from ATP to glutamate. Substitution by histidine will likely weaken such interactions.

Another case report of two siblings with myoclonic epilepsy revealed two novel mutations, K14N and a non-sense mutation leading to a stop codon in the GLUL gene (Bennett et al. 2020). There are several interesting similarities and dissimilarities between these patients and previously described ones (Bennett et al. 2020). As to GS, the most interesting difference is that the latest variants result in a non-lethal phenotype, suggesting a GS residual activity, which was, however, not further verified (Bennett et al. 2020). Prediction of functional effects by PolyPhen-2 (Adzhubei et al. 2010) suggests K14N as "probably damaging". This effect may be explained in that K14 contributes to an ionic-interaction network in the dimerization interface, likely contributing to the inter-subunit stability (Figure 4G). Substitution by asparagine leads to a loss of salt-bridges to D174 and D213, which likely destabilizes GS. As the patients' mutation is compound heterozygous (Bennett et al. 2020), with one allele still carrying fully functional GS, the non-lethal phenotype may also result from a reduced amount of functional GS.

Concluding remarks

GS has a decisive role in the intercellular glutamine cycle, whose regulation is essential for the maintenance of bicarbonate and ammonia homeostasis in the organism. Tyrosine nitration of the enzyme in response to oxidative/

nitrosative stress impairs GS activity in a pH-sensitive way. Combined computational and experimental studies indicate that tyrosine nitration can lead to a fully reversible and pH-sensitive regulation of protein function. GS catalyzes the ligation of glutamate and ammonia in a complex two-step catalytic mechanism. The impact of point mutations leading to congenital GS deficiency has been described in atomistic detail. This understanding could provide the basis to restore impaired GS activity.

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