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Asparagine synthetase: Function, structure, and role in disease

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Asparagine synthetase (ASNS) converts aspartate and glutamine to asparagine and glutamate in an ATP-dependent reaction. ASNS is present in most, if not all, mammalian organs, but varies widely in basal expression. Human ASNS activity is highly responsive to cellular stress, primarily by increased transcription from a single gene located on chromosome 7. Elevated ASNS protein expression is associated with resistance to asparaginase therapy in childhood acute lymphoblastic leukemia. There is evidence that ASNS expression levels may also be inversely correlated with asparaginase efficacy in certain solid tumors as well. Children with mutations in the *ASNS* gene exhibit developmental delays, intellectual disability, microcephaly, intractable seizures, and progressive brain atrophy. Thus far, 15 unique mutations in the *ASNS* gene have been clinically associated with asparagine synthetase deficiency (ASD). Molecular modeling using the *Escherichia coli* ASNS-B structure has revealed that most of the reported ASD substitutions are located near catalytic sites or within highly conserved regions of the protein. For some ASD patients, fibroblast cell culture studies have eliminated protein and mRNA synthesis or stability as the basis for decreased proliferation.

ASNS function

Asparagine synthetase (ASNS)[4] catalyzes the synthesis of asparagine and glutamate from aspartate and glutamine in an ATP-dependent amidotransferase reaction (Fig. 1*A*) (1). The recent discovery of a neurologic disorder associated with asparagine synthetase deficiency (ASD) and its long recognized importance in the treatment of acute lymphoblastic leukemia (ALL) highlight the clinical relevance of ASNS as a topic of current interest.

Many prokaryotes express two forms of ASNS that are characterized by their source of nitrogen donor, either ammonia (AS-A) or glutamine (AS-B (1). The highly conserved residue Cys^2 is required for the nucleophilic deamidation of glutamine, and the mutation of this residue reverts AS-B to use ammonia exclusively as the nitrogen source (2). In mammalian cells, a single form of the ASNS enzyme is expressed that utilizes glutamine as the nitrogen donor in a reaction corresponding to bacterial AS-B (2). Structure–function studies have previously been performed on *Escherichia coli* AS-B and have revealed two distinct catalytic domains that are conserved in the human enzyme (3) (Fig. 1*B*). Enzymatic analyses have shown that the catalytic mechanism requires magnesium ions and ATP (4). The reaction begins in the C-terminal domain with the activation of the aspartate carboxyl group. This ATP-dependent process forms a β-aspartyl-AMP intermediate that is stabilized by proximal residues of the active site. Within the N-terminal domain, the hydrolysis of glutamine yields glutamate and ammonia, the latter of which diffuses through an intramolecular tunnel to perform a nucleophilic attack on the electrophilic β-aspartyl-AMP intermediate, producing asparagine (Fig. 1*A*) (1, 3, 5). Human ASNS is categorized as a class II or N-terminal nucleophile glutamine amidotransferase because the hydrolysis of glutamine occurs in the N-terminal domain of the enzyme (1).

ASNS protein structure

The crystal structure of *E. coli* AS-B (Protein Data Bank (PDB) 1CT9) has been solved (3), but a structure of human ASNS has yet to be published or deposited into the PDB. Therefore, the structural features of the human enzyme are based on homology modeling using the *E. coli* AS-B structure as a template because they share ~40% sequence identity. The canonical human ASNS enzyme consists of 561 amino acid residues with an approximate molecular mass of 65 kDa (Fig. 1*B*). However, the UniProt protein database contains two other putative isoforms of human ASNS that differ in the length of the N-terminal domain: isoform 2 (residues 22–561) and isoform 3 (residues 84–561). The expression and physiological importance of these putative isoforms have not been described. With regard to the two functional domains, the N-terminal domain (residues 1–208) consists of a two-layer, antiparallel β-sheet core surrounded by four α-helices (Fig. 1*C*). This domain contains

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[4] The abbreviations used are: ASNS, asparagine synthetase; ASNase, asparaginase; AAR, amino acid response; ALL, acute lymphoblastic leukemia; ASD, asparagine synthetase deficiency; ATF, activating transcription factor; UPR, unfolded protein response.

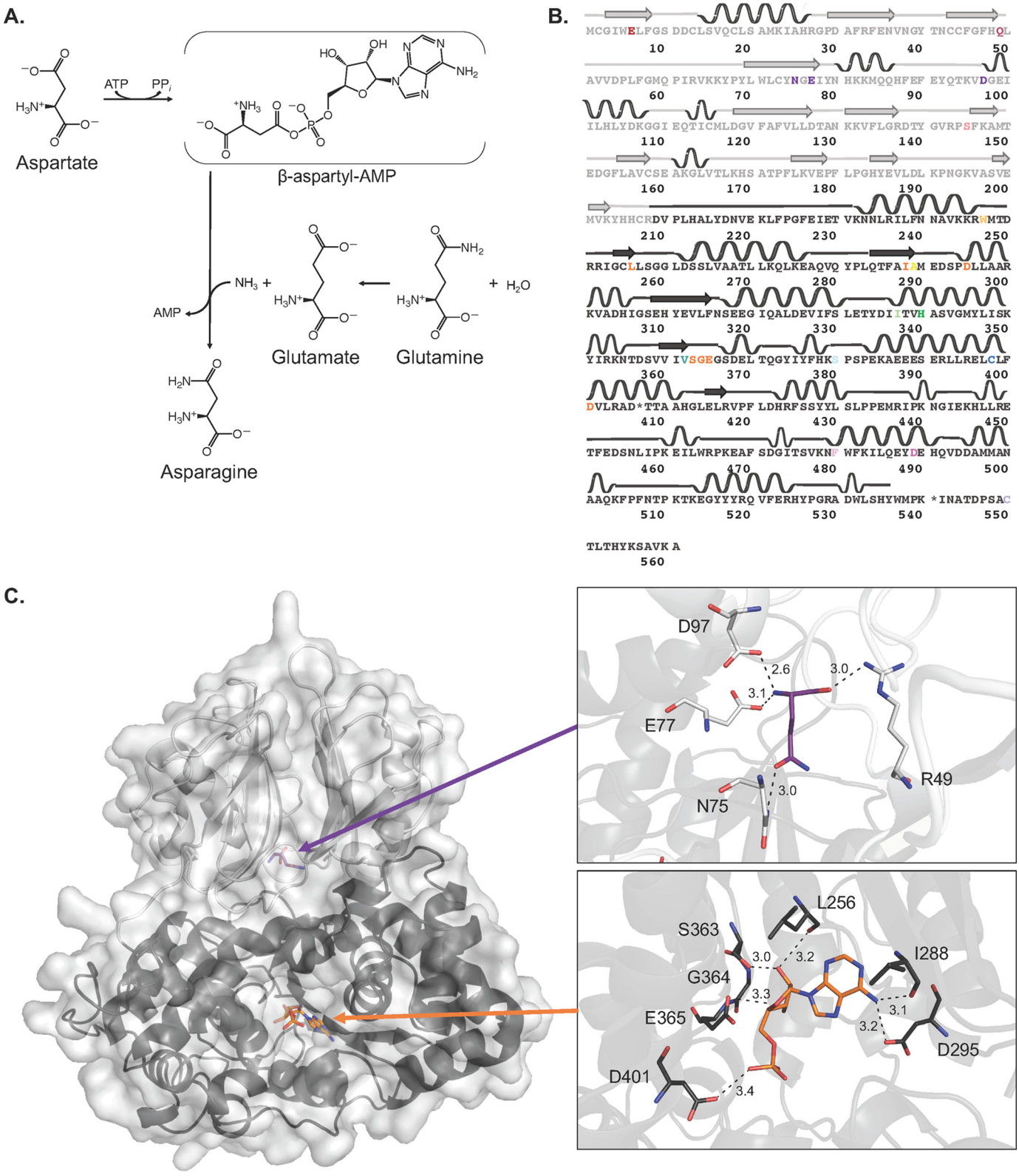

Figure 1. Mechanism and structural features of human asparagine synthetase. *A*, the reaction begins when the aspartate carboxyl is activated by an ATP-dependent process, forming a β-aspartyl-AMP intermediate. Glutamine deamidation releases ammonia, which performs a nucleophilic attack on the aspartyl intermediate to produce asparagine. Glutamate is the second product of the overall reaction. *B*, sequence of human ASNS isoform 1 with residues colored *light* and *dark gray* for the N- and C-terminal domains, respectively. α-Helical and β-sheet secondary structures are shown above the sequence. Residues associated with glutamine and ATP binding are colored *purple* and *orange*, respectively. Clinically identified ASD mutations are colored according to Fig. 3. *C*, the N- and C-terminal domains are represented within the surface structure and colored *light* and *dark gray*, respectively. The substrates glutamine (*purple*) and ATP (*orange*) are indicated by an *arrow* and shown as *sticks*. *Insets* show the substrate-binding pockets. Hydrogen bonds are shown as *black dashes* with distances labeled in angstroms.

the glutamine-binding pocket, consisting of residues Arg^{49}, Asn^{75}, Glu^{77}, and Asp^{97}. As observed in the *E. coli* structure, glutamine is predicted to bind in a manner so that the carboxamide group is oriented toward the interface of the two domains to allow the transfer of an ammonia group from glutamine to aspartate. The C-terminal domain (residues 209–561) is composed primarily of α-helices, but also encompasses a five-stranded, parallel β-sheet that contains the ATP-binding site: residues Leu^{256}, Val^{288}, Asp^{295}, Ser^{363}, Gly^{364}, Glu^{365}, and Asp^{401} (Fig. 1*C*). A distance of ~20 Å separates the two active sites.

Asparagine synthetase and cancer

ASNS expression and asparagine metabolism have received considerable attention in transformed cells, beginning with the observation that childhood ALL is susceptible to treatment by the infusion of bacterial asparaginase (ASNase). Primary ALL cells and many ALL cell lines exhibit little or no detectable ASNS and are sensitive to asparagine depletion (6–8). Standard treatment of childhood ALL includes infusion of bacterial ASNase as a principle component of a combinatorial chemotherapy (9). The circulating ASNase causes rapid depletion of plasma asparagine, followed by the efflux of intracellular asparagine, and thus, starving the leukemia cells to prevent further growth (10, 11). In contrast to the increase in ASNS expression in response to substrate deprivation in most normal cells within the body, there is little or no up-regulation of ASNS protein content in ALL cells, rendering them preferentially sensitive to ASNase (12, 13).

The importance of ASNS expression in solid tumor growth and the sensitivity to ASNase have not been investigated as extensively. A screen of 98 human pancreatic ductal carcinomas established that ASNS protein was low or below detection in about 70% of the samples, suggesting that some pancreatic tumors may be susceptible to ASNase therapy (14). Cui *et al.* (15) showed that pancreatic cancer cells overexpressing ASNS exhibited increased resistance to apoptosis induced by cisplatin. Similarly, using the NCI-60 human cancer cell panel, Lorenzi *et al.* (16) noted a negative correlation between ASNS mRNA levels and susceptibility to ASNase in ovarian cells, as well as increased ASNase sensitivity after siRNA knockdown of ASNS expression. In a second study with a larger number of ovarian cell lines, an inverse correlation between ASNase efficacy and ASNS protein levels was observed (17). These results were consistent with previous observations in human leukemia cells showing that increased ASNase sensitivity correlated with lower ASNS protein expression rather than mRNA content (13).

Protein limitation or an imbalanced dietary amino acid composition leads to intracellular amino acid depletion and activation of *ASNS* gene transcription through a signaling pathway called the amino acid response (AAR) (18). Likewise, endoplasmic reticulum stress also increases ASNS transcription via the unfolded protein response (UPR) (19). These cell stress pathways result in activation of the eIF2 kinases GCN2 (general control nonderepressible) (AAR) and PERK (PKR-like endoplasmic reticulum kinase) (UPR). Phosphorylation of the α-subunit of eIF2 causes a transient, partial suppression of global protein synthesis, but a paradoxical increase in the translation for certain mRNAs, and among these is the transcription factor ATF4 (20). ATF4 binds to an enhancer element within the proximal promoter of the *ASNS* gene and activates transcription (Fig. 2) (21). Ye *et al.* (22) investigated the role of ATF4 in tumor cell survival and proliferation and observed that ATF4 knockdown caused reduced survival in HT1080 fibrosarcoma and DLD1 colorectal adenocarcinoma cells in the absence of nonessential amino acids. Reduced proliferative capacity and increased apoptosis correlated with lower ASNS expression in the ATF4-deficient cells. Supplementation of the tumor cells with asparagine, but not other amino acids, led to increased cell survival. Based on this and other experimental data, Ye *et al.* (22) concluded that induction of ATF4: 1) contributes to tumor cell proliferation during nutrient limitation, 2) is a component of starvation-induced autophagy in cancer cells, and 3) induces ASNS as a key factor in tumor initiation and growth under amino acid–limiting conditions. The exact role of ASNS activity in modulating tumor growth is unknown. One obvious possibility, an impact on protein synthesis, seems tenuous as other amino acid synthetic enzymes are not as highly regulated as ASNS.

Asparagine synthetase deficiency

ASD is a recently characterized neurological disorder having severe impacts on psychomotor development and mortality at an early age. Multiple patient studies have been conducted over the last few years due to the rising awareness of the disease. Symptoms include intellectual disability, microcephaly, severe developmental delay, intractable seizures, progressive brain atrophy, and more rarely, respiratory deficiency (23–30). The disease is associated with homozygous or compound heterozygous mutations within the *ASNS* gene on chromosome 7q2, but the exact mechanisms that cause the overt symptoms of the disease are not well understood (23, 26, 31). The fact that the children are born with epileptic-like seizures and microcephaly suggests that brain ASNS activity is critical for development of this organ. Currently, the disease can only be diagnosed through DNA sequencing. Some, but not all, affected individuals have a measurable decrease in the amount of asparagine in their serum or cerebrospinal fluid, which limits this analysis as a preliminary screen in suspected cases.

Based on animal growth studies, asparagine is traditionally defined as a "nonessential" or "dispensable" amino acid. ASNS is expressed to varying degrees within human organs (http://www.proteinatlas.org/ENSG00000070669-ASNS/tissue),[5] but is particularly high in pancreas (32). However, when considering the requirement for asparagine at the level of individual cells, ASNS deficiency leads to extracellular asparagine dependence, as discussed above for ALL cells. Individuals with ASD express phenotypic impairments in the brain that are not readily apparent in other organs, suggesting a tissue-specific dependence on asparagine for neural development (23). Although asparagine can be obtained through the diet, it is transported by an equilibrating, bidirectional facilitated transporter (System n)

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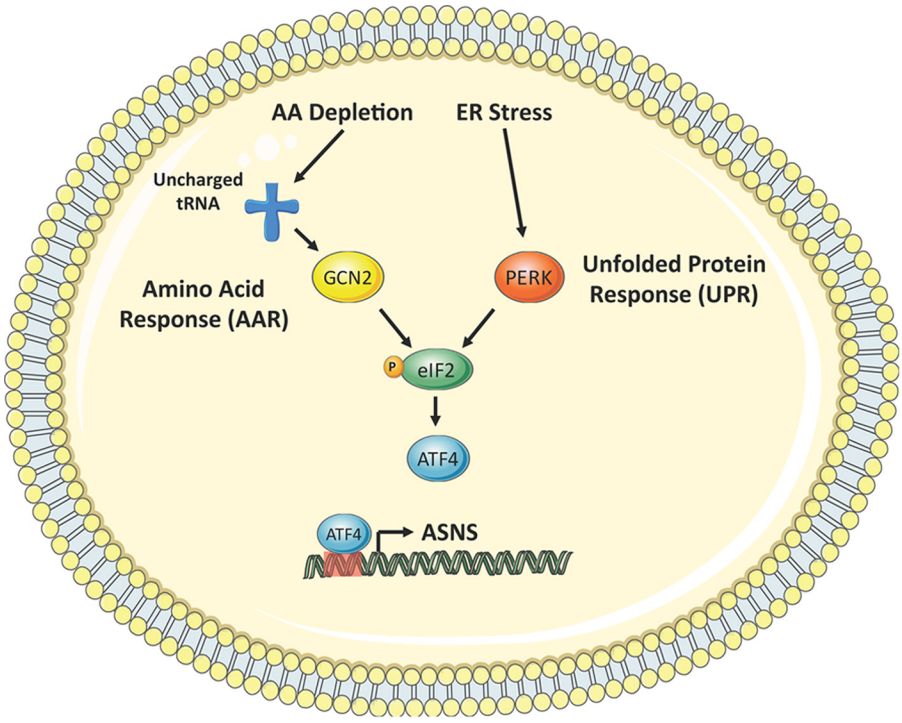

Figure 2. Regulation of ASNS expression. Asparagine depletion activates the AAR, whereas endoplasmic reticulum stress (*ER Stress*) activates the UPR. Each stress condition increases the activity of an eIF2 kinase. Phosphorylation of eIF2 slows global protein synthesis, but paradoxically increases translation of a subset of mRNAs, including that for the transcription factor ATF4. Binding of ATF4 to an enhancer element within the promoter of the ASNS gene induces expression of the enzyme.

across the luminal surface of the endothelial cells that make up the blood–brain barrier. Therefore, asparagine is not actively accumulated within the brain (33). Indeed, Na^+-dependent transport of asparagine across the abluminal membrane of the endothelial layer appears to be designed to remove the amino acid from the brain. Consistent with this hypothesis, the cerebrospinal fluid levels of asparagine, as with many amino acids, are only a fraction of those in the plasma (34, 35). Consequently, a decrease in ASNS catalytic activity in the brain is presumed to cause the disease phenotype (23, 24, 26). Plasma asparagine levels were reduced in 8 out of 16 ASD patients for whom plasma analysis was reported (reviewed in Ref. 31). However, cerebrospinal fluid asparagine content has been analyzed in only four ASD patients, and it was undetectable in two of the four (24, 27, 30). Recent studies with ASD patients' fibroblasts have revealed that lowering the asparagine concentration in the extracellular milieu results in an inability to proliferate (26). These cell culture studies also showed that fibroblasts from asymptomatic heterozygote parents exhibit no ill effects in the presence of sufficient extracellular asparagine, but decline in proliferative capacity when placed in an asparagine-free medium. Collectively, investigation of ASD patients indicates that ASNS activity in the brain is crucial for organ development. Whether this dependence is the direct result of perturbations in asparagine metabolism, or one of the other ASNS reactants, must be established by further analysis and experimentation.

Structural examination of ASD mutations

To date, 15 unique mutations in the *ASNS* gene have been clinically identified in association with ASD (23–31). ASD is characterized as a pan-ethnic disorder because it has been identified within a variety of ethnic groups. Several patients have mutations localized at or near the ATP-binding site, whereas thus far, only one patient has been identified with a mutation in the glutamine-binding site (Table 1). The remaining mutations are located outside the active sites, but in highly conserved regions, which may reduce protein stability without necessarily inhibiting enzyme activity (23). Alignment of the three isoforms of human ASNS and renumbering of the mutations to correlate to the isoform 1 sequence highlights that previously reported mutations Y315C (27) and Y377C (24) are equivalent to Y398C (25), whereas Arg^{324*} would be renumbered to Arg^{407*} (27) (Fig. 1*B*).

Cell culture analyses have shown that several of the aforementioned mutations do not affect mRNA stability (23, 26). Rather, many of the disease-associated mutations are expected to decrease enzyme activity and/or protein stability. Analysis of the human ASNS structural model incorporating the observed amino acid mutations illustrates an overall theme of protein destabilization. The mutations can be classified into four groups based upon their putative effect on the protein structure: change in the amino acid type (hydrophobic to hydrophilic or vice versa), loss of hydrogen bonding or van der Waals interactions, truncation of the protein, and predicted decrease in substrate binding. The introduction of charged or polar residues into hydrophobic regions of the enzyme is expected to destabilize the structure as predicted for the A6E, L145S, A380S, and V489D mutations (23, 30). In addition, the increase in side-chain length of the A6E mutation results in steric hindrance with proximal residue Phe^8. Conversely, the S480F mutation presents a nonpolar amino acid on the surface of pro-

Table 1
Reported mutations in ASD patients
Residue numbers correspond to those for the isoform 1 ASNS sequence (UniProt ID: P08243). H = homozygous, CH = compound heterozygous, * = nonsense mutation, fs = frameshift mutation.

Mutation	Type	Structural consequence	Reference
A6E	CH	Charged amino acid in hydrophobic region, steric clash with Phe[8]	23
R550C		Decrease in side chain length likely to result in loss of interactions	
R49Q	H	Glutamine-binding site, loss of hydrogen bonding	See Footnote 6
L145S	CH	Polar side chain in hydrophobic region	30
L247W		Decrease in van der Waals interactions	
G289A	CH	Proximal to ATP-binding site, steric hindrance with Ser[293]	26
T337I		Proximal to ATP-binding site, hydrophobic patch on protein that may decrease solubility	
R340H	H	Loss of hydrogen bonds, steric clash with Phe[482]	29
F362V	H	Decrease in van der Waals interactions	23
A380S	H	Polar residue in hydrophobic region	31
Y398C	H	Decrease in van der Waals interactions, solvent-accessible thiol group	24, 25, 27
R407*	H	Protein truncation	27
S480F	CH	Nonpolar residue on protein surface that may decrease solubility	28
R550C		Decrease in side chain length likely to result in loss of interactions	
V489D	CH	Charged amino acid in hydrophobic region	30
W541Cfs*5		Protein truncation	
R550C	H	Decrease in side chain length likely to result in loss of interactions	23

tein that may decrease solubility (Fig. 3) (28). Several mutations result in the loss of stabilizing interactions. L247W causes a reorientation of the larger aromatic side chain out of a hydrophobic pocket to avoid steric clash, resulting in the loss of van der Waals interactions (30). Similarly, the reduction in side-chain size is likely to cause a decrease in van der Waals interactions in F362V (23) and a loss of hydrogen bonding in the R340H mutant (29), which is further destabilized by steric hindrance with Phe[482]. In addition to the introduction of a reactive, solvent-accessible thiol group, the loss of van der Waals interactions in Y398C is predicted to disrupt contacts between the N and C domains (24, 25, 27). Although the R550C mutation is not included in the human ASNS model due to the lack of inclusion in the *E. coli* AS-B structure, it is expected to result in destabilization of the C-terminal domain (Fig. 3).

Two observed mutations are predicted to result in the truncation of the human ASNS protein. The nonsense mutation Arg[407]* introduces a premature stop codon (27), whereas the frameshift mutation W541Cfs*5 truncates the enzyme, containing only five codons after the mutated residue 541 (30). The remaining reported mutations are not only predicted to destabilize the structure of the protein, but also potentially decrease the binding of substrate and possibly decrease the catalytic efficiency of the ASNS enzyme. R49Q is the first mutation reported to be in the glutamine-binding pocket of the N-terminal domain.[6] This mutation not only results in a loss of hydrogen bonds with the second β-sheet, but also a loss in hydrogen bonds with the glutamine substrate. The C-terminal domain mutations G289A and T337I are associated with an afflicted child who is a compound heterozygote. Both mutations are located proximal to the ATP-binding pocket (26). The G289A mutation results in a steric clash with Ser[293], whereas the T337I mutation introduces a hydrophobic patch on the protein surface that may decrease solubility. Although these two residues are not predicted to interact directly with the ATP molecule, the mutations are expected to destabilize the region of the ATP-binding site, and therefore, potentially decrease the affinity of ASNS for this substrate (Fig. 3).

Remaining questions

Although many aspects of ASNS enzymology and regulation have been investigated over the last four decades, major gaps remain in our knowledge. The exact contribution of ASNS activity in maintaining the cellular and whole body homeostasis of its four amino acid reactants remains largely unanswered. Although the enzyme name leads one to focus on asparagine synthesis, given the overall reaction, the activity does consume glutamine and aspartate, and produce glutamate. Whether or not ASNS actually impacts the cellular homeostasis of one or more of the other reactants remains speculative. Given the much higher levels of glutamine in most cells, the chance of changes in ASNS activity contributing to the cellular aspartate and glutamate abundance would be more likely. However, the *in vivo* impact during embryonic development or physiological status during periods of up-regulation in response to cellular stresses that activate the AAR or UPR pathways would be valuable studies in tissue-specific ASNS knock-out animals. Furthermore, although limited, the results showing the importance of increased ASNS expression in solid tumor proliferation and the development of resistance to ASNase chemotherapy in childhood ALL illustrate the need for a better comprehension of ASNS regulation in transformed cells.

The recent awareness of the neurological disease ASD highlights the need for a better understanding of the enzymatic consequences of ASNS mutations. The pioneering mouse model studies of Ruzzo *et al.* (23) should be expanded to generate brain-specific transgenic and knock-out animals to investigate the developmental effects of altered ASNS activity. Given that fibroblasts are available from only a few ASD children, the physiologic consequences for some mutations will require expression in ASNS null cells. At the protein level, modeling of the human ASNS protein has provided a means to tentatively map the structural consequences of ASD-associated mutations. However, the X-ray crystal structure of the human enzyme would be useful to confirm the structural effects proposed for

[6] S. J. Sacharow, E. E. Dudenhausen, C. L. Lomelino, L. Rodan, C. Moufawad El Achkar, H. E. Olson, C. A. Genetti, P. B. Agrawal, R. McKenna, and M. S. Kilberg, submitted for review.

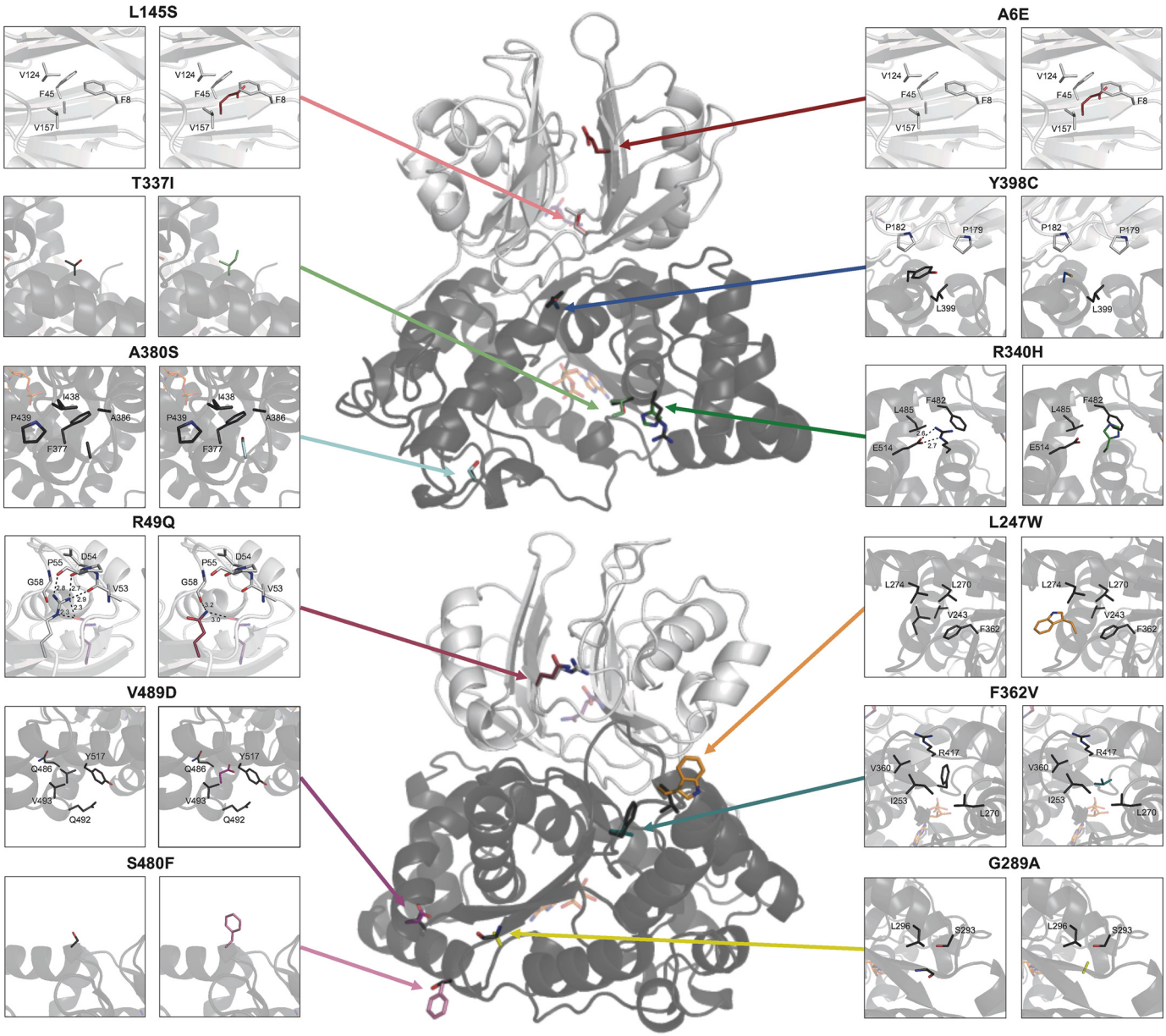

Figure 3. ASD associated mutations in the human ASNS enzyme. Mutations are represented as *sticks* within the predicted ASNS structure. *Top panel*, A6E (*red*), L145S (*salmon*), T337I (*light green*), R340H (*dark green*), A380S (*light blue*), and Y398C (*dark blue*). *Bottom panel* (180° rotation around *y* axis), R49Q (*raspberry*), L247W (*light orange*), G289A (*yellow*), F362V (*teal*), S480F (*light pink*), and V489D (*dark pink*). Interacting residues are also shown as *sticks*, and hydrogen bonds are represented as *black dashes*.

ASD and allow for potential *in silico* drug screening approaches for future anti-tumor use. Furthermore, the current enzymatic assays for ASNS are cumbersome and variable in reproducibility when performed at the relatively low tissue levels of the enzyme. Overexpression of wild-type and mutant recombinant human ASNS protein in quantities suitable for new attempts at analysis would allow for activity comparisons of each mutation. With regard to ASD, the fact that asparagine uptake across the blood–brain barrier is not concentrative may limit dietary amino acid supplement therapy because artificially elevating blood asparagine levels too high may inhibit uptake of other amino acids due to competition for shared transporters (33). In fact, an initial attempt at dietary asparagine therapy resulted in negative consequences (36). Affected ASD children are born with morphological and neurologic defects, indicating significant brain damage during embryonic development, and ASNS has been proven essential during brain development in a mouse model (23). These observations suggest that currently available therapeutic approaches are likely to prove ineffective, as significant and possibly irreversible tissue damage appears to occur during the earliest stages of development.

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