CHAPTER

29

Azetidine-2-Carboxylic Acid and Other Nonprotein Amino Acids in the Pathogenesis of Neurodevelopmental Disorders

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OUTLINE

29.1 INTRODUCTION

There are approximately 900 naturally occurring amino acids, of which only 22 have been evolutionarily selected for inclusion as subunits in the structure of proteins (Rosenthal and Bell, 1979; Rubenstein, 2000). The others, nonprotein amino acids, are found principally in plants and lower marine forms in which they participate in metabolism and provide a means of nitrogen storage. In addition, many are deployed as toxic weapons, deterring intruding vegetation and marauding predators.

Some amino acids have entered the food chain. Notable among this group is azetidine-2-carboxylic acid (Aze), which is the lower homolog of the protein amino acid proline. It readily escapes the gate-keeping function of prolyl aminoacyl-tRNA synthetases and replaces proline in proteins, corrupting their structure, function, and antigenicity. Aze may be an environmental agent that contributes to susceptibility to a wide range of disorders, especially those involving the central nervous system (Rubenstein, 2008).

Proline is an anomalous amino acid (Figure 29.1(c)). Its nitrogen atom is linked to only one hydrogen atom instead of two. It cannot act as a hydrogen bond donor but can serve as an acceptor. The nitrogen is covalently bound to the ring structure, constraining its mobility in peptide bond formation. The φ backbone dihedral angle is about −75°, and proline displays conformational rigidity. Unlike other amino acids, which prefer the *trans* configuration of the hydrogen and oxygen atoms in the peptide bond, proline readily shifts to the *cis* architecture in response to regional changes in spatial charge distribution. Lacking a hydrogen atom, the proline molecule isomerizes by swinging its entire ring into the *cis* position, a conformational change that realigns the local peptide sequence, resulting in folding of the protein (Baldwin, 2008; Smith et al., 2012).

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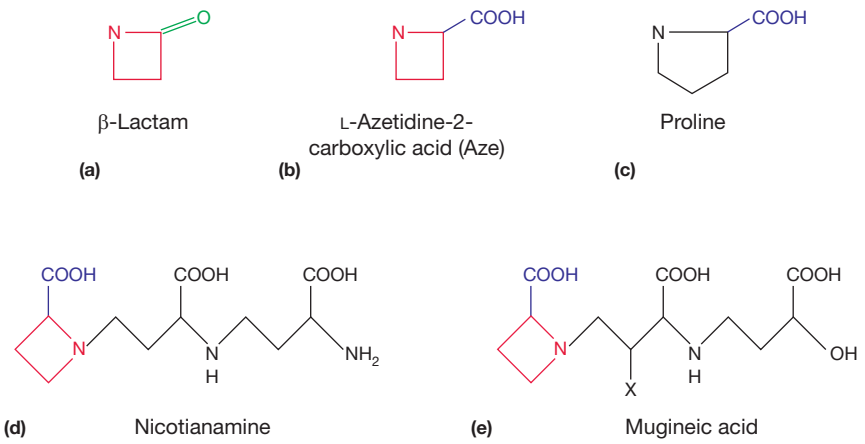

FIGURE 29.1 Small-ring molecules. This figure shows the structures of four-membered rings as free molecules: β-lactam (a), L-azetidine-2-carboxylic acid (Aze) (b), and as constituents of the two plant chelator molecules, nicotianamine (d) and mugineic acid (e). Note the similarity of β-lactam (a) and L-azetidine-2-carboxylic acid (Aze) (b). Proline (c) is the five-membered ring higher homologue of Aze (b). The four-membered rings are red, the ring acyl group is green, and the ring carboxyl groups are blue.

Because of its idiosyncrasies as an amino acid, proline is not found within alpha helices or beta sheets. It can take a position at the ends of these structures and in turns. Repeating prolyl residues may fold into a left-handed helix (PPII), with backbone dihedral angles of about −75°, 150°, and a *trans* configuration of their peptide bonds. A right-handed helix (PPI) forms with dihedral angles of about −75° and 160° when there is a *cis* configuration of their peptide bonds.

Aze, bearing a ring that contains four members instead of five, is otherwise nearly identical to proline (Figure 29.1(b)). There are, of course, subtle differences in bond angles, including those that determine the degree of ring puckering. Azetidine changes its *cis–trans* configuration under slightly different conditions of local charge, and its presence can thus cause misfolding (Tsai et al., 1990).

29.1.1 Ring Strain and Reactivity

Biomolecules comprise atoms in chains that tend to adopt a linear configuration in which their mutually repelling electron clouds maintain maximal distance from each other. This is not possible in small-ring compounds. The conformation of such cyclic molecules subjects them to various forms of strain or structural stress. The increased internal energy acquired during synthesis is released during chemical transactions (Hassner, 1983). Crowding can result in narrowing of preferred bond angles, compression of electron orbitals, and malpositioning of ring members. For these reasons, strain energy is exceedingly high in small cyclic molecules, increasing their chemical reactivity.

Molecules with five or more rings abound in biochemistry, but there appear to be only two four-membered ring compounds that have entered human life, and both have done so as exogenous substances: beta-lactam and Aze.

Beta-lactam closely resembles Aze. It is a cyclic amide with an acyl group on a carbon adjacent to the nitrogen (Figure 29.1(a)). It is the central component of the penicillins. The penicillin molecule binds to and inactivates the bacterial cell wall transpeptidase enzyme that forms an acyl intermediate with a D-alanyl-D-alanyl terminal peptide residue. Penicillin intrudes into the active site of the enzyme, where its beta-lactam impersonates the structure of the normal substrate. It irreversibly inhibits the enzyme, thus preventing cross-linking of the bacterial cell wall (Stryer, 1988). The ring strain of the four-membered beta-lactam contributes to its remarkable reactivity (Hassner, 1983).

Aze is strikingly similar to beta-lactam. Instead of an acyl group, it contains a carboxyl group on ring atom number 2. Like beta-lactam, Aze is highly strained and is thus highly reactive.

29.1.2 The Aminoacyl-tRNA Prolyl Synthetases

There are two aminoacyl-tRNA synthetases that recognize proline, EPRS (cytoplasmic) and PARS2 (nucleus encoded but mitochondrial). EPRS is multifunctional, recognizing glutamic acid as well as proline. The human forms of these synthetases lack editing domains. The chromosomal location of the gene *EPRS* is 1q41-q42 and that of *PARS2* is 1p32.2 (Antonellis and Green, 2008). Certain nonprotein amino acids escape the gate-keeping function of aminoacyl-tRNA synthetases and gain entry into proteins, replacing their homologs and changing the structure, function, and antigenicity of the misassembled molecule. The large number of disorders associated with synthetase dysfunction include the Charcot-Marie-Tooth disease, amyotrophic lateral sclerosis (ALS), leukoencephalopathy, Parkinson's disease, various neoplasms, autoimmune polymyositis, dermatomyositis, rheumatoid and erosive arthritis, and type 2 diabetes (Antonellis and Green, 2008; Beebe et al., 2008; Park et al., 2008).

29.1.3 Azetidine-2-Carboxylic Acid

Notable among nonprotein amino acids with disease-causing potential is azetidine-2-carboxylic acid (Aze), the lower homolog of proline that is identical to proline except in that it contains four instead of five members in its ring (Fowden, 1956). Aze is ubiquitously present in low concentrations in vegetation, where it is a central component of the chelating molecules, nicotianamine and mugineic acid, which capture metals from the soil and transfer them to various parts of the plants (Figure 29.1(d) and 29.1(e)) (Curie et al., 2009; von Wiren et al., 1999).

In certain species, Aze accumulates to exceedingly high levels, notably in the bulbous roots of table beets and sugar beets (*Beta vulgaris*). Such plants deploy the toxic Aze to deter the intrusion of competing vegetation and to poison infecting microorganisms and predating animals. Thus, Aze is present in the food chain via small concentrations in vegetables. However, it is in high concentrations in certain foods, especially in dairy products derived from livestock fed sugar beet by-products (Rubenstein, 2008).

29.1.4 Aze in the Food Chain

The entry of Aze into the food chain is a consequence of geopolitical events of the 19th and 20th centuries (Rubenstein, 2008). Previously sugar beets were not cultivated or used for human consumption. They grew wildly and were foraged by animals, notably red deer in Europe. The sugar beet is an unattractive, large, bulbous plant with a flavor offensive to humans. The root was occasionally used as a therapeutic agent for various illnesses. In 1747, the German apothecary chemist Andreas Margraff discovered low concentrations of sucrose in the root. During the following 50 years, his student and research associate, Franz Achard, succeeded in increasing the concentration of sugar several times over, and at present the sucrose concentration can be as high as 18%. The Napoleonic wars resulted in the blockades of Europe, which severed the supply of Caribbean sugar. The entire continent was deprived of its principal source of sweets for nearly a decade.

To quell unrest, Napoleon issued a decree in 1810 allotting 8000 acres of land near Paris to sugar beet production. He also ordered the construction of facilities to process sugar beets and organized schools to teach sugar beet agronomy. After a slow start, the sugar beet industry grew rapidly, and by the middle of the century it was flourishing. Since then, it has become the fourth leading agricultural enterprise in Europe and a major farming activity in both Canada and the United States. Sugar beets are now grown and processed virtually worldwide, including in Asia, the Middle East, and the Far East. At present, about one-third of the world's sucrose supply comes from sugar beets.

Sugar beet processing involves washing the plants, dicing them, and then boiling them in alkali. The sucrose is extracted after several rounds of distillation. The residue, consisting of plant parts and thick molasses and rich in Aze, is fed to farm animals, especially to dairy cattle, which savor its unusually sweet flavor. A dairy cow may be fed as much as 10 pounds of sugar beet molasses per day. This increases its consumption of grasses and grains so that its daily food intake supports the required volume of its milk production. The health and milk production of dairy cattle begin to falter after about 5 years, at which time they are slaughtered. It is this association between sugar beets and milk and meat production that has led to the introduction of Aze into milk, other dairy products, and, notably, gelatin (Rubenstein et al., 2009).

Aze is also present in high concentrations in table (garden) beets, which are a staple food in Central and Eastern Europe. They are the principal constituent of beet soup (borscht). Sliced beets are also a popular offering in salad bars in the United States (Rubenstein et al., 2006).

29.1.5 The Multiple Sclerosis/Aze Hypothesis

Five independent lines of evidence suggest an association between Aze consumption and the pathogenesis of multiple sclerosis. The central concept is that Aze intrusion into the proline-laden consensual epitope of myelin basic protein leads to the molecule's architectural modification, functional impairment, and eventual emergence as an antigen. Thereafter, immune mechanisms are triggered and progressively damage the myelin sheath.

Correlation does not establish causation; however, the chronologic, geographic, migrational, enzootic, biochemical, and genetic associations warrant further investigation (Bessonov et al., 2010a,b; Rubenstein, 2008; Sobel, 2008).

29.1.6 Chronologic and Geographic Evidence

The emergence of dietary Aze in high concentrations closely preceded in time and place Charcot's description of what appeared to be a new disease, multiple sclerosis. This was in Paris in 1868 (Talley, 2005). The geographic distribution of the disease thereafter coincided with that of sugar beet agriculture, extending at higher latitudes across Europe, North America, and then virtually worldwide, including Sardinia, the Middle East, Scandinavia, and the Orkney and Faroe Islands. Micro hot-spots, such as those in Alberta, Canada, and the Tokachi Province in Japan, add support to the geographical evidence.

Alberta is the epicenter of sugar beet production in Canada and has the highest prevalence of multiple sclerosis in that country. Tokachi province, a small region in northern Japan, produces half of that nation's sugar beet crop and has the highest prevalence of multiple sclerosis among any Asian population (Rubenstein, 2008).

29.1.7 Migrational Evidence

Myelination of the central nervous system begins in early life and continues through adolescence, a fact pertinent to the effect of population migration on the occurrence of multiple sclerosis (Kennedy et al., 2006). An example is the abrupt appearance of the disease in the French West Indies during the 1990s among returning West Indians who had emigrated to France during the 1950s and 1960s (Cabre et al., 2005).

The risk was highest for those who arrived in France before the age of 15. Their mean duration of stay was 12.3 years, and the mean interval between their arrival in France and the appearance of the disease was 19.1 years. This epidemiology recapitulates in mirror image the original emergence and spread of the disease (Rubenstein, 2008).

29.1.8 Enzootic Evidence

Swayback is an enzootic disease closely mimicking multiple sclerosis in clinical and histological characteristics. An outbreak in Alberta in 1972 killed 60 of 100 newborn lambs. An investigation into the circumstances surrounding this event led to the conclusion that the only unique feature was that the ewes and their offspring were fed an unusual diet containing large amounts of sugar beet silage (Cancilla and Barlow, 1969; Chalmers, 1974).

29.1.9 Biochemical Evidence

As already described, the misincorporation of Aze into a protein can result in alteration of protein structure, function, and immunogenicity (Bessonov et al., 2010a,b; Rubenstein, 2008). In the case of multiple sclerosis, the putative site of the misincorporation lies in the myelin basic protein (Boggs, 2006; Harauz et al., 2009). Specifically, the misincorporation would occur in the position of one or more of the four prolyls in the consensual epitopic sequence, which embraces the hexapeptide stretch PRTPPP (residues 96–101, human numbering). This highly conserved proline-rich motif is stochastically vulnerable to the deleterious effects of Aze replacement of proline.

Aze may intrude within other proline-rich molecules involved in myelination. An enormous body of meticulous research has contributed to our knowledge of myelin basic protein, its posttranslational modification, and its roles in signaling and protein–protein binding (Boggs, 2006; Harauz et al., 2009).

The random incursion of Aze into myelin basic protein and related ensemble myelin proteins may account for some of the anatomic, pathologic, and clinical heterogeneity of the disorder. Such misassembly could ultimately ignite or contribute to the progression of immune responses, which can have dire clinical consequences.

The metabolic burdens caused by the inherently error-prone processes of translation are compounded by exposure to an alien amino acid and by an impaired aminoacyl-tRNA synthetase mechanism. Defective editing causes the myriad pathologic effects already listed. The presence of extensive oligodendrocyte apoptosis, together with the absence of lymphocyte and myelin phagocytosis in newly formed multiple sclerosis lesions, supports the concept that autoimmunity plays a critical role in demyelination.

29.1.10 Genetic Evidence

The important role of genetics in the pathogenesis of multiple sclerosis has been authoritatively reviewed by Oksenberg et al. (2008). These considerations have led to a targeted genomic search for SNPs involving genes related to Aze. The work, conducted in collaboration with the laboratory of Dr. Ronald Davis at Stanford, is currently in progress.

29.1.11 Alternative Splicing and Amplification

Alien amino acids ordinarily affect only the molecule that has been misincorporated; however, the misassembly of molecules involved in alternative splicing can alter hundreds of different kinds of 'downstream' proteins that undergo the splicing process. Therefore, the misassembly of alternative splicing proteins can greatly amplify the molecular pathology induced by Aze.

A growing body of evidence has implicated the role of disordered alternative splicing in disease pathogenesis. This mechanism has been identified in a number of developmental disorders, especially those involving the nervous system. Recent reports have focused on the A2BP1 protein, which binds to the C-terminus of ataxin-2.

Ataxin-2 has been found to act as a coregulator of zinc-finger transcriptional activity (Hallen et al., 2011). Defective function of this molecular mechanism can lead to amplified abnormal splicing of a large number of proteins and thus to an exceptionally diverse group of disorders.

Prominent among the diseases associated with abnormal splicing are multiple abnormalities caused by

defective neurodevelopment, including various forms of autism (Abrahams and Geschwind, 2008; Geschwind and Levitt, 2007; Hammock and Levitt, 2011; Martin et al., 2007; Sakai et al., 2011; Voineagu et al., 2011). Exposure to Aze-containing milk and other dairy products early in life may be of relevance, serving as an environmental susceptibility factor in autism spectrum disorders (see Chapter 34 and Rubenstein and Rakic, 2013).

Another neurodegenerative disorder that may be related to ataxin-2 and A2BP1 is ALS (Bonini and Gitler, 2011; Daoud et al., 2011; Lee et al., 2011; Van Damme et al., 2011). It is relevant to point out that the nonprotein amino acid β-*N*-methyl-amino-L-alanine (BMAA) has long been suspected to be an environmental susceptibility factor in ALS, initially in several Western Pacific islands. β-*N*-oxalylamino-L-alanine (BOAA) has been implicated in neurolathyrism endemic in certain regions of East Africa and Southern Asia. Canavanine, a homolog of arginine, is present in hundreds of legumes and in high concentrations in alfalfa seeds and sprouts. It has been suspected of playing a role in the pathogenesis of autoimmune disorders, including systemic lupus erythematosus (Cox et al., 2005; Rubenstein, 2000).

Other nonneurologic disorders associated with ataxin-2/A2BP1 include type 2 diabetes (Lehtinen et al., 2011). Similarly, obesity has been linked to the alternative splicing proteins (Ma et al., 2010). Biliary cirrhosis has also been associated with the same protein (Joshita et al., 2010).

29.1.12 Toxicity of Aze

A large number of early reports confirmed that Aze causes biochemical, morphological, and clinical defects. Among the biochemical abnormalities are misincorporation into vasopressin, hemoglobin, ovalbumin, and proline peptides. Inadvertent administration of Aze has resulted in butyric aminoaciduria. Morphologic abnormalities include abnormalities of somite formation, Golgi structure, osteoblasts, epithelial buds, cartilage, thyroid tissue, cleft palate, vertebral ossification, testes, keratin, hair, collagen, teeth, renal ducts, and mammary epithelium (Rubenstein, 2000; Tsai et al., 1990). Aze has recently been shown to be toxic to neurons and astrocytes in cell cultures, with associated increases in oxidized and ubiquitinated proteins (Dasuri et al., 2011).

Recent studies conducted by the Azetidine Group of collaborating investigators at the Stanford Medical Center have focused on the molecular details of misincorporation. This group includes M. Albertelli, M.J. Butte, R. Davis, J.E. Elias, M. Fontaine, K.V. Grimes, S. Krishnakumar, M. Mindrinos, G. Sonderstrup, R. Sobel, and E. Rubenstein. Some of this work has been summarized in the report by R.A. Sobel et al.

Aze has been found in milk samples from Boulder, CO. The use of sugar beets for dairy cattle feeding ceased in California about 3 years ago, and milk samples from California are currently free of Aze.

The administration (by oral gavage or intraperitoneal injection) of Aze in doses of 300 or 600 mg/kg to newborn CD-1 mice resulted in oligodendrocyte nuclear swelling and hepatic injury. Three of the five mice fed the larger dose by gavage were found dead or euthanized at 12–15 days. Three of the five mice in the 600-mg intraperitoneal group were euthanized or found dead after 20–24 days of Aze administration. The remaining mice in the 600-mg group and all the mice in the 300-mg group and control groups remained healthy. Mice that were euthanized early or found dead showed lethargy and weight loss; two also had tail paresis or paralysis. Some of the livers displayed mottled pale areas of hepatocyte vacuolization (Sobel et al., 2011).

Our collaborator in Canada, G. Harauz, has reported studies of myelin basic protein in recombinant *Escherichia coli*. These showed that Aze resulted in severe diminution in growth rate and that Aze was misincorporated in 3 of 11 possible sites in myelin basic protein. Molecular modeling led to the conclusion that Aze could cause a severe bend in the polypeptide chain and could disrupt a polyproline II structure (Bessonov et al., 2010a,b).

29.1.13 ATAXIN-2 and A2BP1 as Proline-Rich Proteins

These considerations have led to an analysis of compositional bias among the amino acids within ataxin-2 and A2BP1. The study revealed that ataxin-2 is exceptionally proline rich. Of its 1313 residues, a total of 177 are prolyls (13.4%). Positions 47–158 contain 30 prolyls among its 112 amino acids (26.8%), and within this sequence, prolyls comprise 7 of the 10 amino acids in positions 55–64. Among the 184 amino acids in positions 561–734, there are 44 prolyls (23.9%). Finally, there are 34 prolyls (21.7%) among the 157 amino acids in the sequence 929–1085 (UniProt ID Q 99700).

A2BP1 is less proline-rich, containing 40 prolyls among its 370 amino acids (10.8%). Noteworthy is the sequence PPPPIP, which occupies positions 281–286 (GenBank AA143817.1).

29.2 DISCUSSION

It is clear that Aze can displace proline in proteins and that such misassembly can have deleterious effects on protein structure, function, and immunogenicity. Whether Aze, introduced into the diet or into medicinal agents, has contributed to the shifting prevalence and

incidence of clinical disorders is unknown. The complexities associated with an ever-changing biosphere and with population shifts constitute nearly chaotic conditions that challenge epidemiology.

Caution is required before inferences are drawn.

Is it possible that a simple compound, a small and apparently innocent amino acid found in plants, has become a susceptibility factor in the pathogenesis of a broad spectrum of diseases in which genetic predisposition plays a critically important role? Well-planned studies will be required to address this question. These must take into account such confounding factors as dose and time and duration of exposure, from early embryonic life through adulthood.

Some supporting evidence can be accumulated by demonstrating that Aze is in the environment of those affected and that Aze is in their tissues and in the specific cells that are damaged, for instance, in central nervous system cells in the case of neurologic disease. In most instances, this would require examination of autopsy material from patients and from controls. If a suitable animal model exists, the administration of Aze in appropriate doses at appropriate states of development should reproduce illness. Finally, the removal of Aze, perhaps by washout with pure proline, might prove beneficial.

The group at Stanford is testing the reactivity of fresh T cells from patients with various diseases to synthetic proteins in which Aze has replaced proline in differing combinations and permutations.

It is important to proceed with great caution in forming conclusions. It is essential to avoid the needlessly damaging consequences of inappropriate disclosures disseminated by the popular media. The public needs to be reminded periodically about the significance of environmental hazards and that even sunlight, oxygen, and water can be detrimental. It will require ingenuity, collaborative effort, and persistence to identify the environmental agent that may underlie the suffering experienced by millions.

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