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Dedicated to Professor Lech Wojtczak on the occasion of his 80th birthday

Review

Structural aspects of L-asparaginases, their friends and relations★

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Enzymes capable of converting L-asparagine to L-aspartate can be classified as bacterial-type or plant-type L-asparaginases. Bacterial-type L-asparaginases are further divided into subtypes I and II, defined by their intra-/extra-cellular localization, substrate affinity, and oligomeric form. Plant-type L-asparaginases are evolutionarily and structurally distinct from the bacterial-type enzymes. They function as potassium-dependent or -independent Ntn-hydrolases, similar to the well characterized aspartylglucosaminidases with $(\alpha\beta)_2$ oligomeric structure. The review discusses the structural aspects of both types of L-asparaginases and highlights some peculiarities of their catalytic mechanisms. The bacterial-type enzymes are believed to have a disordered active site which gets properly organized on substrate binding. The plant-type enzymes, which are more active as isoaspartyl aminopeptidases, pose a chemical challenge common to other Ntn-hydrolases, which is how an N-terminal nucleophile can activate itself or cleave its own α-amide bond before the activation is even possible. The K^+-independent plant-type L-asparaginases show an unusual sodium coordination by main-chain carbonyl groups and have a key arginine residue which by sensing the arrangement at the oligomeric (αβ)-(αβ) interface is able to discriminate among substrates presented for hydrolysis.

Keywords: L-asparaginase, isoaspartyl peptidase, Ntn-hydrolase

HYDROLYSIS REACTIONS AT THE SIDE CHAIN OF L-ASPARAGINE

The simple hydrolysis reaction of the side-chain amide bond of L-asparagine is catalyzed by a group of amidohydrolases known as L-asparaginases. This reaction can be assayed by measuring the release of ammonia in a simple Nessler test (Derst *et al.*, 1992), or the release of L-aspartate in a glutamate-oxaloacetate amidotransferase/malate dehydrogenase coupled enzymatic test (Tarentino & Maley, 1969). Some of these enzymes can tolerate a modification of the β-amide of the substrate but in general a specialized enzyme is necessary to hydrolyze those bigger substrates. The most important modifications include glycosylation and β-peptide formation. Glycosylated L-asparagine is generated during proteolytic breakdown of glycoproteins, and its degradation is catalyzed by enzymes with aspartylglucosaminidase (AGA) activity. Recycling of defective proteins is also the source of isoaspartyl (or β-aspartyl) peptides, which are resistant to hydrolysis by normal α-peptidases and must be degraded by isoaspartyl peptidases. Both reactions, in common with the simple L-asparaginase reaction, generate L-aspartate as one of the products (Fig. 1). All the above variants of L-asparagine hydrolysis are obviously distinguished from the cleavage of the α-peptide bond formed by

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Abbreviations: AGA, N^4-(β-*N*-acetylglucosaminyl)-L-asparaginase (aspartylglucosaminidase); ALL, acute lymphoblastic leukemia; AtA, *Arabidopsis thaliana* L-asparaginase; EcAIII, *Escherichia coli ybiK* (*iaaA*) gene product; Glu-AdT, Glu-tRNA^{Gln} amidotransferase; HslV, bacterial homolog of eukaryotic proteasome β subunit; HslU, molecular chaperone which binds to HslV; iAsp, isoaspartyl residue; LlA, *Lupinus luteus* L-asparaginase; *O*-MT, L-isoaspartyl(D-aspartyl)-*O*-methyltransferase; Ntn, N-terminal nucleophile; Tas1, threonine aspartase (taspase1).

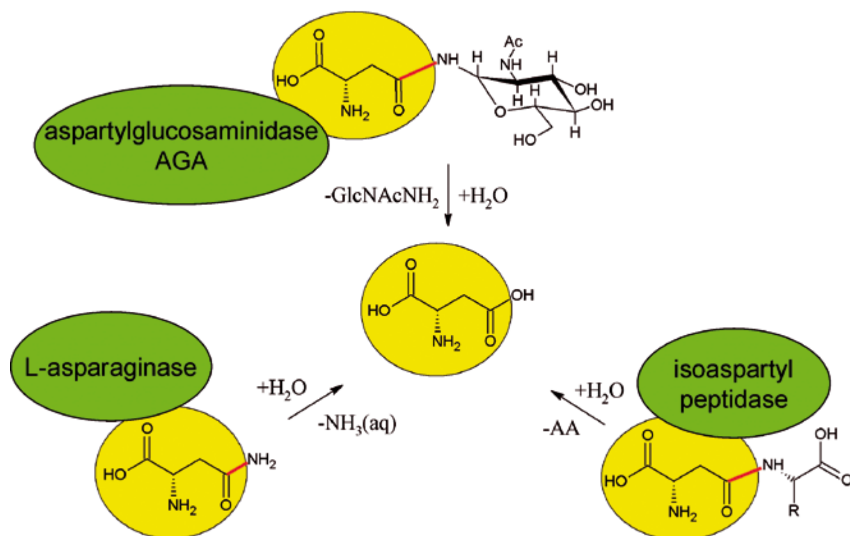

Figure 1. Enzymatic reactions releasing L-aspartate (yellow background) as one of the products and the enzymes (green background) catalyzing them.
The β-amide bond that undergoes hydrolysis is marked in red.

L-asparaginase, which may also release L-aspartate. Surprisingly, some of the α-peptidases bear close structural and mechanistic similarity to certain L-asparaginases, although there is no substrate cross reactivity. The following sections review various structural aspects of these interesting enzymes.

EARLY YEARS OF L-ASPARAGINASE RESEARCH

Enzymatic hydrolysis of L-asparagine to L-aspartate and ammonia was first observed by Lang (1904) who detected asparaginase activity in several beef tissues. Lang's results were confirmed a few years later by Fürth and Friedmann (1910), who found asparagine hydrolysis in horse and pig organs and concluded that all animal tissues possessed the same level of asparaginase activity. Contradicting those conclusions, Clementi (1922) showed that in omnivorous animals (like the pig) the enzyme is present only in the liver, while organs of carnivorous mammals, amphibians and reptiles do not contain L-asparaginase at all, and that only in herbivores it can be found practically in all tissues. Clementi's discovery of L-asparaginase activity in the blood of guinea pig was brought to light again 40 years later by Broome (1961), who attributed the antitumor properties of guinea pig serum, reported by Kidd (1953), to L-asparaginase activity. Broome's intuition turned out to be right, and soon potent antileukemic L-asparaginases were also found in bacteria and introduced into clinical practice, mainly for the treatment of acute lymphoblastic leukemia (ALL). The beneficial role of L-asparaginase administration is usually attributed to the fact that the tumor cells have a compromised ability to generate L-asparagine endogenously, either due to low expression levels of asparagine synthetase (Stams *et al.*, 2005) or insufficient amount of its substrates, aspartate or glutamine (Aslanian & Kilberg, 2001). Because of their dependence on exogenous L-asparagine, the cancerous ALL cells, but not normal cells, can be starved and eliminated by L-asparaginase treatment which depletes the levels of L-asparagine in circulating pools.

Of particular importance has been an enzyme from *Escherichia coli*, EcAII, the first antileukemic L-asparaginase to be used clinically. The severe side effects of L-asparaginase administration have been circumvented by alternating treatment using enzymes from different sources and by conjugating EcAII with polyethylene glycol (Abuchowski *et al.*, 1984). It soon became apparent that, in addition to the secreted (periplasmic) EcAII which has high substrate affinity (K_m=1.15 × 10^{-5} M; Ho *et al.*, 1970), *E. coli* (and other bacteria) also produces a cytosolic enzyme, EcAI, which because of its much lower affinity for L-asparagine (K_m=3.5 × 10^{-3} M; Willis & Woolfolk, 1974) is not effective against cancer. An analogous pattern of intracellular and secreted L-asparaginases has also been discovered in yeasts, although phylogenetic analyses have suggested that the intra- and extra-cellular enzymes evolved in prokaryotes and eukaryotes independently (Bonthron & Jaskolski, 1997).

Crystallographic studies of EcAII were undertaken in the early 1970s (Epp *et al.*, 1971). However, only in 1988 was a partial model of the EcAII structure published (Ammon *et al.*, 1988). While it turned out to be largely inaccurate, it helped to finally crack the L-asparaginase structure in 1993 (Swain *et al.*, 1993). With the archetypal EcAII model available, structural research of bacterial-type L-asparaginase gained momentum. Currently, in the Protein Data Bank (PDB; Berman *et al.*, 2000), there are more than two dozen crystal structures of enzymes from this group, among them such excellent examples as the structure of *Erwinia chrystanthemi* L-asparaginase determined at 1 Å resolution (Lubkowski *et al.*, 2003). It has to be admitted, however, that no eukaryotic enzymes (e.g. from guinea pig or from *Saccharomyces*

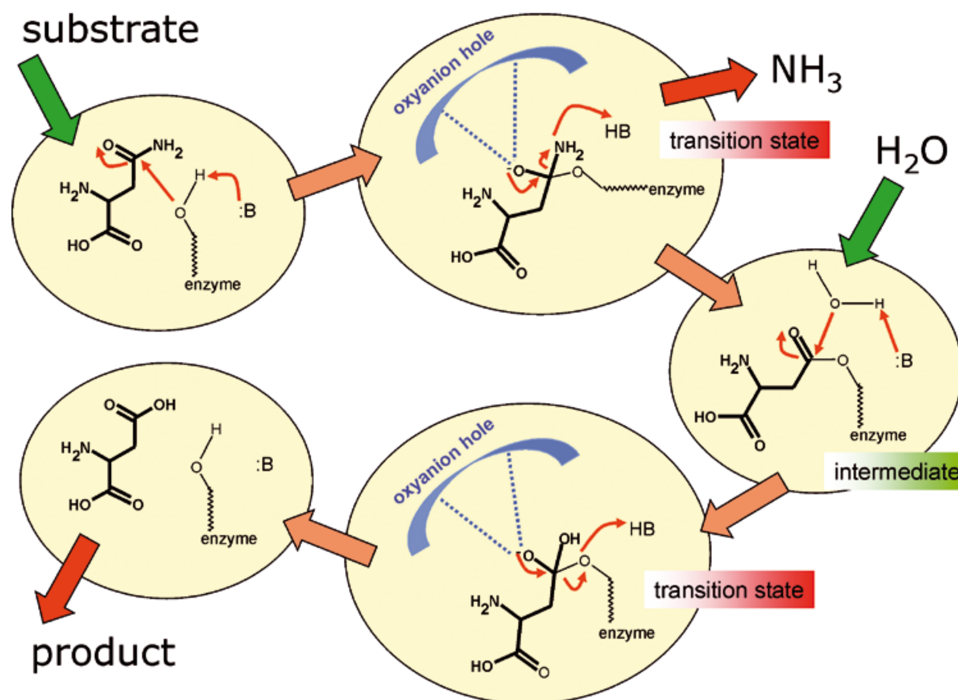

Figure 2. Proposed general mechanism of L-asparaginase reaction.

cerevisiae) from this group have been characterized structurally to date.

GENERAL MECHANISM OF THE REACTION CATALYZED BY L-ASPARAGINASE

The mechanism of L-asparaginases has been compared to that of classic serine proteases, whose activity depends on a set of amino-acid residues, typically Ser-His-Asp, known as the "catalytic triad" (Carter & Wells, 1988). This set includes a nucleophilic residue (Ser), a general base (His), and an additional, acidic, residue (Asp), all connected by a chain of hydrogen bonds. The reaction consists of two steps (Fig. 2). In the first step, the enzyme's nucleophile, activated *via* a strong O-H...B hydrogen bond to an adjacent basic residue, attacks the C atom of the amide substrate, leading through a tetrahedral transition state to an acyl-enzyme intermediate product. The negative charge that develops on the O atom of the amide group in the transition state is stabilized by interactions with adjacent hydrogen-bond donors. The constellation of those donors (which typically are main-chain N–H groups) is known as the "oxyanion hole". The second step of the reaction is similar, but now the attack on the ester C atom is launched by an activated water nucleophile. This useful simple picture is not without doubts, however. One of them concerns the identification of a suitable general base for the activation of the nucleophilic residue.

BACTERIAL-TYPE L-ASPARAGINASES[1]

The structure of EcAII (PDB code 3ECA) revealed a tetrameric protein (a dimer of "intimate" dimers) with 222 symmetry, composed of four identical subunits, 326 residues each (Fig. 3a). In each of the four active sites, a molecule of the reaction product, L-Asp, is bound, defining the enzyme–substrate interactions. Although the intimate dimer is necessary for active site formation, those interactions are mostly formed within one subunit and involve two Thr residues (Thr12 and Thr89 in EcAII numbering), posed on both sides of the side chain of the ligand molecule (Fig. 3b). Since both these Thr residues are required for activity, it has been debated for a long time which of them supplies the –OH nucleophile to attack the C atom of the scissile amide bond. In the end, the consensus favors Thr12, one of the arguments being the observation of a Thr12-acyl product formed on incubation of L-asparagine with a T89V mutant of EcAII (Palm *et al.*, 1996). However, Thr89 is still an inter-

[1]This review uses the historically established but rather unfortunate nomenclature dividing L-asparaginases into "bacterial-type" and "plant-type". The terminological confusion is many-fold. For instance, bacterial-type asparaginases also include archaeal and eukaryotic enzymes and in fact they were first detected in mammals. Likewise, plant-type asparaginases are also found in bacteria. In addition, the plant-type enzymes were later found to be more active as isoaspartyl peptidases. We have tried to find a better terminology but it turns out that there is no nomenclature that would be free from ambiguity or imprecision. In this situation, we have decided to stay with the historically sanctioned, albeit confusing, terminology.

esting alternative because it is part of a very clear T-K-D triad (Fig. 3b), which resembles the classic S-H-D catalytic triad of serine proteases (Carter & Wells, 1988). Despite the chemical analogy, the T-K-D triad would be unusual in that lysine acting as a general base is more typical for catalytic dyad, rather than triad, systems (Paetzel & Dalbey, 1997). In the current interpretation of the catalytic apparatus of EcAII, the nucleophile-activator role has been assigned, somewhat controversially, to Tyr25 (Ortlund *et al.*, 2000). Another unusual feature of the active site of type II bacterial asparaginases is that it gets fully "assembled" only on substrate binding, as its key elements, including the Thr12 nucleophile, are part of a highly flexible loop (Aung *et al.*, 2000).

The crystal structure of type I bacterial L-asparaginase has been solved only recently and is exemplified by the enzyme from *Pyrococcus horikoshii* (Yao *et al.*, 2005). As predicted much earlier (Bonthron & Jaskolski, 1997), the enzyme turned out to be dimeric, corresponding to the intimate dimer of EcAII (Fig. 3c). It is not clear why bacterial type II L-asparaginases function as tetramers whereas dimerization suffices to provide all components of the active site, and if the difference in substrate affinity of the type I and II enzymes is related to their oligomeric forms. Phylogenetic analyses can usually distinguish between type I and type II sequences without ambiguity, despite their relatively high homology. While sequence differences can be correlated with the oligomeric structure, they are not able to explain why the two types have different quaternary structures (Bonthron & Jaskolski, 1997).

EXAMPLES OF OTHER BACTERIAL-TYPE ENZYMES

Homologous bacterial-type L-asparaginases are found in all kingdoms of life. Furthermore, sequence similarity and activity data suggest that in this group also other enzymes can be classified (Borek & Jaskolski, 2001). The most interesting examples include L-glutaminase-asparaginases (Roberts, 1976), lysophospholipases with secondary L-asparaginase activity (Sugimoto *et al.*, 1998), or the GatD subunit of archaeal Glu-tRNA amidotransferase (Glu-AdT; Tumbula *et al.*, 2000). The latter case is related to alternative synthesis of Gln-tRNA^{Gln}, which proceeds *via* transamidation of incorrectly charged Glu-tRNA^{Gln}. The amino group for this reaction is supplied by the GatD subunit of this enzyme complex. The recently published structural data for Glu-AdT from *Pyrococcus abyssi* (Schmitt *et al.*, 2005) confirm the relationship of the GatD module, which also functions as a dimer, to type I bacterial asparaginases.

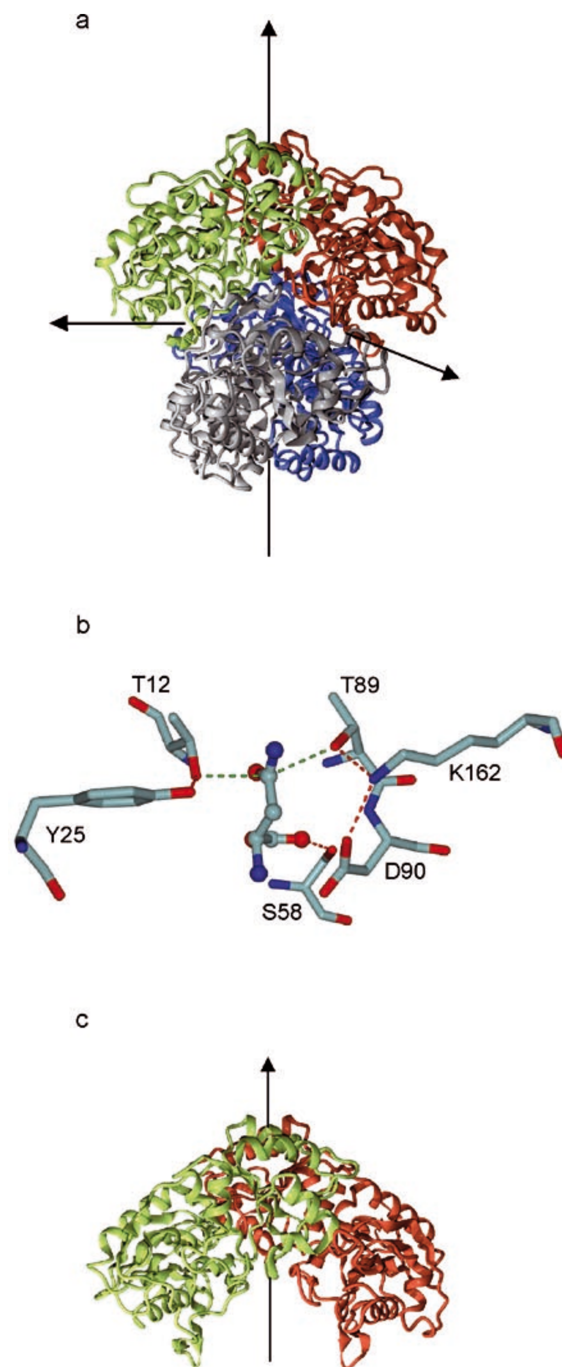

Figure 3. Structural features of bacterial-type L-asparaginases.
(**a**) The quaternary structure of the periplasmic *E. coli* enzyme EcAII (PDB code 3ECA). The green and red (or gray and blue) monomers form the intimate dimer. The arrows correspond to the two-fold axes defining the 222 symmetry of this homotetramer. (**b**) The active site of EcAII with an L-Asn substrate molecule (ball-and-stick) modeled according to the EcAII/L-Asp complex (Swain *et al.*, 1993). The dotted red lines represent hydrogen bonds while the green ones symbolize a potential line of attack of the active-site Thr residues on the substrate's amide C atom. (**c**) The quaternary structure of type-I bacterial L-asparaginase from *Pyrococcus horikoshii* (PDB code 1WLS), corresponding to the cytosolic *E. coli* enzyme EcAI. The protein is a homodimer, corresponding to the intimate homodimer of EcAII in (a).

The hydrolysis of L-glutamine is very similar to the reaction of L-asparaginase, and indeed many of the bacterial-type L-asparaginases have a different degree of both activities. There are, however, enzymes with no cross-reactivity, like the cytosolic enzyme from *Wolinella succinogenes*, which has virtually no L-glutaminase activity (Lubkowski *et al.*, 1996).

ROLE OF L-ASPARAGINASES IN PLANTS

Although homologs of bacterial L-asparaginases exist in such eukaryotes as fungi and mammals, no similar enzymes have been found in plants. On the other hand, the metabolism of plants, which acquire their nitrogen in the roots and transport it in the form of L-asparagine, requires efficient L-asparaginase activity in fast growing tissues where massive protein synthesis takes place. The issue of nitrogen management is of particular interest in legume plants, like yellow lupine (*Lupinus luteus*), which assimilate atmospheric N_2 in symbiosis with root bacteria. The role of L-asparaginase activity in plants has been recognized already in 1927 (Grover & Chibnall, 1927). In the literature published in the 1980s, plant enzymes with L-asparaginase activity were classified as potassium-dependent or potassium-independent (Sodek *et al.*, 1980), although in view of the relatively high sequence similarity (66%) between these two groups, the molecular basis of the K^+-(in)dependence is not clear.

Until recently, no structural data for plant L-asparaginases were available. However, high sequence similarity (60–70%) has suggested common structural classification with bacterial and human aspartylglucosaminidases (AGAs), which are relatively well studied members of the Ntn (N-terminal nucleophile) hydrolase family (Oinonen *et al.*, 1995; Guo *et al.*, 1998; Xuan *et al.*, 1998). As AGAs also exhibit secondary L-asparaginase activity (Noronkoski *et al.*, 1998; Saarela *et al.*, 2004), the structural and functional properties of Ntn-hydrolases provide a convenient starting point for the analysis of plant L-asparaginases.

During the cloning and recombinant expression of a potassium-independent L-asparaginase from *L. luteus* (LlA) (Borek *et al.*, 1999), it was discovered that *E. coli* has a gene (*ybiK*, later annotated as *iaaA*) that codes for a protein with 70% amino acid sequence similarity to this plant enzyme (Borek & Jaskolski, 2000). It became apparent that a larger family of plant-type L-asparaginases should be defined. The first non-plant member in this group was the *E. coli* protein. Since it was later established that it indeed had L-asparaginase activity (*vide infra*), it was named EcAIII (Borek & Jaskolski, 2000; Borek, 2001).

Ntn-HYDROLASES

The term "Ntn-hydrolases" was coined in 1995 (Brannigan *et al.*, 1995) to describe a new general mode of catalysis deduced from the common structural features of glutamine phosphoribosyl pyrophosphate amidotransferase (Smith *et al.*, 1994), penicillin G acylase (Duggleby *et al.*, 1995) and subunit β of the proteasome (Löwe *et al.*, 1995). The distinctive characteristics of this family of enzymes is the involvement of an N-terminal Thr, Ser, or Cys as a nucleophile during the catalytic reaction. All Ntn-hydrolases share amide-bond-oriented activity and a common αββα fold, where two extended and largely antiparallel β-sheets face each other and are flanked on both sides by layers of helices. All these structural features are preserved among Ntn-hydrolases even in situations of negligible sequence similarity. A large subclass in this family is established by Ntn-type glutamine amidotransferases, which use glutamine as the amino group donor in the amidation reaction. The hydrolysis of L-glutamine is carried out by an L-glutaminase domain with Ntn-hydrolase fold (Smith *et al.*, 1994; Isupov *et al.*, 1996; Binda *et al.*, 2000). The group of enzymes with no other than hydrolytic activity includes penicillin G (Duggleby *et al.*, 1995) and penicillin V (Suresh *et al.*, 1999) acylases, cephalosporin acylase (Kim *et al.*, 2000), bile salt hydrolase (Rossocha *et al.*, 2005; Kumar *et al.*, 2006), the proteasome subunit β (Löwe *et al.*, 1995), aspartylglucosaminidases (Oinonen *et al.*, 1995; Guo *et al.*, 1998; Xuan *et al.*, 1998), plant-type L-asparaginases (Borek *et al.*, 2004, Michalska *et al.*, 2005; 2006), and taspase1 or threonine aspartase (Khan *et al.*, 2005).

Ntn-hydrolases are expressed as non-functional precursors with the putative nucleophile occupying an endo position in the polypeptide chain. In order to expose the nucleophile as an N-terminal residue, they require an activation step, usually considered as an autoproteolytic event involving the same catalytic residue that carries out the final enzymatic reaction. The maturation occurs through the removal of an N-terminal propeptide or methionine residue, or as endoproteolytic splitting of the polypeptide chain (sometimes followed by spacer excision), which liberates two subunits (α and β) from the precursor polypeptide. In the latter case, the nucleophlic amino acid becomes the N-terminal residue of subunit β.

Ntn-hydrolases exist in different oligomeric forms, extending from a simple (αβ) heterodimer in penicillin G acylase (Duggleby *et al.*, 1995) or cephalosporin acylase (Kim *et al.*, 2000), up to fourteen subunits in the proteasome (Löwe *et al.*, 1995). The enzymes involved in L-aspartate-derived substrate processing, such as L-asparaginases, aspartylglu-

cosaminidases and taspase1, operate as $(\alpha\beta)_2$ heterotetramers, i.e. as dimers of $(\alpha\beta)$ heterodimers.

The mechanism of Ntn-hydrolases is usually compared to that of serine proteases (Dodson & Wlodawer, 1998), although they do not contain a catalytic triad. Instead, the nucleophilic group is believed to be activated by the free α-amino group of the same residue, functioning as a general base. This interpretation of the mechanism is widely accepted because it explains the need for the nucleophile to be at the N-terminus but detailed analysis of the available structures leaves some serious doubts about its generality, especially in view of the lack of universal conservation of the interactions formed by the catalytic residue.

The sequence-based classification of LlA and EcAIII as Ntn-hydrolases was corroborated at the purification stage, where both proteins displayed a maturation pattern characteristic of Ntn-hydrolases. EcAIII underwent spontaneous breakdown into a larger (α) and smaller (β) subunits in a matter of hours and (recombinant) LlA in a matter of days (Fig. 4a). Mass-spectrometry sequencing revealed a threonine residue at the N-terminus of both subunits β (Thr179 in EcAIII, Thr193 in LlA).

DUAL ACTIVITY OF PLANT-TYPE L-ASPARAGINASES

A comprehensive kinetic study revealed that EcAIII and LlA have very similar activity profiles (Borek *et al.*, 2004). Despite the high sequence similarity to the AGA enzymes, none of the plant-type L-asparaginases is active towards glycosylated L-asparagine (Larsen *et al.*, 2001; Borek *et al.*, 2004) while L-asparaginase activity could be confirmed but only with millimolar affinity for L-asparagine. Unexpectedly, both enzymes showed submillimolar activity towards isoaspartyl peptides. This was also observed for a homologous enzyme from *Salmonella enterica* (Larsen *et al.*, 2001). According to the dominating activity *in vitro*, formally these enzymes should be more properly classified as isoaspartyl aminopeptidases with secondary L-asparaginase activity (Borek *et al.*, 2004).

On the other hand, the results of Bruneau *et al.* (2006) demonstrate that a potassium-independent enzyme from *Arabidopsis thaliana* has a slight preference for L-asparagine over a β-peptide. The different substrate specificity of the *A. thaliana* enzyme and LlA is attributed to their separate evolution. Moreover, it has been shown that a potassium-dependent enzyme from the same organism is strictly specific for L-asparagine and does not hydrolyze isoaspartyl substrates at all. In view of the high sequence similarity between the two *A. thaliana* enzymes and LlA, the above results are somewhat surprising, especially considering that all residues forming the active site in LlA are strictly conserved in both *A. thaliana* proteins.

ROLE OF DUAL ACTIVITY OF PLANT L-ASPARAGINASES

While it is not obvious why *E. coli* should have an enzyme with dual isoaspartyl aminopeptidase/L-asparaginase activity, especially in view of the presence of two other L-asparaginases, a plausible explanation of this dual activity in plants can be given. The L-asparaginase activity is necessary (and indeed detected at elevated levels) during the formation of seeds when new proteins, in particular

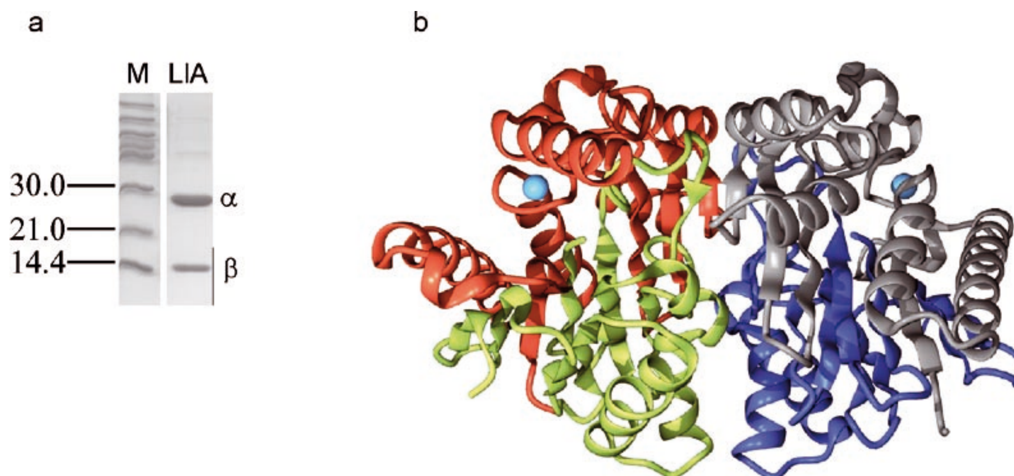

Figure 4. Subunit structure of plant-type L-asparaginases.
(**a**) SDS/PAGE of purified LlA (right lane) showing the presence of the α- and β-subunits with their respective molecular masses of 23 (including His-tag sequence) and 14 kDa (M, molecular mass markers). (**b**) Quaternary structure of the EcAIII $(\alpha\beta)_2$ heterotetramer. The red and gray chains correspond to subunit α, the green and blue ones to subunit β. The blue spheres mark the position of the sodium cation coordinated by the α subunit of each (αβ) unit.

storage proteins, are synthesized. On the other hand, isoaspartyl aminopeptidase activity is necessary to remove the aberrant and harmful isoaspartyl peptides that arise spontaneously and accumulate with time. Those aberrations are especially likely to occur in seed storage proteins, which have to withstand prolonged periods of dormancy.

Isoaspartyl peptides are generated in proteins when the main chain is transferred in a non-enzymatic reaction to the side chain of Asp or Asn. Those transformations proceed *via* L-succinimide formation when the n+1 amide N–H group launches an attack on the side chain of Asp/Asn at position n (Geiger & Clarke, 1987; Fig. 5). In addition to $\alpha \rightarrow \beta$ isomerization, L→D isomerization is another consequence of these rearrangements. Since the formation of β-aspartyl peptides leads to a wrong structure and to dysfunction and is connected with toxic effects, those harmful proteins have to be repaired or destroyed. The repair pathway is initiated by L-isoaspartyl(D-aspartyl)-*O*-methyltransferase, which catalyzes the first step of aspartate recovery (O'Connor & Clarke, 1985). The degradation of β-aspartyl-containing proteins is controlled by the cellular proteolytic machinery, composed mainly of proteases recognizing α-peptide bonds. The remaining β-peptides liberated in the final stages of the degradation processes require specific enzymes, namely isoaspartyl peptidases, for their ultimate destruction.

OVERALL STRUCTURE OF PLANT-TYPE L-ASPARAGINASES

The first crystal structure of a plant-type L-asparaginase was determined for EcAIII by Borek (2001) at 1.65 Å and deposited in the PDB with an accession code 1K2X. In 2004, an essentially the same structure at the same resolution was reported by Prahl *et al.* (2004; 1T3M). In addition, a different crystal form of EcAIII, crystallized at high Ca^{2+} concentration, was also deposited in the PDB (1JN9). All these structures revealed an $(\alpha\beta)_2$ oligomeric protein, arranged as a dimer of two (αβ) heterodimers generated in autocatalytic cleavage of the precursor protein (Fig. 4b). Each of the (αβ) units has the αββα fold typical for Ntn-hydrolases, i.e. consists of two almost exclusively antiparallel β-sheets arranged in a face-to-face manner and flanked on both outer faces by layers of α-helices. The smaller, four-stranded β-sheet is folded exclusively within the β-subunit, while the larger one has eight β-strands contributed by both subunits. In addition, it is complemented by a ninth β-strand contributed by subunit β from the other (αβ) unit. The large β-sheet is, therefore, an important element for the cohesion of the $(\alpha\beta)_2$ oligomer. In its middle, it includes the N-terminal β-strand of subunit β, which harbors the catalytic Thr residue (179 in EcAIII sequence). In each of the α-subunits, a sodium cation is coordinated by a loop adjacent to the catalytic apparatus (*vide infra*).

THE ACTIVE SITE OF PLANT-TYPE L-ASPARAGINASES

The crystal structure of EcAIII crystallized in complex with L-Asp (Michalska *et al.*, 2005) has revealed the architecture and interactions in the active site. As expected, the ligand is located near the catalytic Thr179 residue, at the crest of the large β-sheets. The docking of the product molecule is established by residues contributed exclusively by subunit β. Among them, Arg207 creates the strongest interaction *via* a salt bridge between its guanidinium group and the α-carboxy group of the L-aspartate ligand (Fig. 6a). Residues Asp210 and Gly231 participate in the binding of the substrate α-amino group. Thr230 and Gly231 are components of the oxyanion hole, functioning to stabilize the negative charge of the

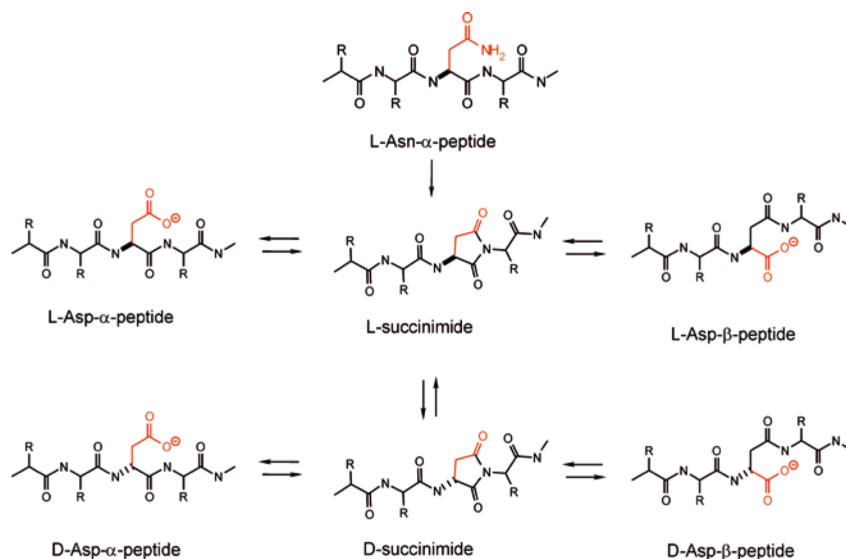

Figure 5. Non-enzymatic modifications of proteins containing L-Asn- and L-Asp-peptides.

tetrahedral transition state during the catalytic reaction. This oxyanion hole is rather unusual, as it involves as one of its elements the side-chain hydroxyl group of Thr230. The residues from the α-subunit involved in sculpting the catalytic pocket are posed to participate in anchoring the other fragment of an isoaspartyl substrate, namely the C-terminal residue in the case of an isoaspartyl dipeptide.

Although in detailed analyses several differences in the secondary and tertiary structures between LlA and EcAIII are noted, the conclusions regarding the catalytic mechanism drawn from the bacterial enzyme are confirmed by the crystal structures of the plant protein, which reveal exact conservation of the architecture of the active site. In particular, although no L-Asp complex of LlA has been crystallized, in the available crystal structures of the plant enzyme the active site is invariably occupied by a negatively-charged entity, either a chloride anion (Michalska *et al.*, 2006) or a formate anion (unpublished results). These anions mimic the α-carboxylate group of the natural substrate/product in the interactions with the side chain of Arg221 (equivalent of EcAIII Arg207) (Fig. 6b, c).

SOME ASPECTS OF THE MECHANISM OF PLANT-TYPE L-ASPARAGINASES

The structural context in which the nucleophilic threonine residue is found in the plant-type enzymes raises several questions about the catalytic mechanism. One of them concerns the role of the free α-amino group as an activator of the –OH nucleophile. The controversy refers mainly to its chemical properties (high likelihood of protonation at physiological pH) and to the unfavorable stereochemistry of its intramolecular hydrogen bonding with the hydroxyl group (Michalska *et al.*, 2005). The latter aspect is connected with the strained geometry of the five-membered ring (including the H atom) closed by the hydrogen bond, in which the O–H…N angle would be about 114°, whereas proton-transfer reactions typically take place in linear D–H…A systems (Olovsson & Jaskolski, 1986). In some other Ntn-hydrolases a water molecule has been proposed to mediate in the $–NH_2…H–OH…H–O–$ interaction (Saarela *et al.*, 1998; Yoon *et al.*, 2004), but in none of the structures of EcAIII and LlA a suitable candidate is seen (Michalska *et al.*, 2005; 2006). Another

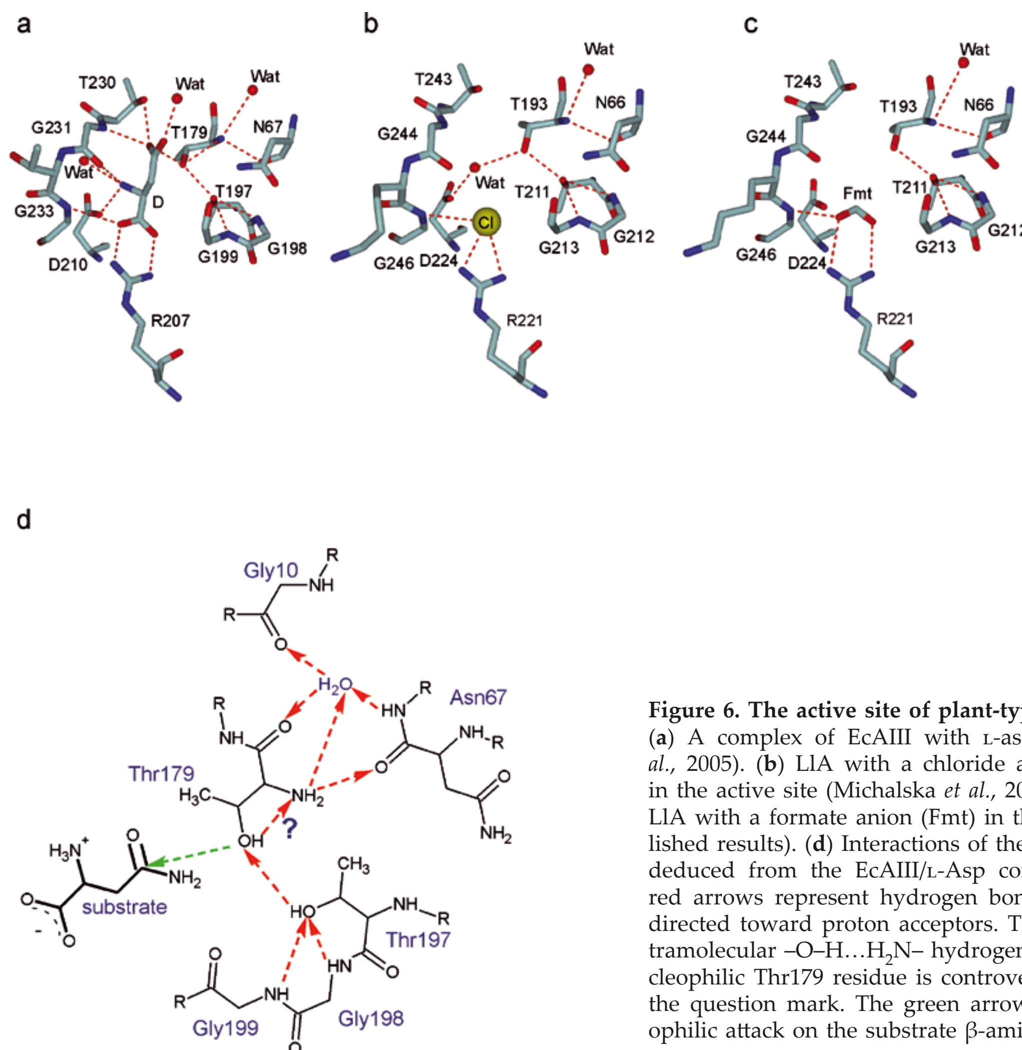

Figure 6. The active site of plant-type L-asparaginases.
(**a**) A complex of EcAIII with L-aspartate (Michalska *et al.*, 2005). (**b**) LlA with a chloride anion (yellow sphere) in the active site (Michalska *et al.*, 2006). (**c**) A complex of LlA with a formate anion (Fmt) in the active site (unpublished results). (**d**) Interactions of the catalytic Thr residue deduced from the EcAIII/L-Asp complex structure. The red arrows represent hydrogen bonds, with arrowheads directed toward proton acceptors. The strength of the intramolecular $–O–H…H_2N–$ hydrogen bond within the nucleophilic Thr179 residue is controversial, as indicated by the question mark. The green arrow indicates the nucleophilic attack on the substrate β-amide carbon atom.

```
                               α                        ↓β
LlA     EAN----RVQVDYSQYNYPEPVKDDAEKELPLTNGDSQIG-----| TVG
AtA     EFN----RVQLDYTVP----------SPKVPDNCGDSQIG-----| TVG
AtAK    EAN----SILFDYRIP--PMGCAGAAATDSPIQMNGLPISIYAPE| TVG
EcAIII  KEG----ATVLDHSGA--------------PLD-EKQKMG-----| TVG
Tas1    RKL----ELAERVDTD--------FMQLKKRRQSSEKENDSGTLD| TVG
hAGA    CQPNYWRNVIPDPSKYCGPYKPPGILKQDIPIHKETEDDR--GHD| TIG
```

Figure 7. Sequence alignment of the autoproteolytic region (α/β boundary) of *Lupinus luteus* potassium-independent L-asparaginase (LlA), *Arabidopsis thaliana* potassium-independent L-asparaginase (AtA), *Arabidopsis thaliana* potassium-dependent L-asparaginase (AtAK), *Escherichia coli* plant-type L-asparaginase (EcAIII), taspase1 (Tas1), and human aspartylglucosaminidase (hAGA).
The vertical line shows the cleavage site. The arrow indicates the catalytic threonine residue. Residues in hAGA proposed to activate the Thr OH nucleophile prior to autoproteolysis are shown in boldface. They are not conserved in the other sequences.

doubt is raised by the interaction of the –OH group of the catalytic threonine with the hydroxyl group of an adjacent threonine residue (Thr197 in EcAIII or Thr211 in LlA), in which the former group definitely acts as a proton acceptor. From the point of view of nucleophilic activation, one would certainly expect the opposite, i.e. release of the hydroxyl proton rather than attraction of an extra one (Fig. 6d). However, the available data do not allow proposing an alternative mechanism.

The dilemma concerning the nucleophile activation is aggravated when one considers how the Thr residue can be activated for a nucleophilic attack on the preceding α-amide bond in the proteolytic maturation event. Here, there is no good candidate group to activate the –OH nucleophile, as the N-terminal amino group is not yet present. In some aspartylglucosaminidases, a His or Asp residue located at the end of subunit α, near the α-β junction, has been postulated to activate the Thr nucleophile (Guan *et al.*, 1998; Xu *et al.*, 1999). However, this cannot be a general mechanism as the candidate residues are not conserved in the sequences of plant-type L-asparaginases (Fig. 7). A separate issue concerns the severe stereochemical strain required if the –OH group of the catalytic threonine is to attack the preceding α-peptide bond in a *cis* reaction (Guan *et al.*, 1998; Suzuki & Kumagai, 2002). It is not completely clear what conformation the polypeptide chain assumes at the α-β boundary prior to maturation. In the crystal structure of LlA (Michalska *et al.*, 2006) a long stretch of residues is seen at the C-terminus of subunit α, but the chain conformation is different from that reported for a catalytically incompetent, immature aspartylglucosaminidase (Qian *et al.*, 2003).

METAL COORDINATION BY K^+-INDEPENDENT PLANT-TYPE L-ASPARAGINASES

Coordination of a sodium cation by a structural loop formed by the α-subunit near the active site is a unique feature of plant-type L-asparaginases, identified so far in all available crystal structures (Fig. 8). Unusually for metal coordination in proteins, the cation is bound exclusively by main-chain carbonyl oxygen atoms from a polypeptide stretch comprising 11 residues (Leu59–Ile69 in LlA), and the coordination sphere has a nearly perfect octahedral geometry. The identity of this metal ion as sodium leaves no doubt, as it has been confirmed at relatively high resolution (1.65 Å) and by various methods, including bond valence tests (Brese & O'Keeffe, 1991; Nayal & Di Cera, 1996; Müller *et al.*, 2003). This coordination site, whose geometry is theoretically also acceptable for calcium, seems to be very tight and specific for Na^+, as experiments with EcAIII crystallization in high excess of Ca^{2+} left the sodium site intact even though additional

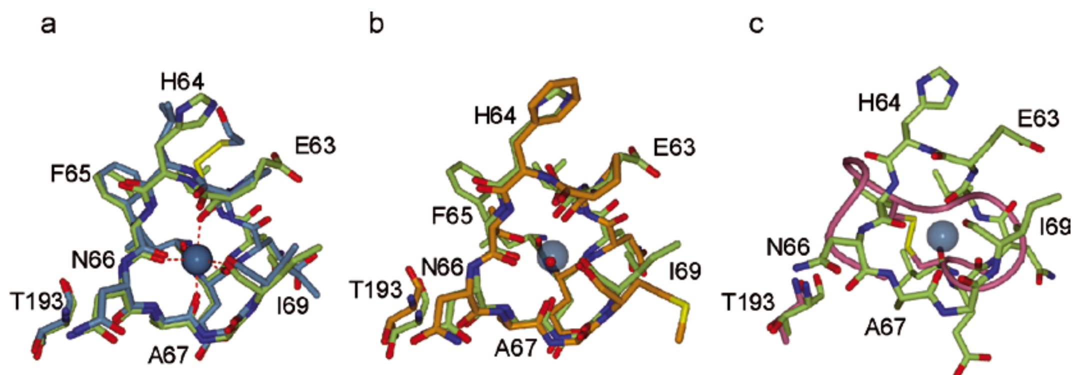

Figure 8. Superposition of the Na^+ binding loops.
The indicated sodium cation (blue sphere) comes from the LlA/Cl^- structure (Michalska *et al.*, 2006). (**a**) LlA (green) and EcAIII (blue). (**b**) LlA (green) and Tas1 (orange). The red sphere corresponds to a water molecule modeled in the Tas1 structure in place of a sodium cation. (**c**) LlA (green) and human AGA (pink). The disulfide bond across the differently shaped loop in the AGA protein is indicated in yellow.

coordination sites became populated with calcium ions (Borek, 2001). It appears that the Na^{+} cation coordinated by plant-type L-asparaginases fulfils a structural purpose, serving as a scaffold for the coordinating loop, which in turn supports the position of the N-terminal nucleophile (Fig. 8). In this way, it might have an indirect bearing on the enzymatic activity.

It is worth stressing that all structurally characterized plant-type L-asparaginases belong to the potassium-independent group. Although the K^{+}-dependent enzymes have a very similar sequence of the "sodium" loop region, it is not known at present if the loop conformation and metal coordination are preserved, or if there is any connection of this region with the reported potassium-dependence. Structural studies are necessary to shed more light on this interesting issue.

Among other members of the Ntn-hydrolase family, an analogous structural motif is present in taspase1 (Tas1) which also bears overall structural similarity to plant-type L-asparaginases. However, despite the conservation of the conformation of the "metal-binding" loop, no metal ion has been modeled in the Tas1 structure (Fig. 8b) and the sodium position is occupied by a most likely misinterpreted water molecule (Khan *et al.*, 2005). Tas1 is an endopeptidase participating in the maturation of MLL protein (myeloid/lymphoid or mixed lineage leukemia protein), whose dysfunction is correlated with the occurrence of certain types of cancer. Tas1 cleaves the precursor MLL chain at two D-G sites. In both cases the cleavage occurs at the α-amide bond after an aspartate residue (Hsieh *et al.*, 2003) and is contrasted with the action of plant-type L-asparaginases, which recognize a β-amide of an aspartate residue. This fundamental difference in substrate specificity might appear unusual in view of the similar architecture of these enzymes but a closer analysis of the active sites provides an explanation of this behavior (*vide infra*).

Interestingly, aspartylglucosaminidases also contain a peculiar-shape loop neighboring the active site. However, its detailed conformation is different from that observed in plant-type L-asparaginases and no metal coordination has been reported in this region in the known AGA structures. Moreover, the loop conformation is variable among aspartylglucosaminidases themselves. In human AGA (Oinonen *et al.*, 1995), the conformation of this loop is maintained by a disulfide bond (Fig. 8c), while it is not known what factors support the loop structure in bacterial AGA (Guo *et al.*, 1998; Xuan *et al.*, 1998).

An octahedral metal-binding site in close proximity to the catalytic threonine residue has also been found in HslV protease, a prokaryotic homolog of the proteasome β subunit (Sousa & McKay, 2001; Song *et al.*, 2003). However, the loop structure is different from that in plant-type L-asparaginases. In HslV, only three ligands are provided by the main-chain carbonyl O atoms, the remaining three being furnished by water molecules. Such a mode of coordination, where the metal ion is not fully embedded within the protein, may explain the enzyme's ability to bind various metal cations. For instance, in the HslV structures, both sodium or potassium ions have been reported. In the yeast proteasome structure, an Mg^{2+} ion occupies an equivalent position (Groll *et al.*, 1997). It has been shown that metal binding to HslV affects the formation of the HslVU complex and its proteolytic activity. This observation is suggestive of the possibility that the metal coordination detected in plant-type L-asparaginases may be of importance for the functioning of these enzymes.

STRUCTURAL DETERMINANTS OF SUBSTRATE SPECIFICITY OF PLANT-TYPE L-ASPARAGINASES

The kinetic studies of plant-type asparaginases showed that L-asparagine was not hydrolyzed if it had a modification of either the α-amino or α-carboxy group, or if it was glycosylated. Moreover, no activity was detected towards L-glutamine or an α-amide formed on either side of an Asn/Asp residue. The specificity for substrates with free α-carboxy group is established by its interaction in the EcAIII/L-Asp structure with the guanidinium group of Arg207 (Arg221 in LlA). This strong salt bridge discriminates against potential substrates with a modification at the α-carboxylate (Fig. 6a). Moreover, the conformation of the arginine side chain determines its distance to the nucleophile. Thus, it controls the length and exact positioning of a substrate in the active site (Fig. 9), preventing access of too long molecules, like L-glutamine. The α-amino group of the substrate is anchored by Asp210 and Gly231. This explains why the enzymes function as aminopeptidases.

Structural comparison with aspartylglucosaminidases provides an explanation why plant-type L-asparaginases are not able to cleave glycosylated L-asparagine. The two aromatic residues (Trp11 and Phe13) which bind the sugar moiety in human and bacterial AGAs, are replaced in LlA and EcAIII by a stretch of small residues (Gly10-Gly11-Ala12-Gly13). This insertion/substitution reduces the size of the catalytic cavity and changes its character to less hydrophobic, thus preventing carbohydrate binding. This fragment of plant-type L-asparaginases is expected to interact with the residue in the β-position of a β-Asp-peptide substrate (Fig. 9).

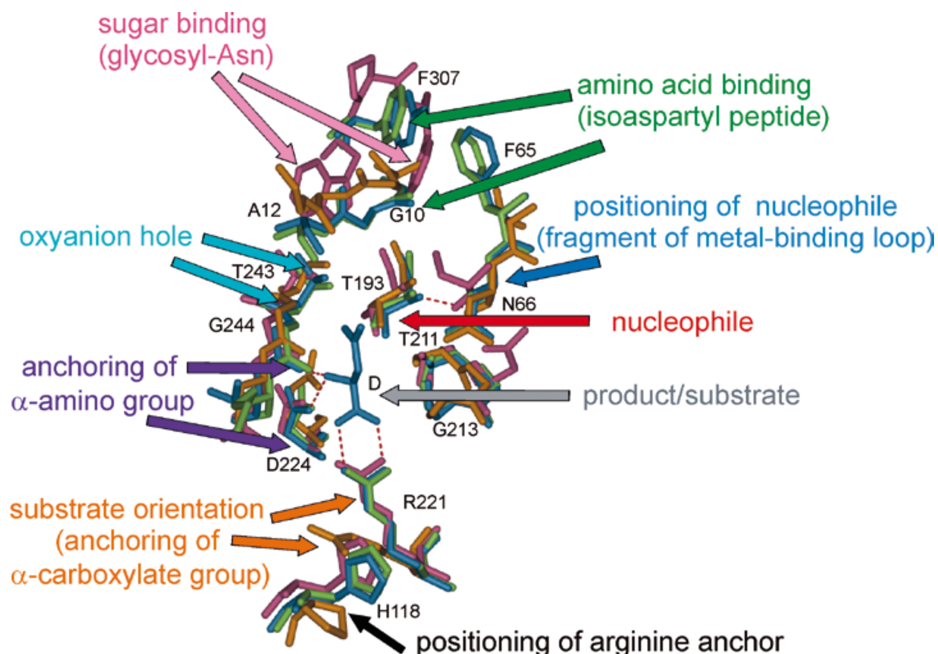

Figure 9. Superposition of the active sites of LlA (green), EcAIII (blue), human AGA (pink), and Tas1 (orange).
The green arrows show the putative docking site for the C-terminal residue of a β-aspartyl dipeptide substrate of plant-type L-asparaginases. The pink arrows indicate the sugar-binding residues (Trp and Phe) in aspartylglucosaminidases. The red arrow indicates the nucleophilic threonine. The L-aspartate product molecule in the EcAIII/L-Asp complex structure is marked "D" and its ion-pair binding to an arginine anchor (orange arrows) is indicated by a gray arrow. The different orientation of the aspartate-docking arginine in Tas1, emphasized by an orange arrow, is enabled by the adjacent Pro162 residue (black arrow) at the (αβ)-(αβ) interface. The cyan arrows indicate the residues forming the oxyanion hole. The violet arrows indicate the docking residues for the α-ammonium group of the substrate/product. The blue arrow indicates a fragment of the loop formed by the α-subunit which serves to orientate the Thr nucleophile. In the plant-type enzymes, this loop is stabilized by a coordinated sodium cation (not shown) and in human AGA by a disulfide bond (not shown). The factors stabilizing this loop in the remaining enzymes are not clear, possibly as a result of incorrect models. The residue numbering follows the LlA sequence.

OLIGOMERIC FORM OF PLANT-TYPE l-ASPARAGINASES

Comparison of the (αβ)-(αβ) interface of plant-type L-asparaginases and taspase1 provides some clues about the ability of these enzymes to distinguish between β- and α-amides of L-aspartate. The arginine responsible for anchoring the substrate's α-carboxy group in EcAIII and LlA belongs to a β-strand located exactly at this interface, namely to that one that extends the larger β-sheet of the second (αβ) heterodimer (*vide supra*). The conformation of that arginine is determined by a neighboring residue from an adjacent β-strand (Fig. 9). If, as is the case in plant-type L-asparaginases, this neighboring residue is a histidine then the arginine is forced to assume an all-*trans* conformation, pointing its guanidinium group directly toward the active site. If the neighbor is a proline, as in taspase1, the side chain of the arginine anchor swings away from the active site, making room for a larger substrate group. Specifically, the different orientation of the arginine element in Tas1 allows the anchoring of the β-carboxylate of an –Xxx-(α-Asp)-Gly-Xxx– substrate.

CONCLUSIONS

Based on the available structural data, enzymes with L-asparaginase activity can be divided into bacterial-type and plant-type. The bacterial-type enzymes are further divided into two subgroups according to the level of L-asparagine affinity, which correlates with their quaternary structure, although the basis of this correlation is not fully understood. Interestingly, bacteria also harbor a gene that encodes a plant-type enzyme. The two types of L-asparaginases are evolutionarily and structurally distinct. They also differ in their substrate affinity and specificity profiles. Moreover, in many cases the L-asparaginase activity is considered a secondary one, dominated by a higher affinity for another substrate. In particular, the plant-type enzymes are more active in hydrolyzing isoaspartyl peptides, which arise spontaneously in natural proteins during extended storage. On the other hand, the plant-type enzymes do not hydrolyze glycosylated L-asparagine, despite high sequence and structural similarity to aspartylglucosaminidases.

The catalytic mechanisms of both types of L-asparaginases have been compared to that of serine

proteases. For this reason, the crystallographic studies have aimed at determining an active site that would resemble the catalytic S-H-D triad of classic serine proteases. However, for neither type of L-asparaginases can such a triad be found in a pure form. In the bacterial-type L-asparaginases, two Thr residues are considered for the role of the catalytic nucleophile, in a T-K-D triad and in a T-Y dyad. The consensus opinion seems to favor the latter alternative even though it is unusual because a tyrosine residue is controversial as a nucleophile activator and because the catalytic apparatus would be located in a highly mobile loop, requiring substrate binding for its stabilization.

The plant-type enzymes belong to the family of Ntn-hydrolases, which means that their nucleophilic (Thr) residue is liberated in an autocatalytic event and that it is activated by its own free α-amino group. This view is not without question marks either, mainly because stereochemical considerations do not favor a strained intramolecular $–O–H…NH_2$ hydrogen bond and cannot explain how a threonine residue can cleave the α-amide bond in front of it on autocatalytic generation of the mature $(\alpha\beta)_2$ form. Despite the doubts remaining, there are also important issues regarding the functioning of plant-type L-asparaginases that have been explained by the available crystal structures. For instance, the lining of the active site's outlet is clearly different in the plant-type enzymes and in aspartylglucosaminidases, explaining their preference for, respectively, isoaspartyl peptides and N-glycosylated L-asparagine. The architecture of the active site makes it also clear that acceptable substrates must have free α-amino and α-carboxy groups. In particular, a key arginine anchor has been identified which through a salt-bridge interaction with the α-carboxylate group is responsible for the positioning of the substrate molecule and whose conformation regulates the presentation of a β- or α-amide for hydrolysis. The arginine conformation in turn is determined by the residues at the (αβ)-(αβ) interface, explaining the oligomeric form of these enzymes. Finally, in the plant-type enzymes a sodium cation coordinated near the active site plays a structural role in the positioning of the N-terminal nucleophile. The sodium binding has been detected in a subclass of plant-type L-asparaginases whose activity is not related to potassium concentration. Sequentially homologous K^+-dependent enzymes also exist and it is tempting to speculate that they might coordinate potassium rather than sodium. This and other exciting aspects of the plant-type enzymes await their elucidation by structural studies.

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