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Catalytic Mechanisms and Biocatalytic Applications of Aspartate and Methylaspartate Ammonia Lyases

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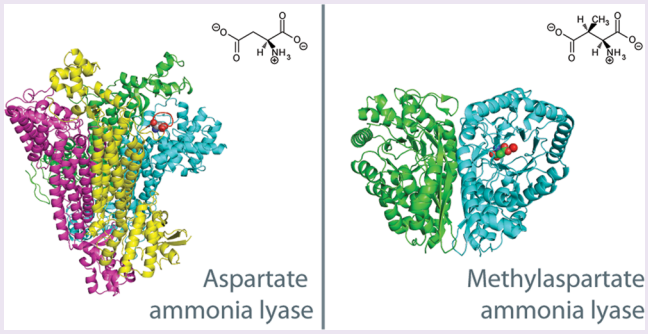
ABSTRACT: Ammonia lyases catalyze the formation of α,β-unsaturated bonds by the elimination of ammonia from their substrates. This conceptually straightforward reaction has been the emphasis of many studies, with the main focus on the catalytic mechanism of these enzymes and/or the use of these enzymes as catalysts for the synthesis of enantiomerically pure α-amino acids. In this Review aspartate ammonia lyase and 3-methylaspartate ammonia lyase, which represent two different enzyme superfamilies, are discussed in detail. In the past few years, the three-dimensional structures of these lyases in complex with their natural substrates have revealed the details of two elegant catalytic strategies. These strategies exploit similar deamination mechanisms that involve general-base catalyzed formation of an enzyme-stabilized enolate anion (*aci*-carboxylate) intermediate. Recent progress in the engineering and application of these enzymes to prepare enantiopure L-aspartic acid derivatives, which are highly valuable as tools for biological research and as chiral building blocks for pharmaceuticals and food additives, is also discussed.

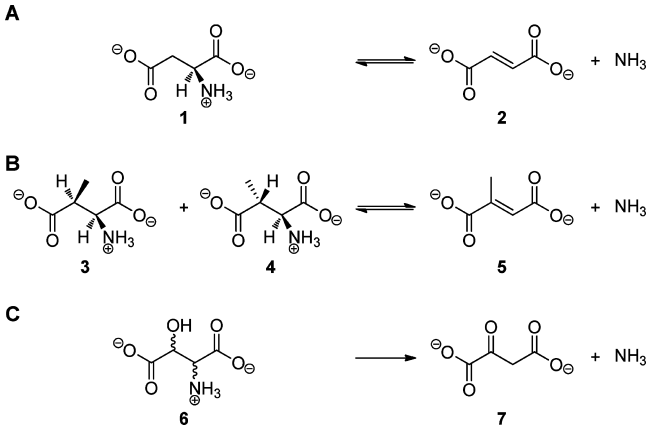


Ammonia lyases are capable of cleaving carbon−nitrogen bonds without employing hydrolysis or oxidation mechanisms.[1] Various ammonia lyases exist in nature, with three types of lyases specific for aspartate or its derivatives as substrates: aspartate ammonia lyase (aspartase), 3-methylaspartate ammonia lyase (MAL), and 3-hydroxyaspartate ammonia lyase.[1]

Aspartate ammonia lyase (aspartase; EC 4.3.1.1) plays an important role in microbial nitrogen metabolism by catalyzing the reversible deamination of L-aspartate (**1**) to yield fumarate (**2**) and ammonia (Scheme 1A). This enzyme was discovered in the early 20th century after it was established that bacteria could transform aspartate to succinate.[2,3] It was shown in 1926 that an equilibrium between L-aspartate, fumarate, and ammonia exists in the presence of resting bacterial cells, and further investigation indicated that a deaminase was responsible for the reversible elimination of ammonia from L-aspartate to give fumarate.[4] Since then, extensive investigations have resulted in the identification and characterization of aspartase from various organisms, including *Escherichia coli*, *Pseudomonas fluorescens*, *Hafnia alvei* (formerly known as *Bacterium cadaveris*), *Bacillus subtilis*, and *Bacillus* sp. YM55-1.[5−13]

3-Methylaspartate ammonia lyase (MAL; EC 4.3.1.2) catalyzes the reversible α,β-elimination of ammonia from L-*threo*-3-methylaspartate (**3**) and L-*erythro*-3-methylaspartate (**4**) to yield mesaconate (**5**) (Scheme 1B). MAL activity was first detected by Barker *et al.*[14] in cell-free extracts of the anaerobic bacterium *Clostridium tetanomorphum* H1. In this bacterium, the MAL-catalyzed deamination reaction forms the second step in the catabolic pathway in which L-glutamate is metabolized *via* L-*threo*-3-methylaspartate to finally yield acetyl-coenzyme A.[14,15]

Scheme 1. Reactions Catalyzed by Ammonia Lyases Specific for Aspartate and Its Derivatives[a]



[a](A) Aspartate ammonia lyase catalyzes the reversible deamination of L-aspartate (**1**) to yield fumarate (**2**) and ammonia. (B) 3-Methylaspartate ammonia lyase catalyzes the reversible deamination of L-3-methylaspartate (*threo* isomer **3** and *erythro* isomer **4**) to yield mesaconate (**5**) and ammonia. (C) 3-Hydroxyaspartate ammonia lyases catalyze the deamination of *threo*- or *erythro*-3-hydroxyaspartate (**6**) to yield oxaloacetate (**7**) and ammonia.

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MAL has subsequently been identified in several other facultative anaerobic organisms, including *Morganella morganii*, *Citrobacter amolonaticus*, *Fusobacterium varium*, and *Hafnia alvei*, which form part of the *Enterobacteriaceae* family.[15−19] MAL also forms part of the anabolic methylaspartate cycle in haloarchaea, in which acetyl-CoA is converted to glyoxylate *via* L-*threo*-3-methylaspartate.[20] In addition to the MALs that have been isolated and characterized from these sources, there are more than 100 putative MAL sequences available in various databases. Raj *et al.* used one of these sequences to study a MAL from the thermophilic bacterium *Carboxydothermus hydrogenoformans* Z-2901.[21]

Two other enzymes, which are classically known as hydratases, have been classified as ammonia lyases by NC-IUBMB: *threo*-3-hydroxyaspartate ammonia lyase (or *threo*-3-hydroxyaspartate dehydratase, EC 4.3.1.16) and *erythro*-3-hydroxyaspartate ammonia lyase (or *erythro*-3-hydroxyaspartate dehydratase, EC 4.3.1.20).[1] As indicated by their names, these enzymes catalyze the deamination of different epimers (*threo* or *erythro*) of 3-hydroxyaspartate (**6**) to yield oxaloacetate (**7**) and ammonia (Scheme 1C). Furthermore, there are two distinct *threo*-3-hydroxyaspartate ammonia lyases known that exhibit specificity toward either the D- or L-enantiomer of *threo*-3-hydroxyaspartate. These pyridoxal-phosphate- and divalent cation-dependent enzymes were first identified in the 1960s and are involved in the glycolate and glyoxylate degradative pathways.[22,23] The first enzyme to be identified was *erythro*-3-hydroxyaspartate dehydratase from *Micrococcus denitrificans* (*Paracoccus denitrificans*).[23,24] Almost 35 years later L-*threo*-3-hydroxyaspartate dehydratase was identified in *Pseudomonas* sp. T62 and a few years later in *Saccharomyces cerevisiae*.[25,26] Recently, enzyme activity toward D-*threo*-3-hydroxyaspartate was identified in *Delftia* sp. HT23, and the enzyme was named D-*threo*-3-hydroxyaspartate dehydratase.[27] As these enzymes are classically characterized as hydratases and since limited information is available for them, they will not be further discussed in this review.

■ ASPARTATE AMMONIA LYASE (ASPARTASE)

Properties and Catalytic Mechanism. Aspartases have been isolated and characterized from different Gram-negative and Gram-positive bacteria, with the aspartase from *E. coli* (AspA) being the focus of most studies. Originally AspA was isolated in a seven-step purification process from *E. coli* with an overall yield of 20%.[5] As science evolved, the isolation of AspA became easier with the enzyme being overexpressed and purified to homogeneity with a yield of ∼100 mg per 10 g of cell pellet.[2] This 52 kDa protein is allosterically activated by L-aspartate and Mg^{2+} ions, which are essential for catalytic activity at alkaline pH.[28−30] AspA shows maximum activity at 55 °C and pH 8.0, but prolonged incubation of the enzyme at this temperature resulted in total loss of activity after 30 min.[2,12]

In recent years, aspartase from the thermophilic bacterium *Bacillus* sp. YM55-1 (AspB) has also been in the spotlight due to its possible use as a biocatalyst for amino acid synthesis. The purification of AspB was reported in 1999, and it was the first thermostable aspartase identified and characterized.[12] AspB has similar subunit molecular weight (51 kDa) and oligomeric state as other aspartases from mesophilic organisms; however, it is structurally more stable at elevated temperatures as well as in the presence of guanidine hydrochloride.[12] AspB shows maximum activity at 65 °C and at pH 8.0.[12] In contrast to its *E. coli* and *P. fluorescence* counterparts, AspB is not allosterically activated by substrate and does not require Mg^{2+} ions for activity at alkaline pH.[12]

The crystal structure of unliganded AspA was elucidated in 1997 to 2.8 Å resolution[31] and showed that each monomer of AspA contains three structurally distinct domains (Figure 1A$_1$). The C-terminal domain is the smallest domain in the subunit and consists mainly of two helix-turn-helix motifs which are orientated approximately 90° relative to each other. The central helix domain is formed from five long α-helices and contains more than half of the total residues of the subunit. Finally, the N-terminal domain consists of a short, two-stranded antiparallel β-sheet followed by five α-helices.[31] AspA belongs to the aspartase/fumarase superfamily, which also includes fumarase C, adenylosuccinate lyase, argininosuccinate lyase, δ-Crystallin, and 3-carboxy-*cis*-*cis*-muconate lactonizing enzyme.[32−37] The members of the aspartase/fumarase superfamily have a similar active site architecture and share a common tertiary and quaternary fold, although they can show pairwise sequence identities as low as 15%. AspA functions as a homotetramer, which contains four composite active sites, each of which is generated by conserved motifs from three monomers (Figure 1A$_2$). A flexible loop of ∼10 amino acid residues forms part of one of these conserved motifs with a signature sequence of GSSxxPxKxN. This specific loop, also called the SS-loop, has been shown to be important for substrate binding and catalysis in some of the enzymes of this superfamily.[32,33,38,39]

Kinetic studies have shown that AspA displays non-Michaelis−Menten kinetics for the deamination reaction at high pH, and there is a time lag before the linear steady-state rate is achieved in time-course kinetics for the amination reaction at high pH.[28,29] From these results it was concluded that AspA is subjected to allosteric activation, in which L-aspartate and Mg^{2+} bind to a separate activator-binding site that is different from the active site. The role of L-aspartate is therefore 2-fold: it acts as substrate, and it serves as an activator for AspA. The location of the activator-binding site has not been identified by the crystal structure of unliganded AspA, but Cys430 has been proposed to be located close to the activator-binding site.[28,31] From the crystal structure of fumarase C, a second binding site was identified, and the amino acid residues that form part of this putative activator-binding site include Arg126 and Asn135.[40] A comparison of the crystal structure of AspA with that of fumarase C indicated Gln129 and Asn138, which are found in a short helix segment, as the corresponding residues in AspA.[31]

A general acid−base reaction mechanism was proposed for aspartases as illustrated in Scheme 2A.[2,11,31,41−43] The active site general base abstracts the *pro*-R proton from the C3 position of **1** resulting in a carbanion, which is stabilized as an *aci*-carboxylate intermediate (**8**). This proposed enolate anion intermediate can rearrange to eliminate ammonia and form the product, fumarate (**2**). The rate-determining step is the cleavage of the C_α−N bond, which may be facilitated by a general acid that donates a proton to the leaving group (NH_3) to form an ammonium ion.[31,41,43] Inhibition studies using 3-nitro-2-aminopropionate as inhibitor also supported the formation of an enzyme-stabilized enolate anion (*aci*-carboxylate) as intermediate, since the resonance-stabilized nitronate (*aci*-form) state of this inhibitor binds very tightly to aspartase as a transition state analogue.[44] pH−rate profiles for AspA and the *H. alvei* aspartase differ from each other, thus making it unclear whether the protonation state of the leaving group is that of the ammonia or the ammonium ion.[11,45] It has been argued that if the

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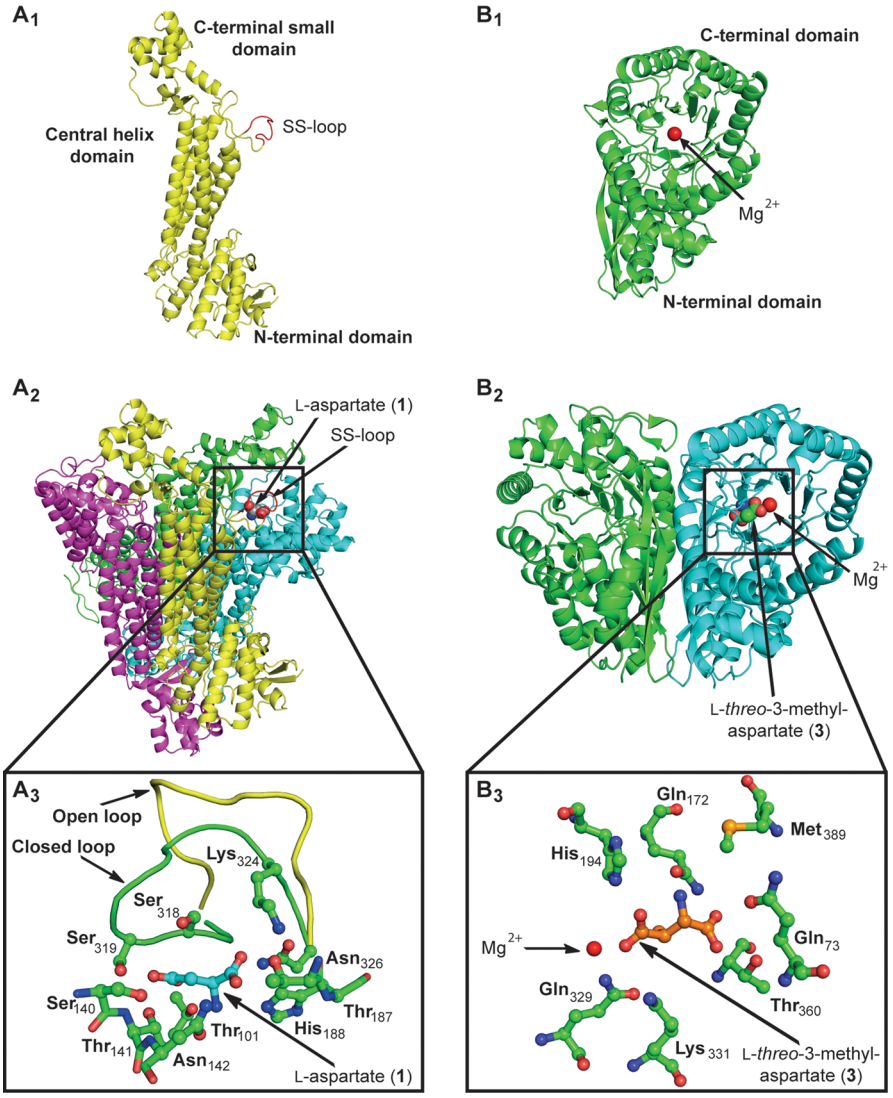

Figure 1. Crystal structures of aspartate and methylaspartate ammonia lyases. (A_1) The structure of the AspB monomer.[39] The three domains are labeled, and the SS-loop is indicated in red. AspA has a similar structure as shown here for AspB.[31] (A_2) Structure of the functional AspB tetramer.[39] The four monomers forming the homotetramer are indicated in magenta, yellow, cyan, and green. The location of one of four active sites is indicated by the bound L-aspartate (**1**) molecule, with the SS-loop indicated in red. (A_3) A close-up of the active site of AspB in complex with L-aspartate (**1**). The different conformations of the SS-loop are shown, with the closed conformation in green and the open conformation in yellow. The amino acid residues that are most important for substrate binding and catalysis are shown. (B_1) Structure of the MAL monomer.[60] The bound Mg^{2+} ion is shown as a red ball. (B_2) Structure of the functional MAL homodimer, in complex with Mg^{2+} (red ball) and L-*threo*-3-methylaspartate (**3**).[60] The two monomers are indicated in green and cyan. The location of one of the two active sites is indicated by the bound ligand. (B_3) A close-up of the active site of *Ca*MAL in complex with Mg^{2+} (red ball) and L-*threo*-3-methylaspartate (**3**).[60] The amino acid residues that are most important for substrate binding and catalysis are shown.

amino group is released as ammonia, there might not be the need for a general acid catalyst.[41,46]

Numerous studies involving site-directed mutagenesis, chemical modification, and mechanism-based inactivation have been done to identify the catalytically important residues of AspA.[2,47,48] Until recently, however, several major issues remained unresolved.[39] For example, the identity of the general base catalyst that abstracts the C3 proton, as well as the identity of other catalytic and substrate-binding residues, was still unclear. The question of whether or not substrate binding induces a conformational change that moves other residues into a favorable position in the active site was also not answered.[39] The structural work on AspA did not address these questions because no crystal structure of AspA in complex with substrate or product was available.[31] In order to resolve these issues recent studies have focused on AspB, since this is a simpler system to work on because of the lack of allosteric activation.[12] In addition, the crystal structure of uncomplexed AspB was already solved at this stage and the location of the putative active site was predicted on the basis of comparisons with AspA and other superfamily members.[49] Manual docking of L-aspartate (**1**) into the putative active site of AspB suggested that the α-carboxylate group of **1** forms hydrogen-bonding interactions with Thr187 and Lys324 (Scheme 2B). On the opposite end of the molecule, the β-carboxylate group forms hydrogen bonds with the hydroxyl groups of Ser140 and Thr141, whereas the amino group of **1** forms hydrogen bonds with the side chains of Thr101, Asn142 and His188.[49] To further verify the role of each residue and to determine the exact mechanism of catalysis, site-directed mutagenesis, pH-rate profile experiments, and inhibition studies

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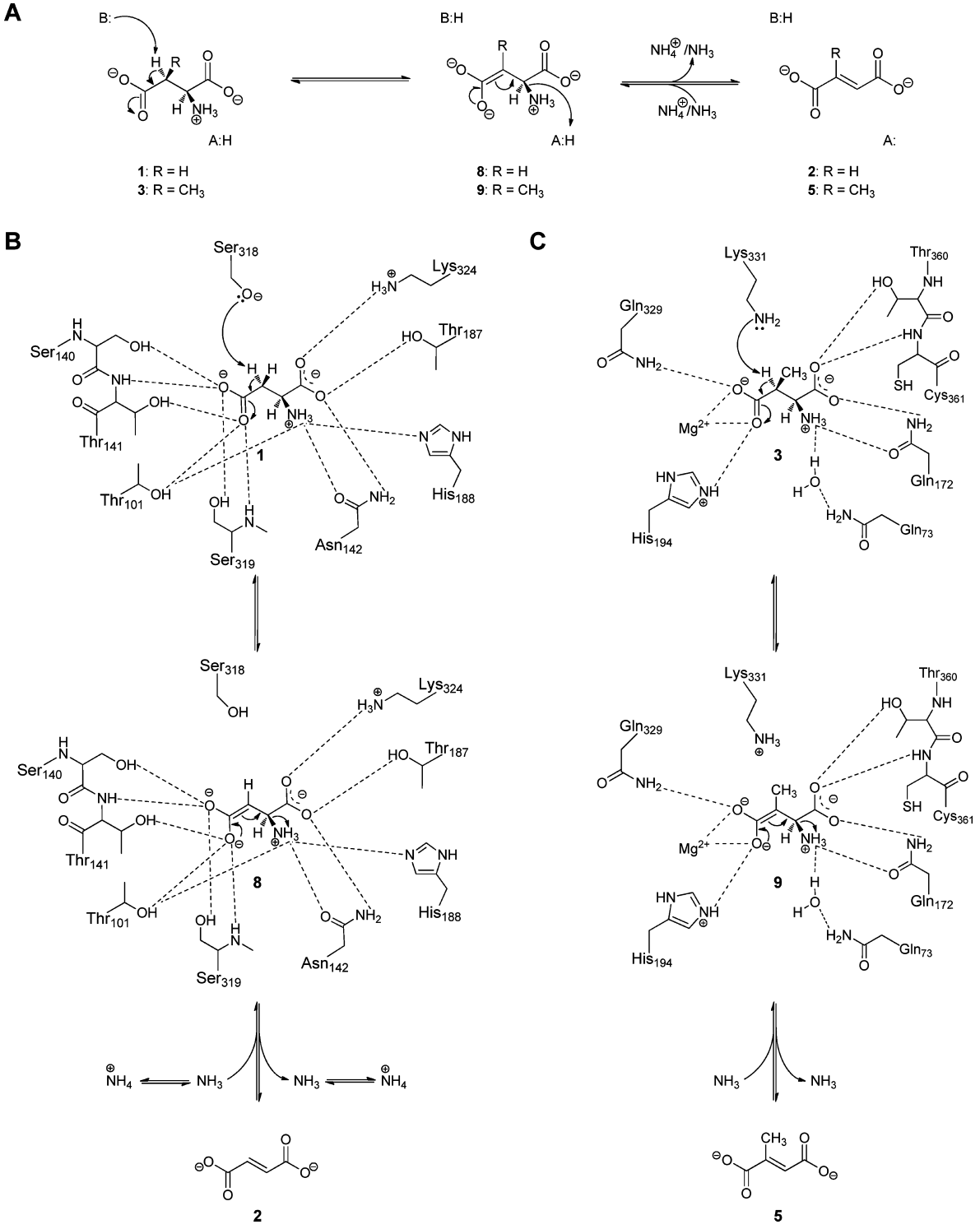
Scheme 2. Schematic Representations of the Proposed Catalytic Mechanisms of Aspartate and Methylaspartate Ammonia Lyases[a]



[a](A) Proposed general reaction mechanism for aspartase and methylaspartase. A base in the active site abstracts the proton from the substrate's C3 position. Consequently, a carbanion (enolate anion intermediate) is formed that is stabilized as the *aci*-acid resonance form. Collapse of the intermediate is followed by C_α–N bond cleavage, which results in the elimination of ammonia and may be facilitated by a general acid in some aspartases. (B) Schematic representation of the catalytic mechanism of AspB. Residue Ser318 acts as the catalytic base and abstracts the *pro*-R proton from the C3 position of **1**. The formed enolate anion intermediate (**8**) is stabilized through hydrogen bonding interactions with amino acid residues that line the active site of AspB. The collapse of **8** results in the cleavage of the C_α–N bond and the formation of **2** and ammonia. (C) Schematic representation of the catalytic mechanism of MAL. Residue Lys331 acts as the catalytic base and abstracts the C3 proton from **3** to form the enolate anion intermediate **9**. This intermediate is stabilized by interactions with both the Mg^{2+} ion and amino acid residues in the active site. Collapse of the intermediate and C_α–N bond cleavage results in **5** and ammonia.

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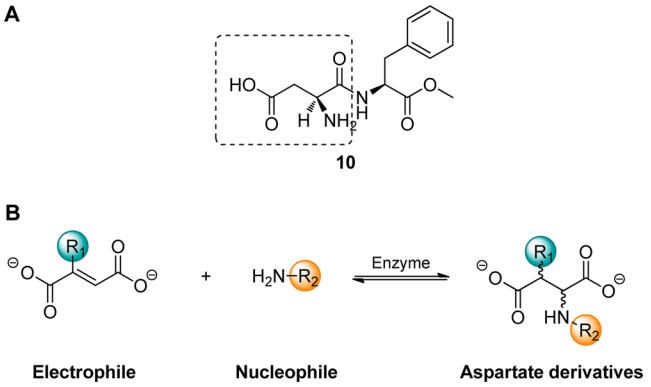
were carried out on AspB.[46] Despite all of these efforts, the specific residue that acts as catalytic base in the first step of the proposed mechanism eluded investigators. The SS-loop in the unliganded AspB structure[49] was also highly disordered and therefore its possible role in substrate binding and catalysis was unclear.

Advances in the elucidation of the catalytic mechanism of aspartase were recently achieved by Fibriansah *et al.*[39] They were successful in solving (2.6 Å resolution) the crystal structure of AspB in complex with L-aspartate (**1**) (Figure $1A_{1-3}$), providing the first detailed view of the substrate-bound active site of an aspartase. This allowed the identification of all active site residues as well as an (improved) understanding of their roles in substrate binding and catalysis (Scheme 2B). Remarkably, AspB forces the bound substrate to adopt a high-energy enolate-like (*aci*-carboxylate-like) conformation that is stabilized by an extensive network of hydrogen bonds between the substrate's β-carboxylate group and residues Thr101, Ser140, Thr141, and Ser319. Furthermore, a large conformational change of the SS-loop takes place from an open conformation in the absence of substrate to one where it closes over the active site in the presence of substrate (Figure $1A_3$). The open conformation of the SS-loop is stabilized by hydrophobic interactions of Ile320 and Met321, which are part of the loop, with a hydrophobic surface patch on the neighboring C-terminal domain. Binding of the substrate is needed in order to close the SS-loop.[39] This conformational change provides additional substrate binding interactions and safeguards the substrate from the solvent during catalysis. As a result the SS-loop, in combination with the C-terminal domain, plays a vital role in the catalytic mechanism of AspB. Most importantly, the closure of the SS-loop positions Ser318 in close proximity to the C3 proton of **1** to enable proton abstraction (Figure $1A_3$ and Scheme 2B). Indeed, mutation of the strictly conserved Ser318 to an alanine resulted in complete loss of AspB activity, suggesting that Ser318 is essential for activity.[39] The exact mechanism by which the intrinsically high pK_a value of Ser318 is lowered to function as a catalytic base is still unclear.

The results of this structural work on AspB[39] thus provide strong support for a catalytic mechanism involving general base-catalyzed formation of the enzyme-stabilized enolate anion intermediate **8** (Scheme 2B). In fact, the highly structured active site environment, the high-energy conformation of the bound substrate, the closed SS-loop conformation, and the extensive hydrogen-bonding network with the β-carboxylate group of the substrate (Scheme 2) show how the active site of aspartase has been optimized for formation and stabilization of the enolate anion (*aci*-carboxylate) intermediate.[39]

Biocatalytic Applications. In industry, aspartase is used as biocatalyst for the preparation of enantiopure L-aspartic acid, an important starting compound for the synthesis of artificial sweeteners such as *N*-(L-α-aspartyl)-L-phenylalanine 1-methylester (**10**, Scheme 3A), which is also known as aspartame.[50,51] Notably, *N*- and 3-substituted derivatives of L-aspartic acid are highly valuable as tools for biological research and as chiral building blocks for a range of pharmaceuticals.[52−54] However, the synthesis of these L-aspartic acid derivatives in enantiopure form remains a major challenge in synthetic chemistry. In principle, the stereo- and regioselective addition of amine nucleophiles to the double bonds of fumarate and its derivatives catalyzed by aspartases appears to be a very attractive strategy for the synthesis of these compounds (Scheme 3B). This strategy, however, is limited by the narrow substrate range of aspartases.[2,55]

Scheme 3. Biocatalytic Preparation of Aspartate Derivatives[a]



[a](A) Structure of the artificial sweetener *N*-(L-α-aspartyl)-L-phenylalanine 1-methylester (**10**) with the L-aspartic acid backbone highlighted. (B) Different combinations of electrophiles and nucleophiles are used with aspartase or methylaspartase as biocatalyst to yield various *N*-, 3-, and *N*,3-(di)substituted aspartate derivatives.

A wide spectrum of compounds (**11**−**63**, Scheme 4A and B) has been tested as potential substrates or inhibitors of AspA.[2,30,56] Only L-aspartate-β-semialdehyde (**11**) is deaminated by AspA, but this compound functions as a suicide substrate and its deamination leads to the irreversible inactivation of the enzyme.[2,56] Compounds **12**−**31** have been found to act as competitive inhibitors of AspA (Scheme 4A), with all of them containing a carboxylic acid or similarly charged functional group, which seems to be a requirement for enzyme binding.[2,30] Compounds **32**−**63** did not act as substrate nor as inhibitor of the enzyme. Some of these compounds (**57**−**63**; Scheme 4B), as well as additional amino acids (**64**−**76**, Scheme 4C), have also been tested as alternative substrates for AspB in order to determine if this aspartase exhibits a different substrate scope. It was found that AspB displays similar substrate specificity as AspA and does not process these non-native amino acids.[55]

In 1963 it was reported by Emery that hydroxylamine can be used as an alternative nucleophile in the addition reaction catalyzed by AspA.[57] This observation prompted Weiner and coworkers to test various alternative nucleophiles as potential substrate in the AspB-catalyzed addition reaction.[55] It was found that AspB accepts hydroxylamine, methoxylamine, hydrazine, and methylamine in the addition to fumarate, yielding L-*N*-hydroxyaspartic acid (**77**), L-*N*-methoxyaspartic acid (**78**), L-2-hydrazinosuccinic acid (**79**), and L-*N*-methylaspartic acid (**80**) (Scheme 5). Whereas compounds **78**−**80** could be isolated in excellent yields, compound **77** could not be isolated due to the instability of the compound.[55] On the basis of the structurally distinct nucleophiles tested, the authors concluded that the nucleophile binding pocket of AspB is designed to bind small amines and excludes any charged or large nucleophiles.[55]

3-METHYLASPARTATE AMMONIA LYASE (MAL)

Properties and Catalytic Mechanism. The MAL enzyme was purified from cell free extracts of several facultative anaerobic bacteria and characterized with regard to its biocatalytic properties.[14,16−19] The best studied MALs are those from *C. tetanomorphum* (*Ct*MAL) and *C. amalonaticus* (*Ca*MAL), the corresponding genes of which have been cloned, sequenced, and functionally expressed in *E. coli*.[18,58] MAL (45 kDa) is a homodimeric protein and depends on divalent (Mg^{2+}) and

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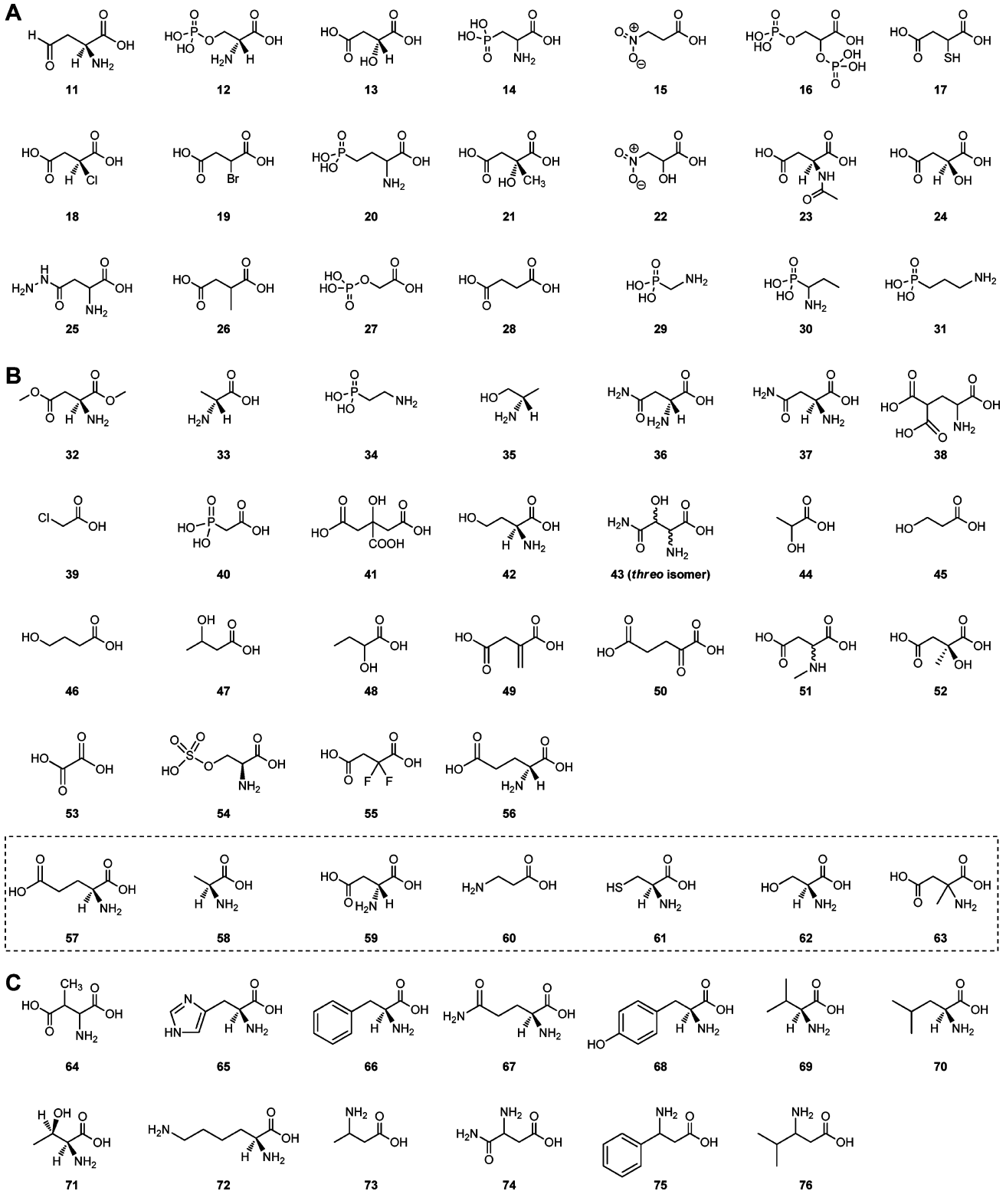
Scheme 4. Compounds Investigated as Potential Inhibitors or Substrates for Aspartase[a]



[a](A) L-Aspartate-β-semialdehyde (**11**) acts as a suicide substrate for AspA.[56] Compounds **12**–**31** were shown to act as competitive inhibitors of AspA.[2,30] (B) Compounds **32**–**63** were shown not to act as competitive inhibitors nor as alternative substrates for AspA.[30] Compounds **57**–**63** in the dashed box have also been tested on AspB but were also not accepted as substrates by this enzyme.[55] (C) Compounds **64**–**76** were shown not to be accepted as alternative substrates by AspB.[55]

monovalent (K^+) cations for activity.[58−60] The crystal structures of *Ct*MAL[59] and *Ca*MAL[60] revealed that the enzyme is not structurally related to aspartases (Figure 1B$_{1-3}$). Instead, the structures showed that MAL belongs to the enolase superfamily, the members of which share a characteristic TIM barrel fold. The monomer of MAL has a small N-terminal domain

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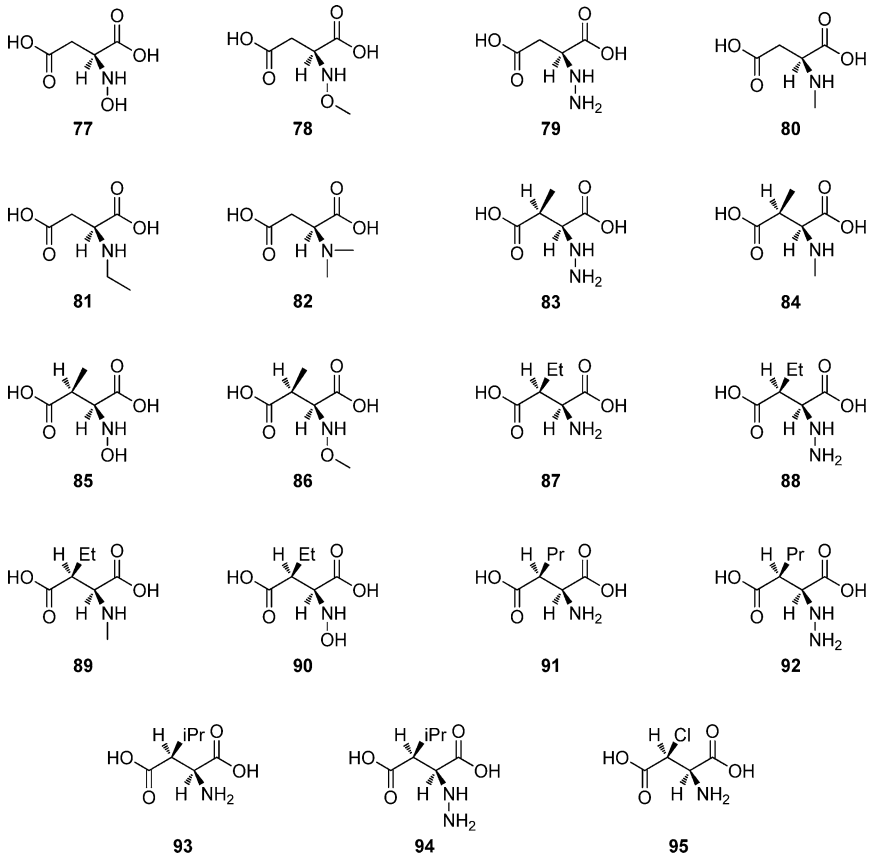
Scheme 5. L-Aspartic Acid Derivatives Obtained from Preparative Reactions[a]



[a] L-Aspartic acid analogues **77**−**95** were prepared using MAL as biocatalyst.[74−76] Derivatives **77**−**80** were also prepared with AspB as biocatalyst.[55]

that wraps around the larger C-terminal domain, which is an 8-fold α/β (TIM) barrel (Figure 1B$_1$).[59,60]

The most recent addition to the library of characterized MALs is MAL from *C. hydrogenoformans* Z-2901 (*Ch*MAL).[21] This organism was isolated from a hot spring in Kunashir Island (Russia) and has an optimal growth temperature of 78 °C.[61,62] The protein (subunit is 46.5 kDa) has a length of 420 amino acids, and the sequence is 53% identical and 73% similar to that of *Ct*MAL. The biological function of this protein in *C. hydrogenoformans* is still unknown. Like *Ct*MAL, *Ch*MAL has an optimum pH for activity of 9. However, whereas *Ct*MAL is active from 10 to 70 °C with an optimum temperature for activity of 50 °C, *Ch*MAL is active between temperatures of 10−90 °C with an optimum temperature for activity of 70 °C.[21] The thermostability of the enzymes also varies dramatically. *Ch*MAL has been shown to be stable at 50 °C for up to 4 h, retaining >95% of its original activity. In contrast, *Ct*MAL retained only half of its original activity after a 30 min incubation period at 50 °C.[21] This study indicated that *Ch*MAL is the most thermostable MAL purified and characterized to date.

MAL catalyzes the reversible *anti*- and *syn*-addition of ammonia to mesaconate (**5**) to give L-*threo*-(2*S*,3*S*)-3-methylaspartate (**3**) as major product and L-*erythro*-(2*S*,3*R*)-3-methylaspartate (**4**) as minor product, respectively (Scheme 1B). The mechanism of action has been debated over the past ∼50 years with various studies aiming to demonstrate the deamination mechanism of MAL.[63−69] Early investigations postulated that MAL catalyzes the deamination reaction through a carbanion mechanism similar to aspartase (Scheme 2A), with cleavage of the C$_\alpha$−N bond as the rate-limiting step.[63,64] This hypothesis was supported by the fact that C3 hydrogen-exchange with solvent occurs more rapidly than cleavage of the C$_\alpha$−N bond. Also, the C3-deuterated substrate **3** used to perform this hydrogen-exchange experiment did not give any primary isotope effect in the deamination reaction.[63,64]

Other studies have questioned this carbanion mechanism by suggesting that MAL is mechanistically similar to phenylalanine ammonia lyase (PAL) and histidine ammonia lyase (HAL) and may catalyze the reaction *via* a covalent enzyme−substrate complex that utilizes an electrophilic dehydroalanine prosthetic group.[58,69,70] MAL is irreversibly inactivated by *N*-ethylmaleimide and phenylhydrazines, and substrate protects the enzyme from inactivation by these compounds. On the basis of these findings, combined with sequence analysis, it has been suggested that post-translational dehydration of an active site serine (Ser173) may be the origin of this prosthetic group.[58,69,70] However, it was later discovered that PAL and HAL do not form a dehydroalanine group through post-translational dehydration but instead form a MIO-group (4-methylene imidazole-1-one) by cyclization and elimination of water in the active site tripeptide Ala-Ser-Gly.[71,72] The crystal structures of uncomplexed *Ct*MAL and *Ca*MAL in complex with the natural substrate **3** were reported in 2002 and proved to be essential for the determination of the exact mechanism of the enzyme.[59,60] It was clear from the structures that MAL is not structurally related to HAL or PAL, and electron density maps did not indicate post-translational modification of any active site residues. Instead, MAL shows structural homology to the enolase superfamily, the members of which share a common mechanism that involves the general-base catalyzed formation of an enolate anion (*aci*-carboxylate) intermediate. The MAL-substrate

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complex structure,[60] in combination with structural alignment of MAL with other members of the enolase superfamily,[59] suggested that Lys331 functions as the *S*-specific base catalyst that is responsible for proton abstraction from C3 of substrate **3** (Figure 1B$_3$ and Scheme 2C). It was further postulated that His194 may act as the *R*-specific base catalyst and abstracts the C3 proton from substrate **4**. This hypothesis was based on structure comparisons between MAL and other enolase superfamily members where the main chain of His194 superimposes on Lys164 of mandelate racemase (a secondary base), as well as on Lys213 in glucarate dehydratase (primary base).[59]

Raj *et al.*[73] investigated the importance of these two proposed catalytic base residues in *Ct*MAL. First, Lys331 was shown to be an essential residue for catalysis, because enzyme activity was lost completely when this amino acid was mutated to a glycine, alanine, histidine, glutamine, or arginine.[73] Second, computer modeling of **4** into the active site of MAL indicated that the C3 proton of **4** is indeed facing toward the imidazole nitrogen atom (Nε2) of His194. Moreover, an alanine mutation at this position disables the protein from accepting **4** as a substrate, but the H194A mutant still has the ability to catalyze the deamination of **3**. It is of synthetic importance that the H194A mutant is completely diastereoselective and aminates **5** to give **3** as the sole product.[73] These studies thus showed that MAL catalyzes the reversible deamination reaction by using Lys331 as the *S*-specific general base/acid catalyst and His194 as the *R*-specific base/acid catalyst. It was also shown that MAL does not catalyze the direct epimerization reaction from **3** to **4**, and therefore the two products form exclusively as a result of the *anti*- and *syn*-addition of ammonia to **5**.[73]

The structure of MAL in complex with substrate[60] further showed that specific hydrogen bonds are made between the substrate's β-carboxylate group and the nitrogen atoms (Nε2) of Gln329 and His194 (Figure 1B$_3$, Scheme 2C). The importance of these residues for MAL activity was confirmed by site-directed mutagenesis.[73] Importantly, the active site Mg^{2+} ion assists these two residues in binding of the substrate's β-carboxylate group, thereby providing efficient stabilization of the enolate anion intermediate. The Mg^{2+} binding site of MAL is located in the mouth of the TIM barrel and consists of Asp238, Glu273, and Asp307.[59,60] Each of these amino acids contributes a single oxygen atom as ligand, and in combination with three water molecules (in the unliganded structure) the normal hexagonal coordination of Mg^{2+} is formed.[59] In the substrate bound active site, one of the six ligands to the Mg^{2+} ion is provided by one of the β-carboxyl oxygen atoms of the substrate, replacing a water molecule.[60] The α-carboxylate of the substrate has hydrogen bond interactions with the main chain NH of Cys361, the oxygen atom (Oγ) of Thr360, and the nitrogen atom (Nε2) of Gln172 (Scheme 2C). The C2 amino group fits nicely into a nucleophile binding pocket and has hydrogen bond interactions with the oxygen atom (Oε1) of Gln172 and, *via* a water molecule, with the nitrogen atom (Nε2) of Gln73. The substrate is further stabilized in the active site by favorable van der Waals packing interactions between its 3-methyl group and the side chains of Leu384 and Gln172.[60] This detailed view of the substrate-bound active site of MAL thus allowed the identification of the active site residues (Figure 1B$_3$) as well as an understanding of their roles in catalysis (Scheme 2C). In conclusion, the MAL structures[59,60] and recent mutagenesis experiments[73] provide strong support for a catalytic mechanism involving general base-catalyzed formation of a highly stabilized enolate anion (carbanion) intermediate (**9**).

Biocatalytic Applications. Several studies have focused on the usefulness of *Ct*MAL as a biocatalyst for asymmetric synthesis of aspartic acid derivatives.[74−76] Gani and co-workers showed that *Ct*MAL accepts different fumarate derivatives in the ammonia addition reaction, yielding a range of 3-substituted aspartic acid derivatives (Scheme 5).[74,75] The same group reported that MAL can also catalyze the addition of a few small amines to fumarate and several 2-alkylfumarates to yield various *N*- and *N*,3-(di)substituted aspartic acid derivatives (Scheme 5).[76] Hence, *Ct*MAL has a broader substrate spectrum than the aspartases AspA and AspB and can be used to synthesize amino acids **77**−**95** (Scheme 5). Like *Ct*MAL, the thermostable *Ch*MAL also accepts various substituted fumarates and amines, and exhibits high regio- and stereoselectivity.[21] This makes *Ch*-MAL an attractive enzyme for biocatalytic applications, as well as a promising scaffold for protein engineering.

ENGINEERING OF ASPARTATE AND METHYLASPARTATE AMMONIA LYASES

A synthetic strategy in which aspartate and methylaspartate ammonia lyases are used as biocatalysts for the preparation of enantiomerically pure L-aspartic acid derivatives is of high interest (Scheme 3B). However, this strategy is currently limited by the narrow substrate range of these enzymes. It would therefore be very attractive to extend the accessible range of aspartic acid derivatives by the redesign of these lyases to convert new unnatural substrates, which would enlarge their biocatalytic applicability.[77] In fact, aspartase has been subjected to protein engineering by various investigators, but with limited success. Asano *et al.*[78] used directed evolution in an attempt to enlarge the substrate scope of AspA. Interestingly, they found that mutation of Lys327 to an asparagine enables AspA to catalyze the deamination of L-aspartic acid α-amide (β-asparagine), albeit at a very low level.[78] Unfortunately, no other AspA variants with a modified substrate scope have been reported. In our laboratory, we have attempted to broaden the substrate scope of aspartase AspB by rational design and structure-based saturation mutagenesis. However, we were unsuccessful in these engineering attempts, which may have to do with the complex movement of the SS-loop upon substrate binding, making protein engineering of aspartases a formidable challenge.

In contrast to aspartases, where substrate-dependent closure of the SS-loop positions catalytic and substrate-binding residues in a suitable orientation for catalysis, MAL has an intact catalytic machinery in the uncomplexed enzyme and does not undergo large conformational changes upon substrate binding. This implies that MAL is a more promising template for redesign to convert new unnatural substrates.[79] Raj *et al.*[73] have shown that the diastereoselectivity of the enzyme can be altered by mutation of active-site residues, allowing the preparation of exclusively L-*threo*-3-methylaspartate (**3**, Scheme 1B). The crystal structure of substrate-bound MAL shows that the surface lining of the 3-methyl binding pocket, which is formed in part by Leu384, Phe170, and Tyr356, is composed almost entirely of side chains with little or no interaction between the substrate's 3-methyl group and the main chain atoms.[60] Recently, Poelarends and co-workers have engineered this alkyl binding pocket of MAL and obtained a single active site mutant (L384A) that has a wide electrophile scope including fumarate derivatives with alkyl, aryl, alkoxy, aryloxy, alkylthio, and arylthio substituents at the C2 position (Scheme 6).[80] These investigators also engineered the nucleophile (amine) binding pocket of MAL, which is formed in part by Gln73 and Gln172,

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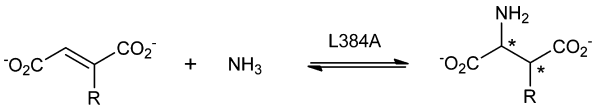
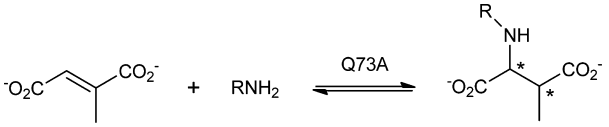
Scheme 6. Electrophile Spectrum of an Engineered MAL Variant: MAL-L384A Mutant As Catalyst in the Ammonia Addition to Fumarate and Its 2-Substituted Derivatives



R = H, methyl, ethyl, propyl, butyl, pentyl, hexyl, benzyl, ethoxy, phenoxy, benzyloxy, ethylthio, phenylthio, benzylthio

Scheme 7. Nucleophile Spectrum of an Engineered MAL Variant: MAL-Q73A Mutant As Catalyst in the Amine Addition to Mesaconate



R = H, methyl, ethyl, propyl, butyl, pentyl, hexyl, benzyl, isopropyl, cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, (cyclopropyl)methyl, ethoxy, 2-hydroxyethyl, 3-hydroxypropyl, 2-methoxyethyl, *N*-methyl-2-aminoethyl, 2-aminoethyl, 3-aminopropyl

yielding a single active site mutant (Q73A) that exhibits a broad nucleophile scope including various structurally diverse linear and cyclic alkylamines (Scheme 7).[80] Hence, MAL appears to be the most attractive enzyme to create new biocatalysts for the asymmetric synthesis of enantiomerically pure L-aspartic acid derivatives.

CONCLUDING REMARKS

Aspartase and MAL have been under investigation extensively for a number of decades, but the details of their catalytic mechanisms have been elucidated only in recent years. Information on the properties of these enzymes has been extended, and the isolation and characterization of enzymes from thermophilic organisms have added more robust enzymes to the arsenal of researchers. There are still limitations around the range of substrates that can be accepted. Hence, there is a need to apply directed evolution techniques to provide biocatalysts with a much broader substrate scope. However, the complex movement of the SS-loop from an open conformation to one that closes over the active site upon substrate binding makes engineering of the substrate specificity of aspartases a difficult task. We will therefore continue our efforts to further expand the substrate spectrum of MAL, which appears to be a more promising template for protein engineering.

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Notes

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KEYWORDS

Ammonia lyase: an enzyme that catalyzes the formation of an α,β-unsaturated bond by the elimination of ammonia from a substrate molecule.; Amino acid: a molecule containing an amine group, a carboxylic acid group, and a side-chain that is specific to each amino acid.; Aspartate: an α-amino acid with the chemical formula $HOOCCH(NH_2)CH_2COOH$.; Biocatalysis: the use of natural catalysts, such as enzymes or cells, to perform chemical transformations on organic compounds.; Catalytic mechanism: the mechanism by which a catalyst increases the rate of a chemical transformation.; Crystal structure: a unique arrangement of atoms or molecules within a crystalline liquid or solid.; Deaminase: an enzyme that catalyzes the removal of an amine group from a substrate molecule.; Methylaspartate: an α-amino acid with the chemical formula $HOOCCH(NH_2)CH(CH_3)COOH$.

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