

Structure and Function of Amino Acid Ammonia-lyases

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Histidine ammonia-lyase (HAL) and methylaspartate ammonia-lyase (MAL) belong to the family of carbon-nitrogen lyases (EC 4.3.1). The enzymes catalyze the α,β -elimination of ammonia from (S)-His to yield urocanic acid, and (S)-threo-(2S,3S)-3-methylaspartic acid to mesaconic acid, respectively. Based on structural analyses, the peptide at the active center of HAL from *Pseudomonas putida* is considered to be post-translationally dehydrated to form an electrophilic 4-methylidene-imidazole-one (MIO) group. A reaction mechanism was proposed with the structure. On the other hand, the structure of MAL from *Citrobacter amalonaticus* was found to be a typical TIM barrel structure with Mg^{2+} coordinated to the 4-carbonyl of the substrate methylaspartate. Unlike HAL, MIO was not observed in MAL, and the reaction of MAL appears to be completely different from phenylalanine ammonia-lyase (PAL), HAL, and other amino acid ammonia-lyases. A reaction mechanism is proposed in which the hydrogen at the β to the amino group of the substrate is abstracted forming an enolate type intermediate and then ammonia is released.

Keywords: Amino acid ammonia-lyase; Histidine ammonia-lyase; 3-Methylaspartate ammonia-lyase; X-ray crystallography

INTRODUCTION

A group of enzymes called amino acid ammonia-lyases has intrigued enzymologists and organic chemists, since the reactions cannot be done non-enzymatically, and the enzymes catalyze the addition of ammonia to achiral olefinic acids to form chiral L-amino acids (Hanson and Havir, 1973). These enzymes include histidine ammonia-lyase (HAL; EC 4.3.1.3), phenylalanine ammonia-lyase (PAL; EC 4.3.1.5), aspartate ammonia-lyase (AAL; EC 4.3.1.2), and 3-methylaspartate ammonia-lyase (3-methylaspartase, MAL; EC 4.3.1.1), etc. These enzymes pose a mechanistic challenge as to how they remove ammonia from L-amino acids by trans

elimination involving abstraction of a non-acidic and inactive β hydrogen (Walsh, 1979).

In 1960, a Japanese company Tanabe Seiyaku started the industrial production of (S)-Asp from fumaric acid by immobilized cells of *Escherichia coli* containing AAL as one of the examples of the industrial use of the enzyme (van der Werf *et al.*, 1994). It has also been discovered by Japanese researchers that PAL catalyzes not only the degradation of (S)-Phe, but also the synthesis of (S)-Phe from *trans*-cinnamic acid in a high concentration of ammonia up to 5 M (Yamada *et al.*, 1981).

In this review, we outline how the structures have been elucidated and the possible reaction mechanisms of amino acid ammonia-lyases studied by the groups of Rétey in Germany, and Rice in England and ourselves. There are excellent recent reviews on HAL and PAL by Rétey *et al.* (Langer *et al.*, 2001; Rétey, 2003), and AAL by Viola (2000).

HISTIDINE AMMONIA-LYASE (HAL) AND PHENYLALANINE AMMONIA-LYASE (PAL)

HAL catalyzes the degradation of (S)-His to urocanic acid and ammonia, and has been purified from *Pseudomonas* sp. and other biological sources. Ables *et al.*, have shown that HAL may have an active and essential electrophilic group at the active site because it was inactivated by $NaBH_4$, but also a carbonyl group, because the enzyme was inactivated by nucleophiles such as phenylhydrazine, $NaHSO_3$, NH_2OH , and KCN (Smith *et al.*, 1967). Later, they inactivated HAL with [^{14}C]-nitromethane and reduced with $NaBH_4$, and found that the radioactivity was incorporated into 4-amino-2-hydroxybutyric acid, 2,4-diaminobutyric acid and β -alanine after hydrolysis. They considered that the electrophilic group of HAL could be dehydroalanine and the amino group of (S)-His may form a Schiff base with

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an unidentified carbonyl group (Givot *et al.*, 1969). This hypothesis was also supported by the fact that (S)-His prevented the inactivation and 0.9 mol [^3H]Ala per mole of the enzyme was obtained, when HAL was inactivated and reduced with NaB^3H_4 and then hydrolyzed (Wickner, 1969).

A similar observation was obtained with PAL, when it was inactivated with NaB^3H_4 or [^{14}C]cyanide and then hydrolyzed with HCl and subjected to amino acid analysis. The radioactivity was present in Ala or Asp, respectively. Four moles of K^{14}CN per mole of the tetrameric enzyme was incorporated, and with an intermediate formation of β -cyanoalanine, [^{14}C]Asp was obtained as a product (Consevege and Philips, 1985; Hodgins, 1971) Hanson and Havir (1970) have postulated a possible reaction mechanism of HAL and PAL (Fig. 1), in which the initiation step is a nucleophilic attack of the substrate amino group on the electrophilic group of the enzyme (dehydroalanyl residue).

How might such an electrophilic prosthetic group function in the catalytic deamination reaction (Hanson and Havir, 1973; Walsh, 1979)? Attack of the substrate amino group at the β -carbon of the dehydroalanyl residue could occur in a net 1,4-addition step. To activate the initial complex for loss of the amino moiety, a 1,3-prototropic shift could occur, producing an enamine with the conjugated α,β -unsaturated carbonyl system. The enamine acts as an electron sink to cleave the C–N bond, and *trans*-cinnamic acid is formed. Expulsion of ammonia could proceed together with regeneration of the initial dehydroalanyl residue at the active site by a 1,3-prototropic shift.

Origin of the Electrophilic Group

Dehydroalanine could be formed by the dehydration of the precursor serine in the enzyme. It has also

been reported to occur in the peptide lantibiotics such as Nisin (Sahl *et al.*, 1995).

Comparison of the primary structures of HAL and PAL from various sources showed that four Ser residues are conserved (Taylor *et al.*, 1991). When the conserved Ser residues were changed to Ala by site-directed mutagenesis, only the Ser143Ala mutant was inactivated, therefore Ser143 was considered to be the precursor of the prosthetic group in HAL and Ser202 in PAL (Langer *et al.*, 1994). Furthermore, the sequence containing the active Ser, Gly–Ser–Val–Gly–Ala–Ser–Gly–Asp–Leu–Ala–Pro–Leu (conserved amino acid residues are shown underlined) was highly conserved in most of HAL and PAL (Poppe, 2001). Since Ser202 in Parsley PAL was considered to be the precursor of the prosthetic group (Schuster and Rétey, 1995), the Ser was changed to Thr and Gly with the loss of most of the activity. Although it was not shown that the prosthetic group was dehydroalanine, it has been strongly suggested the Ser residue is the precursor of dehydroalanine (Langer *et al.*, 1994).

Structure of HAL and Discovery of MIO

The structure of HAL from *Pseudomonas putida* was solved to 1.8 Å resolution (Schwede *et al.*, 1999). HAL is a homo tetrameric enzyme with 509 amino acids per peptide and is subdivided into two subdomains. The N terminus region (40%) is a globular domain with 8 helices and 4 short β sheets. The C terminal domain is composed of a core of 5 long parallel α helices surrounded by 6 further helices. Although there is no notable similarity between the primary structure of HAL and other ammonia-lyases, its tertiary structure resembles that of tetrameric lyases such as fumarase (Weaver *et al.*, 1995), AAL (Fujii *et al.*, 2003; Shi *et al.*, 1997), arginosuccinate lyase (Turner *et al.*, 1997).

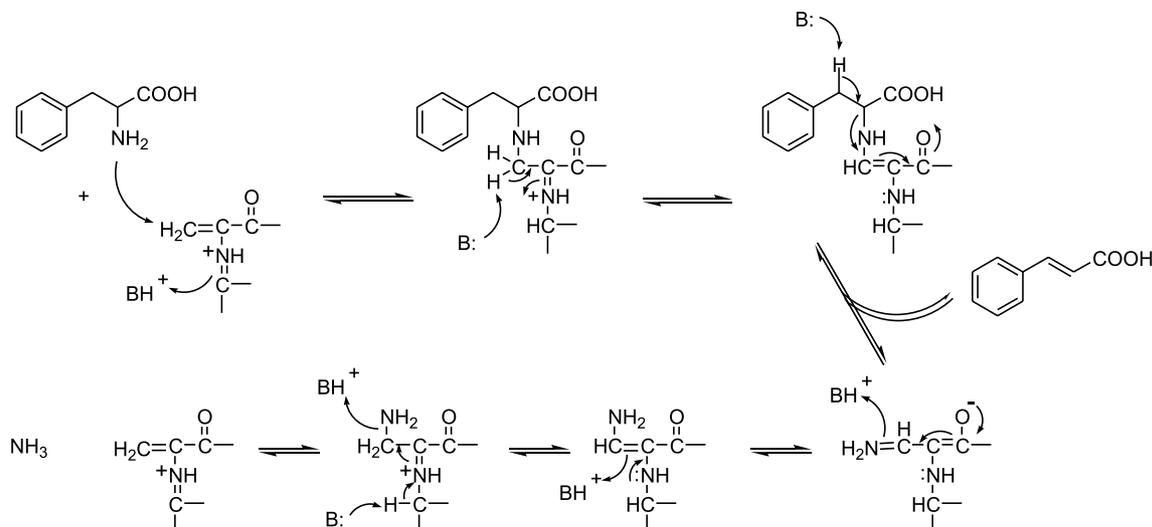


FIGURE 1 Proposed reaction mechanism of PAL with active site dehydroalanine (Hanson and Havir, 1970; Walsh, 1979).

The most striking discovery in the X-ray crystallographic analysis of HAL is the existence of the catalytically important electrophilic group, 4-methylidene imidazole-1-one (MIO) (Fig. 2). MIO can be considered as a modified dehydroalanine with strong electrophilicity (Poppe, 2001; Rétey, 2003). Interestingly, when various HAL genes were expressed in translation systems such as *E. coli* and *COS* cells, the Ala142–Ser143–Gly144 residue was autocatalytically modified to MIO. It is considered that dehydration reactions occur after the exomethylene structure of MIO is formed by a cyclization reaction (Baedeker and Schulz, 2002) (Fig. 2). MIO has a maximum absorption at 308 nm and shoulder at 315 nm. Although the X-ray structure analysis of PAL has not been carried out yet, it is expected that MIO will be present in PAL (Röther *et al.*, 2000). A homology model and reaction mechanism of HAL has been presented (Röther *et al.*, 2002).

Reaction Mechanisms of HAL and PAL

There are still several questions about the role of dehydroalanine as, before the success of X-ray structural analysis, research had not answered how the β -hydrogen (*Re* side) of the amino acids is activated, how HAL catalyzes not only the exchange of β -hydrogen but also that of the 5-hydrogen in the imidazole ring (Furuta *et al.*, 1992; Langer *et al.*, 1995), and why (*R*)-Cys and (*S*)-homocysteine strongly inhibit HAL, while other amino acids do not. Langer *et al.* (2001) have discovered that nitro-(*S*)-His is active as a very poor substrate of the HAL mutants Ser143Ala, Ser143Thr, and NaBH₄-reduced HAL, knowing that (*S*)-5-nitro-[β -²H₂]-His shows no isotopic effect because of the electron-withdrawing effect of the nitro group. This means that abstraction of the β -proton is not a rate-limiting step with 5-nitro-(*S*)-His. Thus the reactivity of the mutants

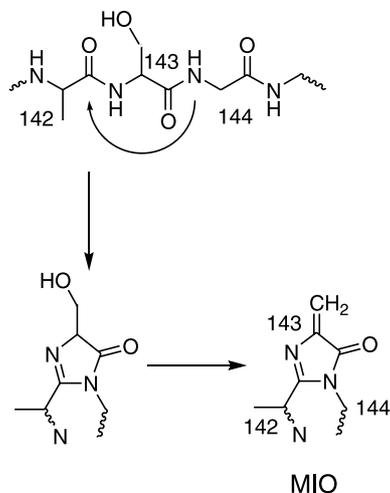


FIGURE 2 Formation and reaction of 4-methylidene-imidazol-5-one (MIO) in HAL (Retéy *et al.*, 1999).

lacking dehydroalanine and the NaBH₄-treated enzyme with 5-nitro-(*S*)-His as substrate ruled out the mechanism in which the α -amino group of (*S*)-His attacks this electrophilic group as proposed in Fig. 1.

With these observations, together with the X-ray crystallographic analysis, a reaction mechanism for HAL was proposed (Schwede *et al.*, 1999) (Fig. 3). When (*S*)-His is bound to the enzyme, the electron pair from the imidazole ring of (*S*)-His attacks the methylidene group of MIO. The cation formed at the imidazole ring increases the acidity of the hydrogen atom at the β position of (*S*)-His. A basic group in the enzyme abstracts the proton at the β position forming urocanic acid and ammonia, regenerating an electrophilic prosthetic group. This mechanism is also considered to be effective in the reaction of phenylalanine and PAL (Röther *et al.*, 2002).

Uses of PAL

HAL is active toward substrates such as 5-nitro-His, 5- or 2-fluoro-His, whereas PAL has a narrower substrate specificity. It was shown that PAL catalyzes the stereospecific addition of ammonia to acrylyc acid, when the concentration of ammonia was raised up to 5 M (Yamada *et al.*, 1981). Recently, fluorinated and chlorinated (*S*)-Phe and β -(5-pyrimidinyl)-(*S*)-Ala were synthesized (Gloge *et al.*, 2000).

3-METHYLASPARTATE AMMONIA-LYASE (MAL)

MAL is an enzyme, which catalyzes reversible amination-deamination between several 3-substituted (*S*)-aspartic acid and corresponding fumaric acid derivatives. The enzyme activity was first detected in a cell-free extract of the obligate anaerobic bacterium *Clostridium tetanomorphum* H1 by Barker *et al.* (1959). The enzyme is believed to be distributed very narrowly in obligate anaerobic microorganisms (Hanson and Havir, 1974), which are very hard to handle. Characterization of the different MALs from bacterial strains other than obligate anaerobes might provide a relevant contribution, not only to the

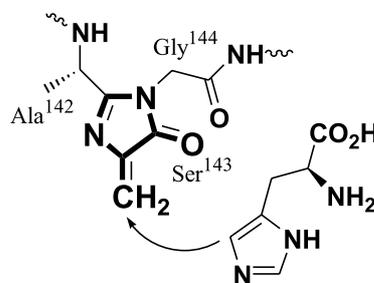


FIGURE 3 Proposed reaction mechanism of HAL (Schwede *et al.*, 1999).

knowledge of the physiological role of the enzymes but also to evolutionary studies in bacterial genome.

Screening and the Enzymatic Properties of MAL (Asano and Kato, 1994; Kato and Asano, 1997)

We screened for new sources of MAL to synthesize (S)-Asp derivatives by an enzymatic stereoselective addition of ammonia to various fumaric acid derivatives, which are easily obtainable by chemical methods. We tried to isolate MAL not only from aerobic microorganisms, but also from facultative anaerobes which had not previously been targets of microbial screening as MAL sources. Screening was carried out by enrichment culture in a medium containing (S)-Glu under anaerobic conditions created by sealing the surface of the medium in the culture tubes or flasks with liquid paraffin. Four facultative anaerobes belonging to the family of *Enterobacteriaceae* were isolated as powerful MAL producers from soil samples collected at Toyama Prefecture and identified as *Citrobacter freundii*, *Morganella morganii*, *Citrobacter amalonaticus*, and *Enterobacter* sp. from their morphological and physiological characteristics. We also screened from stock cultures and discovered for the first time that MAL producers are relatively widely distributed in *Enterobacteriaceae*. We could not isolate clostridial strains or any other obligatory anaerobes which showed this enzyme activity by our screening procedures. The enzymes were strongly induced by (S)-Glu or *threo*-(2S,3S)-3-methyl-Asp only when they were grown statically or anaerobically with all the isolates. When aerated, only a little activity was detected in the cell-free extracts, although the cells grew well.

Use of MAL in the Enzymatic Synthesis of (S)-Asp Derivatives (Asano and Kato, 1994)

By using cell-free extracts of the strains, mesaconic acid, ethylfumaric acid and chlorofumaric acid were aminated to give optically pure *threo*-(2S,3S)-3-methyl-Asp, *threo*-(2S,3S)-3-ethyl-Asp and *threo*-

(2R,3S)-3-chloro-Asp in good yields, respectively (Table I). This method is quite useful for preparing the (S)-Asp derivatives having a two-consecutive asymmetric centers without any protection of the substrates.

Physiological Aspects of MAL (Asano and Kato, 1994; Kato and Asano, 1997)

We also examined the physiological function of MAL in *Enterobacteriaceae*. The production of the enzyme was dependent on oxygen limitation during growth and was arrested by aeration. The addition of external electron acceptors such as dimethylsulfoxide could support cell growth and the production of the enzyme. Activities of glutamate mutase (EC 5.4.99.1) and (S)-citramalate hydrolyase (EC 4.2.1.34), key enzymes of the mesaconate pathway of (S)-Glu fermentation in the genus *Clostridium*, were detected in the cells of the active strains grown under oxygen limited conditions. Based on these results, the mesaconate pathway is proposed to explain the (S)-Glu fermentation process observed in *Enterobacteriaceae* and MAL could be a marker enzyme for this pathway.

Enzymatic Properties of MAL (Kato and Asano, 1995a,b, 1998)

MALs were purified 28, 39, 24 fold, respectively from the cell-free extracts of *C. freundii*, *M. morganii*, and *C. amalonaticus* by various column chromatographies and subsequently crystallized. Their molecular weights were calculated to be 79,000, 70,000, and 84,000 respectively, and those of the subunits were 40,000, 44,000, and 42,000 respectively. The properties of the enzymes were similar to that from the obligatory anaerobic microorganism *Clostridium tetanomorphum*, although the substrate specificities differed. The enzyme from *Enterobacteriaceae* catalyzed the reversible amination-deamination reactions between (2R,3S)-3-chloro-Asp and chlorofumaric acid, but the Clostridial enzyme carried out only the amination reaction of chlorofumaric acid. On the other hand, the latter enzyme catalyzed the reversible reaction between (S)-Asp and fumaric acid, but the former enzyme catalyzed only the amination reaction of fumaric acid. The Enterobacterial enzyme favours the amination reaction over the deamination, as the *K_m* values in the amination reaction are lower than those in the deamination reaction. The enzymes from *Enterobacteriaceae* were similar to each other in various enzymological properties, and required Mg²⁺ and similar divalent cations or alkali metal cations such as K⁺, and inhibited by typical SH reagents, chelating reagents, heavy metals and several divalent cations as well as

TABLE I Synthesis of several 3-substituted (S)-aspartic acids from the corresponding fumaric acid derivatives (HO₂C-(R)C=CH-CO₂H) by the cell-free extract of isolated strains.

Strain	Substrate (R =)		
	CH ₃	C ₂ H ₅	Cl
	Yield/Conc. (%) / (g l ⁻¹)	Yield/Conc. (%) / (g l ⁻¹)	Yield/Conc. (%) / (g l ⁻¹)
<i>C. freundii</i> YG-0504	27/31	66/74	18/14
<i>M. morganii</i> YG-0601	54/61	58/65	51/38
<i>C. amalonaticus</i> YG-1002	69/78	57/64	49/36
<i>Enterobacter</i> sp. YG-1202	38/42	54/61	41/31

TABLE II Properties of MAL from *Citrobacter amalonaticus*.

Molecular weight	Native	90,936
	Subunit	45,468
Number of subunits		2
pH optimum	Deamination	9.7
	Amination	8.5
Cation requirement	Divalent	Mg > Mn > Co
	Monovalent	K > Rb > Cs
<i>K_m</i> (mM) value for		
(2 <i>S</i> ,3 <i>S</i>)-3-methylaspartic acid		0.76
(2 <i>R</i> ,3 <i>S</i>)-3-chloroaspartic acid		7.9
Aspartic acid		no reaction
Mesaconic acid		0.130
Mg ²⁺		0.081
K ⁺		3.60
NH ₄ Cl		85.0

clostridial MAL. The optimum pH of the enzyme was 8.0–8.5 for the amination reaction, and pH 9.7 for the deamination reaction. The optimum temperature was at 45–50°C. The properties of MAL from *C. amalonaticus* strain YG-1002 are shown in Table II.

The MAL gene (*mal*) from *C. amalonaticus* strain YG-1002 was cloned and sequenced. The predicted polypeptide has 62.5% identity with MAL from *C. tetanomorphum*. ORF 1, which showed similarities with subunit E of glutamate mutases of *Clostridium* strains was found upstream of the *mal* gene, suggesting that MAL and glutamate mutase co-exist in the genome of the strain. The enzyme was overexpressed in *E. coli* under the control of the *lac* promoter. Under the optimized cultivation conditions, the amount of MAL in a cell-free extract of the recombinant *E. coli* was 51,800 U l⁻¹ culture, which is about 50-fold that of wild type strain, comprising over 40% of the total extractable cellular proteins.

Crystallization and X-ray Structural Analysis of MAL (Levy *et al.*, 2001, 2002)

We have been successful in the structure determination of MAL from *Citrobacter amalonaticus*. MAL from *C. amalonaticus* was expressed in *E. coli* in the presence of selenomethionine, purified to homogeneity and crystallized. Three crystal forms were obtained from identical crystallization conditions, two of which, forms A and B, diffract to high resolution and a third that diffracted poorly. Crystals of form A diffract to beyond 2.1 Å and have been

TABLE III Structural studies of MAL.

Crystals: Grown from 32% ammonium sulfate, pH 8 for 72 h.
Form A: Tetragonal form
Cell dimensions: A (<i>a</i> = 66.0 Å, <i>b</i> = 66.0 Å, <i>c</i> = 233.1 Å)
(P ₄ 2 ₂ , with a monomer in the asymmetric unit) to 2.16 Å resolution
Form B: Orthorhombic form
Cell dimensions: B (<i>a</i> = 129.5 Å, <i>b</i> = 238.9 Å, <i>c</i> = 66.3 Å)
(C ₂ 2 ₂ , with a dimer in the asymmetric unit) to 1.3 Å resolution

characterized as belonging to space group P₄2₂ with cell dimensions *a* = *b* = 66.0, *c* = 233.0 Å, $\alpha = \beta = \gamma = 90^\circ$ and a monomer in the asymmetric unit. Crystals of form B diffract to beyond 1.5 Å and belong to space group C₂2₂ with cell dimensions *a* = 130.0, *b* = 240.0, *c* = 67.0 Å, $\alpha = \beta = \gamma = 90^\circ$ and a dimer in the asymmetric unit. The structure was determined by multiwavelength anomalous diffraction (MAD) techniques using data collected from a single form A selenomethionine-labeled crystal to 2.16 Å resolution. Data to a resolution of 1.3 Å were obtained from B form crystals. The structure of the B form was solved by molecular replacement analysis. The data from forms A and B gave identical molecular structures (Table III). The following discussion is based on the structure from the B crystal form. The subunit of MAL is composed of two domains (1–160 aa and 170–411 aa) connected by a loop of extended β structure (161–169) (Fig. 4).

Biochemical studies of MAL by Gani and co-workers have suggested that MAL like PAL, utilizes a dehydroalanine prosthetic group for catalysis, following modification of an active site serine (Ser173 in *C. amalonaticus* MAL (Pollard *et al.*, 1999)). Furthermore, they cited a considerable primary deuterium isotope effect for (2*S*,3*S*)-3-methylaspartic acid, the natural substrate, indicative of the presence of a covalently bound intermediate. However, these findings are in contrast to the earlier reports of Bright (1964), who found no primary deuterium isotope effect for the deamination reac-

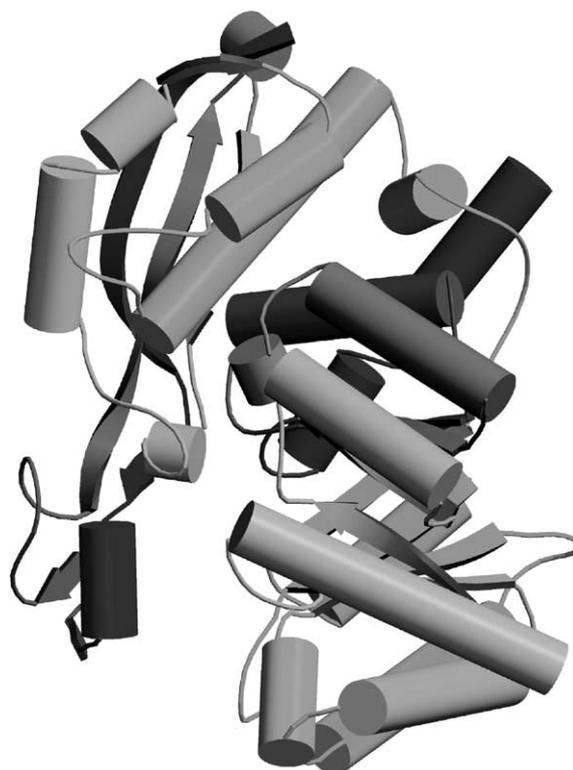
FIGURE 4 MAL monomer (Levy *et al.*, 2002).

TABLE IV Structure of MAL.

1. Each subunit consists of two domains (1–160 aa, 170–411 aa) linked by a single extended β -type structure
2. Domain I: (1–160 aa) a three-stranded antiparallel β sheet and an antiparallel, four a helix bundle
3. Domain II: (170–411 aa) an eight-stranded TIM barrel structure
4. Active site: Mg^{2+} is octahedrally coordinated by one carboxyl oxygen of Asp238, Glu273, and Asp307, two H_2O , which are both linked to the carboxyl of Glu308, and one of the carboxyl oxygens of the substrate
5. The dimer appears to be formed by interaction between the C-terminal helix of the TIM barrel domain

tion of *C. tetanomorphum* MAL. Consistent with this, our X-ray analysis suggested that Ser173, which had been claimed to be modified to dehydroalanine appeared to be unmodified.

To identify the active site of MAL, a form B crystal (C222) was soaked in cryoprotectant containing 10 mM (2*S*,3*S*)-3-methyl-Asp plus 5 mM $MgCl_2$. An electron density map of the substrate 3-methyl-Asp and Mg^{2+} complexed with the enzyme revealed that Mg^{2+} was coordinated to one of the carboxyls of the acidic residues of the side chain Asp238, Glu273 and Asp307, and two molecules of water connected to Glu308, and to one of the carboxyls of the substrate 3-methyl-Asp (Table IV, Figs. 5 and 6).

MAL as One of the Enolase Superfamily

The primary structure of MAL does not resemble those of HAL or PAL. Although Gani *et al.* have reported that Ser173 of clostridial MAL is converted to dehydroalanine by post-translational modification, we have observed that *C. amalonaticus* MAL is not modified. Ser173 lies at the end of one of the β strands of the TIM barrel and away from the active center. There is no post-translational modifi-

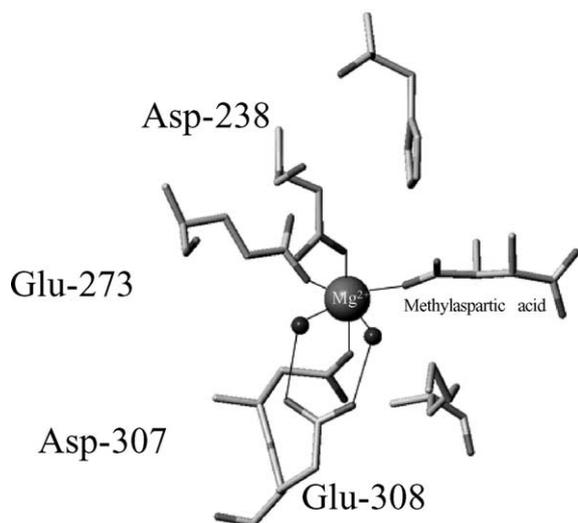


FIGURE 5 Coordination of Mg^{2+} with one carboxyl oxygen of Asp238, Glu273, Asp307, and methylaspartic acid, and two H_2O linked to Glu308 (Levy *et al.*, 2002).

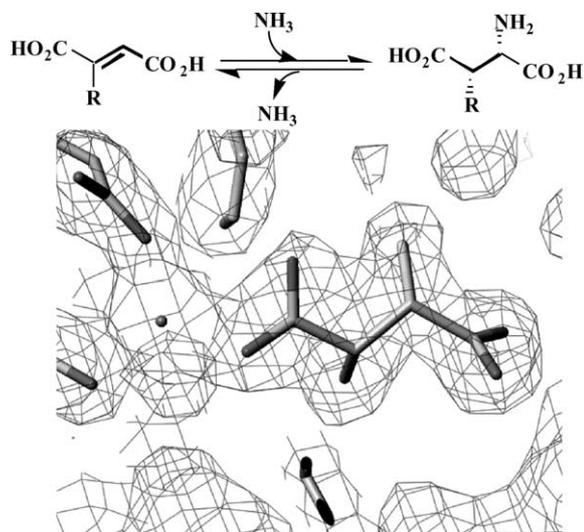


FIGURE 6 Electron density for the bound substrate, 3-methylaspartic acid with Mg^{2+} (Levy *et al.*, 2002).

cation of MAL, such as observed in HAL, and the X-ray studies have suggested that the mechanism of MAL is different from PAL, HAL and the other amino acid ammonia lyases. The TIM barrel structure of MAL closely resembled some of the enolase superfamily (Babbitt *et al.*, 1996), mandelate racemase from *P. putida*, muconolactonase from *P. putida*, and enolase from *Saccharomyces cerevisiae* (Pollard *et al.*, 1999). In the enzymes of the enolase superfamily, the substrate binds to a common site in the C terminus of the TIM barrel structure. Although there are some differences between these enzymes, a conserved acidic residue is located to coordinate with the divalent metal ion (Asp198 in muconate lactonizing enzyme, Asp195 in mandelate racemase, Asp246 in enolase). Carboxyl groups (Asp238, Glu273, Asp307) linked to divalent cations are seen in other enzymes. The common mechanism in the enolase superfamily is the formation of an enolate intermediate by the abstraction of a proton from the 3-position of the carboxylic acid.

In the complex of MAL and the substrate *threo*-(2*S*,3*S*)-3-methyl-ASP, Lys331 is located at an ideal position to act as a base to abstract a proton. In mandelate racemase, a similar residue to Lys331 has been identified. An electrophilic residue, neutralizing the acidic carboxyl group of the substrate is His194 (Lys164 in mandelate racemase, Lys167 in muconate lactonizing enzyme) which may act to abstract proton from the 3-position of the substrate.

On the basis of this analysis, the likely mechanism for MAL involves abstraction of the 3-proton of the substrate by Lys331, stabilization of the enolic intermediate by the metal ion and possibly His194, and collapse of the intermediate with elimination of ammonia to give mesaconic acid (Fig. 7). When the primary structures of MAL and enzymes of the enolase superfamily are compared, there is only very

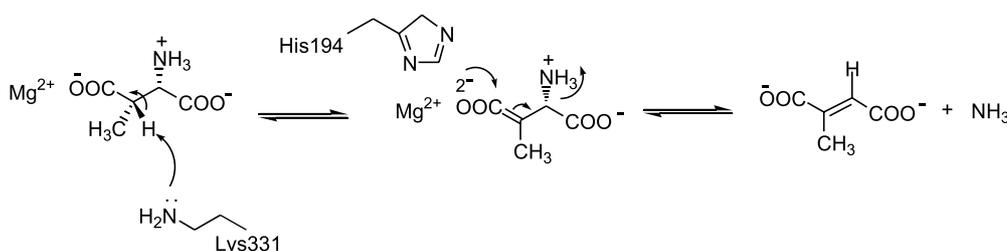
FIGURE 7 Proposed reaction mechanism of MAL (Levy *et al.*, 2002).

TABLE V Amino acid ammonia-lyases.

Enzyme	Typical source	Molecular weight	X-ray	Prosthetic group
Histidine ammonia-lyase	<i>P. putida</i>	53,700 × 4	Solved	MIO
Phenylalanine ammonia-lyase	Parsley	77,828 × 4	Not solved	MIO?
Aspartic acid ammonia-lyase	<i>E. coli</i>	52,224 × 4	Solved	Divalent cation?
3-Methyl-aspartic acid ammonia-lyase	<i>C. amalonaticus</i>	45,468 × 2	Solved	Mg ²⁺

MIO: 4-Methylidene-imidazol-5-one.

low similarity with less than 10 amino acid residues in common. However, catalytically important amino acids are conserved.

The Active Site and Substrate Specificity

MAL from *C. amalonaticus* catalyzes the α,β -elimination of ammonia from 3-substituted (*S*)-Asp such as *threo*-(2*S*,3*S*)-3-methyl-Asp and *threo*-(2*S*,3*S*)-3-ethyl-Asp, but unsubstituted (*S*)-Asp is not a substrate. It is apparent from the structure that there is a small cavity adjacent to the carbon at the 3 position that could accommodate the increase in side chain bulk from a methyl to an ethyl as encountered in going from *threo*-(2*S*,3*S*)-3-methyl-Asp to *threo*-(2*S*,3*S*)-3-ethyl-Asp. The enzyme surface lining the 3-methyl binding site is composed almost entirely of side chains (including those of Tyr356, Phe170 and Leu384), with little or no interaction between the 3-methyl group and main chain atoms. There is thus considerable potential for the engineering of this site to alter the substrate specificity of MAL, providing a route for the enzymatic production of novel 3-substituted (*S*)-aspartic acids.

CONCLUSIONS

We have presented the structures, reaction mechanisms and the functions of several amino acid ammonia-lyases. The analysis presented here clearly indicates that nature has evolved two different strategies for carrying out the related chemistries of the ammonia-lyases, each of which exploits the inherently different potential of the substrates in dictating the chosen chemistry. One is with the MIO structure seen in HAL, and the other is with TIM barrel structure we discovered in MAL (Table V).

Interestingly, the α,β -elimination of ammonia from (*S*)-Asp is catalyzed by AAL (Fujii *et al.*, 2003; Shi *et al.*, 1997), the structure of which is similar to that of HAL (Schwede *et al.*, 1999). This would suggest that AAL has a similar chemistry to that of HAL and PAL, although there is currently no evidence for a posttranslationally modified residue at the active site of AAL. Our analysis on the chemistry of MAL suggests that the α,β -elimination of ammonia from (*S*)-Asp could, in principle, proceed via an enolic intermediate. However, since it is unclear whether a metal is involved in catalysis by AAL, a mechanism like HAL and PAL is perhaps more probable. Further studies on AAL will be valuable in establishing which of the two mechanisms are used.

The reaction mechanism of these enzymes may answer the question as to why nature did not provide other ammonia-lyases acting on different amino acids. Of course new enzymes acting on other amino acids may be discovered in the future or some enzymes might evolve, although it is clear that the enzyme requires a functional group to activate the β hydrogen of the substrate amino acids. These are aromatic groups in the reactions of HAL and PAL, and β carboxylate groups in those of MAL and AAL (Asano, 2002).

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