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Aspartase/Fumarase Superfamily: A Common Catalytic Strategy Involving General Base-Catalyzed Formation of a Highly Stabilized *aci*-Carboxylate Intermediate

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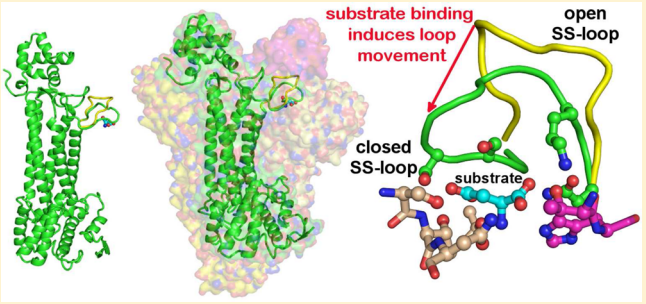
ABSTRACT: Members of the aspartase/fumarase superfamily share a common tertiary and quaternary fold, as well as a similar active site architecture; the superfamily includes aspartase, fumarase, argininosuccinate lyase, adenylosuccinate lyase, δ-crystallin, and 3-carboxy-*cis*,*cis*-muconate lactonizing enzyme (CMLE). These enzymes all process succinyl-containing substrates, leading to the formation of fumarate as the common product (except for the CMLE-catalyzed reaction, which results in the formation of a lactone). In the past few years, X-ray crystallographic analysis of several superfamily members in complex with substrate, product, or substrate analogues has provided detailed insights into their substrate binding modes and catalytic mechanisms. This structural work, combined with earlier mechanistic studies, revealed that members of the aspartase/fumarase superfamily use a common catalytic strategy, which involves general base-catalyzed formation of a stabilized *aci*-carboxylate (or enediolate) intermediate and the participation of a highly flexible loop, containing the signature sequence GSSxxPxKxN (named the SS loop), in substrate binding and catalysis.



The aspartase/fumarase superfamily is a group of homologous proteins that share a characteristic tertiary and quaternary fold, as well as a similar active site architecture. The best studied members of this superfamily are aspartase, fumarase, argininosuccinate lyase, adenylosuccinate lyase, δ-crystallin, and 3-carboxy-*cis*,*cis*-muconate lactonizing enzyme (CMLE). These characterized members all process succinyl-containing substrates leading to the formation of fumarate as the common product (Scheme 1a). The only exception is the CMLE-catalyzed reaction, which results in the reversible formation of a lactone (Scheme 1b). Recent structural and mechanistic studies uncovered intriguing similarities as well as some fascinating differences for these superfamily members.

Aspartases or aspartate ammonia lyases (EC 4.3.1.1) catalyze the reversible amination of fumarate to L-aspartic acid (Scheme 1a). They play an important role in microbial nitrogen metabolism and have been purified from a number of Gram-positive and Gram-negative bacteria. The best studied examples are AspA from *Escherichia coli*[1−3] and AspB from *Bacillus* sp. YM55-1.[4−8] For both aspartases, the crystal structure of the unliganded enzyme has been determined,[3,6] and most recently, the structure of AspB in complex with L-aspartate was determined.[9]

Fumarases or fumarate hydratases (EC 4.2.1.2) are ubiquitous enzymes as they form part of the citric acid cycle. They catalyze the reversible addition of water to fumarate to give L-malate (Scheme 1a). Two distinct classes of fumarases are known in prokaryotes. The class I fumarases are iron-dependent dimeric proteins, while the class II fumarases resemble the eukaryotic fumarases and are members of the aspartase/fumarase superfamily. The class II fumarase from *E. coli* (Fum C)[10,11] and fumarase from *Saccharomyces cerevisiae*[12] have been structurally characterized.

Adenylosuccinate lyase (ADL, EC 4.3.2.2) plays a major role in both cellular metabolism and replication by catalyzing two similar but nonsequential reactions in the *de novo* biosynthesis pathway for purine nucleotides.[13] In the first reaction, ADL converts 5-aminoimidazole(*N*-succinylocarboxamide) ribotide (SAICAR) into 5-aminoimidazole-4-carboxamide ribotide (AICAR) and fumarate. In the second reaction, ADL converts adenylosuccinate (ADS) into adenosine monophosphate (AMP) and fumarate (Scheme 1a). Consistent with its major role in metabolism, a deficiency of ADL activity in humans causes muscle wasting, psychomotor retardation, and mental disorder with autistic features.[14−16] The structures of the ADL

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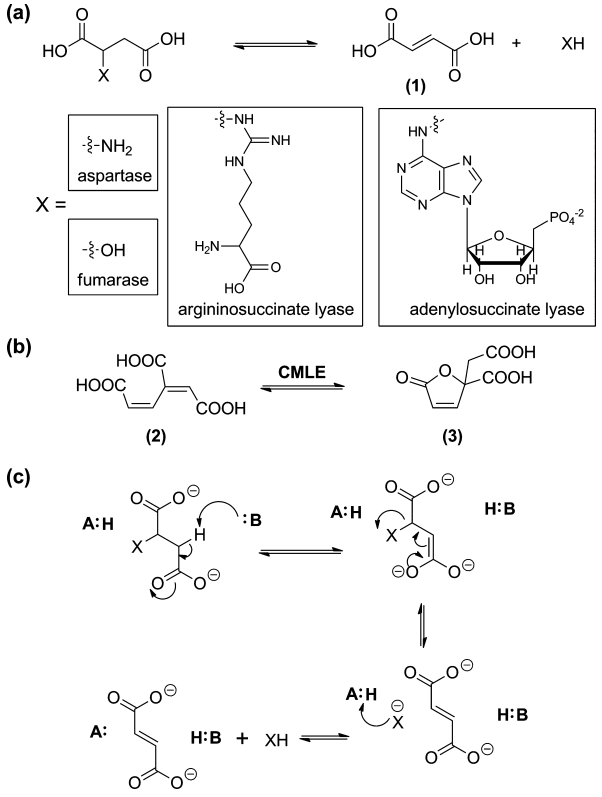
Scheme 1. (a) Conversion of Succinyl-Containing Substrates into Fumaric Acid (1) Catalyzed by Aspartase, Fumarase, Argininosuccinate Lyase, or Adenylosuccinate Lyase, (b) Conversion of 3-Carboxy-*cis*,*cis*-muconate (2) into a Muconolactone (3) Catalyzed by CMLE, and (c) General Acid−Base Catalytic Mechanism for the *anti* 1,2-Addition−Elimination Reaction (adapted from ref 45)



enzymes from *Thermotoga maritima* (tmADL),[17] *E. coli* (ecADL),[18,19] and *Pyrobaculum aerophilum* (paADL)[20] have been determined. Biochemical studies have been reported for the ADL enzymes from *Bacillus subtilis* (bsADL)[21−26] and *Plasmodium falciparum* (pfADL).[27]

δ-Crystallin is the major soluble protein in the eye lenses of birds and terrestrial reptiles. It is directly related to the enzyme argininosuccinate lyase (ARL, EC 4.3.2.1) and is thought to have evolved from it through a process called gene sharing.[28−30] Two isoforms of δ-crystallin are known, δ1- and δ2-crystallin. Duck δ1- and δ2-crystallin have 94% identical sequences and are 69 and 71% identical to human ARL, respectively. The duck δ1 isoform has no ARL activity,[31] while the duck δ2 isoform has retained ARL activity and has been used to study the mechanism of ARL activity in δ-crystallin.[28,32] ARL is a key enzyme in the biosynthesis of arginine in all organisms and is involved in the urea cycle in ureotelic species. It catalyzes the reversible conversion of L-argininosuccinate into L-arginine and fumarate (Scheme 1a). In humans, a deficiency of the enzyme causes argininosuccinic aciduria, a urea cycle disorder that causes ammonia to accumulate in the blood, leading to neurological problems and liver damage.[33] The structures of both isoforms of δ-crystallin[34] and of several ARL enzymes have been reported. In fact, the structure of δ2-crystallin from turkey eye lens was the first reported crystal structure of a member of the aspartase/fumarase superfamily.[35] The structures of the ARL enzymes from human[36] and *E. coli* (ecARL)[37] have also been determined.

3-Carboxy-*cis*,*cis*-muconate lactonizing enzymes (CMLEs) catalyze the reversible conversion of 3-carboxy-*cis*,*cis*-muconate into a muconolactone (Scheme 1b). This reaction is a key step in the protocatechuate branch of the β-ketoadipate pathway in both prokaryotic and eukaryotic microorganisms. For eukaryotic CMLEs such as *Neurospora crassa* CMLE (NcCMLE),[38] the lactonization product is 3-carboxymuconolactone, whereas for prokaryotic CMLEs such as *Pseudomonas putida* CMLE (PpCMLE), the lactonization product is 4-carboxymuconolactone.[39] Only the prokaryotic CMLEs belong to the aspartase/fumarase superfamily. The crystal structures of PpCMLE[40] and *Agrobacterium radiobacter* CMLE (ArCMLE)[41] have been determined, which showed that the tertiary, quaternary, and active site structures of prokaryotic CMLEs are similar to those of other members of the aspartase/fumarase superfamily. Because CMLE is the only member of this superfamily that catalyzes an intramolecular variant of the 1,2-addition−elimination reaction, it will not be further discussed here.

These homologous enzymes have been studied for the past nine decades, and it has been suggested that they belong to the same superfamily since as early as 1988.[42] In this review, we focus on recent structural studies that have provided new and detailed insights into the substrate binding modes and catalytic mechanisms of these enzymes. These studies showed that members of the aspartase/fumarase superfamily use a common catalytic strategy, which involves general base-catalyzed formation of a stabilized *aci*-carboxylate (or enediolate) intermediate and the participation of a highly flexible loop in substrate binding and catalysis. Some members of the superfamily (AspA and FumC) were reported to have a second site where the substrate (or another dicarboxylic acid) can bind (i.e., an activator binding site).[1,43,44] In this review, however, we focus strictly on the active site, where the actual catalysis takes place.

A GENERAL ACID−BASE CATALYTIC MECHANISM

On the basis of pH−rate profiles and inhibition studies, it has been shown that aspartase,[1,7,45] fumarase,[46] argininosuccinate lyase,[47] and adenylosuccinate lyase[48] all use a common acid−base catalytic mechanism. The general mechanism proposed for the *anti* 1,2-addition−elimination reaction is given in Scheme 1c. In the first step, the general base of the enzyme abstracts the *pro-R* hydrogen from the C3 atom (Cβ) of the substrate. The resultant carbanion is stabilized as the *aci*-carboxylate (or enediolate) intermediate with two negative charges on the β-carboxylate group. The existence of such an intermediate was supported by kinetic isotope experiments and inhibition studies with nitro analogues of the respective substrates.[1,7,46,48−51] Moreover, a recent study, in which the structure of AspB complexed with L-aspartate was determined, showed that aspartase forces the bound substrate to adopt a high-energy enediolate-like conformation.[9] Collapse of the intermediate is followed by cleavage of the C−N bond of the substrate (C−O bond, for fumarase), producing fumarate and the corresponding second product. This C−N or C−O bond cleavage step is the rate-determining step of the reaction,[1,27,32,46,50] which may be facilitated by a general acid that donates a proton to the leaving group. Given that the reaction occurs via *anti* elimination, two separate enzymic groups are required for proton abstraction and donation. The removal of the basic proton at the C3 position of the substrate and the stabilization of the *aci*-

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carboxylate intermediate are the most intriguing aspects of the mechanism. The role of the general acid, which is an essential catalytic residue for most of the superfamily members (argininosuccinate lyase, adenylosuccinate lyase, and fumarase), is ambiguous in aspartases, for which it is not clear whether the protonation state of the leaving group is that of ammonia or the ammonium ion.[6,7,50]

A COMMON TERTIARY AND QUATERNARY FOLD

Members of the aspartase/fumarase superfamily share a common tertiary and quaternary fold, despite the fact that pairwise sequence identities can be as low as 15%. However, there are three stretches of conserved amino acid residues denoted C1−C3, which have been implicated in catalysis (Figure 1). The structure of turkey δ-crystallin was the first

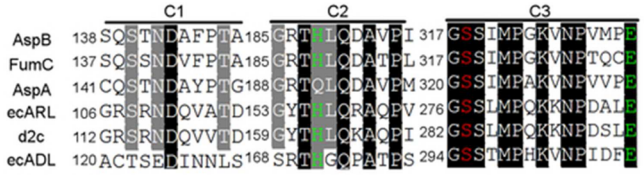

Figure 1. Sequence alignment of the three conserved regions, C1−C3, in *Bacillus* sp. YM55-1 aspartase (AspB), *E. coli* fumarase (FumC), *E. coli* aspartase (AspA), *E. coli* argininosuccinate lyase (ecARL), duck δ2-crystallin (d2c), and *E. coli* adenylosuccinate lyase (ecADL). The strictly conserved residues are highlighted in black and those less conserved in gray. The catalytic base residue (serine) is colored red, while the catalytic acid residue (histidine) and its charge relay pair residue (glutamate) are colored green. The figure was prepared using GeneDoc.[59]

structure from the superfamily to be determined.[35] It was found to be a tetramer, and the monomeric subunit was shown to have a new fold consisting of three mainly α-helical domains. Since then, structures have been determined for several members of the superfamily.[3,6,10,12,17−20,34−37,40,41,52] In all cases, tetramers were observed and the monomeric subunit was composed of three mainly α-helical domains: the N-terminal domain (D1), the central helix domain (D2), and the C-terminal domain (D3) (Figure 2a−d). The five long α-helices of the central helix domain of one subunit interact with those of the other three subunits to form a well-packed 20-helix bundle in the tetrameric molecule (Figure 2e). The first conserved region, C1, is located at the start of D2, while the other two regions, C2 and C3, are located in the loop regions between the helices forming the D2 domain. Conserved regions C1−C3, which are spatially separated from each other in the monomer, come together from three different subunits in the tetramer to form the active site; there are four active sites in the tetrameric molecule (Figure 2e).

Conserved region C3 contains the signature sequence GSSXXPXKXNPXXXE. Part of this region (GSSXXPXKXN) is a loop (named the SS loop), which plays an important role in catalysis. The SS loop, which is located at the opening of the active site, is highly flexible and one of the most structurally variable regions within each member of the superfamily. Moreover, binding of the substrate at the active site induces a conformational change in the SS loop from an open conformation to one in which the loop closes over the active site. This loop movement is considered essential for catalysis, was first observed in δ-crystallin,[34] and has subsequently been observed in other members of the superfamily (Figure 2f).[9,19,37]

SUBSTRATE BINDING AND STABILIZATION OF THE *ACI*-CARBOXYLATE INTERMEDIATE

All characterized members of the superfamily catalyze 1,2-addition−elimination reactions via an *aci*-carboxylate intermediate (Scheme 1c). Hence, a common structural feature expected in the active site of these enzymes would be a positively charged residue (or residues) for stabilizing the negative charge on the *aci*-carboxylate intermediate. Indeed, a highly conserved lysine residue is found in all members of the superfamily (Figure 1). This lysine (Lys324 in AspB, Lys324 in FumC, Lys301 in ecADL, Lys283 in ecARL, and Lys289 in δ2-crystallin) was initially proposed to be crucial for stabilization of the additional negative charge that develops on one of the β-carboxylate oxygens upon proton abstraction.[43,53,54] However, it has now been shown that this lysine residue interacts with the α-carboxylate group of the substrate (Figure 3a,b).[9,19] In fact, the conserved lysine forms part of the SS loop, and as such, it may have a crucial role in movement of the loop from an open to a closed conformation upon substrate binding. In all members of the superfamily in which this conserved lysine was mutated, an almost complete loss of activity was observed, emphasizing the important role of this residue in catalysis. The other residues implicated in binding of the α-carboxylate group of the substrate are Asn142, Thr187, and Asn326 of AspB; Asn141, Thr187, and Asn326 in FumC; Thr170, Gln247, and Asn303 in ecADL; and Asn110, Thr155, and Asn285 in ecARL (Figure 3).

The structure of aspartate-bound AspB[9] shows that the bound aspartate molecule adopts a high-energy enediolate-like (or *aci*-carboxylate-like) conformation that is stabilized by an extensive network of hydrogen bonds between residues Thr101, Ser140, Thr141, and Ser319 and the substrate's β-carboxylate group (Figure 3b). Similar interactions are observed in the structure of adenylosuccinate-bound ecADL(H171A),[19] with the corresponding H-bond donors being His91, Thr122, Ser123, and Ser296 (Figure 3a). A detailed comparison of these structures has further revealed that in the ecADL active site, the succinyl moiety of the bound adenylosuccinate has an enediolate-like conformation similar to that of the bound aspartate molecule in the AspB complex structure.[9] These observations provide support for the suggestion that enzymes that abstract a proton from a highly basic sp^3-hybridized carbon center may achieve this, in part, through binding-induced distortion of this carbon to a geometry resembling sp^2 hybridization.[49] In addition to its role in substrate binding, the extensive hydrogen bonding network with the β-carboxylate group of the substrate is important for stabilizing the negative charge on the *aci*-carboxylate intermediate. The enediolate-like conformation of the bound substrate, in combination with the strong hydrogen bonding network with the β-carboxylate group, is expected to decrease the pK_a ($\geq$32) of the proton at the C3 position such that it is equal or less than that of the active site base catalyst.[49,55] An extensive network of H-bond interactions between corresponding active site residues and the substrate's β-carboxylate group is also predicted for other superfamily members.[37,56]

THE SS LOOP AND THE CATALYTIC BASE

The SS loop containing the signature sequence GSSXXPXKXN is one of the most conserved regions in the superfamily (Figure 1). Hence, this loop has been proposed to play an important role in catalysis. However, the exact roles of the SS loop and the

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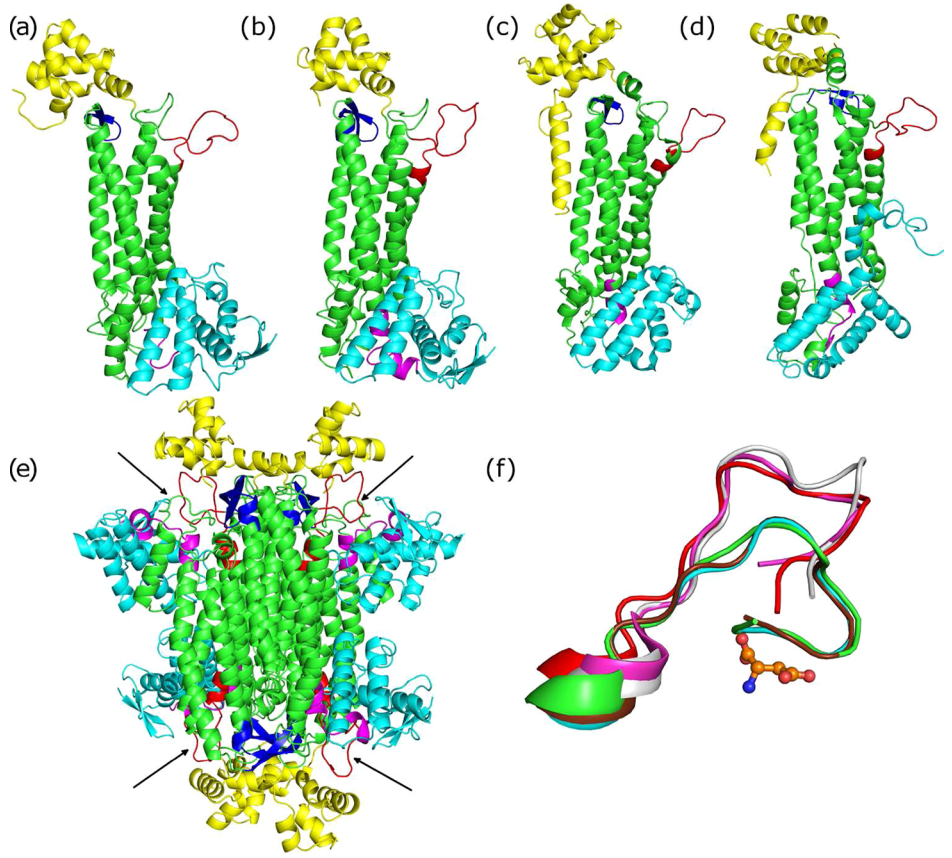

Figure 2. (a–d) Ribbon representations of the monomers of aspartase (AspB, PDB entry 3R6V), fumarase (FumC, PDB entry 1YFE), argininosuccinate lyase (δ2-crystallin, PDB entry 1HY1), and adenylosuccinate lyase (ecADL, PDB entry 2PTR), respectively. The three structural domains, D1–D3, are colored cyan, green, and yellow, respectively. The highly conserved regions, C1–C3, are colored magenta, blue, and red, respectively. (e) Ribbon representation of the FumC tetramer showing the various domains and the conserved regions coming together to form four equivalent active sites (indicated by the arrows). The coloring scheme is the same as that for panels a–d. (f) Structural comparison of the SS loop in the open conformation in AspB (red, PDB entry 3R6Q), δ2-crystallin (magenta, PDB entry 1HY1), and FumC (gray, PDB entry 1YFE) and in the closed conformation in AspB (green, PDB entry 3R6V), δ1-crystallin (cyan, PDB entry 1HY0), and ecADL (brown, PDB entry 2PTR). The aspartate in the ligand-bound AspB structure is shown as a ball and stick model to demonstrate that the loop approaches the substrate in the closed conformation. This figure was made using PyMOL.[60]

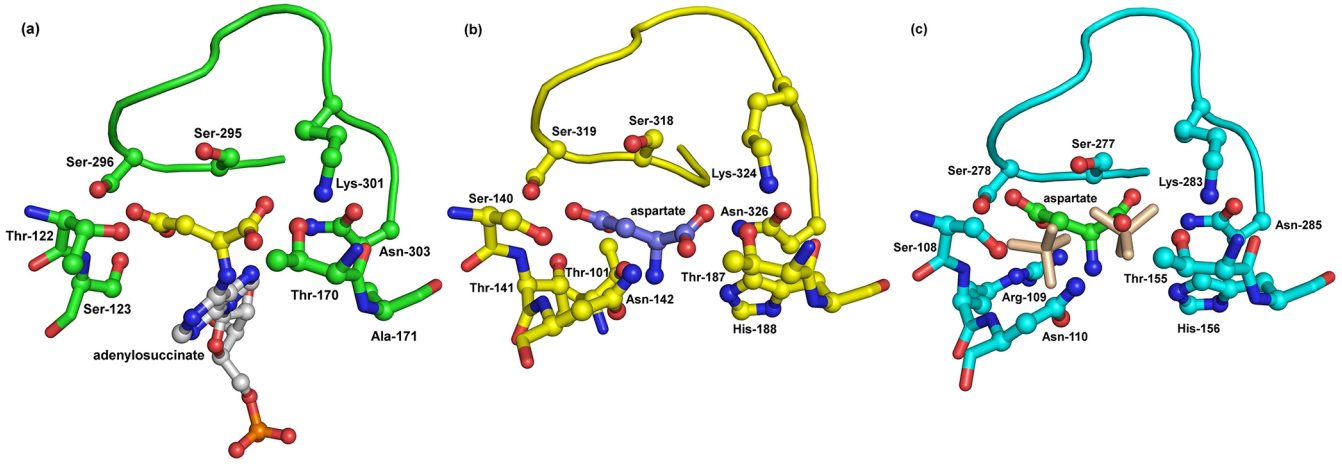

Figure 3. (a) Active site structure of ecADL(H171A) with bound ADS (PDB entry 2PTR). The succinyl moiety of ADS is colored yellow. Residue Gln247 is not shown for the sake of clarity. (b) Active site structure of AspB in complex with L-aspartate, which is colored blue (PDB entry 3R6V). (c) Overlay of the active site of ecARL (PDB entry 1TJ7) with two bound phosphate ions (colored wheat) and that of AspB in complex with L-aspartate. For the sake of clarity, the AspB active site residues have been removed and only the bound L-aspartate molecule (green) is shown, illustrating the similar positions taken by the two phosphate ions in the ecARL active site and the substrate's carboxylate groups in the AspB active site. This figure was made using PyMOL.[60]

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two highly conserved serine residues in substrate binding and catalysis have only recently been elucidated. A comparison of the structure of unliganded ecADL with that of ecADL-(H171A) in complex with ADS showed that upon substrate binding, the SS loop moves from an open conformation in the absence of substrate to one that closes over the active site in the presence of substrate (Figure 2f).[19] In the closed conformation of the SS loop, the strictly conserved SS loop residue Ser295 is properly placed to abstract the *pro-R* proton from the Cβ atom of the substrate and thus likely functions as the catalytic base (Figure 3a).[19] Recent structural work on AspB revealed a similar conformational change in the SS loop upon substrate binding (Figure 2f). In the closed conformation with L-aspartate bound in the active site of AspB, residue Ser318 (corresponding to Ser295 in ecADL) is at a suitable position to act as the catalytic base, abstracting the Cβ proton of the substrate in the first step of the reaction mechanism, leading to the *aci*-carboxylate intermediate (Scheme 1c and Figure 3b).[9]

The mechanistic role of the SS loop and conserved catalytic serine in δ2-crystallin (which has argininosuccinate lyase activity) was uncertain as crystal structures obtained with bound argininosuccinate did not show the closed loop conformation.[53,57] In fact, Ser283 (corresponding to Ser295 in ecADL) in δ2-crystallin was initially proposed to act as the catalytic acid.[53] However, a comparison of a substrate docking model of AspB with the structure of δ1-crystallin complexed with sulfate, in which a closed SS loop conformation is observed, showed that Ser281 in δ1-crystallin (Ser283 in δ2-crystallin) cannot act as a general acid because of the relatively large distance from this residue to the nitrogen atom of the leaving group.[6] It was further argued that the orientation of Ser281 is suitable for abstraction of the *pro-R* proton from the Cβ atom of the substrate. Further evidence of a role of the serine as a catalytic base was provided by the structure of ecARL with two phosphate ions bound in the active site, showing a closed SS loop conformation.[37] An argininosuccinate binding mode in the active site of ecARL was generated by superposing the carboxylic acid moieties of *trans*-succinate on the two phosphate ions. On the basis of this model, it has been suggested that the succinate moiety of argininosuccinate rotates to a new position when the SS loop closes over the active site, and in this proposed orientation for the substrate, the *pro-R* proton at the Cβ position points toward Ser277 (Ser283 for δ2-crystallin). Hence, the serine residue could initiate catalysis by abstracting this proton.[37] An alignment of the recently determined L-aspartate-bound AspB structure with the ecARL structure indeed shows that the carboxylate groups of L-aspartate occupy positions similar to those of the two phosphates in the ecARL structure (Figure 3c). In the case of fumarases, unliganded enzyme structures and structures of the enzyme in complex with various competitive inhibitors (e.g., citrate) have been determined.[10,11] However, no closure of the SS loop upon binding of inhibitors was observed, and the exact mechanistic role of the SS loop in the reaction catalyzed by fumarases has remained elusive.

That the serine in the SS loop is indeed a key catalytic residue is further supported by a number of mutagenesis studies. When Ser318 in AspB was mutated to an alanine, a complete loss of activity was observed.[9] Mutagenesis of the corresponding serines in other members of the superfamily (Ser289 in human ADL, Ser262 in bsADL, and Ser283 in δ2-crystallin) has also shown that this residue is essential for catalysis.[26,53] However, to function as an efficient catalytic base, the side chain of serine needs to be activated and form an oxyanion species. This would require a substantial perturbation of the pK_a (~15) of serine in the enzyme active site and suggests the presence of enzymic groups that stabilize the serine oxyanion. Stabilization of the oxyanion (and the resulting decrease in the pK_a value of the Ser-OH species) is likely realized by interactions with backbone amides as has been proposed for Ser295 in ecADL.[19] Interestingly, for pfADL, it has been proposed that the β-carboxylate group of the substrate assists in catalysis by acting as a base to generate the serine oxyanion.[27] However, the precise mechanism by which the conserved serine is activated to function as a catalytic base has not yet been elucidated.

CATALYTIC ACID RESIDUE

Another highly conserved residue in the superfamily is a histidine (His188 in FumC, His171 in ecADL, and His156 in ecARL) in conserved region C2, which forms a charge relay with a highly conserved glutamate residue (Glu331 in FumC, Glu308 in ecADL, and Glu290 in ecARL) in conserved region C3 (Figure 1). This His-Glu charge relay pair is strictly conserved in fumarases, argininosuccinate lyases, δ-crystallins, and adenylosuccinate lyases. This histidine residue was initially proposed to act as the general base, which abstracts the Cβ proton from the substrate, in these enzymes.[1,10,17,25,56,57] The charge relay with the glutamate residue was thought to facilitate this role. However, recent structural studies of ecADL,[19] phosphate-bound forms of ecARL,[37] and the enzyme−substrate complex of AspB[9] revealed that the conserved histidine residue is not in a suitable position to abstract the Cβ proton of the substrate and hence cannot function as the base catalyst (Figure 3a−c). Instead, these studies have shown that the histidine is positioned close to the leaving group (Cα functional group) of the substrate. This suggests that the histidine may function as the general acid, donating a proton to the leaving group. In fumarase, the conserved histidine appears to be essential as an acid catalyst, facilitating the release of the OH group as water.[46]

Intriguingly, the histidine residue in the C2 region is not conserved in all members of the superfamily and is replaced with a glutamine in most known aspartases, including AspA (Figure 1). Notably, the histidine is present in AspB and the aspartase from *B. subtilis*. However, an engineered variant of AspB in which His188 was mutated to an alanine still displayed significant activity, suggesting that the histidine is not an absolute requirement for C−N bond cleavage in this enzyme.[7] It is important to emphasize that for aspartases it is not clear whether the protonation state of the leaving group is that of ammonia or the ammonium ion.[6] The incomplete conservation of the histidine residue in the superfamily and its dispensable role in some members of the superfamily suggest that the reaction mechanism in the releasing stage of the Cα functional group of the substrate may be different for distinct members of the superfamily.

CONCLUSIONS AND FUTURE PERSPECTIVES

The examples of members of the aspartase/fumarase superfamily discussed here clearly show that these enzymes use a common catalytic strategy to process different succinyl-containing substrates. This strategy employs a common binding mode for the succinyl moiety of the substrate, uses general base-catalyzed formation of an enzyme-stabilized *aci*-carboxylate intermediate, and involves the participation of a highly

flexible loop in substrate recruitment and catalysis. Remarkably, a serine residue, which is part of this flexible loop, acts as the catalytic base. The question remains, though, how this strictly conserved serine is activated to function as the catalytic base. An active site histidine is thought to act as the catalytic acid. However, the incomplete conservation of this residue and its dispensable role in some members of the superfamily suggest that the mechanism of elimination of the Cα functional group of the substrate may be different for distinct members of the superfamily.

A notable gap in the structural studies is the absence of crystal structures of active enzymes in complex with their natural substrate, with the exception of that of the L-aspartate–AspB complex.[9] Additional structures of native enzyme–substrate complexes will undoubtedly give a better idea of how the pieces fit together. Furthermore, detailed mechanistic and structural analyses have only been conducted for a few members of the superfamily, and many more members of the superfamily await the determination of their mechanisms and structures. For example, sequence analysis suggested that ethylenediamine-*N*,*N*′-disuccinic acid lyase (EDDS lyase), which catalyzes two sequential 1,2-elimination reactions to convert EDDS into ethylenediamine and fumaric acid,[58] also belongs to the aspartase/fumarase superfamily. EDDS lyase has exciting potential for use as a biocatalyst in the preparation of biodegradable metal chelators such as EDDS and its derivatives.[58] We have therefore initiated mechanistic and structural studies of this lyase with the aim of elucidating the details of its unusual two-step addition–elimination reaction mechanism. Many more fascinating members of the superfamily may be discovered by exploring the wealth of sequence information produced by the genome sequencing projects.

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Notes

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