Structural Basis for the Catalytic Mechanism of Aspartate Ammonia Lyase

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ABSTRACT

Aspartate ammonia lyases (or aspartases) catalyze the reversible deamination of L-aspartate into fumarate and ammonia. Lack of crystal structures of complexes with substrate, product or substrate analogs so far precluded to define their precise mechanism of catalysis. Here, we report crystal structures of AspB, the aspartase from Bacillus sp. YM55-1, in an unliganded state and in complex with L-aspartate at 2.4 Å and 2.6 Å resolution, respectively. AspB forces the bound substrate to adopt a high energy, enediolate-like conformation that is stabilized, in part, by an extensive network of hydrogen bonds between residues Thr101, Ser140, Thr141 and Ser319 and the substrate’s β-carboxylate group. Furthermore, substrate binding induces a large conformational change of the SS-loop (residues G_{317}SSIMPGKV_{326}) from an open conformation to one that closes over the active site. In the closed conformation, the strictly conserved SS-loop residue Ser318 is at a suitable position to act as a catalytic base, abstracting the Cβ-proton of the substrate in the first step of the reaction mechanism. The catalytic importance of Ser318 was confirmed by site-directed mutagenesis. Site-directed mutagenesis of SS-loop residues, combined with structural and kinetic analysis of a stable proteolytic AspB fragment, further suggests an important role for the small C-terminal domain of AspB in controlling the conformation of the SS-loop and, hence, in regulating catalytic activity. Our results provide evidence for the notion that members of the aspartase/fumarase superfamily use a common catalytic mechanism involving general base-catalyzed formation of a stabilized enediolate intermediate.
Introduction

Aspartate ammonia lyases (also known as aspartases; EC 4.3.1.1) play an important role in microbial nitrogen metabolism by catalyzing the reversible conversion of L-aspartate to fumarate and ammonia. In industry, these enzymes are used as biocatalysts for the production of enantiopure L-aspartate, an important starting compound for the synthesis of food additives and artificial sweeteners (1). Aspartases belong to the aspartase/fumarase superfamily, which also includes fumarase C, adenylosuccinate lyase, argininosuccinate lyase, δ1-crystallin and 3-carboxy-cis,cis-muconate lactonizing enzyme, all of which have been structurally characterized by X-ray crystallography (2-7). Members of this superfamily share a common tertiary and quaternary fold, as well as a similar active site architecture, despite the fact that pairwise sequence identities can be as low as 15%. They are biologically active as homotetramers and contain four composite active sites, each generated by conserved sequence motifs from three monomers. One of these conserved regions is a flexible loop of about 10 residues, containing the signature sequence GSSxxPxxKxN (therefore named the SS-loop), which for some aspartase/fumarase superfamily members has been shown to play an important role in substrate binding and catalysis (3,8). Because of the structural similarities, and since they all process succinyl-containing substrates leading to the formation of fumarate as the common product, it is believed that members of the aspartase/fumarase superfamily use a similar mechanism of catalysis involving general base-catalyzed formation of a stabilized enediolate (i.e. an aci-carboxylate) as reaction intermediate (3,6,8-11).

Aspartases have been purified and characterized from various Gram-negative and Gram-positive bacteria, including Escherichia coli, Hafnia alvei, Pseudomonas fluorescens, Bacillus subtilis and Bacillus sp. YM55-1 (12-18). The aspartase from E. coli, AspA, has been studied most extensively, and its crystal structure has been elucidated (19). AspA is allosterically activated by its substrate and Mg\textsuperscript{2+} ions, which are required for activity at alkaline pH. Based on biochemical and biophysical studies, and by relating the enzyme to other members of the aspartase/fumarase superfamily, a reaction mechanism has emerged for the AspA-catalyzed deamination of L-aspartate. In this proposed mechanism, AspA catalyzes an anti-elimination reaction, where in the first step an active site base abstracts a proton from the C\textbeta atom of L-aspartate to form an enzyme-stabilized enediolate intermediate, which has two negative charges on its \beta-carboxyl group (i.e. an
Aspartate ammonia lyase AspB from *Bacillus* sp. YM55-1

aci-carboxylate) (14, 18, 20). Collapse of this putative enediolate intermediate eliminates ammonia and yields the product, fumarate. In the absence of structural data of AspA in complex with a substrate, product or competitive inhibitor, the exact role of each active site residue in substrate binding and catalysis is not known yet. In particular, the identity of the catalytic base that abstracts the Cβ proton and the functional role of the SS-loop have remained elusive.

The only other aspartase for which a crystal structure has been reported, albeit also in an unliganded state, is AspB from the thermophilic bacterium *Bacillus* sp. YM55-1 (21). Lack of allosteric regulation, combined with a high activity, excellent enantioselectivity and relatively high thermostability, makes AspB very suitable as biocatalyst in organic synthesis (13). Notably, the broad nucleophile specificity of AspB in the reverse conjugate addition reaction, accepting alternative nucleophiles, such as methylamine, hydroxylamine, hydrazine and methoxylamine, has been exploited for the synthesis of enantiopure *N*-substituted aspartic acids (22). AspB is composed of 468 amino acid residues and is active as a homotetramer (13). Site-directed mutagenesis, kinetic analysis and structural modeling of substrate binding have provided insights into the functional role of various active site residues (21, 23). However, no obvious residue in the active site of AspB could be identified that may serve as catalytic base in the first step of the proposed reaction mechanism. Also, the SS-loop in the unliganded AspB structure (residues 317-328) is highly disordered, and, like for AspA, its role in substrate binding and catalysis is unclear.

To provide a better understanding of the functional roles of the active site residues and the SS-loop in substrate binding and catalysis, and to identify the general base catalyst in AspB, we have determined the crystal structure of wild-type AspB in complex with L-aspartate at 2.6 Å resolution. Comparison between this structure and a newly determined structure of unliganded AspB at 2.4 Å resolution, reveals a large conformational change of the SS-loop, from an open conformation in the absence of substrate to one where it closes over the active site in the presence of substrate. The functional role of the SS-loop in AspB, as well as that of the nearby C-terminal domain, was further analysed by site-directed mutagenesis and limited proteolysis. The obtained results provide clear and new insights into the mechanism of catalysis by AspB, and strongly suggest that Ser318 functions as the general base catalyst.
Experimental procedures

Expression vector for wild-type AspB production

To allow the overproduction of native, wild-type AspB (without any tags), the aspB gene was amplified by PCR from plasmid pUCBA (24) using two synthetic primers, and cloned in the vector pBAD/Myc-His A (Invitrogen). The forward primer (5’-ATACCATGGATACCGATGTTCG-3’) contains a NcoI restriction site (in bold) followed by 13 bases that correspond to the coding sequence of the aspB gene. The reverse primer (5’-CATCTGCAATTTTCTTTCCACGCAATTCC-3’) contains a PstI restriction site (in bold) followed by 18 bases that correspond to the complementary sequence of the aspB gene (including the stop codon sequence). The resulting PCR product and the pBAD/Myc-His A vector were digested with NcoI and PstI restriction enzymes, purified, and ligated using T4 DNA ligase. Aliquots of the ligation mixture were transformed into competent E. coli TOP10 cells. Plasmid DNA was isolated from several transformants and analyzed by restriction analysis for the presence of the insert. The cloned aspB gene was sequenced to verify that no mutations had been introduced during the amplification of the gene. The newly constructed expression vector was named pBAD(AspB).

Expression and purification of wild-type AspB

To produce native, wild-type AspB, the pBAD(AspB) plasmid was transformed into E. coli TOP10 cells. A single freshly transformed colony was picked using a sterile loop and used to inoculate 10 mL of LB/ampicillin medium. After overnight growth at 37 °C, the culture was diluted to an A<sub>600</sub> of ~0.02 in 1 L of LB/ampicillin medium in a 5 L Erlenmeyer flask. The culture was incubated at 37 °C with vigorous shaking until an A<sub>600</sub> of 0.5-0.6 was reached, after which arabinose (0.04%, w/v) was added. Cells were incubated further for 8-10 h at 37 °C and then harvested by centrifugation (6000 g for 15 min). The cell pellet was stored at -20 °C until further use.

In a typical purification experiment, ~3 grams of cell pellet (from a 1 L culture) were thawed and suspended in lysis buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA). A protease inhibitor cocktail tablet (Roche Applied Sciences) was added to the mixture and cells were treated (20 min on ice) with lysozyme (0.1 mg/ml). Cells were disrupted by sonication for 4 x 1 min (with 4-6 min rest in between each cycle) at a 60 W output, after which unbroken cells and debris were removed by centrifugation (10,000g, 50 min). The supernatant was collected and L-aspartic acid was added to a final concentration
of 0.1 M. Ammonium sulphate was added (11%, w/v) and the sample (~20 mL) was heated at 72 °C for 9 minutes and then immediately cooled in an ice/water bath. Precipitated and denatured proteins were removed by centrifugation (10,000g, 50 min) and the supernatant was loaded onto a phenyl-sepharose column (Sigma-Aldrich) equilibrated with buffer A (50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 0.83 M (NH₄)₂SO₄). The column was washed with two column volumes of buffer A, after which retained protein was eluted with buffer B (50 mM Tris/HCl, pH 7.5, 1 mM EDTA). Collected fractions were analysed by SDS-PAGE and checked for activity. Fractions containing AspB were pooled and the buffer was exchanged with buffer B. The protein sample was then loaded on a Q-sepharose anion-exchange column (GE Healthcare), which had previously been equilibrated with Buffer B. Retained protein was eluted with a linear concentration gradient of NaCl in buffer B (0-0.5 M). Fractions containing aspartase activity were pooled and concentrated to ~4 mg/ml, and then applied to a Sephadex S200 16/60 gel filtration column (GE Healthcare), which had previously been equilibrated with buffer C (25 mM Tris/HCl, pH 7.5). Fractions were analyzed by SDS-PAGE, and those that contained purified AspB were pooled and concentrated to ~10 mg/mL. Freshly purified AspB was directly used for crystallization trials.

**Cloning, expression and purification of AspB-His mutants**

Site-directed mutagenesis was performed by overlap extension PCR (25) using plasmid pBAD(AspB-His) as the template (22). The final PCR products were purified and cloned in the pBAD-myc-His A vector, which allows for the production of mutant proteins with a C-terminal polyhistidine tag. Each mutant gene was completely sequenced (with overlapping reads) to verify that only the intended mutation had been introduced. His-tagged mutant proteins were produced and purified using previously reported procedures (22,23).

**Enzyme kinetic measurements**

Kinetic assays were performed at 25 °C in 50 mM NaH₂PO₄ buffer, pH 8.5, by monitoring the increase in absorbance at 240 nm corresponding to the formation of fumarate (ε = 2530 M⁻¹cm⁻¹) as previously described (22,23). Each kinetic assay was carried out twice, and the differences in kcat and kcat/Km values were less than 25%.
Structure determination of AspB and AspB/L-Asp

Attempts to crystallize AspB at previously reported conditions (21) were unsuccessful. Therefore, a search for new crystallization conditions was performed by sparse-matrix sampling using various commercially available crystallization kits. Screening was performed at room temperature in 96-well sitting-drop crystallization plates using a Mosquito (TTP LabTech) robot for drop dispensing. An initial crystallization hit was obtained with the JCSG+ (Qiagen) crystallization screen, which was optimized manually using the hanging-drop vapour diffusion method, varying precipitant concentration, pH, temperature and testing the effect of additives. Droplets were composed of 1 µl AspB protein stock solution and 1 µl crystallization solution and were equilibrated against 500 µl of the crystallization solution. The final crystallization solution contained 0.25 M NaSCN, 10 mM CaCl$_2$, 0.1 M HEPES, pH 7.0, 20% PEG3350. Crystals grew at 4 ºC within 2 days as single plates with maximum dimensions of 100 µm x 100 µm x 5 µm.

Prior to diffraction data collection, a single crystal was transferred into crystal mother liquor containing 12% PEG400 as cryo protectant. Mounted in a nylon loop, the crystal was subsequently dipped into liquid nitrogen. The substrate-bound state was trapped by soaking an AspB crystal for a few seconds in cryo protectant solution supplemented with 100 mM L-aspartate, followed by immediately flash-cooling in liquid nitrogen. All these procedures were carried out at 4 ºC. Diffraction data for unliganded-AspB and AspB/L-Asp were collected at 100 K on single crystals at beamline ID14-1 of the ESRF, Grenoble, using an ADSC Quantum Q210 detector. The two data sets were indexed and integrated with the program XDS (26), and scaled using the program SCALA (27) from the CCP4 package [http://www.ccp4.ac.uk]. Data collection statistics are shown in Table 1.

The unliganded triclinic structure of AspB was determined by molecular replacement using the program MOLREP (28). A single subunit from the orthorhombic AspB structure (pdb accession number 1J3U, chain A) (21) was used as a search model. The presence of two independent tetramers in the P1 unit cell was verified by analysis of self-rotation patterson maps and calculation of the Matthews coefficient (solvent content is 58%). The protein structure was optimized by restrained refinement using REFMAC5 (29), alternated with manual model building using Coot (30). At the final stages of refinement, water molecules were added to the model based on strict geometrical and electron density criteria. For AspB/L-Asp, an initial map calculated with phases derived from the
unliganded triclinic structure of AspB, showed poor density for the SS-loops of chains D and H. Manual rebuilding of the loop regions, followed by restrained-refinement using REFMAC5, improved the electron density. Several additional cycles of manual model building and refinement, including the placement of water molecules into the structure, were carried out prior to addition of L-aspartate to the model. Each tetramer had a L-aspartate molecule bound in one of its active-site pockets, as revealed by inspection of $2F_o - F_c$ and $F_o - F_c$ fourier maps (Figure 1). The final refinement statistics for both structures are shown in Table 1.

![Figure 1](image)

**Figure 1.** $2F_o - F_c$ electron density maps of the SS-loop and bound L-Asp. (A) SS-loop of subunit C in unliganded-AspB and (B) in L-Asp bound AspB. (C) Bound L-Asp in the ABC active site of the AspB/L-Asp structure. Maps are contoured at 1σ. AspB residues and L-Asp are drawn in ball-and-stick representation and labeled.
Limited proteolysis of AspB

α-Chymotrypsin (Sigma, stock solution: 1 mg/ml in 1 mM HCl; 1 mM CaCl$_2$) was added to purified AspB (in 25 mM Tris buffer, pH 7.5, ~6.8 mg/ml) at a 1:1000 w/w ratio. The mixture was incubated at room temperature for two days and the proteolysis reaction was stopped by addition of PMSF at a final concentration of 1 mM. The proteolytic cleavage was followed by SDS-PAGE, revealing the production of a ~44 kDa stable proteolytic fragment. Without additional purification, the chymotrypsin-treated protein was used for crystallization, mass spectrometry and activity analysis. For MALDI-TOF mass spectrometry the fragment was mixed 1:1 v/v with a solution of α-cyano-4-hydroxycinnamic acid (10 mg/ml in 70% acetonitrile, 0.1% trifluoroacetic acid), spotted onto a stainless steel MALDI target and analyzed on a 4800 Proteomics analyzer (AB SCIEX) in the linear mode following standard procedures. The mass of the intact chymotrypsin-cleaved AspB fragment was characterized as 44231 (±12) Da, which matches the calculated mass of an AspB fragment comprising residues 1-401 (44235 Da).

Structure determination of chymotrypsin-treated AspB

Chymotrypsin-treated AspB (chymo-AspB) was crystallized by using the hanging-drop vapour diffusion method. The protein solution was mixed with crystallization solution which contained 0.2 M calcium acetate, 0.1 M HEPES pH 7.5 and 40% PEG400. Thick elongated plate-like crystals appeared in a few days and grew to a maximum size of 60 µm x 300 µm x 10 µm in about one week. For data collection, crystals were mounted in a nylon loop and frozen directly in liquid nitrogen. Diffraction data extending to 3.0 Å resolution were collected from a frozen crystal at beamline ID14-2 at the ESRF, Grenoble, equipped with an ADSC Quantum 4 detector. The data set was indexed and integrated using the program Mosflm (31) and scaled using the program SCALA from the CCP4 package (see Table 1 for data collection and processing statistics).

The structure of chymo-AspB (space group $P2_1$, eight molecules per asymmetric unit) was determined by molecular replacement with the program MOLREP. The unliganded AspB structure, with residues 402-466 removed, was used as search model. The obtained solution was initially refined by applying rigid-body and NCS-restrained refinement in REFMAC5. The model was completed by several rounds of restrained refinement using REFMAC5, interspersed with manual model building using COOT. Model refinement statistics are presented in Table 1.
**Structure analysis**

Structure validation was performed with Molprobity (32). Structure superpositions and calculation of RMSDs was done using Superpose (33).

**Figures**

Figures were prepared using the program PyMOL [http://pymol.sourceforge.net].

**Results**

**Structure determination of unliganded and L-Asp-bound AspB**

AspB was crystallized in a ligand-free state at a different condition and in a different crystal form from that reported previously (21). Crystals belong to space group $P1$ and contain two physiologically relevant tetramers per unit cell. The triclinic structure of AspB was determined at 2.4 Å resolution by molecular replacement, using the previously reported orthorhombic structure as a search model, and refined to an R-free of 25.3% and R-factor of 19.9%. The L-aspartate-bound state of AspB was captured by performing a quick soak of the triclinic crystals in 100 mM L-aspartate (L-Asp) at 277 K, immediately followed by flash-cooling in liquid nitrogen. Inspection of $F_o-F_c$ difference electron density maps revealed that in each of the two tetramers in the unit cell one of the four active sites contained an L-Asp molecule. The structure of L-Asp-bound AspB was determined and refined at 2.6 Å resolution to an R-free of 25.0% and R-factor of 19.3% (see Table 1 for a summary of the relevant crystallographic statistics). The subunits in the refined apo-AspB and AspB/L-Asp structures all contain 462 residues as there was insufficient electron density present to model residues 1-4 and 467-468 in each monomer. The overall structures of the two crystallographically independent tetramers in the unit cell are identical (the pairwise Cα-backbone root-mean-square-deviation (RMSD) is 0.07 and 0.11 Å for the two unliganded and L-Asp-bound AspB tetramers, respectively), as are the overall binding modes of the two aspartate molecules. For sake of clarity we will therefore mostly restrict our description and analysis to a single tetramer.

**Overall structure and substrate-induced conformational changes**

The unliganded AspB structure determined in the present study is identical to that reported previously by Fujii et al. (21). Briefly, AspB exists as a homo-tetramer with 222 point-symmetry (Figure 2). Each of the subunits has approximate dimensions of
40 Å x 50 Å x 100 Å and is composed of three structurally distinct domains: the so-called “N-terminal large domain” (residue 5-139), containing a two-stranded antiparallel β-hairpin followed by five α-helices, the “central helix domain” (residues 140-393), mainly containing six long anti-parallel α-helices, and the “C-terminal small domain” (residues 394-466), which consists of seven small α-helices. In the tetramer the central helix domain of each subunit interacts co-axially with those of the other three subunits to form a well-packed bundle of 20 α-helices. The N- and C-terminal domains of adjacent subunits meet at the ends of this helical bundle forming four beak-like protrusions (Figure 2B). Inside these protrusions are the four independent active sites of the AspB tetramer. Each active site is located at a pocket formed by two conserved N-terminal segments (residues 96-110 and 135-143) and a central domain segment (residues 183-194) of two adjacent subunits (for a representative active site we will refer to these subunits as A and B), that is complemented by a nearby SS-loop (residues 317-328) from a third subunit (referred to as C). In the unliganded AspB tetramer, each SS-loop interacts with the C-terminal domain of a neighboring subunit (e.g. the SS-loop of subunit C interacts with the C-terminal domain of subunit A), locking it in an open conformation away from the active site pocket. However, with substrate present in the active site, as observed in the L-Asp-bound AspB tetramer, the SS-loop adopts a dramatically different conformation, in which it no longer contacts the C-terminal domain, but instead closes over the active site allowing it to participate in substrate binding. The closed and open Cα backbone conformations of the SS-loop have an RMSD of ~6 Å with Ser319 showing the largest displacement of ~10 Å (Figure 2C).

As mentioned above, only one of the active sites in the tetramer of the AspB/L-Asp structure has a substrate bound (for convenience we have labeled this as the ABC active site, referring to its participating subunits). In the crystal, the ABC active site (and the equivalent EFG active site of the other, crystallographically independent tetramer) is adjacent to a solvent channel and not making any crystal packing contacts. In contrast, the other active sites are in areas where the tetramers make multiple crystal contacts, thereby restricting their conformational flexibility. Interestingly, the substrate-induced conformational change of the SS-loop at the ABC active site coincides with a significant increase in overall temperature factor of the C-terminal domain in subunit A, which
Figure 2. Overall structure of AspB.
(A) Ribbon representation of an AspB subunit with a rainbow color gradient from the N terminus (blue) to the C terminus (red). Secondary structure elements are indicated with numbers for α-helices and with letters for β-strands.
(B) Ribbon representation of the L-Asp bound AspB tetramer. Subunits A, B, C and D are labeled and are colored pale-yellow, cyan, red and purple, respectively. The bound L-Asp is drawn as a CPK model, colored in yellow, blue and red for carbon, nitrogen, and oxygen atoms, respectively.
(C) Superposition of the SS-loops at the ABC active site in the unliganded and L-Asp-bound AspB structures, showing the open-to-closed conformational change (green, unliganded-open conformation; red, liganded-closed conformation). Hydrogen bonds between Ser319 and L-Asp are depicted as dashed lines.
changes from ~57 Å$^2$ in the unliganded AspB structure to ~95 Å$^2$ in the AspB/L-Asp complex (the other C-terminal domains in the tetramer show a similar B-factor of ~40 Å$^2$ in both structures, see Figure 3). A similar effect is observed for the other tetramer present in the crystal. The higher mobility of the C-terminal domain at the substrate-bound active site is also apparent from the significant weaker electron density associated with this domain, as compared to the C-terminal domains that are adjacent to the unliganded active sites. Apparently, in the open conformation the SS-loop stabilizes the adjacent C-terminal domain, which becomes highly mobile when this loop detaches upon substrate binding. Apart from the conformational change of the SS-loop, and the increase in mobility of the adjacent C-terminal domain, no significant changes are observed in the AspB structure upon substrate binding.

![Figure 3](image.png)

**Figure 3.** Temperature factor distribution in the unliganded AspB and AspB/L-Asp tetrameric structures. Residue-averaged temperature factors range from 5 Å$^2$ (dark blue) to 127 Å$^2$ (red). The most ordered regions of AspB (lowest temperature factors) are located in the central tetramerization domains. The C-terminal domain adjacent to the L-Asp-bound active site in AspB/L-Asp shows the highest temperature factors, indicative of its high disorder.
**L-Asp is bound in a high energy, enediolate-like conformation**

The bound L-Asp substrate in the active site of AspB is stabilized by an extensive number of interactions (Figure 4), most of which have previously been predicted based on modeling and mutagenesis studies (21,23). Interestingly, and not noted previously, the substrate binding interactions force the side chain of the bound L-Asp to adopt an energetically unfavorable rotamer in which the Cα, Cβ and β-carboxylic atoms are almost co-planar, thus resembling the conformation of the putative enediolate intermediate in catalysis.

**Figure 4.** L-Asp binding mode. (A) Stereo view of the AspB active site with bound L-Asp (yellow) showing the residues that form hydrogen bonds with the substrate. The coloring scheme is the same as in Figure 1. (B) Schematic representation of the interactions between L-Asp and AspB. Hydrogen bonds are represented as dashed lines. The putative role of Ser318 as catalytic base, abstracting the pro-R hydrogen from the Cβ-carbon of the substrate, is depicted with an arrow.
As explained above, the residues of AspB that bind the substrate originate from three different subunits and are part of four conserved regions: residues 96-110 and 135-143 from subunit B, residues 183-194 from subunit A and residues 317-328 (the SS-loop) from subunit C. The α-amino group of L-Asp is hydrogen bonded to the side chains of Thr101, Asn142 and His188, while the α-carboxylate group forms hydrogen bonds with the side chains of Asn142, Thr187 and Lys324. The positively charged side chain of Lys324 further stabilizes the negative charge on the α-carboxylate group by an electrostatic interaction, which is crucial for the functioning of AspB as shown previously by site-directed mutagenesis (23). The oxygens of the β-carboxylate group of L-Asp (Oδ1 and Oδ2) are in hydrogen bond distance to four side chain hydroxyl groups and two main chain amides of AspB (Thr101, Ser140, Thr141 and Ser319). However, considering that in its ground state the Oδ1 and Oδ2 atoms of aspartate are sp² hybridized, some of the potential hydrogen bonds show an unfavourable geometry. All hydrogen bonds would be appropriate, though, for stabilizing a high energy conformation of the side chain in which the Oδ1 and Oδ2 atoms are sp³ hybridized, consistent with a polarization of the β-carboxylate C-O bonds as is expected to occur in the enediolate-like transition state and enediolate intermediate (18,20,23). Hence, in addition to its role in substrate binding, this extensive hydrogen-bonding network with the β-carboxylate group appears to be important for stabilizing the additional negative charge that develops on one of the carboxylate oxygens upon proton abstraction. Notably, there is no nearby positively charged residue to assist in the stabilization of the negatively charged β-carboxylate group. However, similar as suggested previously based on substrate and product-bound structures of E. coli adenylosuccinate lyase (3) the negative charge(s) on the β-carboxylate group of the substrate or enediolate intermediate may be further stabilized by an electrostatic interaction with the dipole moment of helix α6 (Figure 5A). The AspB/L-Asp structure further provides clear evidence that the SS-loop contributes significantly to substrate binding. In addition to the above mentioned interactions of Ser319 and Lys324 with the two carboxylate groups of the substrate, the carbonyl oxygen of Gly317 and the side chain of Ser318 make van der Waals contacts with the Cα and Cβ carbons of L-Asp. Noteworthy, the Oγ oxygen of Ser318 is positioned at a distance of only 3.6 Å from the Cβ carbon of L-Asp and at an angle that would be suitable for abstracting the Cβ pro-R proton.
Figure 5. Structural comparison of AspB with other aspartase/fumarase superfamily members.

(A) Overlay of the active sites of AspB/L-Asp (coloring scheme as in Figure 2, L-Asp in yellow) and ecADL-H171A/ADS (grey, ADS in green), showing the conserved binding mode of the common succinyl-moieties. Residue labels of ecADL are given in parentheses. Dashed lines indicate the interactions of Ser318 with the amide backbones of Ile320 and Met321, and with the Cβ carbon of L-AspB.

(B). Comparison of SS-loop conformations in structures of different aspartase/fumarase superfamily members, i.e. of unliganded-AspB (light-green), L-Asp bound AspB (red), citrate bound fumarase C from E. coli (PDB ID 1FUO, magenta) (10), unliganded duck δ2-crystallin (PDB-ID: 1HY1, cyan) (2), ADS-bound ecADL-H171A (PDB-ID: 2PTR, grey) (3), ecADL-H171N bound with AMP and fumarate (PDB-ID: 2PTQ, light-brown) (3), and duck δ1-crystallin with bound sulfate ion (PDB-ID: 1HY0, dark-brown) (2).

Structural comparison with other aspartase/fumarase superfamily members

The biological significance of the AspB/L-Asp structure was further analysed by comparing it to the structure of the His171Ala mutant of E. coli adenylosuccinate lyase with bound adenylosuccinate (ecADL-H171A/ADS) (3), one of the few aspartase/fumarase superfamily members for which a structure of its complex with uncleaved natural substrate has been reported. This comparison revealed that in one of the two crystallographically independent substrate molecules in the ecADL-H171A/ADS
crystal, the succinyl moiety has a similar enediolate-like conformation as the bound L-Asp molecules in the AspB crystal (Figure 5A). The second bound adenylosuccinate (ADS) molecule in ecADL-H171A/ADS exhibits a low energy rotamer conformation of its succinyl moiety, but the adjacent SS-loop is highly disordered and not showing a closed conformation, suggesting that the observed binding mode of this substrate molecule is less tight and probably nonproductive. For the formerly mentioned ADS molecule the binding mode of its succinyl moiety in the active site of ecADL-H171A is highly similar to that of L-Asp in AspB, with the two carboxylate groups forming an almost identical hydrogen bonding network with residues His91, Thr122, Ser123, Thr170, Ser296 and Lys301 (the equivalents of Thr101, Ser140, Thr141, Thr187, Ser319 and Lys324 in AspB). Moreover, the SS-loop adjacent to the bound substrate in ecADL-H171A adopts a closed conformation, like the SS-loop in the L-Asp-bound active site of AspB, with Ser295 (the equivalent of Ser318 in AspB) positioned close to the Cβ carbon of the succinyl moiety. It thus appears that the key features of the substrate binding mode in AspB/L-Asp, i.e. an extensive hydrogen bonding network to stabilize and polarize the substrate’s β-carboxylate group, closure of the SS-loop and the positioning of one of the conserved SS-loop serine residues close to the substrate’s Cβ atom, are all conserved in ecADL-H171A/ADS, emphasizing the close relationship of AspB and ecADL.

**Conformational flexibility of the SS-loop and its importance for activity**

The present AspB structures reveal a large conformational change of the SS-loop from an open conformation in the absence of substrate to one that closes over the active site in the presence of substrate. The closed conformation of the SS-loop in AspB/L-Asp is similar to that observed in the structures of ecADL and duck δ1-crystallin with bound substrate, product or substrate-mimicks (Figure 5B). The open conformation of the SS-loop in the absence of bound substrate is similar to that in the previously reported unliganded structures of AspB, duck δ2-crystallin and citrate-bound E. coli fumarase C. In the unliganded structures of other aspartase/fumarase superfamily members, including E. coli AspA, the SS-loop is highly disordered. In contrast, in our AspB structures the SS-loops associated with the empty active sites are relatively ordered and adopt the same open conformation, as shown by an average pairwise backbone RMSD of 0.7 Å² (calculated for residues 317-324). The open conformation of the SS-loop is stabilized by hydrophobic contacts of the side chains of Ile320 and Met321 with a hydrophobic surface patch on the
adjacent C-terminal domain (formed by residues Ile406, Ile409, Tyr418, Ala421, Ala422, Ala425, and Tyr429, Figure 6).

**Figure 6.** Stabilization of the open SS-loop conformation. Stereo view of the open SS-loop (red, subunit C, ribbon representation) and its contacts with the C-terminal domain (yellow, subunit A, surface representation) in the unliganded AspB structure. Subunit B is not shown for clarity. The side chains of Ile320, Met321, and Pro322 in the SS-loop are shown in ball-and-stick representations. The hydrophobic residues of the C-terminal domain of subunit A that contact these three SS-loop residues are colored in grey and identified by black labels. Also shown are Ser318 and Ser319. Dashed lines indicate the interactions of Ser318 with the amide backbones of Ile320 and Met321.

The functional importance of the interactions of the SS-loop in the open and closed conformations was studied by creating five single site-directed mutations, i.e. Ser318Ala, Ser319Ala, Ile320Ala, Met321Ala and Pro322Ala. Each mutant was purified to homogeneity and analysed for its deamination activity towards L-aspartic acid (Table 2). The Ser319Ala mutant, like the Pro322Ala mutant, shows kinetic parameters similar to those of wild-type AspB, indicating that the contribution of the conserved Ser319 residue to the hydrogen-bonding network with the β-carboxylate group of L-Asp is not that significant. In contrast, mutation of Ser318 to an alanine causes a complete inactivation of the enzyme, consistent with a direct role of this residue in AspB catalysis, most likely as the general base catalyst, like has been proposed for Ser295 in ecADL (3). Surprisingly, for the Ile320Ala and Met321Ala mutants also a large decrease in catalytic efficiency was observed, primarily due to a large increase in $K_m$ values. In the closed conformation of the
SS-loop, residues Ile320 and Met321 point away from the active site and are largely solvent exposed, thus one would not expect such a large decrease in activity and substrate binding affinity upon mutation. However, the mutation of Ile320 or Met321 to an alanine residue may destabilize the open conformation of the SS-loop by disrupting the interaction with the C-terminal domain. We therefore suggest that the decreased catalytic efficiency of these AspB mutants may be caused by an increased disorder of the SS-loop, thereby preventing efficient loop closure, which, in turn, may affect substrate binding and positioning.

**Proteolytic removal of the C-terminal domain inactivates AspB**

The highly reduced catalytic efficiency of the Ile320Ala and Met321Ala mutants suggests that the interactions between the open SS-loop and the C-terminal domain of AspB are important for its enzymatic function. To provide further evidence to support this hypothesis, AspB was subjected to limited proteolysis, aiming at the full or partial deletion of the C-terminal domain. Using \( \alpha \)-chymotrypsin it was possible to generate a stable proteolytic fragment of AspB, which remained tetrameric but showed a subunit size of 44 kDa (as judged from SDS-PAGE and gel filtration), consistent with the absence of the C-terminal domain (Figure 7A). Further analysis of chymotrypsin-treated AspB (hereafter named chymo-AspB) by mass spectrometry revealed that cleavage had occurred after residue Tyr401, which is located within a surface exposed helix (\( \alpha \)14) at the beginning of the C-terminal domain. Kinetic analysis showed that chymo-AspB exhibits no detectable activity towards L-Asp (data not shown).

To identify the effects of removing the C-terminal domain on the rest of the AspB structure, chymo-AspB was crystallized and its crystal structure determined and refined at 3.0 Å resolution to an R-free of 24.0 % and R-factor of 29.7 % (Table 1). Other than the absence of the C-terminal domains, no significant overall structural differences were observed in the chymo-AspB tetramer compared to the unliganded, full-length AspB tetramer (the average tetrameric C\( \alpha \)-backbone RMSD is 0.4 Å for four times 382 common residues, excluding the SS-loops). Near the active site, the only local conformational consequence of removing the C-terminal domain concerns the SS-loop. In two of the subunits of the chymo-AspB tetramer poor electron density is observed for the SS-loop, indicative of a high degree of disorder.
Figure 7. Preparation and crystallographic analysis of chymo-AspB. (A) SDS-PAGE gel showing the appearance of a stable 44 kDa AspB fragment after incubation with α-chymotrypsin at room temperature. Lane 1: control AspB (without chymotrypsin, calculated molecular weight is 51.6 kDa); lane 2: AspB after 2 days incubation with chymotrypsin at a ratio of 1:10⁴ (w/w, AspB:chymotrypsin); lane 3: AspB after 2 days incubation with chymotrypsin at a ratio of 1:10³. (B) Crystal packing of chymo-AspB. The chymo-AspB backbones are drawn as lines and colored dark gray for tetramer ABCD and its symmetry-related equivalents (top and bottom layer) and light gray for tetramer EFGH and its symmetry related equivalents (middle layer). The unit cell is drawn and labeled (only yz plane, the x-axis is approximately perpendicular to the figure). Also shown is the fulllength AspB tetramer (ribbon representation, colored as in Figure 1), superimposed to tetramer EFGH of chymo-AspB. The superposition reveals that the C-terminal domains are indeed absent in chymo-AspB, as they would clash with neighboring molecules in the crystal.

In the other two subunits the SS-loop adopts a defined conformation, but that conformation is stabilized by crystal contacts of residues Ile320, Met321 and Pro322 with residues of a neighboring tetramer, and differs from the open or closed conformation in the full length AspB structures. Thus it seems that, like for the Ile320Ala and Met321Ala mutants, the negative effect on AspB activity upon removal of the C-terminal domain is related to a disruption of the SS-loop/C-terminal domain interactions, resulting in an increased disorder of the SS-loop.
Discussion

The structure of the AspB/L-Asp complex determined in this study provides strong experimental support for the proposal that conversion of L-Asp to fumarate and ammonia proceeds via general base-catalyzed formation of an enzyme-stabilized enediolate intermediate (Figure 8). The highly structured active site environment, the observed high energy conformation of the bound substrate, the closed SS-loop conformation and the extensive hydrogen-bonding network with the substrate’s β-carboxylate group show how the active site of AspB is primed for stabilizing an enediolate-like transition state and enediolate intermediate.

Our results further reveal that the SS-loop serves a crucial role in the catalytic mechanism of AspB. Its closure over the active site provides additional substrate binding stabilization and ensures that the substrate is sequestered from the solvent during catalysis. A similar closure of the SS-loop has been observed in the crystal structures of ecADL with bound substrate or product, and duck δ1-crystallin with bound sulfate (2,3). However, more often the SS-loop is found to be disordered or in an open conformation in structures of other aspartase/fumarase superfamily members, even in the presence of substrate in the active site (5,8,10). Closure of the SS-loop is thus not only dependent on the presence of a succinyl-moiety (or other ligand) in the enzyme active site. In fact, as shown in this study for AspB, the C-terminal domain may play a crucial role in controlling the conformation change of the SS-loop, and thus in regulating the enzyme activity. Hence, the factors that govern closure of the SS-loop are highly complex. Most importantly, we observed that closure of the SS-loop positions Ser318 near the Cβ proton of L-Asp, in a suitable orientation to allow proton abstraction in the first step of the reaction. Although the involvement of serine as a catalytic base is highly unusual given its intrinsically high pK\textsubscript{a} value, such a role for Ser318 in AspB is supported by several additional observations. First, the participation of a basic group in AspB catalysis has been revealed by kinetic studies (23), and there are no AspB residues other than Ser318 in close vicinity of the Cβ carbon of L-Asp that could act as catalytic base. Secondly, the complete lack of activity of the Ser318Ala mutant and the strict conservation of this residue across all members of the aspartase/fumarase superfamily strongly indicate that Ser318 has an essential role in AspB catalysis. Finally, a role as catalytic base has also been proposed for the SS-loop Ser295 of ecADL (3), and the structural comparison of AspB/L-Asp and substrate-bound ecADL.
shows that Ser318 of AspB and Ser295 of *E. coli* ADL are structurally and functionally equivalent.

**Figure 8.** A schematic representation of the proposed catalytic mechanism of AspB.
The question remains, though, how Ser318 is activated to function as a catalytic base. A possible mechanism would be the deprotonation of Ser318 by a neighboring base to form a Ser-O⁻ oxyanion, which then abstracts the Cβ-proton of L-Asp. Such a mechanism, however, requires a substantial perturbation of the pKₐ of Ser318 by its microenvironment in the substrate-bound active site (the predicted intrinsic pKₐ of the serine-OH group is 15). Stabilization of the serine oxyanion (and resulting decrease of the pKₐ value of the Ser-OH species) may be realized due to interactions with the backbone amides of Ile320 and Met321 (Figure 5A), similar to what has been proposed for Ser295 in ecADL (3). Furthermore, a role of the substrate in activating the SS-loop serine could be considered, with the β-carboxylate group acting as a base to generate the serine oxyanion, like has been proposed for Plasmodium falciparum adenylosuccinate lyase (34). In pH profiles (pH 5.8-8.8) of this lyase, the group corresponding to the catalytic base gets ionized in the enzyme-substrate complex but not in the free enzyme. This suggests that in the reaction catalyzed by P. falciparum adenylosuccinate lyase the substrate (succinyl-adenosine monophosphate) assists in the ionization of the catalytic base. However, in the case of AspB previous kinetic studies revealed that the group corresponding to the catalytic base does already ionize in the substrate-free enzyme (pKₐ ~7.1), and its pKₐ value undergoes only a relatively small extra decrease in the enzyme-substrate complex (pKₐ ~6.2) (23). In the open SS-loop conformation Ser318 of AspB forms similar interactions with the amides of Ile320 and Met321 as in the closed SS-loop conformation (Figure 6), suggesting that these interactions are the main contributors to the serine oxyanion stabilization. An additional contribution from the substrate cannot be excluded, though. Given the large displacement of Ser318 upon substrate binding, the already perturbed pKₐ of the catalytic base in the free enzyme may obscure the effect of L-Asp on ionization of the catalytic base when positioned in the active site upon SS-loop closure. Hence, the current data do not allow us to determine the exact mechanism how Ser318 is activated as a catalytic base and whether substrate assists in ionizing the catalytic serine residue.

In conclusion, the high-resolution structure of the AspB/L-Asp complex described herein provides the first detailed view of the substrate-bound active site of an aspartase, allowing a better understanding of the roles of the various active site residues in substrate binding and catalysis. Our results further give credence to the notion that all aspartase/fumarase superfamily members use a common catalytic mechanism, which
involves general base-catalyzed formation of an enzyme-stabilized enediolate intermediate and the participation of the SS-loop in substrate binding and catalysis.

Acknowledgements

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References


Chapter 3

Aspartate ammonia lyase AspB from *Bacillus* sp. YM55-1


Chapter 3  Aspartate ammonia lyase AspB from Bacillus sp. YM55-1


### Table 1. Data Collection and Refinement Statistics

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<td>0.108 (0.505)</td>
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* Data between parentheses are for the highest resolution shell

† $R_{work} = \frac{\sum_{hkl} \|F_{obs}\| - \|F_{calc}\| \|F_{obs}\|}{\sum_{hkl} \|F_{obs}\|}$, where the crystallographic R-factor was calculated with 95% of the data used in the refinement; $R_{free} = \frac{\sum_{hkl} \|F_{obs}\|}{\sum_{hkl} \|F_{obs}\|}$, where the crystallographic R-factor based on 5% of the data withheld from the refinement for cross validation.
Table 2. Kinetic parameters for the deamination of L-aspartic acid by wild-type AspB-His and the SS-loop mutants

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<tr>
<th>Enzyme</th>
<th>$K_m$ (mM)</th>
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<th>$k_{cat}/K_m$ [M$^{-1}$s$^{-1}$]</th>
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<td>Wild type§</td>
<td>15 ± 2</td>
<td>40 ± 7</td>
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<tr>
<td>S318A</td>
<td>-</td>
<td>-</td>
<td>&lt;$10^3$</td>
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<tr>
<td>S319A</td>
<td>28 ± 4</td>
<td>17 ± 1</td>
<td>$6.1 \times 10^3$</td>
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<td>I320A</td>
<td>&gt;100</td>
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<td>M321A</td>
<td>&gt;100</td>
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<td>P322A</td>
<td>11 ± 1</td>
<td>21 ± 0.5</td>
<td>$1.9 \times 10^3$</td>
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§The kinetic data were obtained from Puthan Veetil et al. (23)

Errors are standard deviations from each fit.
Chapter 3

Aspartate ammonia lyase AspB from *Bacillus* sp. YM55-1