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Biocatalytic Enantioselective Synthesis of *N*-Substituted Aspartic Acids by Aspartate Ammonia Lyase

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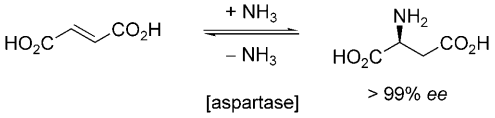
Abstract: The gene encoding aspartate ammonia lyase (*aspB*) from *Bacillus* sp. YM55-1 has been cloned and overexpressed, and the recombinant enzyme containing a C-terminal His_6 tag has been purified to homogeneity and subjected to kinetic characterization. Kinetic studies have shown that the His_6 tag does not affect AspB activity. The enzyme processes L-aspartic acid, but not D-aspartic acid, with a K_m of ≈ 15 mM and a k_{cat} of $\approx 40\ s^{-1}$. By using this recombinant enzyme in the reverse reaction, a set of four *N*-substituted aspartic acids were prepared by the Michael addition of hydroxylamine, hydrazine, methoxylamine, and methylamine to fumarate. Both hydroxylamine and hydrazine were found to be excellent substrates for AspB. The k_{cat} values are comparable to those observed for the AspB-catalyzed addition of ammonia to fumarate ($\approx 90\ s^{-1}$), whereas the K_m values are only slightly higher. The products of the enzyme-catalyzed addition of hydrazine, methoxylamine, and methylamine to fumarate were isolated and characterized by NMR spectroscopy and HPLC analysis, which revealed that AspB catalyzes all the additions with excellent enantioselectivity (>97% *ee*). Its broad nucleophile specificity and high catalytic activity make AspB an attractive enzyme for the enantioselective synthesis of *N*-substituted aspartic acids, which are interesting building blocks for peptide and pharmaceutical synthesis as well as for peptidomimetics.

Keywords: amino acids · biotransformations · enzyme catalysis · kinetics · lyases

Introduction

Aspartate ammonia lyases (also referred to as aspartases; EC 4.3.1.1) are microbial enzymes that play a key role in nitrogen metabolism by catalyzing the reversible elimination of ammonia from L-aspartate to yield fumarate (Scheme 1).



Scheme 1. General reaction of aspartase.

Several related aspartases have been purified and characterized from Gram-positive and -negative bacteria, including *E. coli*,[1] *Hafnia alvei*,[2] *Pseudomonas fluorescens*,[3] *Bacillus subtilis*,[4] and *Bacillus* sp. YM55-1.[5] The aspartase from *E. coli* (AspA) has been studied most extensively, and its crystal structure has been elucidated.[6] AspA functions as a homotetramer with each monomer consisting of 478 amino acid residues, and the enzyme is allosterically activated by its substrate (L-aspartate) and Mg^{2+} ions, which are required for activity at alkaline pH. It is proposed that AspA carries out an *anti* elimination reaction in which an active-site base abstracts a proton from C-3 of L-aspartate to form an enzyme-stabilized enolate intermediate.[7] This forms upon elimination of ammonia the product, fumarate. The rate-determining step is the cleavage of the carbon–nitrogen bond, which may be facilitated by a general acid that protonates the leaving ammonia group. Although several active-site res-

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idues have been investigated by site-directed mutagenesis, kinetic analysis, and chemical modification, the essential catalytic base and presumed catalytic acid have not yet been identified.[6–8]

AspA is one of the most specific enzymes known.[9] Extensive studies over the last 80 years have shown that no other substrates can replace L-aspartic acid in the deamination reaction.[10] Only the suicide substrate L-aspartate-β-semialdehyde is deaminated by AspA, but the enzyme is at the same time irreversibly deactivated.[11] In contrast, many competitive inhibitors have been reported and these all require a carboxylic acid or similarly charged functional group at each end of the carbon chain.[7] Interestingly, Emery reported in 1963 that hydroxylamine is an alternative nucleophile for aspartase, but the *N*-hydroxyaspartic acid produced was too unstable for isolation.[12] The high selectivity of AspA for its natural substrates limits the practical application of this enzyme. The reverse reaction catalyzed by AspA, that is, the amination of fumarate, is used commercially in the industrial production of the artificial sweetener aspartame (*N*-L-α-aspartyl-L-phenylalanine 1-methyl ester).[13,14] An excess of ammonia is used in this process to drive the equilibrium from fumarate towards L-aspartic acid. The yields are usually quantitative and L-aspartic acid is obtained with an enantiomeric excess of $>99\,\%$.

In our studies we have focussed on aspartase (AspB) from the thermophilic bacterium *Bacillus* sp. YM55-1.[5] This aspartase is an interesting enzyme for industrial application because of its high activity, relative thermostability, and lack of allosteric regulation by substrate or metal ions. The crystal structure of AspB was elucidated in 2003 by Fujii et al.[15] The overall topology and active-site structure of AspB are similar to those observed in AspA and fumarase C (FumC) from *E. coli*, which confirms its membership in the aspartase/fumarase superfamily of enzymes. Like AspA, AspB functions as a homotetramer in which each subunit is composed of 468 amino acid residues. To date, there is no crystal structure available of AspB (or any other aspartase) complexed to a substrate or product, and details of the catalytic mechanism have not yet been elucidated.

Herein, we report the cloning and overexpression of the *aspB* gene in *E. coli* and the efficient one-step affinity purification of the recombinant His_6-tagged enzyme. By using ^{1}H NMR spectroscopy, the purified enzyme was extensively screened for its ability to process alternative substrates. We have identified four amines that can replace ammonia as the substrate in the AspB-catalyzed Michael addition to fumarate. The resulting *N*-substituted aspartic acids are interesting building blocks for peptide synthesis and peptidomimetics.

Results and Discussion

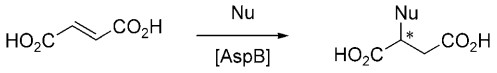
Expression, purification, and characterization of AspB: The *aspB* gene was amplified from plasmid pUCBA and cloned in-frame with both the initiation ATG start codon and the sequences that code for the *myc* epitope and polyhistidine region of the expression vector pBAD/*Myc*-His A, which resulted in the construct pBAD(AspB-His). Sequencing of the cloned gene verified that no mutations had been introduced during the amplification and cloning procedures. The *aspB* gene in pBAD(AspB-His) is under transcriptional control of the *araBAD* promoter and recombinant aspartase containing the C-terminal fusion peptide was produced upon induction with arabinose in soluble and active form in *E. coli* TOP10. Expression of the *aspB* gene was most efficient when cells were cultivated at 37 °C and when 0.04 % (w/v) arabinose was used. The recombinant enzyme was purified by a one-step protocol (the C-terminal His_6 tag forms a metal binding site for affinity purification on metal-chelating resin), which typically provided 20–30 mg of homogeneous enzyme per liter of culture. The His_6-tagged AspB was found to migrate during non-denaturing polyacrylamide gel electrophoresis with a similar mobility to that of native AspB carrying no fusion tag (G. J. Poelarends (2008), unpublished results), which suggests that gross conformational changes do not occur and that the homotetrameric quaternary structure of native AspB is maintained in the His_6-tagged enzyme.

A mixture containing His_6-tagged AspB and L-aspartic acid was monitored by ^{1}H NMR spectroscopy to verify that the product of the reaction is fumarate, as previously reported for the aspartase enzyme from *E. coli*. The enzymatic conversion of L-aspartic acid yields fumarate, as indicated by a singlet at $\delta = 6.39$ ppm. This spectrum is identical to an independent sample,[16] which confirms that fumarate is the product of the AspB-catalyzed conversion of L-aspartic acid. The rate of deamination of L-aspartate by His_6-tagged AspB was monitored spectrophotometrically by following the formation of fumarate at 240 nm in 50 mM NaH_2PO_4 buffer (pH 8.5) at 25 °C. A k_{cat} value of $(40 \pm 7)\ s^{-1}$ and a K_m of (15 ± 2) mM were found, which results in a k_{cat}/K_m of $\approx 2.7 \times 10^3\ M^{-1}\,s^{-1}$. Similar kinetic parameters have previously been found for purified AspB in its native form.[5]

Because the C-terminal fusion peptide does not interfere with the structural and enzymatic properties of AspB, and because purification of His_6-tagged AspB is very efficient, this AspB variant has been used for all of the experiments described below.

Screening for alternative substrates: *Amino acids*: Several amino acids were tested as potential substrates of AspB. The deamination reactions were monitored by using a colorimetric assay that follows ammonia production upon incubation with 25 mM substrate in 100 mM Na_2HPO_4 buffer (pH 9.0) at 37 °C. The compounds D-aspartic acid, L-cysteine, L-histidine, L-phenylalanine, L-glutamine, L-tyrosine, L-serine, L-alanine, L-valine, L-leucine, L-threonine, L-lysine, α-methyl-DL-aspartic acid, β-methyl-DL-aspartic acid, L-glutamate, β-alanine, β-DL-aminobutyric acid, β-asparagine, β-phenylalanine, and β-leucine were not processed by AspB. This screening thus failed to identify any alternative amino acid substrate that can replace L-aspartic acid. The specificity for L-aspartic acid has also been clearly demonstrated for the corresponding aspartase (AspA) from *E. coli*.[7]

Nucleophiles: It has previously been determined that *E. coli* aspartase can catalyze the reverse reaction, the *trans* addition of ammonia to fumarate, with high stereoselectivity, exclusively yielding L-aspartic acid.[7] This observation prompted us to examine whether AspB catalyzes the stereoselective amination of fumarate and whether different nucleophiles can be used in this enzyme-catalyzed Michael addition reaction (Scheme 2). The addition reactions were



Scheme 2. AspB-catalyzed addition of various nucleophiles to fumarate.

monitored by ^{1}H NMR spectroscopy under a variety of conditions, including different enzyme concentrations and different pH values (7.0, 8.0, and 9.5). Representative conversions for each reaction are summarized in Table 1. Interestingly, we observed a robust activity of AspB with hydroxylamine and hydrazine, and a small but significant activity with methoxylamine and methylamine. In the absence of AspB, these amines do not react with fumarate.

Table 1. Addition of various nucleophiles to fumarate catalyzed by AspB in phosphate buffer in an NMR tube with a volume of 0.6 mL.[a]

Entry	Nucleophile	pH	Time	Conversion [%]
1	H_2NOH[b]	7.0	20 min	100
2	$MeONH_2$[c]	7.0	12 days	100
3	$MeONH_2$[c]	8.0	6 days	100
4	H_2NNH_2[c]	7.0	1 days	100
5	$MeNH_2$[d]	8.0	7 days	100
6	$EtNH_2$[d]	8.0	14 days	0
7	$EtNH_2$[d]	9.5	14 days	0
8	$EtNH_2$[e]	8.0	14 days	0
9	NaN_3[c]	7.0	14 days	0
10	NaCN[c]	7.0	14 days	0
11	NaCN[c]	8.0	14 days	0
12	NaOCN[c]	7.0	14 days	0
13	NaOCN[c]	8.0	14 days	0
14	Gly[d]	8.0	14 days	0
15	Gly[d]	9.5	14 days	0
16	Gly[e]	8.0	14 days	0
17	formamide[d]	8.0	14 days	0
18	formamide[d]	9.5	14 days	0
19	formamide[e]	8.0	14 days	0

[a] Unless indicated otherwise, 250 μmol nucleophiles, 25.0 μmol fumarate, and 50 mM phosphate buffer in D_2O were used. [b] 1.12 μmol AspB, 200 μmol nucleophile, 20.0 μmol fumarate. [c] 9.34 μmol AspB. [d] 8.95 μmol AspB. [e] 17.9 μmol AspB.

Hydroxylamine appears to be the best alternative substrate for AspB (Table 1, entry 1). The ^{1}H NMR spectrum recorded 20 min after the addition of the enzyme showed the complete disappearance of the signals corresponding to fumarate ($\delta = 6.41$ ppm) and the formation of new signals corresponding to the expected *N*-hydroxyaspartic acid ($\delta =$ 3.65 (dd, 1H), 2.50 (dd, 1H), 2.29 ppm (dd, 1H); see the Supporting Information). The NMR spectra recorded at a later stage were rather complex, which we assume results from chemical decomposition of the *N*-hydroxyaspartic acid. The instability of this class of compounds has previously been reported by Emery[12] and Ottenheijm and Herscheid.[17] Methoxylamine is a poor substrate for AspB. The spectrum recorded 1 day after the addition of enzyme showed hardly any conversion of the starting material, but after 2 weeks of incubation at pH 7.0, the spectrum showed complete loss of the signals corresponding to fumarate (Table 1, entry 2) and the formation of new signals corresponding to the expected *N*-methoxyaspartic acid ($\delta = 2.39$ (dd, 1H), 2.58 (d, 1H), 3.59 (s, 3H), 3.85 ppm (dd, 1H); see the Supporting Information). We repeated the screening at pH 8.0, and it was established that after 1 week the reaction was complete (Table 1, entry 3). When hydrazine was used as the nucleophile, complete disappearance of the starting material and the formation of the expected 2-hydrazinosuccinic acid ($\delta = 2.31$ (dd, 1H), 2.49 (dd, 1H), 3.49 ppm (dd, 1H); see the Supporting Information) was observed after incubation for 1 day (Table 1, entry 4). By using methylamine as the nucleophile, the reaction was complete after 1 week of incubation at pH 8.0 and the expected *N*-methylaspartic acid ($\delta = 2.62$ (s, 3H), 2.88 (d, 2H), 3.75 ppm (dd, 1H); see the Supporting Information) was formed (Table 1, entry 5).

The data clearly show that AspB efficiently processes small substituted amines such as hydroxylamine and hydrazine, but displays very low (methoxylamine and methylamine) or no (ethylamine, glycine, and formamide) activity with larger amine nucleophiles. Small charged nucleophiles (azide, cyanide, and cyanate) are also not processed. Taken together, these observations suggest that the nucleophile binding pocket of AspB is designed to bind small amine compounds and excludes any charged or large nucleophiles.

In the following sections we describe the results of more detailed studies of the AspB-catalyzed addition reactions of four alternative nucleophiles, including the steady-state kinetic parameters and characterization of the generated *N*-substituted aspartic acid products.

Kinetic measurements: The rate of amination of fumarate catalyzed by AspB was measured by following the decrease in absorbance at 270 nm in phosphate buffer (pH 8.0) at 22 °C. Both hydroxylamine and hydrazine are good substrates for the enzyme and the kinetic parameters are summarized in Tables 2 and 3. The data clearly show that AspB processes both amines with similar catalytic efficiency. Whereas the k_{cat} values are comparable to those observed for the AspB-catalyzed addition of ammonia to fumarate, the K_m values are slightly higher (1.8-fold for hydroxylamine and 3.6-fold for hydrazine, Table 2). As a result, the k_{cat}/K_m values are 1.6- and 3.4-fold lower for hydroxylamine and hydrazine compared with ammonia, respectively.

The kinetic parameters also show that similar K_m values for fumarate are found for the three AspB-catalyzed amination reactions (Table 3). The k_{cat} values obtained with a fixed concentration of amine nucleophile (Table 3) are slightly lower than those obtained with a fixed and saturat-

Table 2. Kinetic parameters for the AspB-catalyzed addition of different amine nucleophiles to fumarate.[a]

Entry	Nucleophile	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$L\,mol^{-1}\,s^{-1}$]
1	NH_3[b]	85±40	89±12	1040
2	H_2NOH[b]	151±22	99±5.5	654.3
3	H_2NNH_2[c]	308±49	94±7.6	304.1

[a] Steady-state kinetic parameters were measured using a fixed concentration of 20 mM fumarate in 50 mM phosphate buffer (pH 8.0) at 22 °C. Errors are standard deviations. [b] [AspB] = 0.041 μM. [c] [AspB] = 0.082 μM.

Table 3. Kinetic parameters for the AspB-catalyzed amination of fumarate using different nucleophiles at a fixed concentration.[a]

Entry	Nucleophile	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$L\,mol^{-1}\,s^{-1}$]
1	NH_3[b]	1.61±0.76	59±17	36414
2	H_2NOH[b]	2.78±0.56	92±11	33166
3	H_2NNH_2[c]	1.54±0.24	31±6.8	20193

[a] Steady-state kinetic parameters were measured at fixed nucleophile concentrations ([NH_3] = 200 mM, [H_2NOH] = 400 mM, and [H_2NNH_2] = 750 mM) in 50 mM phosphate buffer (pH 8.0) at 22 °C. Errors are standard deviations. [b] [AspB] = 0.0179 μM. [c] [AspB] = 0.0358 μM.

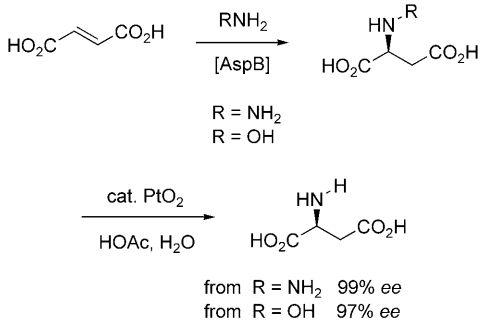
ing concentration of fumarate (Table 2).[18] Nevertheless, these observations suggest that the three different amine nucleophiles are optimally positioned in the active site of AspB to undergo an amination reaction, and that the binding and positioning of fumarate is not significantly affected by the binding of the different amine nucleophiles.

Under the conditions used, the observed initial rates for the AspB-catalyzed addition of methoxylamine or methylamine to fumarate were too low to measure accurate kinetic parameters. Although a structural basis for this observation is not yet known, the lower rates observed with methoxylamine and methylamine suggest that these slightly larger amines are not optimally bound in the active site of AspB to undergo an addition reaction.

Isolation and characterization of the products of the AspB-catalyzed amination reactions: The AspB-catalyzed amine additions to fumarate were performed routinely in 5 mM phosphate buffer at pH 8.0 and 37 °C. The enzyme concentration was varied depending on the rate of the reaction. To isolate and characterize *N*-hydroxyaspartic acid, the addition of hydroxylamine to fumarate was scaled up to 1.0 mmol of substrate. By using 9.34 μmol (0.9 mol%) of enzyme the reaction was complete within 15 min, as assessed both by UV analysis and ^{1}H NMR spectroscopy. Several attempts to purify the enzymatically formed *N*-hydroxyaspartic acid by ion-exchange column chromatography on cationic Dowex 50 resin, SPE-SCX cation exchange, and basic Amberlite IRA 140 all failed. Identification of *N*-hydroxyaspartic acid as the product of the AspB-catalyzed addition of hydroxylamine to fumarate was established by ^{1}H NMR spectroscopy by comparison with chemically synthesized *N*-hydroxyaspartic acid. Both enzymatically and chemically synthesized *N*-hydroxyaspartic acid showed the same unstable behavior. After 1 week of incubation, the signals in the ^{1}H NMR spectrum corresponding to *N*-hydroxyaspartic acid were no longer present. Ottenheijm and Herscheid previously described *N*-hydroxy acids as highly unstable.[17] The *N*-hydroxy group is readily converted into the corresponding nitroso compound either by air oxidation or disproportionation, which subsequently undergoes rapid decarboxylation to the aldoxime. However, the presumed decomposition products could neither be isolated nor identified.

As the decomposition of *N*-hydroxyaspartic acid is initiated by the oxidation of its hydroxy functionality, protection of the hydroxy group should give a stable compound. Several attempts to protect the hydroxy group through benzylation, silylation, and acylation failed and could not overcome the rapid decomposition. In an alternative approach, the carboxylic acid groups were esterified with concentrated H_2SO_4 in MeOH overnight, which yielded only 4% of the product with 40% *ee*. The harsh conditions probably lead to racemization at the stereogenic center. Mild esterification with trimethylsilyldiazomethane also failed.

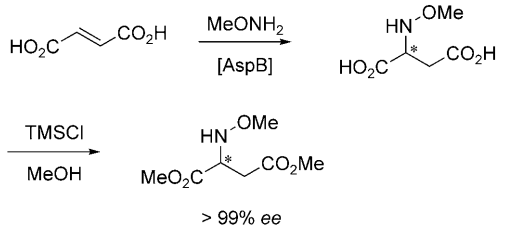
To establish the enantiomeric excess of the enzymatically produced *N*-hydroxyaspartic acid, hydrogenolysis of the N–O bond of *N*-hydroxyaspartic acid with a catalytic amount of PtO_2 and a few drops of acetic acid was performed. This yielded (*S*)-aspartic acid, which was isolated in 80% yield and 97% *ee* (Scheme 3).[19] To summarize, we could identify optically active *N*-hydroxyaspartic acid as the product of the AspB-catalyzed addition of hydroxylamine to fumarate, but failed to stabilize and isolate this interesting product.



Scheme 3. Enantioselective hydrazine and hydroxylamine addition to fumarate and hydrogenolysis to L-Asp.

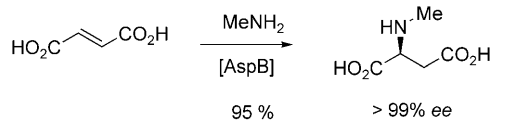
The enzymatic addition of hydrazine to fumarate was scaled up to 5.0 mmol. The stable product 2-hydrazinosuccinic acid was successfully purified in quantitative yield by ion-exchange chromatography on cationic Dowex 50 resin. To determine the enantiomeric excess, the crude 2-hydrazinosuccinic acid was reduced with a catalytic amount of PtO_2 to give (*S*)-aspartic acid, which was purified by ion-exchange chromatography on cationic Dowex 50 resin. Subsequent HPLC analysis established the product to have 99% *ee* (Scheme 3).

As the enzymatic addition of methoxylamine to fumarate occurs slowly, we used a high concentration of biocatalyst (1.8 mol% of AspB). The reaction was scaled up to 1.0 mmol and was allowed to proceed for 1 week until fumarate was completely converted. The resulting *N*-methoxyaspartic acid could not be purified by chromatography on a cationic Dowex 50 resin. Therefore the crude methoxyaspartic acid was esterified using TMSCl in MeOH, which yielded *N*-methoxyaspartic dimethyl ester in 11% yield over two steps with an enantiomeric excess >99% (Scheme 4).



Scheme 4. Enantioselective addition of methoxylamine to fumarate and esterification.

The enzymatic addition of methylamine gave full conversion of fumarate after 6 days of incubation by using 1.4 mol% of AspB. The *N*-methylaspartic acid was purified by column chromatography on silica gel to give a yield of 95%[20] and the pure acid had >99% *ee* (Scheme 5). Determination of the optical rotation gave a value of $[\alpha]_D^{20} = +24.5$ ($c = 0.26$ in 1 N HCl), which corresponds to an *S* configuration according to literature.[21]



Scheme 5. Enantioselective AspB-catalyzed addition of methylamine to fumarate.

The biocatalytic production of these four *N*-substituted aspartic acids has previously been demonstrated for 3-methylaspartate ammonia lyase (methylaspartase).[22] In contrast to that study, we have presented a detailed analysis of these reactions, including an HPLC analysis of the products and the determination of kinetic parameters.

Conclusion

We have exploited the nucleophile promiscuity of aspartase (AspB) from *Bacillus* sp. YM55-1 for catalyzing stereoselective amination reactions to produce the *N*-substituted aspartic acids (*S*)-*N*-hydroxyaspartic acid, (*S*)-2-hydrazinosuccinic acid, (*S*)-*N*-methylaspartic acid, and *N*-methoxyaspartic acid with excellent enantioselectivities. These non-proteinogenic amino acids are interesting building blocks for peptidomimetics, synthetic enzymes, and pharmaceuticals. For example, 2-hydrazinosuccinic acid is an important structural unit because it represents a turn mimic in peptides,[23] and several peptides containing this structural motif show antibiotic activity.[24] When included in peptides, *N*-methylaspartic acid leads to enhanced proteolytic stability and an increase in lipophilicity.[25]

Although this investigation has set the stage for the development of a biocatalytic process for the stereoselective synthesis of *N*-substituted aspartic acid derivatives, the biocatalytic scope of AspB is rather limited.[26] Based on the structural characterization of the enzyme, we have started protein engineering experiments aimed at evolving AspB activity towards a broader range of amine compounds.

Experimental Section

Construction of the expression vector for the production of AspB: The *aspB* gene was amplified by PCR by using two synthetic primers, the coding sequence for the aspartase in plasmid pUCBA[5] as the template, and PCR reagents supplied in the Expand High Fidelity PCR system following the protocol supplied with the system (F. Hoffman-La Roche, Ltd.). The forward primer (5′-ATA**CCATGG**ATACCGATGTTCG-3′) contains a *Nco*I restriction site (in bold) followed by 13 bases that correspond to the coding sequence of the *aspB* gene. The reverse primer (5′-CAT**AAGCTT**TTTTCTTCCAGCAATTCC-3′) contains a *Hin*dIII restriction site (in bold) followed by 18 bases that correspond to the complementary sequence of the *aspB* gene. The resulting PCR product and the pBAD/*Myc*-His A vector (Invitrogen) were digested with *Nco*I and *Hin*dIII restriction enzymes, purified, and ligated using T4 DNA ligase. Aliquots of the ligation mixture were transformed into competent *E. coli* TOP10 cells. Transformants were selected at 37°C on LB/ampicillin plates. Plasmid DNA was isolated from several colonies 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-His).

Expression and purification of AspB-His$_6$: The AspB enzyme was produced in *E. coli* TOP10 using the pBAD expression system. Fresh TOP10 cells containing pBAD(AspB-His) were collected from a LB/ampicillin plate using a sterile loop and used to inoculate 1 L of a LB/ampicillin medium that contained 0.04% (w/v) arabinose. After overnight growth at 37°C and 200 rpm, the cells were harvested by centrifugation (10 min at 6000 rpm at 4°C in a JA-10 rotor) and stored at −20°C until further use.

In a typical purification experiment, cells of three 1 L cultures were thawed, combined, and suspended in lysis buffer (15 mL, 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). Cells were disrupted by sonication for 10×1 min (with 3–5 min rest in between each cycle) at a 60 W output after which unbroken cells and debris were removed by centrifugation (30 min at 15000 rpm at 4°C in a JA17 rotor). The supernatant was filtered through a 0.45 μm pore diameter filter and incubated with nickel nitriloacetic acid (4×1 mL slurry in small columns at 4°C for 2 nights), which had previously been equilibrated with lysis buffer. The nonbound proteins were eluted from the column by gravity flow. The columns were first washed with lysis buffer (10 mL per column) and then with buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0; 10 mL per column). Retained proteins were eluted with buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0; 3.0 mL per column). Fractions (≈0.5 mL) were analyzed by SDS-PAGE on gels containing 12% acrylamide, and those that contained purified aspartase were pooled and concentrated to a protein concentration of about 9.4 mg mL^{-1}

in 50 mM NaH_2PO_4 (pH 8.0) using an Amicon-stirred cell equipped with a YM30 (30000 MW cut-off) ultrafiltration membrane. The purified enzyme was stored at −80 °C until further use.

Colorimetric assay for ammonia detection: The deamination of potential amino acid substrates was monitored by following ammonia production upon incubation with the different compounds. Accordingly, an appropriate amount of enzyme was incubated in a microtitre plate with an amino acid (150 μL of a 25 mM solution) in 100 mM Na_2HPO_4 buffer (pH 9.0). After incubation of the plate at 37 °C for 18 h, a drop of 1.5 M trichloroacetic acid followed by 100 μL of Nessler's reagent were added. A red-brown color indicated the presence of AspB activity.

Kinetic studies: To determine the kinetic parameters for the AspB-catalyzed deamination of L-aspartate, the kinetic assays were performed at 25 °C by following the increase in absorbance at 240 nm, which corresponds to the formation of fumarate ($\varepsilon = 2530\ M^{-1}cm^{-1}$). An aliquot of AspB was diluted in 50 mM NaH_2PO_4 buffer (pH 8.5) to yield a final enzyme concentration of 0.08 μM and incubated for 60 min at 25 °C. Subsequently, a 1 mL portion was transferred to a 10 mm quartz cuvette and the enzyme activity was assayed by the addition of a small quantity (1–10 μL) of sodium L-aspartate from a stock solution. The stock solution was made up in 50 mM NaH_2PO_4 buffer (pH 8.5). The concentrations of L-aspartate used in the assay ranged from 5–100 mM.

To determine the kinetic parameters for the AspB-catalyzed addition of amines to fumarate, the kinetic assays were performed at 22 °C and the decrease in absorbance was followed at 270 nm, which corresponds to the depletion of fumarate ($\varepsilon = 555\ M^{-1}cm^{-1}$).[27] To determine the values of K_m for fumarate, an aliquot of AspB was diluted in 50 mM NaH_2PO_4 buffer (pH 8.0) containing a fixed concentration of amine (ammonia 200 mM, hydroxylamine 400 mM, hydrazine 750 mM, all titrated to pH 8.0) to yield a final enzyme concentration of 0.0179 μM (0.0358 μM when using hydrazine). Subsequently, a 1 mL portion was transferred to a 10 mm quartz cuvette and the enzyme activity was assayed by the addition of a small quantity of fumarate from a stock solution made up in 50 mM NaH_2PO_4 buffer (pH 8.0). The concentrations of fumarate used in the assay ranged from 0.10–3.75 mM. To determine the values of K_m for the amine nucleophiles, the incubation mixtures contained various concentrations of nucleophiles ranging from 20–571 mM, 0.041 μM of AspB (0.081 μM when using hydrazine), and 50 mM phosphate buffer (pH 8.0). A 350 μL portion of this solution was transferred to a 1 mm quartz cuvette, and reactions were started by adding 20 mM of fumarate. The kinetic data were fitted by nonlinear regression data analysis using the Grafit program.[28]

General procedure for the nucleophile screening: The nucleophile screening was carried out on a Varian Inova 500 NMR spectrometer using a pulse sequence for selective presaturation of the water signal. The reactions were performed in a NMR tube with a volume of 550 μL at room temperature, and ^{1}H NMR spectra were recorded after 1 day, and 1, 2, and 3 weeks. A sample initially contained 9.34 or 8.95 μmol of AspB, 0.25 mmol of fumarate, and 10 equiv of nucleophile. The nucleophile was dissolved in 50 mM phosphate buffer containing D_2O in order to reduce the water signal in the spectrum. The total volume was adjusted to 550 μL by using buffer in D_2O. At the same time a blank sample without AspB was monitored. The reactions were monitored at different pH values (pH 7.0, 8.0, and 9.5).

2-Hydrazinosuccinic acid: A solution of 6 N HCl was added to a solution of hydrazine (35% in water, 5.20 mL, 50.0 mmol) in 5 mM phosphate buffer in a Greiner tube until pH 8.0 was reached. A 500 mM stock solution of fumarate (10 mL, 5.00 mmol) at pH 8.0 was added and the Greiner tube was filled up to 25 mL with 5 mM phosphate buffer (pH 8.0). After addition of AspB (80.0 μL, 0.014 mmol) from a frozen stock, the tube was shaken at 95 rpm at 37 °C. The reaction was allowed to continue to completion, as confirmed spectrophotometrically by the disappearance of the absorption at 240–270 nm. The solvent was evaporated and the oily residue was purified by ion-exchange column chromatography on a cationic Dowex 50 resin (H^+, 20–50 mesh, washed with water) by elution with 2.5% NH_3 solution. After evaporation of the solvent and lyophilization, the product (0.799 g, 5.39 mmol, 108%)[29] was obtained quantitatively as a colorless gum. $[\alpha]_D^{20} = -16.1$ ($c = 0.557$ in 1N HCl); ^{1}H NMR (500 MHz, D_2O): $\delta = 2.31$ (dd, $^2J(2,2) = 16.1$ Hz, $^3J(2,1) = 8.3$ Hz, 1H; CH_2), 2.49 (dd, $^2J(2,2) = 16.1$ Hz, $^3J(2,1) = 4.5$ Hz, 1H; CH_2), 3.49 ppm (dd, $^3J(1,2) = 8.5$ Hz, $^3J(1,2) = 4.5$ Hz, 1H; CH); ^{13}C NMR (75.0 MHz, D_2O): $\delta = 36.9$ (CH_2), 62.8 (CH), 175.8 (CO_2H), 178.4 ppm (CO_2H); MS (ESI): *m/z*: 149.1 $[M+1]^+$, 171.1 $[M+Na]^+$, 147.1 $[M-1]^+$.

***N*-Methylaspartic acid**: A solution of 6 N HCl was added to a solution of methylamine (40% in water, 4.20 mL, 50.0 mmol) in 5 mM phosphate buffer in a Greiner tube until pH 8.0 was reached. A 500 mM stock solution of fumarate (10 mL, 5.00 mmol) at pH 8.0 was added, and the Greiner tube was filled up to 50 mL with 5 mM phosphate buffer (pH 8.0). After addition of AspB (400 μL, 0.072 mmol) from a frozen stock, the tube was shaken at 100 rpm at 37 °C for 7 days. The reaction was followed to completion, as confirmed spectrophotometrically until no absorption at 240–270 nm was observed. The solvent was evaporated and the white residue purified by flash chromatography on silica gel (HOAc/EtOAc/MeOH/H_2O = 3:3:3:2). After evaporation of the solvent and lyophilization, the product (95%)[29] was obtained as white gum. $[\alpha]_D^{20} = +24.6$ ($c = 0.256$ in 1 N HCl); ^{1}H NMR (300 MHz, D_2O): $\delta = 2.62$ (s, 3H; CH_3), 2.88 (d, $^3J(2,1) = 8.5$ Hz, 2H; CH_2), 3.75 ppm (dd, $^3J(1,2) = 8.8$ Hz, $^3J(1,2) = 8.8$ Hz, 1H; CH); ^{13}C NMR (125 MHz, D_2O): $\delta = 31.2$ (CH_2), 34.7 (CH_3), 60.1 (CH), 172.9 (CO_2H), 176.8 ppm (CO_2H); MS (ESI): *m/z*: 146.2 $[M-1]^+$; HPLC (Astec CLC-L, 2 mM $CuSO_4$ in H_2O/MeOH 90:10, flow 1.0 mL min^{-1}, 40 °C): 9.2 (L-*N*-MeAsp), 11.4 min (D-*N*-MeAsp); 99.5% *ee*.

***N*-Methoxyaspartic acid**: Methoxylamine hydrochloride (4.18 g, 50.0 mmol) was dissolved in 5 mM phosphate buffer (10 mL) and 5 N NaOH was added until tube pH 8.0 was reached. A 500 mM stock solution of fumarate (10 mL, 5.00 mmol) at pH 8.0 was added and the Greiner tube was filled up to 50 mL with 5 mM phosphate buffer (pH 8.0). After addition of AspB (500 μL, 0.090 mmol) from a frozen stock, the tube was shaken at 100 rpm at 37 °C. The progress of the reaction was monitored spectrophotometrically. After 9 days, no absorption was observed at 240–270 nm, which indicated that the reaction had been completed. The solvent was evaporated and the white residue lyophilized and used without further purification. ^{1}H NMR (400 MHz, D_2O): $\delta = 2.39$ (dd, $^2J(2,2) = 15.2$ Hz, $^3J(2,1) = 8.0$ Hz, 1H; CH_2), 2.58 (d, $^2J(2,2) = 15.2$ Hz, $^3J(2,1) = 5.2$ Hz, 1H; CH_2), 3.59 (s, 3H; OCH_3), 3.85 ppm (dd, $^3J(1,2) = 7.6$ Hz, $^3J(1,2) = 5.2$ Hz, 1H; CH); ^{13}C NMR (75.4 MHz, D_2O): $\delta = 34.5$ (CH_2), 61.5, 63.0, 179.9 (CO_2H), 180.2 ppm (CO_2H).

***N*-Methoxyaspartic acid dimethyl ester**: A suspension of the crude lyophilized *N*-methoxyaspartic acid (5.00 mmol) containing buffer salts in MeOH (18 mL) was cooled with ice and TMSCl (4.00 mL, 31.5 mmol, 6.3 equiv) was added dropwise. A precipitate formed and the solution was stirred at room temperature overnight. After removal of the solvent, the product was purified by flash chromatography on silica gel (pentane/EtOAc = 2:1) to yield a colorless oil (0.107 g, 0.560 mmol, 11%). $[\alpha]_D^{20} = -3.6$ ($c = 1.4$ in $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.71$ (dd, $^2J(2,2) = 16.3$ Hz, $^3J(2,1) = 7.3$ Hz, 1H; CH_2), 2.83 (dd, $^2J(2,2) = 16.3$ Hz, $^3J(2,1) = 6.0$ Hz, 1H; CH_2), 3.51 (s, 3H; OCH_3), 3.71 (s, 3H; CO_2CH_3), 3.76 (s, 3H; CO_2CH_3), 4.03 (dd, $^3J(1,2) = 6.8$ Hz, $^3J(1,2) = 6.8$ Hz, 1H; CH), 6.26 ppm (br s, 1H; NH); ^{13}C NMR (75.0 MHz, D_2O): $\delta = 34.0$ (CH_2), 52.0 (OCH_3), 52.4 (OCH_3), 59.8 (CH_3), 62.4 (CH), 171.2 (CO_2Me), 172.0 ppm (CO_2Me); MS (EI): *m/z*: 191 $[M]^+$, 160 $[M-OMe]^+$, 132 $[M-CO_2Me]^+$; HRMS: calcd. for $C_6H_{10}NO_4$: 160.0610; found: 160.0612; HPLC (Chiralpak OD-H, heptane/*i*PrOH 95:5, flow 0.5 mL min^{-1}): 21.9 min; 99% *ee*.

***N*-Hydroxyaspartic acid**: A solution of 5 N NaOH was added to a solution of hydroxylamine hydrochloride (0.69 g, 10.0 mmol) in 5 mM phosphate buffer in a Greiner tube until pH 8.0 was reached. A 1 M stock solution of fumarate (1 mL, 1.00 mmol) at pH 8.0 was added and the Greiner tube was filled up to 15 mL with 5 mM phosphate buffer at pH 8.0. After the addition of AspB (50 μL, 0.009 mmol) from a frozen stock, the tube was shaken at 100 rpm at 37 °C for 30 min. The solvent was evaporated and a white gum obtained. ^{1}H NMR (400 MHz, D_2O): $\delta = 2.29$ (dd, $^2J(2,2) = 15.8$ Hz, $^3J(2,1) = 9.0$ Hz, 1H; CH_2), 2.50 (dd, $^2J(2,2) = 15.8$ Hz, $^3J(2,1) = 4.2$ Hz, 1H; CH_2) 3.65 ppm (dd, $^3J(1,2) = 8.6$ Hz, $^3J(1,2) = 4.6$ Hz, 1H; CH). Attempts at purification by ion-exchange chromatography led to decomposition of the material.

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[20] Because *N*-methylaspartic acid could not be completely dried by lyopilization, the yield was determined by ^{1}H NMR spectroscopy using formic acid as the reference.

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