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Characterization of a thermostable methylaspartate ammonia lyase from *Carboxydotherrmus hydrogenoformans*

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Abstract Methylaspartate ammonia lyase (MAL; EC 4.3.1.2) catalyzes the reversible addition of ammonia to mesaconate to give (2*S*,3*S*)-3-methylaspartate and (2*S*,3*R*)-3-methylaspartate as products. MAL is of considerable biocatalytic interest because of its potential use for the asymmetric synthesis of substituted aspartic acids, which are important building blocks for synthetic enzymes, peptides, chemicals, and pharmaceuticals. Here, we have cloned the gene encoding MAL from the thermophilic

bacterium *Carboxydotherrmus hydrogenoformans* Z-2901. The enzyme (named *Ch*-MAL) was overproduced in *Escherichia coli* and purified to homogeneity by immobilized metal affinity chromatography. *Ch*-MAL is a dimer in solution, consisting of two identical subunits (~49 kDa each), and requires Mg²⁺ and K⁺ ions for maximum activity. The optimum pH and temperature for the deamination of (2*S*,3*S*)-3-methylaspartic acid are 9.0 and 70°C ($k_{\text{cat}}=78 \text{ s}^{-1}$ and $K_{\text{m}}=16 \text{ mM}$). Heat inactivation assays showed that *Ch*-MAL is stable at 50°C for >4 h, which is the highest thermal stability observed among known MALs. *Ch*-MAL accepts fumarate, mesaconate, ethylfumarate, and propylfumarate as substrates in the ammonia addition reaction. The enzyme also processes methylamine, ethylamine, hydrazine, hydroxylamine, and methoxylamine as nucleophiles that can replace ammonia in the addition to mesaconate, resulting in the corresponding *N*-substituted methylaspartic acids with excellent diastereomeric excess (>98% de). This newly identified thermostable MAL appears to be a potentially attractive biocatalyst for the stereoselective synthesis of aspartic acid derivatives on large (industrial) scale.

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Keywords Methylaspartate ammonia lyase · Thermostable enzyme · *Carboxydotherrmus hydrogenoformans* · Enzyme catalysis · Amino acids

Introduction

Enantiomerically pure amino acids constitute a significant part of the chiral building blocks for a range of natural

products, pharmaceuticals, and agrochemicals (Schulze and Wubbolts 1999; Pollard and Woodley 2007; Panke et al. 2004). The synthesis of enantiomerically pure amino acids still remains a challenging task with traditional chemical catalysts (Schoemaker et al. 2003; Weiner et al. 2010). In some cases, the use of biocatalysts is an attractive alternative option (Wohlgemuth 2010). Recently, the ammonia lyases and aminomutases (which exhibit ammonia lyase activity) have gained a lot of interest for the asymmetric synthesis of chiral α - and β -amino acids, with the advantage that they use readily available unsaturated acids as substrates (Turner 2010; Verkuijl et al. 2010; Szymanski et al. 2009; Wu et al. 2009, 2010; Weiner et al. 2008). However, most known ammonia lyases show low stability and therefore they are not compatible with the harsh reaction conditions that are usually required for industrial processes, such as high temperature, high pH, and high ammonia concentrations needed to catalyze the reverse reactions (Turner 2010). Hence, there is a clear need for the discovery and characterization of novel ammonia lyases with increased stability.

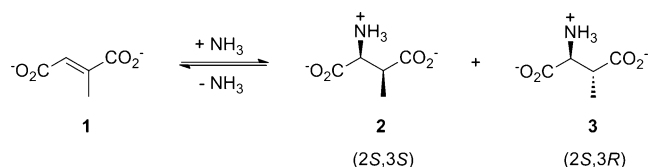
3-Methylaspartate ammonia lyase (MAL; EC 4.3.1.2) catalyzes the reversible amination of mesaconate (**1**) to give (2*S*,3*S*)-3-methylaspartate (**2**) as a major product and (2*S*,3*R*)-3-methylaspartate (**3**) as a minor product (Scheme 1) (Barker et al. 1959; Goda et al. 1992). MAL was discovered initially in *Clostridium tetanomorphum* and subsequently also in several other anaerobic bacteria, where the enzyme forms part of the glutamate catabolic pathway that converts (*S*)-glutamic acid via **2** to finally yield acetyl-CoA (Kato and Asano 1997). Recently, it has been shown that MAL also forms part of the methylaspartate cycle in haloarchaea, in which acetyl-CoA is oxidized to glyoxylate via methylaspartate. This is a novel pathway of carbon assimilation in addition to the already known glyoxylate cycle and ethylmalonyl-CoA pathway (Khomyakova et al. 2011).

Microbial genome and metagenome sequencing projects have revealed that there is a large diversity of protein sequences that based on sequence similarities to *C. tetanomorphum* MAL (*Ct*-MAL) might be classified as putative MALs. However, so far, MALs have been isolated and characterized from only a few different organisms, including *C. tetanomorphum*, *Bacterium cadaveris*, *Morganella morganii*, *Citrobacter amalonaticus*, *Escherichia coli*, and *Fusobacterium varium* (Barker et al. 1959; Kato and Asano

1997, 1995a, b; 1998; Asano and Kato 1994). The best studied MALs are those from *C. tetanomorphum* and *C. amalonaticus*, for both of which the structures have been solved by X-ray crystallography (Levy et al. 2002; Asuncion et al. 2002). Based on these structural studies (Levy et al. 2002; Asuncion et al. 2002) and recent mutagenesis experiments (Raj et al. 2009), a mechanism has emerged for the MAL-catalyzed deamination reaction. In this proposed mechanism, a (*S*)- or (*R*)-specific catalytic base (Lys-331 and His-194, respectively; *Ct*-MAL numbering) abstracts the C-3 proton of the respective stereoisomer of 3-methylaspartate to generate an enolate anion intermediate that is stabilized by coordination to the essential active site Mg²⁺ ion. Collapse of this intermediate eliminates ammonia and yields the product, mesaconate.

Ct-MAL has been shown to accept a range of different nucleophiles (amines) and electrophiles (substituted fumarates). The broad substrate scope of *Ct*-MAL has been exploited for the stereoselective synthesis of various *N*-, 3-, and *N*,3-(di)substituted aspartic acids (Akhtar et al. 1987; Botting et al. 1988; Gulzar et al. 1997). A recent mutagenesis study on *Ct*-MAL has shown that the H194A, Q329A, and K331A mutants display distinct diastereoselectivities and may be useful for the diastereoselective synthesis of (2*S*,3*S*)-3-methylaspartic acid (Raj et al. 2009). Its broad substrate scope, high activity, and stereoselectivity make *Ct*-MAL an attractive biocatalyst for organic synthesis. However, the enzyme is not stable upon long-term storage (at +4 or -80°C) and rapidly loses activity at elevated temperatures (Barker et al. 1959). Hence, in order to use the MAL reaction for biocatalytic applications, it is essential to identify putative MAL isozymes with increased stability.

In this study, we screened the microbial genomes available in the NCBI database for homologues of *Ct*-MAL with the aim to identify MALs from thermophilic microorganisms. Here, we report the identification of a MAL enzyme (designated *Ch*-MAL) from the thermophilic bacterium *Carboxydotherrmus hydrogeniformans* Z-2901, which was isolated from a hot spring in Russia and grows at very high temperatures (Svetlichny et al. 1991; Wu et al. 2005), which shares significant sequence identity (53%) with *Ct*-MAL. The gene encoding *Ch*-MAL was cloned and the corresponding enzyme overproduced, purified, and subjected to kinetic and biochemical characterization. *Ch*-MAL shows optimal activity at 70°C and pH 9.0 and is stable at 50°C for >4 h, which is the highest thermal stability observed among reported MALs. Like *Ct*-MAL, *Ch*-MAL has a broad substrate scope, accepting various substituted fumarates and amines, and exhibits high activity and diastereoselectivity. This makes *Ch*-MAL an attractive enzyme for biocatalytic applications, as well as a promising scaffold for engineering to yield highly stable and efficient



Scheme 1 MAL-catalyzed reversible amination of mesaconate to yield (2*S*,3*S*)- and (2*S*,3*R*)-3-methylaspartate

enzymes for the asymmetric synthesis of new aspartic acid derivatives.

Materials and methods

Materials

All chemicals were purchased from Sigma-Aldrich unless stated otherwise. The sources for the media components, buffers, solvents, Pre-packed PD-10 Sephadex G-25 columns, and molecular biology reagents, including PCR purification, gel extraction, and Miniprep kits, are reported elsewhere (Raj et al. 2009). Oligonucleotides for DNA amplification were synthesized by Operon Biotechnologies (Cologne, Germany).

Bacterial strains, plasmids, and growth conditions

E. coli strain TOP10 (Invitrogen) was used for cloning, isolation of plasmids, and in combination with the pBAD/*Myc*-His A vector (Invitrogen) for recombinant protein production. The genomic DNA of *C. hydrogenoformans* Z-2901, the source of the *Ch*-MAL gene, was kindly provided by Professor Frank Robb (Center of Marine Biotechnology, University of Maryland, USA). *E. coli* TOP10 cells were grown in Luria–Bertani (LB) medium containing 100 µg/mL ampicillin (Ap). *Ct*-MAL was produced in *E. coli* TOP10 and purified to homogeneity using a previously published protocol (Raj et al. 2009).

General methods

BLASTP searches of the National Center for Biotechnology Information (NCBI) databases were performed using the *Ct*-MAL amino acid sequence (GenBank number AAB24070.1) as the query sequence. Amino acid sequences were aligned using a version of the ClustalW multiple-sequence alignment routines available in the computational tools at the EMBL-EBI website. Techniques for restriction enzyme digestions, ligation, transformation, and other standard molecular biology manipulations were based on methods described elsewhere (Sambrook et al. 1989) or as suggested by the manufacturer. PCR was carried out in a DNA thermal cycler (model GS-1) obtained from Biolegio (Nijmegen, The Netherlands). DNA sequencing was performed by Macrogen (Seoul, South Korea). Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions on gels containing polyacrylamide (10%). The gels were stained with Coomassie brilliant blue. Protein concentrations were determined by the Waddell method (Waddell 1956). Kinetic data were obtained on a V-650 spectropho-

tometer from Jasco (IJsselstein, The Netherlands). High performance liquid chromatography (HPLC) was performed using a Waters 510 HPLC Pump (Waters Corporation, USA) in combination with a variable wavelength UV detector 875-UV (Jasco) and a BD112 recorder (Kipp and Zonen, The Netherlands). ¹H NMR spectra were recorded on a Varian Inova 500 (500 MHz) spectrometer using a pulse sequence for selective suppression of the proton signals for water by presaturation methods. ¹H chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane and are calibrated on protons in the NMR solvents (H₂O: δ=4.79).

Construction of the expression vector for the production of *Ch*-MAL

The *Ch*-MAL gene was amplified from genomic DNA of *C. hydrogenoformans* Z-2901 (Wu et al. 2005) using two synthetic primers and high fidelity Phusion polymerase by following the protocol supplied with the polymerase (Finnzymes, Espoo, Finland). The forward primer (5'-G GAG CGG TGG **CAT ATG** AGA ATA AAA GAT G -3') contains an *Nde*I restriction site (in bold) followed by 13 bases corresponding to the coding sequence of the *Ch*-MAL gene. The reverse primer (5'- CC TTC CGG **AAG CTT** ACC AAC TTT TTT CTG AAA TGT GAC C -3') contains a *Hind*III restriction site (in bold) followed by 25 bases corresponding to the complementary sequence of the *Ch*-MAL gene. The resulting PCR product and the pBADN/*Myc*-His A vector were digested with *Nde*I and *Hind*III 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/Ap plates. Plasmid DNA was isolated from several colonies and analyzed by restriction analysis for the presence of the insert. The cloned *Ch*-MAL gene was sequenced to verify that no mutations had been introduced during the amplification of the gene.

Expression and purification of *Ch*-MAL

The *Ch*-MAL enzyme was produced in *E. coli* TOP10 using the pBADN expression system. Fresh TOP10 cells containing the appropriate expression plasmid were collected from a LB/Ap plate using a sterile loop and used to inoculate LB/Ap medium (15 mL). After growth for 8 h at 37°C, a sufficient quantity of the culture was used to inoculate 1 L of fresh auto-induction (ZYM) medium (10 g/L trypton, 5 g/L yeast extract, 25 mM Na₂HPO₄ buffer, 25 mM KH₂PO₄, 5 mM Na₂SO₄, pH 6.7), containing 0.5% (v/v) glycerol, 0.05% (w/v) glucose, MgSO₄ (2 mM), ampicillin (100 µg/mL), and arabinose (0.05% w/v), in a 3-L Erlenmeyer flask to an

initial A_{600} of ~ 0.1 (Studier 2005). Cultures were grown for 24 h at 22°C with vigorous (170 rpm) shaking. Cells were harvested by centrifugation (6,000×g, 15 min). Protein purification was performed using an immobilized metal affinity chromatography procedure as previously described (Raj et al. 2009). The elution buffer was exchanged against Tris–HCl buffer (50 mM, pH 8.0), containing $MgCl_2$ (2 mM) and KCl (0.1 mM), using a pre-packed PD-10 Sephadex G-25 gel filtration column. The purified enzyme was stored at +4°C or –80°C until further use. At both temperatures, the enzyme can be stored for several months without significant loss of activity.

Determination of the molecular mass of *Ch*-MAL

The native molecular mass of *Ch*-MAL was determined by gel filtration chromatography. The purified enzyme (1 mg/mL) was applied to a Superdex 200 column (10/300; GE Healthcare, USA), previously equilibrated with Tris–HCl buffer (50 mM, pH 8.0), containing $MgCl_2$ (2 mM) and KCl (0.1 mM). The column was eluted with the same buffer at a flow rate of 0.5 mL/min. The column was calibrated with the reference proteins aldolase (MW 158 kDa), bovine serum albumin (MW, 66.7 kDa), and ovalbumin (MW, 44 kDa) (GE Healthcare). The subunit molecular mass of *Ch*-MAL was determined by mass spectrometry using an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) connected to an LC system via a TurboIonSpray source. For this, the Tris–HCl buffer of the protein sample was exchanged to NH_4CO_2H (5 mM, pH 7.0) using a Nanosep centrifugal device (PALL Life Sciences). Data were collected and analyzed using Analyst 1.5.1 data acquisition software (Applied Biosystems/MDS Sciex).

Enzyme assays

The rate of the MAL-catalyzed amination of **1** was monitored by following the depletion of **1** at 270 nm ($\epsilon = 482 \text{ M}^{-1} \text{ cm}^{-1}$) in Tris–HCl buffer (500 mM, pH 9.0) containing $MgCl_2$ (20 mM) and NH_4Cl (400 mM) at 30°C (Raj et al. 2009). The rate of the MAL-catalyzed deamination of **2** was monitored by following the formation of **1** at 240 nm ($\epsilon = 3,850 \text{ M}^{-1} \text{ cm}^{-1}$) in Tris–HCl buffer (500 mM, pH 9.0), containing $MgCl_2$ (20 mM) and KCl (1 mM), at either 30°C or 70°C (Botting et al. 1988; Raj et al. 2009). At 70°C, the pH of the Tris buffer was adjusted to the desired pH of 9.0. Like *Ct*-MAL, *Ch*-MAL requires both Mg^{2+} and K^+ ions for its deamination activity, whereas for the amination activity only Mg^{2+} is needed. Optimal activity was obtained with 20 mM $MgCl_2$ and 1 mM KCl, and these concentrations were used for all enzyme assays.

Determination of pH and temperature optima of MAL

The pH optima of *Ch*-MAL and *Ct*-MAL were determined in Tris–HCl buffer (500 mM), containing $MgCl_2$ (20 mM) and KCl (1 mM), with pH values ranging from 6.0 to 9.5 at 30°C. A sufficient quantity of enzyme was added and its activity assayed using **2** (15 mM) as the substrate. Stock solutions of **2** were made in Tris buffer (500 mM), and the pH of the stock solutions were adjusted to the desired pH (6.0–9.5). The normalized initial reaction rates were plotted against pH.

The temperature optima for *Ch*-MAL and *Ct*-MAL were determined in Tris–HCl buffer (500 mM, pH 9.0), containing $MgCl_2$ (20 mM) and KCl (1 mM), using a temperature range of 10–90°C. At each temperature, the pH of the Tris buffer was adjusted to the desired pH (9.0). A sufficient quantity of enzyme was added and its activity assayed using **2** (15 mM) as the substrate. Stock solutions of **2** were made in Tris–HCl buffer (500 mM, pH adjusted to 9.0). The normalized initial reaction rates were plotted against temperature.

Thermostability assay

The thermostability of *Ch*-MAL or *Ct*-MAL was examined in Tris–HCl buffer (500 mM, pH 9.0), containing $MgCl_2$ (20 mM) and KCl (1 mM). An appropriate amount of enzyme was incubated in the assay buffer (25 mL) at 50°C. Samples (1 mL) were withdrawn every 5 min, and the residual activity was measured using **2** (15 mM) as the substrate. The initial reaction rates were plotted against time.

Product analysis of the amination of **1** by MAL

1H NMR spectra monitoring the *Ch*-MAL- or *Ct*-MAL-catalyzed amination of **1** were recorded according to a protocol reported elsewhere (Raj et al. 2009), with the following modifications. Reaction mixtures consisted of NH_4Cl (500 μ L of 1 M stock solution in water, pH 9.0, containing 20 mM $MgCl_2$), D_2O (100 μ L), and **1** (100 μ L of a 500 mM stock solution in water, pH 9.0). Reactions were initiated by the addition of 10–15 μ L of freshly purified enzyme (400 μ g of either *Ct*-MAL or *Ch*-MAL), and each reaction mixture was incubated at 22°C. 1H NMR spectra were recorded 2 h, 7 days, and 14 days after the addition of enzyme. Product amounts were estimated by integration of the signals corresponding to **2** and **3**. The 1H NMR signals for **1**, **2**, and **3** were previously reported (Raj et al. 2009).

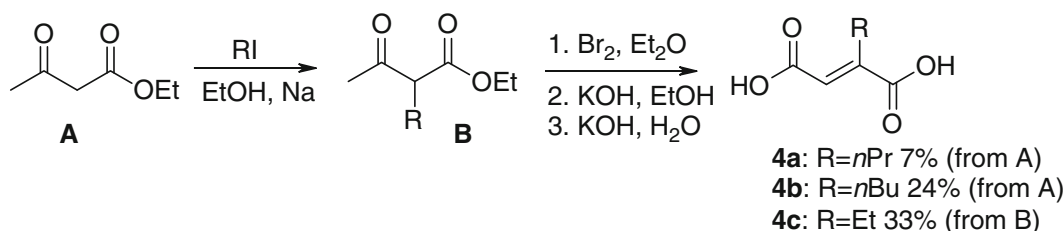
Procedure for the nucleophile screening

The nucleophile scope of *Ch*-MAL was examined by using 1H NMR spectroscopy. *Ch*-MAL was incubated (in separate reactions) with different amines in the presence of **1**. The reaction mixtures consisted of amine (500 μ L of a

1 M stock solution in water, pH 9.0, containing 20 mM MgCl_2 , **1** (100 μL of a 500 mM stock solution in water, pH 9.0), and D_2O (100 μL). Reactions were initiated by the addition of 10–15 μL of freshly purified *Ch*-MAL (400 μg), and each reaction mixture was incubated at 22°C. ^1H NMR spectra were recorded 2 h, 7 days, and 14 days after the addition of enzyme. To identify the products of the

reactions and to assign their relative (*threo* or *erythro*) configuration, the ^1H NMR spectra were compared to those generated for the same reactions catalyzed by *Ct*-MAL (performed as described above for *Ch*-MAL) as well as to previously published ^1H NMR spectral data (Akhtar et al. 1987; Gulzar et al. 1997). Relative product distributions were estimated by integration of the corresponding signals.

Synthesis of 2-substituted fumaric acids



4a: 2-Propylfumaric acid The synthesis of **4a** (and **4b** and **4c**) is largely based on a previously published protocol (Akhtar et al. 1987). Accordingly, sodium (10 mmol, 230 mg) was dissolved in ethanol (6.0 mL). Ethyl acetoacetate (9.0 mmol, 1.14 mL) was added dropwise over 5 min at 5°C, followed by *n*-propyl iodide (12 mmol, 1.17 mL). The reaction mixture was refluxed for 2 h and was then cooled to room temperature, poured on water (30 mL), and extracted with Et_2O (3×25 mL). Organic fractions, containing a mixture of mono- and dialkylated product, were dried and concentrated to ~15 mL volume. Bromine (4.0 g) was added slowly at room temperature, and the reaction mixture was refluxed for 3 h. The volatiles were evaporated, and the residue was slowly added to a solution of KOH (4.0 g) in ethanol (15 mL). The resultant mixture was heated at reflux for 30 min, after which 20 mL of water was added and the refluxing continued for another 20 min. The reaction mixture was washed with EtOAc (2×40 mL). The aqueous phase was acidified with aqueous HCl (12 N) to pH < 1 and extracted with Et_2O (4×50 mL). The collected organic fractions were dried, decolorized with activated carbon, and the solvent was evaporated. The resultant residue was triturated with pentane to yield white crystals (100 mg, 7%) [Mp., 173.5–174.7°C (lit., 172–174°C, Akhtar et al. 1987); ^1H NMR (400 MHz, $\text{D}_2\text{O}+\text{K}_2\text{CO}_3$): δ 0.70 (t, 3H, $^3J=7.6$ Hz, CH_3), 1.19–1.25 (m, 2H, CH_3CH_2), 2.63 (t, 2H, $^3J=7.2$ Hz, $\text{CH}_2\text{C}=\text{C}$), 6.18 (s, 1H, vinyl H)]. ^1H NMR was consistent with literature data (Akhtar et al. 1987).

4b: 2-Butylfumaric acid This was prepared according to the same procedure as 2-propylfumaric acid [yield, 24%; yellow crystals; Mp., 171.7–172.5°C (lit., 170–171°C, Akhtar et al.

1987); ^1H NMR (400 MHz, $\text{D}_2\text{O}+\text{K}_2\text{CO}_3$): δ 0.71 (t, 3H, $^3J=7.2$ Hz, CH_3), 1.06–1.24 (m, 4H, $\text{CH}_3\text{CH}_2\text{CH}_2$), 2.31 (t, 2H, $^3J=7.2$ Hz, $\text{CH}_2\text{C}=\text{C}$), 6.17 (s, 1H, vinyl H)]. ^1H NMR was consistent with literature data (Akhtar et al. 1987).

4c: 2-Ethylfumaric acid This was prepared according to the same procedure as 2-propylfumaric acid, starting with commercially available ethyl 2-ethylacetoacetate [yield, 33%; yellow crystals; Mp., 197–198°C (lit., 195°C, Akhtar et al. 1987); ^1H NMR (400 MHz, D_2O): δ 0.89 (t, 3H, $^3J=7.2$ Hz, CH_3), 2.49 (d, 2H, $^3J=7.2$ Hz, CH_2), 6.57 (s, 1H, vinyl H)]. ^1H NMR was consistent with literature data (Akhtar et al. 1987).

Procedure for the electrophile screening

The electrophile scope of *Ch*-MAL was analyzed by ^1H NMR spectroscopy. In separate experiments, *Ch*-MAL was incubated with fumarate (or substituted fumarates) and ammonia. Reaction mixtures consisted of NH_4Cl (500 μL of 1 M stock solution in water, pH 9.0, containing 20 mM MgCl_2), D_2O (100 μL), and (substituted) fumarate (100 μL of a 500-mM stock solution in water, pH 9.0). Reactions were initiated by the addition of 10–15 μL of freshly purified *Ch*-MAL (400 μg), and each reaction mixture was incubated at 22°C. ^1H NMR spectra were recorded 2 h, 7 days, and 14 days after the addition of enzyme. To identify the products of the reactions and to assign their relative (*threo* or *erythro*) configuration, the ^1H NMR spectra were compared to those obtained for the same reactions catalyzed by *Ct*-MAL (performed as described above for *Ch*-MAL) as well as to previously published ^1H

NMR spectral data (Akhtar et al. 1987; Gulzar et al. 1997). Product amounts were estimated by integration of the corresponding signals.

The enantiomeric excess of the product of the MAL-catalyzed addition of ammonia to fumarate (i.e., aspartate) was determined by chiral HPLC using a Chirex 3126-(D)-penicillamine column (250×4.6 mm, Phenomenex, USA) with 2 mM CuSO₄ solution-methanol (90:10) as eluent at a flow rate of 1 mL/min. Retention times were as follows: (S)-aspartate, 23.4 min; (R)-aspartate, 31 min).

Preparative scale synthesis of *threo*-(2*S*,3*S*)-*N*,3-dimethylaspartic acid

Purified *Ch*-MAL was used for the synthesis of *threo*-(2*S*,3*S*)-*N*,3-dimethylaspartic acid. A solution (20 mL) of **1** (0.2 g, 1.54 mmol), methylamine (1.04 g, 15.4 mmol), and MgCl₂ (20 mM) was prepared. The pH was adjusted to 9.0 by the addition of small aliquots of an aqueous NaOH (1 M) solution. The reaction was started by the addition of *Ch*-MAL (10 mg), and the reaction mixture was incubated at 50°C. The progress of the reaction was monitored using ¹H NMR (500 MHz) spectroscopy. After 8 days, the reaction was terminated, and the reaction mixture was lyophilized, after which the product was purified using cation exchange chromatography (Dowex, 50W X8, 100–200 mesh size, Merck). A Dowex column (15.0 g resin per 1 g of mesaconate) was prepared by pretreatment of the resin with a solution of aqueous NH₃ (2 M, four column volumes), aqueous HCl (1 N, two column volumes), and distilled water (four column volumes). The lyophilized reaction mixture was suspended in aqueous HCl (1 N, 20 mL) and loaded on the column. The column was washed with distilled water (one column volume), and the product was eluted with aqueous NH₃ (2 M, two column volumes). The ninhydrin-positive fractions were pooled and concentrated under reduced pressure, followed by lyophilization. The product was obtained as a white solid (152 mg) {yield, 61%; ¹H NMR (200 MHz, D₂O): δ 1.13 (d, 3H, ³J=7.6 Hz, CHCH₃), 2.75 (s, 3H, CH₃NH), 2.82–2.95 (dq, 1H, ³J=7.6 Hz, ³J=3.0 Hz, CHCH₃), 3.79 (d, 1H, ³J=3.0 Hz, CHNH); ¹³C NMR (50 MHz, D₂O): δ 11.7, 33.0, 40.8, 65.4, 172.2, 180.9; high resolution mass spectra (HRMS) (ESI+): *m/z* calc. for C₆H₁₂NO₄, 162.0766 [M+H]⁺; found, 162.0760}.

Results

Identification of a thermostable MAL

A sequence similarity search in the NCBI microbial genome database was performed with the BLASTP program using the *Ct*-MAL amino acid sequence as the

query. This search yielded several bacterial proteins that shared high sequence identity (>50%) with *Ct*-MAL. The top 20 hits included a sequence from the thermophilic bacterium *C. hydrogenoformans* Z-2901, which was isolated from a hot spring in Kunashir Island (Russia) and grows optimally at 78°C (Svetlichny et al. 1991; Wu et al. 2005). This bacterial protein (designated *Ch*-MAL) is annotated as a putative methylaspartate ammonia lyase and was selected for further study.

The *Ch*-MAL protein is predicted to be 420 amino acids in length. Unlike the *Ct*-MAL gene, the gene encoding *Ch*-MAL is not located next to a gene encoding a putative glutamate mutase (Kato and Asano 1997). In fact, the genomic context of the gene encoding *Ch*-MAL does not provide any clues about the biological function of this protein in *C. hydrogenoformans* Z-2901. The sequence of *Ch*-MAL is 53% identical and 73% similar to that of *Ct*-MAL. A sequence alignment shows that nine of the ten active site residues of *Ct*-MAL are conserved in *Ch*-MAL (Asuncion et al. 2002) (Fig. S1, supporting information). The non-conserved residue, Thr-360, is replaced by Ser-359 in *Ch*-MAL. As there are no significant active site differences between *Ch*-MAL and *Ct*-MAL, a catalytic mechanism, with important roles for Lys-331 and His-194 (*Ct*-MAL numbering) as the *S*- and *R*-specific base/acid catalysts, similar to that of *Ct*-MAL (Raj et al. 2009) may be expected for *Ch*-MAL. To obtain insight into the functional properties of this *Ct*-MAL homologue, *Ch*-MAL was overproduced, purified, and subjected to kinetic and biochemical characterization (see below).

Expression and purification of *Ch*-MAL

The gene coding for *Ch*-MAL was amplified from genomic DNA of *C. hydrogenoformans* Z-2901 and cloned into the expression vector pBADN/*Myc*-His A, resulting in the construct pBAD(*Ch*-MAL). The *Ch*-MAL encoding gene in pBAD(*Ch*-MAL) is under transcriptional control of the *araBAD* promoter, and the recombinant enzyme was produced upon induction with arabinose in *E. coli* TOP10 as a C-terminal His₆-tag fusion protein. Optimal expression of the *Ch*-MAL gene was achieved when the TOP10 cells were cultivated at 22°C in an auto-induction medium (Studier 2005) in the presence of 0.05% (*w/v*) arabinose. The recombinant enzyme was purified by a one-step Ni-sepharose affinity chromatography protocol, which typically provides ~200 mg of homogeneous enzyme per liter of culture. The purified *Ch*-MAL has a molecular mass of ~50 kDa when analyzed by SDS-PAGE. *Ch*-MAL was further analyzed by electrospray ionization mass spectrometry (ESI-MS) and gel filtration chromatography. Analysis of *Ch*-MAL by ESI-MS showed, upon deconvolution, one major peak that corre-

sponds to a mass of 49,291 (± 3) Da. A comparison of this value to the calculated mass (49,288 Da) indicates that, in contrast to Cys-361 in *Ct*-MAL (Asuncion et al. 2002), no oxidation of the active site cysteine (Cys-360) in *Ch*-MAL had occurred upon purification. Gel filtration chromatography resulted in elution of *Ch*-MAL as a single symmetrical peak, which corresponds to a native molecular mass of ~ 100 kDa. A comparison of this value to that of the subunit mass suggests that, like *Ct*-MAL (Asuncion et al. 2002), *Ch*-MAL is a homodimeric protein.

Ammonia lyase activity of *Ch*-MAL

To examine whether *Ch*-MAL exhibits ammonia lyase activity, the enzyme was incubated with **2** (in 500 mM Tris–HCl buffer, pH 9.0), and the reaction was monitored by a previously described spectroscopic assay (Raj et al. 2009). The results show that *Ch*-MAL deaminates **2** to yield **1**, and maximum activity is achieved in the presence of both Mg^{2+} (≥ 20 mM) and K^+ (≥ 1 mM) ions in the assay buffer. Having established that *Ch*-MAL exhibits methylaspartate ammonia lyase activity, kinetic parameters were measured (at 30°C) and compared to those previously measured for *Ct*-MAL (Table 1). *Ch*-MAL catalyzes the deamination of **2** with a catalytic efficiency (k_{cat}/K_m) of 3.5×10^3 $M^{-1} s^{-1}$, which is ~ 25 -fold lower than the value measured for the same reaction catalyzed by *Ct*-MAL.

Optimum pH and temperature for the ammonia lyase activity of *Ch*-MAL

The optimum pH for the *Ch*-MAL-catalyzed deamination of **2** was determined at 30°C in Tris–HCl buffers (500 mM, containing 20 mM $MgCl_2$ and 1 mM KCl) with pH values ranging from 6.0 to 9.5. The *Ch*-MAL enzyme is active in the complete pH range tested and shows maximum activity at pH 9.0 (Fig. 1a). A similar pH optimum was found for *Ct*-MAL (Fig. 1a). The optimum temperature for the

Table 1 Kinetic parameters for the *Ct*-MAL and *Ch*-MAL catalyzed deamination of (2*S*,3*S*)-3-methylaspartic acid (**2**)

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($M^{-1} s^{-1}$)
<i>Ct</i> -MAL (at 30°C) ^a	89 \pm 4	1.0 \pm 0.1	8.9 $\times 10^4$
<i>Ch</i> -MAL (at 30°C)	16 \pm 1	4.6 \pm 0.4	3.5 $\times 10^3$
<i>Ch</i> -MAL (at 70°C)	78 \pm 4	16 \pm 3	4.9 $\times 10^3$

The steady state kinetic parameters were determined in Tris–HCl buffer (500 mM, pH 9.0) containing $MgCl_2$ (20 mM) and KCl (1 mM) at 30°C or 70°C

^a These kinetic data were obtained from Raj et al. (2009). Errors are standard deviations from each fit

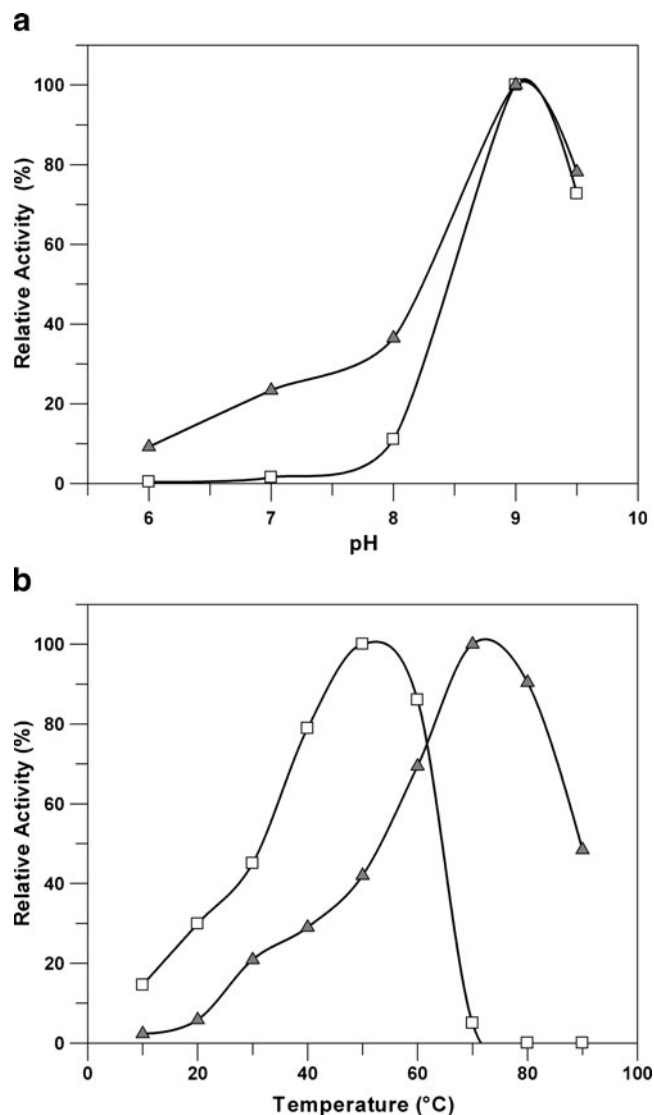


Fig. 1 Effect of **a** pH and **b** temperature on the deamination activity of *Ch*-MAL (filled triangles) and *Ct*-MAL (empty squares)

deamination of **2** by *Ch*-MAL was determined in Tris–HCl buffer (500 mM, pH 9.0, containing 20 mM $MgCl_2$ and 1 mM KCl) at different temperatures ranging from 10 to 90°C. *Ch*-MAL was active at all temperatures analyzed and showed the highest activity at 70°C (Fig. 1b). For comparison, *Ct*-MAL was found to be active at temperatures ranging from 10 to 70°C with maximum activity at 50°C (Fig. 1b). The observation that *Ch*-MAL shows maximum activity at 70°C, prompted us to measure kinetic parameters at this temperature. At 70°C, *Ch*-MAL catalyzes the deamination of **2** with a k_{cat} of 78 s^{-1} and a K_m of 16 mM, which results in a k_{cat}/K_m that is slightly higher (1.4-fold) than that measured at 30°C (Table 1). The observed 4.9-fold increase in k_{cat} confirms that *Ch*-MAL is more active at elevated temperatures. At 70°C, *Ct*-MAL is almost completely inactive (Fig. 1b).

Thermostability of *Ch*-MAL

The preceding results demonstrate that *Ch*-MAL and *Ct*-MAL have different temperature–activity profiles, with *Ch*-MAL being active at higher temperatures. To compare the thermostability of these two MALs, they were incubated at 50°C (in separate incubations), and samples were withdrawn every 5 min to measure residual ammonia lyase activity. Interestingly, *Ch*-MAL stayed fully active upon incubation at 50°C. Even after 4 h of incubation, the enzyme retained more than 95% of its initial activity toward **2** (Fig. 2). In contrast, the ammonia lyase activity of *Ct*-MAL decreased rapidly upon incubation at 50°C, and within 30 min half of its initial activity was lost. After 4 h, *Ct*-MAL retained only ~4% of its initial activity toward **2**. Hence, in contrast to *Ct*-MAL, *Ch*-MAL is highly stable at 50°C.

Amination activity of *Ch*-MAL

The rate of amination of **1** by *Ch*-MAL was monitored by following the depletion of **1** at 270 nm in Tris–HCl buffer (500 mM, pH 9.0) containing MgCl₂ (20 mM) and NH₄Cl (400 mM) at 30°C (Botting et al. 1988; Raj et al. 2009). *Ch*-MAL catalyzes the amination of **1** with an apparent k_{cat}/K_m value of $2.5 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, which is only 3.5-fold lower than the value measured for the same reaction catalyzed by *Ct*-MAL (Table 2). The *Ch*-MAL-catalyzed amination of **1** was also monitored by ¹H NMR spectroscopy to verify that the products of the reaction are **2** and **3** (Scheme 1). The *Ch*-MAL catalyzed amination of **1** indeed yields **2** and **3**, as indicated by signals in the NMR spectra consistent with the structures of these amino acid products (Fig. 3a). Although the ¹H NMR spectra showed signals for both **2** and **3**, those

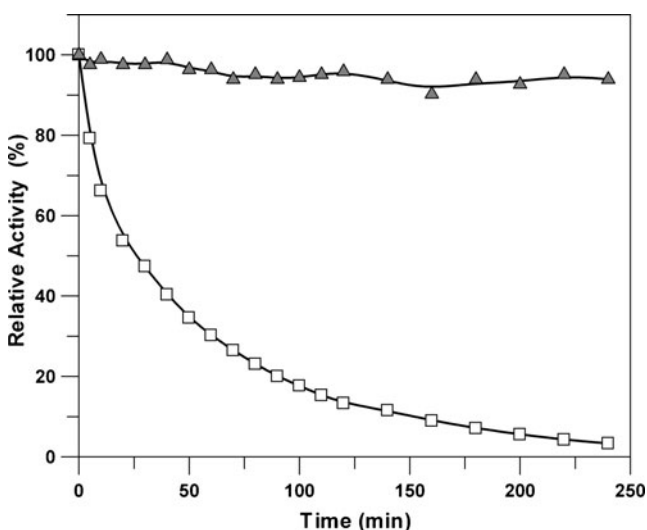


Fig. 2 The thermostability of *Ch*-MAL (filled triangles) and *Ct*-MAL (empty squares) upon incubation at 50°C

Table 2 Apparent kinetic parameters for the *Ch*-MAL and *Ct*-MAL catalyzed amination of mesaconate (**1**)

Enzyme	k_{cat} (s ⁻¹)	K_m for 1 (mM)	k_{cat}/K_m (M ⁻¹ s ⁻¹)
<i>Ct</i> -MAL ^a	61±1	0.7±0.02	8.7×10^4
<i>Ch</i> -MAL	15±1	0.6±0.1	2.5×10^4

The steady state kinetic parameters were determined in Tris–HCl buffer (500 mM, pH 9.0) containing MgCl₂ (20 mM) and NH₄Cl (400 mM) at 30°C

^a These kinetic data were obtained from Raj et al. (2009). Errors are standard deviations from each fit

corresponding to **2** predominated in the initial spectra, whereas signals for **3** increased in the later spectra. Hence, **2** is the kinetically preferred product. After a 14-day-incubation period at 22°C, a final conversion of ~76% was achieved, giving a ~1:1 ratio of **2**:**3** (Fig. 3a; Table 3). For comparison, the *Ct*-MAL-catalyzed amination of **1** was also followed by ¹H NMR spectroscopy, showing the same kinetic profile of product (**2** and **3**) formation (Fig. 3b; Table 3). These results suggest that, like *Ct*-MAL, *Ch*-MAL likely catalyzes the rapid *anti*-addition and the much slower *syn*-addition of ammonia to **1**, yielding **2** and **3**, respectively (Raj et al. 2009; Akhtar et al. 1987).

Electrophile scope of *Ch*-MAL

It has previously been determined that *Ct*-MAL accepts fumarate and several of its 2-substituted derivatives as alternative substrates (Akhtar et al. 1987; Botting et al. 1988). These observations prompted us to examine whether *Ch*-MAL also catalyzes the amination of these electrophiles. The *Ch*-MAL-catalyzed addition reactions were monitored by ¹H NMR spectroscopy. To identify the products of the reactions and to establish their relative configuration (*threo* or *erythro*), we compared the ¹H NMR spectra of the products of the *Ch*-MAL-catalyzed addition reactions to those obtained for the same reactions catalyzed by *Ct*-MAL, the products of which have previously been identified and their absolute configuration assigned (Akhtar et al. 1987). Representative conversions for each reaction are summarized in Table 3. Both MALs efficiently catalyze the amination of fumarate and ethylfumarate (in addition to the natural substrate methylfumarate). While *Ct*-MAL has reasonable activity toward propylfumarate, this compound is a poor substrate for *Ch*-MAL. Butylfumarate is not accepted as substrate by either MAL.

Fumarate appears to be the best non-natural substrate for *Ch*-MAL, showing ~97% conversion after 2 h of incubation. The ¹H NMR spectrum recorded 7 days after the addition of enzyme showed the nearly complete disappearance of the signals corresponding to fumarate and the formation of new signals corresponding to the expected

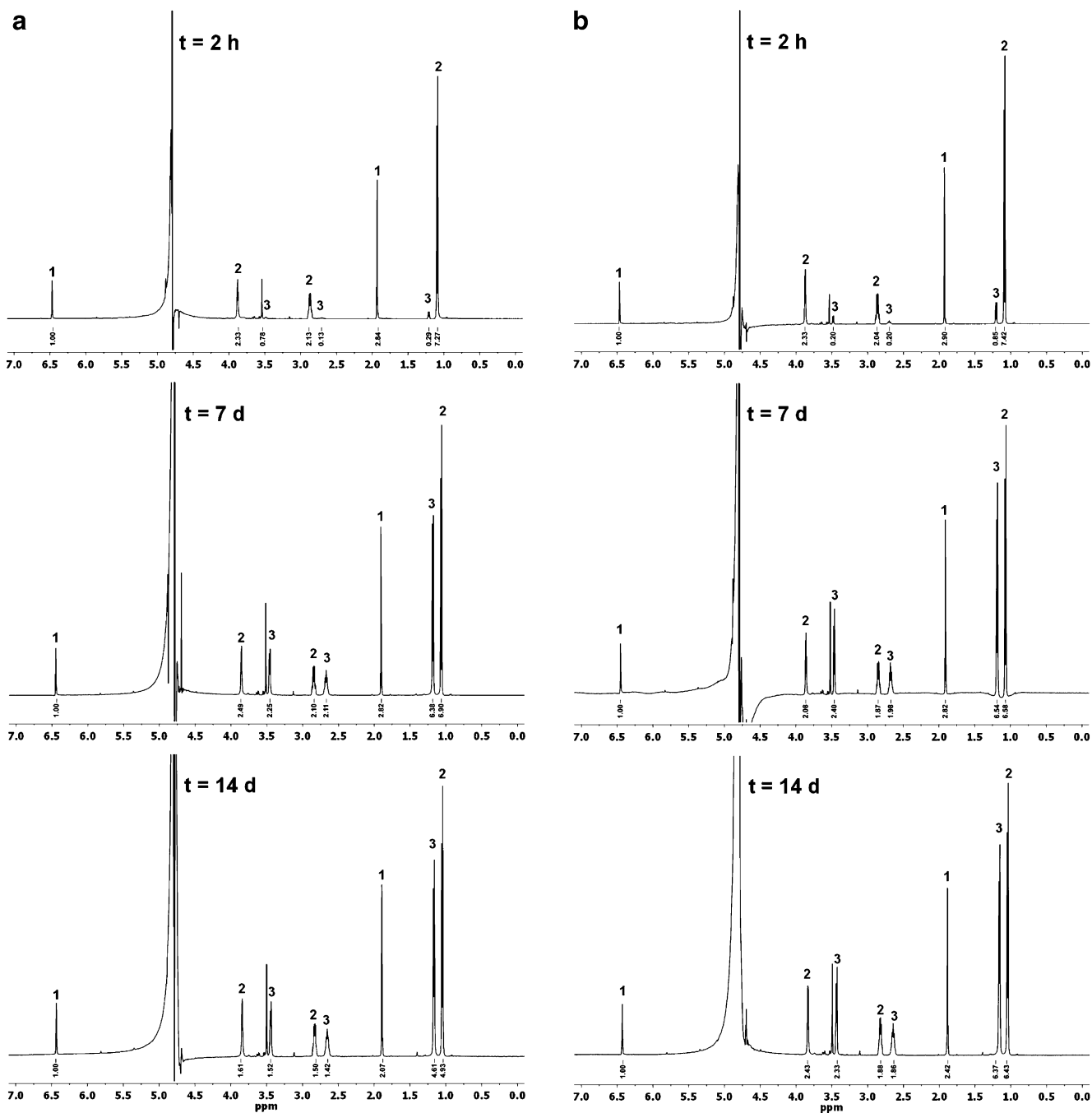


Fig. 3 ^1H NMR spectra monitoring the amination of **1** by **a** *Ch*-MAL and **b** *Ct*-MAL. The ^1H NMR signals for **1**, **2**, and **3** were previously reported (Raj et al. 2009). Impurity (Tris): $\delta = 3.5$ (s)

product, aspartate (Fig. S2). The absolute configuration of this product was determined by using chiral HPLC, with authentic (*R*)- and (*S*)-aspartate for comparison, and found to be (*S*)-aspartate (>99% ee). Ethylfumarate is also a good substrate for *Ch*-MAL, showing ~60% conversion after 2 h of incubation. After a 14-day incubation period, a final conversion of ~65% was achieved, and the spectrum showed signals corresponding to the expected products

threo-(2*S*,3*S*)-3-ethylaspartate (P1) and *erythro*-(2*S*,3*R*)-3-ethylaspartate (P2). The ratio of S/P1/P2 was determined to be 35:60:5 (Fig. S3). Propylfumarate is a poor substrate for *Ch*-MAL; the spectrum recorded after 14 days of incubation showed only ~7% conversion of substrate with formation of *threo*-(2*S*,3*S*)-3-propylaspartate as the expected product (Fig. S4). The tentative assignment of the absolute configuration of the 3-ethylaspartate and 3-

Table 3 Addition of ammonia to various electrophiles catalyzed by *Ch*-MAL or *Ct*-MAL

Electrophile	<i>Ch</i> -MAL% conversion (days of incubation)	<i>Ch</i> -MAL ee ^a or d.r. ^b of product	<i>Ct</i> -MAL% conversion (days of incubation)	<i>Ct</i> -MAL ee ^a or d.r. ^b of product
Fumarate	99 (7)	ee>99%	99 (7)	ee>99%
Methylfumarate	76 (14)	d.r.=52:48	81 (14)	d.r.=51:49
Ethylfumarate	65 (14)	d.r.=95:5	71 (14)	d.r.=72:28
Propylfumarate	~7 (14)	d.r.=100:0	61 (14)	d.r.=100:0

^aThe enantiomeric excess of the (*S*)-aspartic acid product was determined by chiral HPLC

^bThe diastereomeric ratio (*threo:erythro*) of the 3-substituted aspartic acid products was determined by ¹H NMR spectroscopy

propylaspartate products was made on the basis of analogy to the known configuration of the products of the corresponding *Ct*-MAL catalyzed reactions (Akhtar et al. 1987).

Nucleophile scope of *Ch*-MAL

We next screened *Ch*-MAL for its ability to add different unnatural amines to mesaconate (**1**). The reactions were followed using ¹H NMR spectroscopy. The products were identified (and their relative configuration established) by comparing the ¹H NMR spectra of the *Ch*-MAL-catalyzed addition reactions to those obtained for the same reactions catalyzed by *Ct*-MAL, the products of which have previously been identified and their absolute configuration assigned (Gulzar et al. 1997). Representative conversions for each reaction are summarized in Table 4. Both MALs efficiently catalyze the addition of hydroxylamine and hydrazine (in addition to the natural nucleophile, ammonia) to mesaconate. Methylamine, ethylamine, and methoxylamine are also processed by both MALs, but at a much lower catalytic rate. Propylamine is not accepted as alternative nucleophile by either MAL.

Hydroxylamine and hydrazine seem to be the best alternative nucleophiles for *Ch*-MAL, showing ~80% and ~60% conversion after 2 h of incubation, respectively. For

both these amines, ¹H NMR spectra recorded 7 days after the addition of enzyme showed the nearly complete disappearance of the signals corresponding to mesaconate (**1**) and the formation of new signals corresponding to the expected products, *threo*-(2*S*,3*S*)-*N*-hydroxy-3-methylaspartate and *threo*-(2*S*,3*S*)-2-hydrazino-3-methylaspartate, respectively (Figs. S8 and S9) (Table 4). Methylamine and ethylamine are also accepted as substrates, showing ~12% and <1% conversion after 2 h of incubation, respectively. After a 14-day-incubation period, respective conversions of ~70% and ~30% were achieved (Table 4), and the ¹H NMR spectra showed signals corresponding to the expected products, *threo*-(2*S*,3*S*)-*N*,3-dimethylaspartate and *N*-ethyl-3-methylaspartate, respectively (Figs. S5 and S6). The relative configuration of the latter product has not been determined. Methoxylamine is a poor substrate for *Ch*-MAL. The spectrum recorded after 14 days of incubation showed only ~9% conversion of substrate with formation of *threo*-(2*S*,3*S*)-*N*-methoxy-3-methylaspartate as the expected product (Fig. S7). The tentative assignment of the absolute configuration of the amino acid products was again made on the basis of analogy.

Preparative scale synthesis of *threo*-(2*S*,3*S*)-*N*,3-dimethylaspartic acid

In order to demonstrate the potential of *Ch*-MAL for chemical synthesis, the enzyme was used to synthesize *threo*-(2*S*,3*S*)-*N*,3-dimethylaspartic acid at preparative scale. Accordingly, *Ch*-MAL was incubated with methylamine (1.04 g, 15.4 mmol) and mesaconate (0.2 g, 1.54 mmol) at 50°C, and the reaction was monitored by ¹H NMR spectroscopy (Fig. 4a); after 8 days of incubation, a final conversion of ~75% was achieved. The product was purified using a Dowex cation exchange column, giving a final yield of 61% (white solid), and identified as *threo*-(2*S*,3*S*)-*N*,3-dimethylaspartic acid by ¹H NMR (Fig. 4b), ¹³C NMR, and HRMS (Gulzar et al. 1997). The enzyme showed high diastereoselectivity in the formation of *threo*-(2*S*,3*S*)-*N*,3-dimethylaspartic acid (>98% de, as assessed by ¹H NMR spectroscopy).

Table 4 Addition of various amines to mesaconate catalyzed by *Ch*-MAL or *Ct*-MAL (reactions were followed by ¹H NMR spectroscopy)

Amine	<i>Ch</i> -MAL% conversion (days of incubation)	<i>Ct</i> -MAL% conversion (days of incubation)
Ammonia	76 (14)	81 (14)
Methylamine ^a	70 (14)	64 (14)
Ethylamine ^a	30 (14)	14 (14)
Methoxylamine ^a	9 (14)	67 (14)
Hydroxylamine ^a	99 (7)	96 (7)
Hydrazine ^a	100 (7)	100 (7)

^aThe enzyme showed high diastereoselectivity in the formation of the corresponding amino acid product (>98% de)

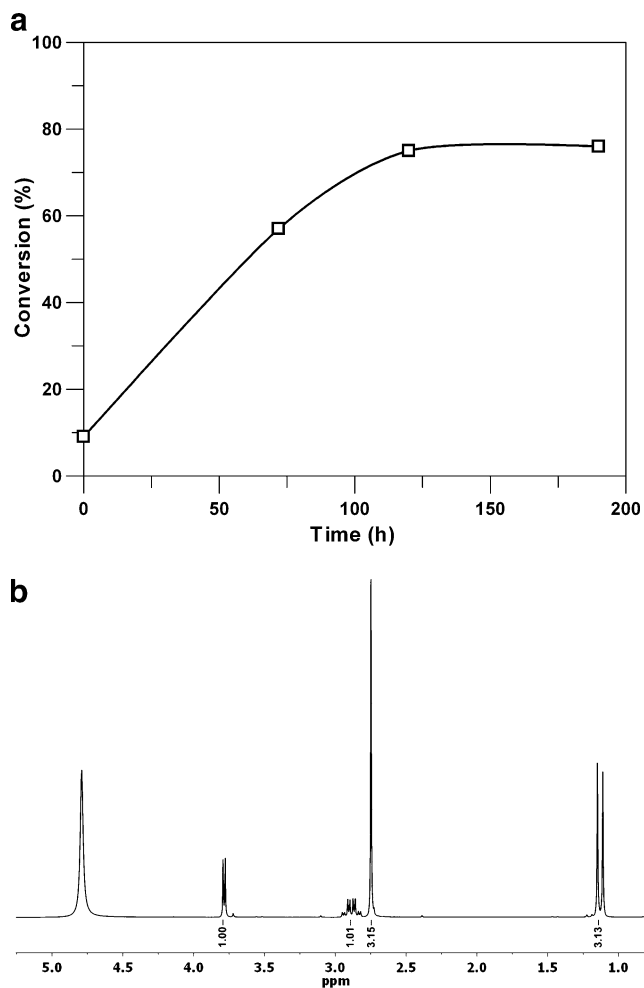


Fig. 4 Enzymatic synthesis of *threo*-(2*S*,3*S*)-*N*,3-dimethylaspartic acid. **a** Progress curve of the *Ch*-MAL-catalyzed methylamine addition to mesaconate (**1**) as monitored by ^1H NMR spectroscopy. **b** ^1H NMR spectrum of purified *threo*-(2*S*,3*S*)-*N*,3-dimethylaspartic acid. The ^1H NMR signals for this amino acid are reported elsewhere (Gulzar et al. 1997)

Discussion

The thermophilic, Gram-positive bacterium *C. hydrogenoformans* Z-2901 has attracted much interest because of its unique biology: It grows at very high temperature, it lives almost entirely on a diet of carbon monoxide, and it converts water to hydrogen gas as part of its metabolism. The genome sequence of this extreme thermophile contains two open reading frames coding for putative MALs (Wu et al. 2005). It can be anticipated that the MALs from *C. hydrogenoformans* exhibit increased thermostability compared to known MALs from mesophilic hosts (Barker et al. 1959; Kato and Asano 1997, 1995a, b, 1998; Asano and Kato 1994).

Herein, we have described the cloning, recombinant expression and purification of *Ch*-MAL from *C. hydrogenoformans* Z-2901, which provided an opportunity to

characterize the substrate specificity and thermostability of a MAL isozyme from an extreme thermophile. The enzyme can be highly overproduced in *E. coli* in a soluble and active form and rapidly purified with the help of a C-terminal hexahistidine tag. The thermostability, activity, and substrate specificity of *Ch*-MAL were compared to *Ct*-MAL, the best studied MAL from a mesophilic host. As might be expected, *Ch*-MAL and *Ct*-MAL have different temperature-activity profiles, with *Ch*-MAL being active at much higher temperature. *Ch*-MAL catalyzes the deamination of (2*S*,3*S*)-3-methylaspartate (**2**, Scheme 1) with a k_{cat}/K_m of $3.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ at 30°C and $4.9 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ at 70°C, which is somewhat lower (18- to 25-fold) than the value measured for the same conversion catalyzed by *Ct*-MAL at 30°C. The lower catalytic efficiency of the *Ch*-MAL catalyzed reaction results mainly from a higher K_m value, which suggests less optimal binding of **2** in the active site of *Ch*-MAL. A comparison of the kinetic parameters for the *Ch*-MAL and *Ct*-MAL catalyzed amination of **1** (Table 2) shows similar catalytic efficiencies for both enzymes. These results indicate that *Ch*-MAL functions as an effective 3-methylaspartate ammonia lyase, catalyzing the reversible addition of ammonia to mesaconate to give (2*S*,3*S*)-3-methylaspartate and (2*S*,3*R*)-3-methylaspartate as products. Activity measurements further showed that *Ch*-MAL is highly thermostable and retains >95% of its initial activity after heating for 4 h at 50°C. Importantly, and in contrast to *Ct*-MAL (Barker et al. 1959), *Ch*-MAL can be stored at 4°C for several months without significant loss of activity. Thus, *Ch*-MAL indeed exhibits enhanced stability compared to *Ct*-MAL and other MALs from mesophilic hosts (Barker et al. 1959; Kato and Asano 1995a, b, 1997, 1998; Asano and Kato 1994).

Ch-MAL has been shown to catalyze the regioselective addition of ammonia to several substituted fumarates, resulting in the corresponding 3-substituted aspartic acids with the *threo*-isomers being the kinetically preferred products (Table 3). In addition, the enzyme is highly enantioselective in the addition of ammonia to fumarate, leading to the formation of (*S*)-aspartic acid with >99% ee. While *Ch*-MAL efficiently processes fumarate and small substituted fumarates such as methylfumarate and ethylfumarate, it displays low (propylfumarate) or no (butylfumarate) activity with larger substrates. This suggests that the binding pocket for the C-2 alkyl substituent in *Ch*-MAL is designed to bind small alkyl chains and excludes large groups. A similar electrophile scope and isomer preference was found for *Ct*-MAL (Table 3). *Ch*-MAL (and *Ct*-MAL) also accepts various unnatural amines in the addition to mesaconate, yielding the corresponding *N*-substituted methylaspartic acids (Table 4). The enzyme efficiently processes small substituted amines such as hydroxylamine and hydrazine, but displays low (methylamine, ethylamine,

and methoxylamine) or no (propylamine) activity with larger amine nucleophiles. These observations suggest that the amine binding pocket of *Ch*-MAL (and *Ct*-MAL) is designed to bind small amine compounds and excludes large nucleophiles. In contrast to the enzyme-catalyzed ammonia addition reactions, both MALs showed high diastereoselectivity in the amine additions to mesaconate, resulting in the formation of the *N*-substituted methylaspartic acids with high diastereomeric excess (>98% de, as assessed by ¹H NMR spectroscopy) (Akhtar et al. 1987; Botting et al. 1988; Gulzar et al. 1997).

In conclusion, *Ch*-MAL from *C. hydrogenoformans* Z-2901 can be overproduced in *E. coli* and purified in high yield. This MAL is a promising new biocatalyst because it is highly thermostable and accepts various substituted fumarates and amines to produce a range of aspartic acid derivatives. These chiral amino acids are important building blocks for synthetic enzymes, peptides, chemicals, and pharmaceuticals (Kahn 1993; Burger and Spengler 2000; Hughes et al. 2000). To further enlarge the substrate scope of MAL and improve its stereoselectivity in the amination reactions, protein engineering experiments, guided by the previously published crystal structure of *C. amalonaticus* MAL in complex with substrate **2** (Levy et al. 2002), have been initiated in our laboratory. If successful, these efforts may help to increase the number and diversity of enzyme applications in industry.

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