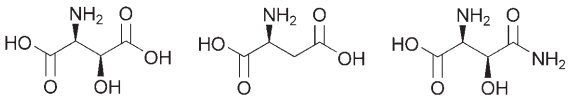
DOI: 10.1002/cbic.200700557

# Non-Heme Hydroxylase Engineering For Simple Enzymatic Synthesis of L-*threo*-Hydroxyaspartic Acid

Matthias Strieker,[a, b] Lars-Oliver Essen,[a] Christopher T. Walsh,[b] and Mohamed A. Marahiel*[a]

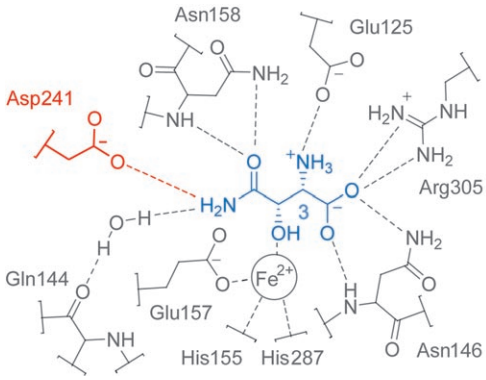
L-*threo*-Hydroxyaspartic acid (L-THA, **1**), the β-hydroxylated form of L-aspartic acid (**2**), is of current medicinal interest because it inhibits L-asparagine synthetase,[1] is a key constituent




of several proteins in the blood-clotting cascade,[2] and inhibits the function of excitatory amino acid (EAA) transporters as an L-glutamic acid mimic.[3] The last of these functions is of greater importance, as L-glutamate plays a key role as a primary neurotransmitter in the mammalian central nervous system (CNS) and participates in diverse and complex neuronal communication by activating a broad assortment of the EAA receptors.[4] With its potential to overactivate these receptors, L-glutamate can contribute to CNS damage in acute injuries or chronic diseases.[5] Thus, regulation of extracellular L-glutamate concentration, carried out by the EAA transporters, is crucial. A readily available source of **1** could help in further investigations of these transporters and of the complexity of L-glutamate-mediated signaling processes.

Various synthetic routes to complex diastereomeric mixtures of *erythro*- and *threo*-hydroxyaspartic acid have been described previously.[4] These preparations are circuitous and expensive, making a more efficient synthesis of **1** desirable. Enzymatic catalysis provides an alternative approach, but, to the best of our knowledge, no hydroxylase that directly catalyzes the β-hydroxylation of 2 to 1 has been described. We therefore applied a rational protein design approach to fulfill this task.

Protein engineering, based on 3D-structural information, has become an accepted tool for the manipulation of enzymes for biocatalysis,[7] and we used this method to alter the substrate specificity of an asparagine oxygenase (AsnO) from L-Asn to L-Asp. AsnO, involved in the biosynthesis of calcium-dependent antibiotics (CDAs) in *Streptomyces coelicolor*,[8] is an $Fe^{2+}$- and α-ketoglutarate-dependent (αKG-dependent) hydroxylase, which exclusively catalyzes the synthesis of L-*threo*-hydroxyasparagine (**3**), which is used as a CDA building block.[9] AsnO therefore provides an amino acid with the desired stereochemistry, but does not accept **2** as a substrate. In previous studies,[9] the crystal structure (PDB ID: 2OG7) of AsnO in complexation with 3 and succinate was solved, and the substrate binding residues were identified (Scheme 1). The side chain of resi-




**Scheme 1.** Representation of the active site of asparagine oxygenase (AsnO) in complexation with **3** (shown in blue). The mutation of residue Asp241, which coordinates the $NH_2$ of the carboxamide group of **3**, to Asn alters the substrate specificity of the enzyme from Asn to Asp with retention of stereochemistry.

due Asp241 binds to the $NH_2$ of the carboxamide group of the L-Asn substrate. We hypothesized that site-directed mutagenesis of this residue to asparagine would yield a binding site for an aspartate side chain. Retention of stereochemistry would also be expected, as the α-carboxy and α-amino coordinating residues (Glu125, Asn146, Arg305) are unaffected by the mutagenesis of Asp241.

The site-directed mutagenesis of residue Asp241 to Asn was conducted by use of the *asnO*-containing expression vector as the template.[9] The variant—AsnO D241N—was expressed as a $His_7$-tagged fusion and purified as soluble protein, with yields of 5–6 mg per liter of bacterial culture. After Ni-NTA affinity chromatography, SDS-PAGE analysis indicated >95% purity (see Figure S1 in the Supporting Information). To evaluate its activity on an analytical scale, purified enzyme was incubated with 2, $(NH_4)_2Fe(SO_4)_2$ (as source of the ferrous iron cofactor), and αKG cosubstrate at different temperatures. The reaction was monitored, and the identity of the reaction product was verified by HPLC-MS analysis by scanning for the masses of 2 ($[M+H]^+ = 134.05$ Da) and its hydroxylated form **1** ($[M+H]^+ = 150.04$ Da). The HPLC-MS chromatogram (Figure S2) of the assay after incubation of 2 with AsnO D241N revealed nearly quantitative conversion into **1** at 16 °C.

[a] *M. Strieker, Prof. Dr. L.-O. Essen, Prof. Dr. M. A. Marahiel*
*Chemistry and Biochemistry Department, Philipps-University Marburg*
*Hans-Meerwein-Strasse, 35032 Marburg (Germany)*
*Fax: (+49) 6421-282-2191*
*E-mail: marahiel@chemie.uni-marburg.de*

[b] *M. Strieker, Prof. Dr. C. T. Walsh*
*Biological Chemistry and Molecular Pharmacology, Harvard Medical School*
*240 Longwood Avenue, Boston, MA 02115 (USA)*

*Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.*

In the control reaction without enzyme added, only the substrate L-Asp was observed. Complete conversion was only observed at 16 °C; higher temperatures yielded 80, 67, 55 and 32% conversions for 20, 25, 30 and 37 °C (data not shown), most probably due to reduced enzyme stability.

We were encouraged by these results to carry out the enzymatic reaction on a preparative scale [enzyme (10 μM), L-Asp (14.2 mM), αKG (18 mM) in buffer (10 mL) at 16 °C for 14 h]. The reaction mixture was analyzed by HPLC, with the assistance of a chiral column to distinguish between possible *threo* and *erythro* products, and absorbance was measured at 220 nm and compared with authentic L-3-hydroxyaspartic acid standards (Tocris Bioscience, Ellisville, USA). The overlaid HPLC traces (Figure 1 A) of L-*erythro*-hydroxyaspartic acid (orange), L-aspartic acid (**2**, green), L-*threo*-hydroxyaspartic acid (**1**, red), and the enzyme assay (black) revealed that the only reaction product co-eluted with the L-THA standard. L-*erythro*-Hydroxyaspartic acid was not observed. Integration of the starting material and product peaks gave evidence of 70% conversion of L-Asp into L-THA.

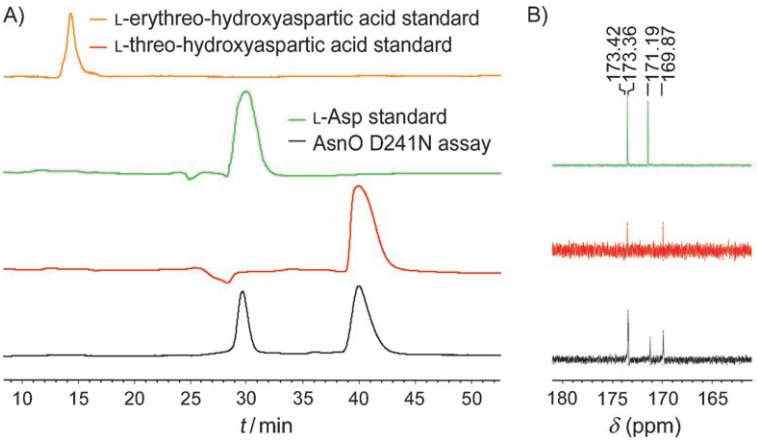



**Figure 1.** Analysis of preparative assay by chiral HPLC. A) The HPLC chromatogram shows the standards of L-*erythro*-hydroxyaspartic acid (orange), L-aspartic acid **2** (green), and L-THA **1** (red). The main peak of the assay (black) co-eluted with **1**, and its integral revealed 70% conversion at 16 °C. B) $^{13}$C NMR spectra of 1,4-$^{13}$C-labeled **2** (green), unlabeled L-THA standard (red), and reproduction of the assay with 1,4-$^{13}$C-labeled **2** (black). The peak at 169.97 ppm verified that L-THA had been generated, and the integral confirmed 70% conversion.

The identity of **2** and its conversion into **1** in the reaction mixture were validated by employing 1,4-$^{13}$C-labeled **2** (Cambridge Isotopes, Andover, USA) in the assay for NMR analysis. The labeled aspartic acid was used to observe specific $^{13}$C NMR signals for **2** and **1** as well as to suppress interfering carbon signals from αKG and succinate, its decomposition product. The NMR spectrum of the reaction mixture (Figure 1 B in black) shows that the two signals ($\delta$ = 173.36, 171.19 ppm) of L-Asp (Figure 1 B, green) are shifted towards the L-THA signals (Figure 1 B, red, $\delta$ = 173.42, 169.87 ppm). Integration of the $^{13}$C signals revealed 70% conversion, in agreement with product quantification from the HPLC assays. Quantitative conversion could not be achieved by addition of more enzyme, most probably due to product inhibition. However, in this simple one-step enzyme reaction, enantiomerically pure 1 was isolated in 68% yield (14.8 mg, 0.1 mmol) after preparative HPLC and freeze-drying of the L-THA-containing fractions. $^1$H NMR and mass data were consistent with those of the authentic standard.

To determine the kinetic parameters of the variant, assays with 5 μM enzyme were quenched at appropriate time points for different substrate concentrations (50 μM to 2 mM), by addition of perfluoropentanoic acid, which also acts as an ion pairing reagent for HPLC-MS analysis. The reaction follows Michaelis–Menten kinetics (Figure S 3) with an apparent $K_M$ of 0.46 ± 0.03 mM and $k_{cat}$ = 1.0 ± 0.1 min$^{-1}$, affording a catalytic efficiency of $k_{cat}/K_M$ = 2.2 ± 0.4 min$^{-1}$ mM$^{-1}$. In total, the biochemical characterization of AsnO D241N shows that the variant is capable of hydroxylating 2 on a preparative scale in a reasonable timespan. For comparison, wild-type AsnO shows an almost identical $K_M$ (0.48 ± 0.07 mM) for L-Asn, but has a 300 times higher $k_{cat}$ (298 ± 19 min$^{-1}$).[9] The side chain swap therefore maintains the substrate affinity but results in less efficient hydroxylation. We anticipate that less obvious secondary interactions are responsible for the accelerated reaction in the wild type. Thus, further evolution of the AsnO D241N variant could raise its catalytic efficiency.

In addition, the substrate specificity of the variant was evaluated by incubation of the enzyme with different amino acids overnight at 16 °C (Table 1). LC-MS analyses of these assays indicated that the engineered protein is highly specific and exclusively accepts **2** as its cognate substrate. Neither the AsnO wild-type substrate L-Asn- nor L-Asp-related amino acids such as L-Glu or the D-Asp enantiomer, nor representatives of other amino acid classes were accepted as substrates for protein-mediated hydroxylation.

In summary, we have designed and biologically characterized a highly specific hydroxylase that catalyzes the direct hydroxylation of the aliphatic β-position of L-Asp for the

**Table 1.** Evaluation of the substrate specificity of AsnO D241N.

| Amino acid | $[M+H]^+$ substrate | $[M+H]^+$ product (calcd)[a] | Mass (obs.)[b] | Hydroxylation |
|---|---|---|---|---|
| L-Asp | 134.0 | 150.0 | 150.0 | yes |
| D-Asp | 134.0 | 150.0 | 134.0 | no |
| L-Asn | 133.1 | 149.1 | 133.0 | no |
| L-Glu | 148.1 | 164.1 | 148.2 | no |
| L-Gln | 147.1 | 163.1 | 147.2 | no |
| L-Ile | 132.1 | 148.1 | 132.2 | no |
| L-Phe | 166.1 | 182.1 | 166.1 | no |
| L-Trp | 205.1 | 221.1 | 205.3 | no |
| L-Val | 118.1 | 134.1 | 118.0 | no |

[a] Calculated masses of the hydroxylation products. [b] Masses observed after overnight incubation of AsnO D241N and the corresponding amino acid. Analyses were carried out by LC-MS.

enantiomerically pure generation of the medicinally interesting L-THA.

## Experimental Section

**Site-directed mutagenesis**: The mutagenesis was carried out with the *QuickChange II Kit* (Stratagene) in accordance with the manufacturer's manual with the synthetic oligonucleotide primers 5′-CCCCGACCTGCGGGTGAACCTGGCGGCCACCGAGC-3′ and 5′-GCTCGGTGGCCGCCAGGTTCACCCGCAGGTCGGGG-3′ (the mutagenesis site is underlined). The previously described *asnO* expression vector[9] served as the template. DNA dideoxy sequencing confirmed the identity of the constructed mutation-carrying plasmid.

**Protein purification**: Positive clones were used to transform *E. coli* BL21(DE3) (Novagen). The transformed cells were grown at 34 °C to an optical density of 0.5 (600 nm), induced with isopropyl β-D-thiogalactopyranoside (0.1 mM), and again grown at 28 °C for 3 h. The cells were harvested by centrifugation and lysed in pH 8 buffer containing Hepes (50 mM) and NaCl (300 mM). The recombinant protein was purified by Ni-NTA affinity chromatography by use of a FPLC system (Amersham Pharmacia Biotech) with increasing imidazole concentration (up to 250 mM). Fractions containing the AsnO D241N (38.9 kDa) were identified by 12% SDS-PAGE analysis (Figure S1), pooled, and subjected to buffer exchange into HEPES (25 mM), NaCl (50 mM), pH 7.0 with HiTrap desalting columns (Amersham Pharmacia Biotech). The concentration of the purified protein was determined spectrophotometrically with use of the calculated extinction coefficient at 280 nm. After being flash-frozen in liquid nitrogen, the protein was stored at −80 °C.

**Analytical assay, kinetics and substrate specificity**: The recombinant AsnO D241N (5 μM) was incubated for 16 h at 16 °C with **2** (1 mM), the co-factor $(NH_4)_2Fe(SO_4)_2$ (1 mM), and cosubstrate αKG (2 mM) in HEPES buffer (pH 7.5, 50 mM, 0.1 mL). Controls were carried out in the absence of AsnO D241N. The reaction was stopped by addition of a nonafluoropentanoic acid solution (4% *v/v*, 20 μL), and the mixture was then analyzed for possible hydroxylation by reversed-phase HPLC-MS analysis on a Hypercarb column (Thermo Electron Corporation, pore diameter of 250 Å, particle size of 5 μm, 100% carbon). Mobile phases were: A) aqueous nonafluoropentanoic acid (20 mM), and B) acetonitrile. The following gradient was applied: 0–10% B in 12 min, with a flow rate of 0.2 mL min$^{-1}$ at 17.5 °C. Identification of **2** was achieved by high-accuracy MS on an API Qstar Pulsar I device (Applied Biosystems). Kinetics were carried out accordingly, the substrate concentration being varied between 50 μM and 2 mM. The reaction was stopped in the linear range (after 5 and 30 min) and analyzed as described above. Integration of the mass signals of **1** and **2** gave relative conversion values from which starting velocities were calculated. Substrate specificity assays were carried out as described above with the corresponding amino acid (see Table 1).

**Preparative assay**: AsnO D241N (10 μM) was incubated with **2** (18.9 mg, 142 μmol), the co-substrate αKG (26 mg, 18 mmol), and ammonium iron(II) sulfate hexahydrate (0.39 mg, 100 μmol) as source of the ferrous iron co-factor. All solids were dissolved in Hepes buffer, (pH 7.0, 25 mM, 9 mL). The reaction was started by addition of enzyme stock solution (same buffer, 100 μM, 1 mL) and was allowed to stir for 14 h at 16 °C. Compound **1** was isolated by HPLC separation on a Chirex 3126 column (Phenomenex, D-penicillamine, 150×4.6 mm) with an isocratic mobile phase of $CuSO_4$ (1 mM) in $H_2O$ (95%) and isopropyl alcohol (5%) on a Beckman Coulter Gold System with UV detector 168 (Fullerton, USA). The L-THA-containing fractions were pooled and passed through an NTA-Agarose resin by gravity flow to remove the copper ions and freeze-dried to yield **1**.

**NMR spectroscopy**: All $^1$H and $^{13}$C NMR spectra were acquired on a Varian 600 MHz spectrometer (Palo Alto, USA). The reproduction of the preparative assay with 1,4-$^{13}$C-labeled **2** was carried out as described above, except that phosphate solution in $D_2O$ (pH 7.0, 20 mM) was used as buffer.

## *Acknowledgements*

*We gratefully acknowledge A. Schäfer and Dr. A. Koglin for technical assistance and Dr. E. M. Nolan for carefully proof-reading the manuscript. We thank the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie for financial support.*

**Keywords:** enzyme catalysis · hydroxylases · non-proteinogenic amino acids · NRPS · protein engineering

[1] M. Mokotoff, J. F. Bagaglio, B. S. Parikh, *J. Med. Chem.* **1975**, *18*, 354–358.
[2] K. G. Mann, R. J. Jenny, S. Krishnaswamy, *Ann. Rev. Biochem.* **1988**, *57*, 915–956.
[3] a) K. Shimamoto, Y. Shigeri, Y. Yasuda-Kamatani, B. Lebrun, N. Yumoto, T. Nakajima, *Bioorg. Med. Chem. Lett.* **2000**, *10*, 2407–2410; b) R. J. Bridges, C. S. Esslinger, *Pharmacol. Ther.* **2005**, *107*, 271–285.
[4] a) J. Cartmell, D. D. Schoepp, *J. Neurochem.* **2000**, *75*, 889–907; b) D. D. Schoepp, *J. Pharmacol. Exp. Ther.* **2001**, *229*, 12–20.
[5] J. W. Olney, *Curr. Opin. Pharmacol.* **2003**, *3*, 101–109.
[6] a) H. D. Dakin, *J. Biol. Chem.* **1921**, *48*, 273–291; b) L. Benoiton, S. M. Birnbaum, M. Winitz, J. P. Greenstein, *Arch. Biochem. Biophys.* **1959**, *81*, 434–438; c) A. M. Hafez, T. Dudding, T. R. Wagerle, M. H. Shah, A. E. Taggi, T. Lectka, *J. Org. Chem.* **2003**, *68*, 5819–5825.
[7] a) U. T. Bornscheuer, M. Pohl, *Curr. Opin. Chem. Biol.* **2001**, *5*, 137–143; b) K. Hult, P. Berglund, *Curr. Opin. Biotechnol.* **2003**, *14*, 395–400.
[8] Z. Hojati, C. Milne, B. Harvey, L. Gordon, M. Borg, F. Flett, B. Wilkinson, P. J. Sidebottom, B. A. Rudd, M. A. Hayes, C. P. Smith, J. Micklefield, *Chem. Biol.* **2002**, *9*, 1175–1187.
[9] M. Strieker, F. Kopp, C. Mahlert, L.-O. Essen, M. A. Marahiel, *ACS Chem. Biol.* **2007**, *2*, 187–196.

Received: September 17, 2007
Published online on January 21, 2008