Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Overexpression, purification, crystallization and data collection of 3-methylaspartase from *Clostridium tetanomorphum*

Miryam Asuncion,[a]† John N. Barlow,[b]† John Pollard,[b] Adam G. Staines,[a] Stephen A. McMahon,[a]‡ Wulf Blankenfeldt,[a] David Gani[b] and James H. Naismith[a]*

[a]The Centre for Biomolecular Sciences, The University, St Andrews KY16 9ST, Scotland, and [b]Department of Chemistry, The University of Birmingham, Edgbaston, Birmingham, England

† These authors made equal contributions.
‡ Current address: Department of Chemistry, University of California at San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0314, USA.

Correspondence e-mail: naismith@st-and.ac.uk

Received 26 January 2001
Accepted 16 February 2001

3-Methylaspartase (E.C. 4.3.1.2) catalyses the reversible *anti* elimination of ammonia from L-*threo*-(2*S*,3*S*)-3-methylaspartic acid to give mesaconic acid as well as a slower *syn* elimination from the (2*S*,3*R*)-epimer, L-*erythro*-3-methylaspartic acid. The *anti*-elimination reaction occurs in the second step of the catabolic pathway for glutamic acid in *Clostridium tetanomorphum*. The reverse reaction is of particular interest because the addition of ammonia to substituted fumaric acids is highly stereoselective and gives highly functionalized amino acids. The mechanism of the transformation is unusual and of considerable interest. 3-Methylaspartase from *C. tetanomorphum* has been overexpressed and purified from *Escherichia coli*. Crystals of the enzyme have been obtained by sitting-drop vapour diffusion. Two native data sets have been collected, one in-house on a rotating-anode generator to 3.2 Å and one at the European Synchrotron Radiation Facility to 2.0 Å. A 2.1 Å data set has been collected on a crystal of selenomethionine protein. Combining the data sets identify the space group as $P2_12_12$, with unit-cell parameters a = 110.3, b = 109.9, c = 67.2 Å, $\alpha = \beta = \gamma = 90^\circ$. The asymmetric unit contains two monomers with 42% solvent. A self-rotation function indicates the presence of a twofold axis, consistent with a biological dimer.

1. Introduction

The synthesis of optically pure chiral compounds remains a fundamental challenge in synthetic chemistry. One approach is to use stereospecific or highly stereoselective enzyme catalysts to control, for example, the stereochemical course of addition reactions (Akhtar, Botting *et al.*, 1987; Akhtar, Cohen *et al.*, 1987) or the selection of specific enantiomers of the substrate in kinetic resolutions. However, for many synthetically useful reactions there are no available enzyme catalysts. The alteration of substrate selectivity and reaction specificity by the rational redesign of protein structure has proven difficult. An alternative method is to evolve novel enzyme activities using directed evolution. The combined use of both approaches can be particularly powerful, as recently demonstrated by Altamirano *et al.* (2000). Selected enzyme residues are chosen for random mutagenesis on the basis of function (*e.g.* substrate binding or catalysis) and the required enzyme activity selected using a suitable screen. Of course, the ability to assign function to enzyme amino-acid residues is facilitated with a three-dimensional structural model.

3-Methylaspartic acid ammonia lyase (3-methylaspartase) catalyses the reversible *anti* elimination of ammonia from L-*threo*-(2*S*,3*S*)-3-methylaspartic acid to give mesaconic acid (Fig. 1). It lies on the main catabolic pathway for glutamate in *C. tetanomorphum* (Barker *et al.*, 1958) and a number of other anaerobic microorganisms. Monovalent and divalent cation cofactors are required for full catalytic activity, with potassium and magnesium ions serving as the best metal-ion cofactors (Botting & Gani, 1992). It has been proposed, on the basis of chemical modification studies, that Ser173 is dehydrated post-translationally to dehydroalanine (Goda *et al.*, 1992; Pollard *et al.*, 1999). The unusual dehydroalanine prosthetic group could function as a Michael acceptor in the deamination reaction (Gulzar *et al.*, 1995). Strong support for a similar post-translational modification in the related enzyme histidine ammonia lyase (histidase) has been provided by X-ray crystallography (Schwede *et al.*, 1999). In histidase, an internal Ala-Ser-Gly tripeptide (residues 142–144) undergoes intrachain cyclization accompanied by dehydration of Ser143 to form a 4-methylidene-imidazol-5-one moiety (Fig. 2). Interestingly, Ser173 of 3-methylaspartase is also present in an Ala-Ser-Gly tripeptide. However, there is no structural evidence for a dehydroalanine residue at present.

3-Methylaspartase is a good target for protein-engineering studies. Engineered 3-methylaspartase could potentially be used to

extend the accessible range of synthetic homochiral substituted aspartic acids (Akhtar, Botting *et al.*, 1987; Gulzar *et al.*, 1997). The native enzyme is known to display limited utility in such syntheses. There is also the possibility of switching the stereochemical course of the amination reaction with respect to the configuration at C-3 (Archer *et al.*, 1993). Recent studies show that two different bases deprotonate the C-3 position in the processing of the (2*S*,3*S*)- and (2*S*,3*R*)-diastereomers of 3-methylaspartic acid (Gani *et al.*, 1999). Thus, identification of these two bases would allow their specific substitution and potentially complete control of the stereospecificity of the corresponding amination reactions to give *anti* or *syn* addition products. Access to these synthetically useful compounds by conventional synthesis is extremely difficult and not well developed. However, engineering of 3-methylaspartase is greatly hindered by the lack of structural information and the absence of homologues in the Protein Data Bank. We report here the overexpression, crystallization and data collection for crystals of 3-methylaspartase and its selenomethionine form. The crystal structure will facilitate future efforts to engineer 3-methylaspartase by directed evolution.

2. Materials and methods

2.1. Expression and purification

Recombinant 3-methylaspartase from *C. tetanomorphum* has been cloned and expressed by Goda *et al.* (1992) previously. In order to increase the expression levels of protein it was re-cloned into a different expression vector, pJGetit, a laboratory-prepared variant of the commercially available pET vector (kindly donated by Dr R. K. Allemann, University of Birmingham). The sequence of the cloned gene was confirmed to be identical to the chromosomal copy. The resulting construct was used to transform *E. coli* BL21 (λDE3). Transformed cells were grown at 310 K and 150 rev min^{-1} in Luria–Bertani medium containing 50 µg ml^{-1} carbenicillin to an A_{600} of 0.5. Induction was with 0.4 m*M* IPTG at 310 K for 3 h. Cells were harvested at 6500*g* for 15 min and stored at 193 K. Analysis of cell extracts by SDS–PAGE confirmed the high expression levels of 3-methylaspartase.

Thawed wet cell paste (18 g from 6 l of cells) was suspended in lysis buffer [25 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 5 m*M* DTT, 10%(*v*/*v*) glycerol] to give a final volume of 70 ml. Protease inhibitors (5 m*M* PMSF, Sigma) and hen egg-white lysozyme (100 µg ml^{-1}) were added and the suspension stirred (277 K for 45 min). Magnesium chloride (10 m*M*) and a small spatula of DNase I were then added and the suspension stirred (277 K for 40 min). The cell suspension was sonicated (six cycles of 30 s) and centrifuged (30 min at 20 000*g*) at 277 K. The resulting supernatant was diluted three times prior to loading onto a Fast Q High Performance column (Amersham-Pharmacia Biotech; 300 ml) and equilibrated with two column volumes of buffer *A* (25 m*M* Tris–HCl pH 8.0, 5 m*M* DTT). After sample loading, the column was washed with buffer *A* until the A_{280} had decreased to ∼0. 3-Methylaspartase was eluted with an NaCl gradient (0–500 m*M* over 1.5 l at 5 ml min^{-1}; the high-salt buffer *B* was made up as buffer *A* but contained 1 *M* NaCl). 3-Methylaspartase eluted at 125 m*M* NaCl. Fractions were assayed for 3-methylaspartase using the method of Goda *et al.* (1992). Active fractions were examined by 12.5% SDS–PAGE, pooled, concentrated to ∼15 mg ml^{-1} and stored at 277 K overnight. Protein concentration was determined using the method of Bradford (1976) with bovine serum albumin as standard.

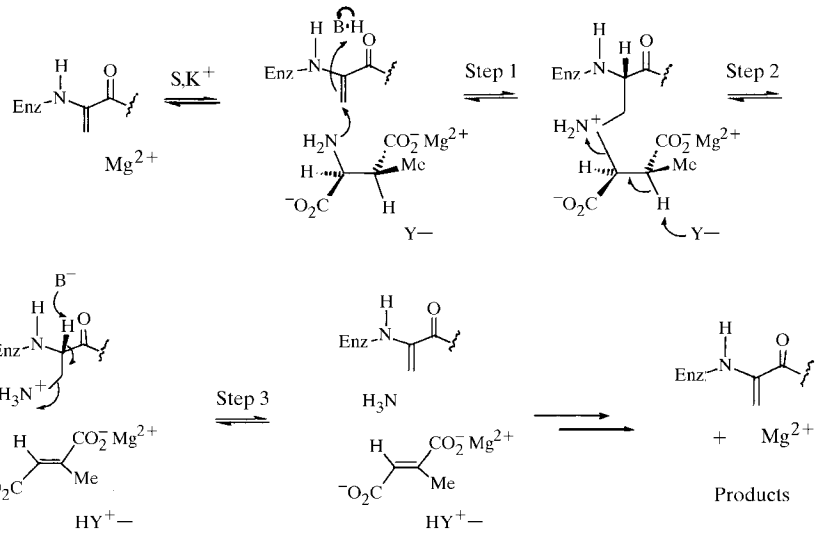

Figure 1
Proposed mechanism for the *anti* elimination of ammonia from (2*S*,3*S*)-3-methylaspartic acid.

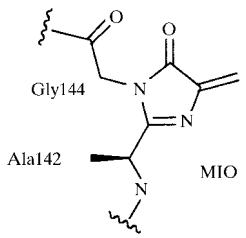

Figure 2
Structure of 4-methylidene-imidazol-5-one moiety proposed for histidine ammonia lyase.

A second chromatographic step was performed on an S-200 gel-filtration column (Amersham-Pharmacia Biotech; 2.6 × 100 cm) equilibrated at 277 K with two column volumes of 50 m*M* Tris–HCl pH 8.0. The 3-methylaspartase concentrate was loaded at 1 ml min^{-1} and eluted at 2.5 ml min^{-1}. Fractions containing 3-methylaspartase migrated as a single band as determined by SDS–PAGE. The pure fractions were pooled, concentrated to 15 mg ml^{-1} with an Amicon concentrator, aliquoted and stored at 193 K. The measured k_{cat} (240 ± 20 s^{-1}) and K_M (0.58 ± 0.02 m*M*) were similar to those previously determined for recombinant 3-methyl-aspartase. Mass analysis of purified protein gave an observed mass of 45 538 Da (calculated, 45 534 Da). Light-scattering analysis indicated that the 3-methylaspartase existed as a dimer in solution.

The selenomethionine variant of 3-methylaspartase was expressed in the methionine auxotroph *E. coli* B834 following the protocol of Doublie (1997) and was purified using the same protocol as used for the wild type. Mass analysis of purified selenomethionine-variant 3-methylaspartase indicated a mass of 46 341 Da (calculated, 46 331 Da). To further check the efficiency of selenomethionine incorporation, mass analysis of a tryptic digest of 3-methylaspartase was performed and confirmed complete incorporation.

2.2. Crystallization

Initial crystallization conditions were obtained from Crystal Screen I from Hampton Research (Jancarik & Kim, 1991; Cudney *et al.*, 1994) at 298 K. The sitting-

Table 1
Data collection from *C. tetanomorphum* 3-methylaspartase.

Values in parentheses refer to the highest resolution shell.

	Native	Native	SeMet
Wavelength (Å)	0.933	1.54	1.54
Temperature (K)	130	298	130
Detector	CCD	R-AXIS IV++	DIP2000
Resolution (Å)	50.6–2.0 (2.11–2.0)	24–3.2 (3.26–3.2)	25-2.4
Space group	$P2_12_12$	$P2_12_12$	$P2_12_12$
Unit-cell parameters (Å)	a = 110.3, b = 109.9, c = 67.2	a = 109.0, b = 106.6, c = 67.5	a = 109.3, b = 108.8, c = 67.1
Unique reflections	53841	10586	31901
Average redundancy	4.2 (4.0)	3.4 (2.1)	6.5 (5.1)
$I/\sigma(I)$	7.0 (3.4)	4.2 (1.1)	10.1 (3.1)
Completeness (%)	96.8 (98.1)	79 (68)	87 (65)
$R_{\rm merge}$ (%)	7.4 (19.9)	10.9 (16.0)	13.3 (44.1)

drop vapour-diffusion method (Ducruix & Giegé, 1992) with 4.5 µl protein sample (7.5 mg ml^{-1}) and 4.5 µl precipitant was used throughout. Crystals were obtained using 20–25%(*w*/*v*) PEG 6000, 100 m*M* sodium acetate pH 7.0, 25 m*M* Tris–HCl pH 7.0 as precipitating solution and using 16–22% of ethylene glycol as additive.

2.3. Data collection

A 3.2 Å resolution data set was collected in house from a single crystal mounted in a glass capillary. The data were collected on an R-AXIS IV++ detector with a Rigaku rotating-anode generator (5 kW, 100 mA) as 1° 10 min exposures in three overlapping segments totalling 240° of data (130° of unique data). The oscillation images were indexed and integrated with the program *DENZO* (Otwinowski & Minor, 1996) and scaled with *SCALEPACK* (Otwinowski & Minor, 1996). A 2.0 Å resolution data set from a frozen crystal of the protein was measured at the ESRF, Grenoble. Crystals were cryoprotected by soaking in a solution containing 20%(*w*/*v*) PEG 6000, 100 m*M* sodium acetate pH 7.0, 25 m*M* Tris–HCl pH 7.0, 16% ethylene glycol and 20% glycerol for 20 s prior to rapid cooling to 120 K for data collection. The data were collected as 109 1° oscillation 20 s exposures on ID14-EH2 using a Quantum 4 CCD detector. Data were processed with *MOSFLM* (Leslie, 1992) and scaled with the *CCP*4 program *SCALA* (Collaborative Computational Project, Number 4, 1994). Crystals of the selenomethionine-containing protein were obtained under similar conditions to those of the native protein. They differ only in their slower rate of growth (five weeks compared with two weeks). A single crystal of the selenomethionine protein was flash-frozen and an in-house data set collected on a Nonius DIP2000 image-plate rotating-anode system. Data were collected as 240 non-overlapping 10 min 1° exposures. Data were merged and scaled using *MOSFLM* (Leslie, 1992). The data are of poor quality owing to the high mosaic spread of the crystal (1.5°). Full details of data collection are given in Table 1. Together, the three data sets measure more than 15 reflections along each of the three axes. The pattern of absences shows that the space group is $P2_12_12$.

The use of beamlines at the ESRF Grenoble, France is gratefully acknowledged. We thank Marie-France Giraud and Susan Richardson for assistance.

References

Akhtar, M., Botting, N. P., Cohen, M. A. & Gani, D. (1987). *Tetrahedron*, **43**, 5899–5908.
Akhtar, M., Cohen, M. A. & Gani, D. (1987). *Tetrahedron Lett.* **28**, 2413–2416.
Altamirano, M. M., Blackburn, J. M., Aguayos, C. & Fersht, A. R. (2000). *Nature (London)*, **403**, 617–622.
Archer, C. H., Thomas, N. R. & Gani, D. (1993). *Tetrahedron Asym.* **4**, 1141–1152.
Barker, H. A., Smyth, R. D., Wilson, R. M. & Weissbach, H. (1958). *J. Biol. Chem.* **234**, 320–328.
Botting, N. P. & Gani, D. (1992). *Biochemistry*, **31**, 1509–1520.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–252.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Cudney, R., Patel, S., Weisgraber, K., Newhouse, Y. & McPherson, A. (1994). *Acta Cryst.* D**50**, 414–423.
Doublie, S. (1997). *Methods Enzymol.* **276**, 523–530.
Ducruix, A. & Giegé, R. (1992). *Crystallization of Nucleic Acids and Proteins, a Practical Approach.* Oxford: IRL Press.
Gani, D., Archer, C. H., Botting, N. P. & Pollard, J. (1999). *Bioorg. Med. Chem.* **7**, 977–990.
Goda, S., Minton, N. P., Botting, N. P. & Gani, D. (1992). *Biochemistry*, **31**, 10747–10756.
Gulzar, M. S., Akhtar, M. & Gani, D. (1997). *J. Chem. Soc. Perkin Trans.* I, pp. 649–655.
Gulzar, M. S., Morris, K. & Gani, D. (1995). *J. Chem. Soc. Chem. Commun.* pp. 1061–1062.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Leslie, A. G. W. (1992). *Jnt CCP4/ESF–EACMB Newsl. Protein Crystallogr.* **26**.
Otwinowski, Z. & Minor, W. (1996). *Methods Enzymol.* **276**, 307–326.
Pollard, J., Richardson, S., Lasry, P., Neal, T., Akhtar, M., Botting, N. P. & Gani, D. (1999). *Bioorg. Med. Chem.* **7**, 949–975.
Schwede, T. F., Rétey, J. & Schulz, G. E. (1999). *Biochemistry*, **38**, 5355–5361.