Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray analysis of *Citrobacter amalonaticus* methylaspartate ammonia lyase

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Received 8 August 2001
Accepted 8 October 2001

Methylaspartate ammonia lyase (MAL) catalyses the reversible α,β-elimination of ammonia from L-*threo*-(2*S*,3*S*)-3-methylaspartic acid to give mesaconic acid. Crystals of *Citrobacter amalonaticus* MAL have been obtained by the hanging-drop method of vapour diffusion using ammonium sulfate as the precipitant. Three crystal forms were obtained from identical crystallization conditions, two of which (forms *A* and *B*) diffract to high resolution, whilst the third form diffracted poorly. Crystals of form *A* diffract to beyond 2.1 Å and have been characterized as belonging to one of the enantiomorphic space groups $P4_122$ or $P4_322$, with unit-cell parameters $a = b = 66.0$, $c = 233.1$ Å, $\alpha = \beta = \gamma = 90^\circ$ and a monomer in the asymmetric unit. Crystals of form *B* diffract to beyond 1.5 Å and belong to space group *C*222, with unit-cell parameters $a = 128.3$, $b = 237.4$, $c = 65.8$ Å, $\alpha = \beta = \gamma = 90^\circ$ and a dimer in the asymmetric unit. Determination of the structure of MAL will be an important step in resolving current conflicts concerning the enzyme mechanism which differ between one which places MAL as a member of the superfamily of ammonia lyases whose catalytic activity requires a cofactor formed by post-translational modification of the enzyme and another which links MAL to the enolase superfamily.

1. Introduction

Methylaspartate ammonia lyase (MAL) catalyses the reversible α,β-elimination of ammonia from L-*threo*-(2*S*,3*S*)-3-methylaspartic acid to give mesaconic acid. The enzyme was first isolated from *C. tetanomorphum* and is narrowly distributed throughout both obligate and facultative anaerobes (Asano & Kato, 1994), where it forms a component of glutamate catabolism (Kato & Asano, 1997).

MAL from *C. amalonaticus* (Kato & Asano, 1998) is dimeric in nature, with a subunit molecular mass of 45.5 kDa, and shows approximately 60% sequence identity to the enzyme from *C. tetanomorphum*. *C. amalonaticus* MAL appears to require both divalent and monovalent cations such as Mg^{2+} and K^+ for activity (Kato & Asano, 1995) and is stable at temperatures up to 323 K and over a wide pH range (5.5–10.0), with the pH optimum for the deamination reaction of (2*S*,3*S*)-3-methylaspartic acid lying at the upper end of this range. A possible application of MAL lies in the chiral synthesis of some 3-substituted (*S*)-aspartic acids from their corresponding fumaric acid derivatives (Kato & Asano, 1995).

Currently, the mechanism of MAL is the subject of some debate. Based on the chemistry of the reaction catalysed by MAL, it is not surprising that the enzyme has been linked to the family of ammonia lyases, which include phenylalanine ammonia lyase (PAL) and histidine ammonia lyase (HAL) (Pollard *et al.*, 1999). Biochemical studies of phenylalanine ammonia lyase suggest that the mechanism of this enzyme requires the post-translational modification of an active-site serine to a catalytically essential dehydroalanine prosthetic group (Schuster & Rétey, 1995), the essential role of which is to form an intermediate in which a covalent linkage is formed between the prosthetic group and the substrate. Structural studies of histidine ammonia lyase, which was proposed to utilize a dehydroalanine prosthetic group for catalysis (Langer, Lieber *et al.*, 1994; Langer, Reck *et al.*, 1994), have revealed the presence of a novel electrophilic prosthetic group, 4-methylidene-imidazole-5-one, formed by post-translational modification of an active-site serine (Schwede *et al.*, 1999). Gani and coworkers have suggested that MAL utilizes a dehydroalanine prosthetic group in its reaction mechanism (Pollard *et al.*, 1999). However, in studies elsewhere, low-level sequence similarities have suggested that MAL is a member of the muconate-lactonizing enzyme (MLE) subgroup of the enolase superfamily (Babbitt *et al.*, 1996). If MAL is a member of the enolase superfamily then it would be predicted to have a TIM-barrel architecture and the enzyme

mechanism would proceed *via* the ability of the enzyme to catalyze the abstraction of the proton α to a carboxylic acid to form an enolic intermediate without the requirement of any additional cofactors. In contrast, the reaction catalysed by PAL and HAL cannot be accommodated by a mechanism related to the enolase superfamily, as neither of their substrates (phenylalanine or histidine, respectively) contains a carboxylate group α to the abstracted proton and an enolic intermediate cannot therefore be formed. The determination of the structure of MAL should resolve the conflict in the MAL mechanism and provide important clues towards exploiting the biotechnological potential of the enzyme.

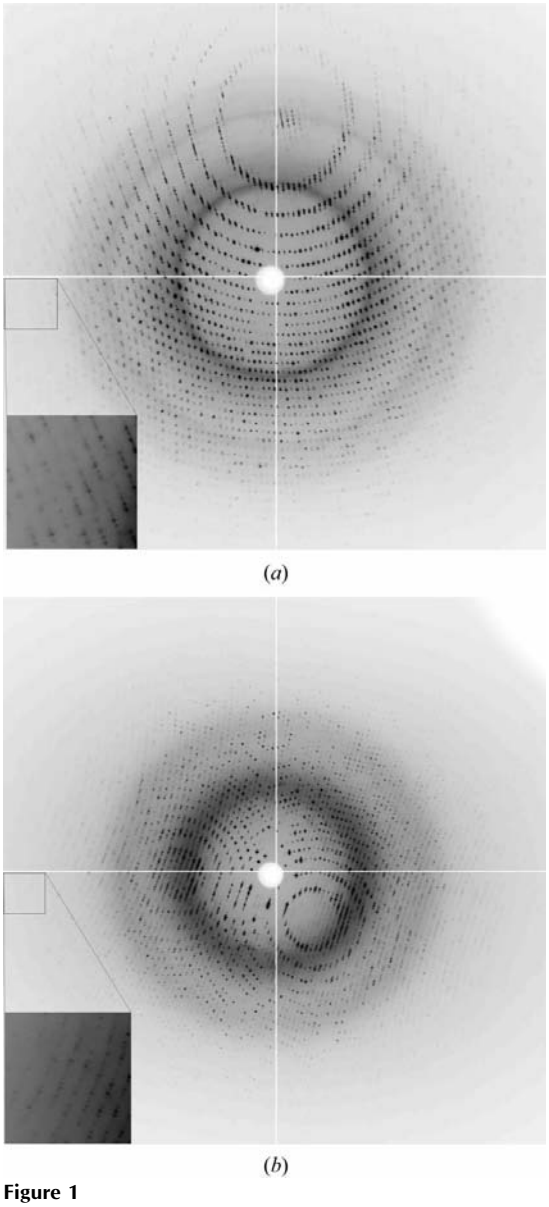

Figure 1
Representative oscillation images of *C. amalonaticus* MAL crystals: (*a*) form *A* crystals, (*b*) form *B* crystals. The resolution at the edge of the enlarged square in each image corresponds to 2.1 and 1.5 Å, respectively.

2. Materials and methods

2.1. Preparation of MAL

The gene for *C. amalonaticus* MAL was cloned into the expression vector pUC18 under the control of the IPTG-inducible *lac* promotor together with an ampicillin-resistance gene and transformed into *Escherichia coli* line JM109. The *E. coli* JM109 cells harbouring the plasmid containing the *C. amalonaticus* MAL gene were grown at 310 K with shaking at 200 rev min^{-1} in the presence of ampicillin (100 µg ml^{-1}) until an OD_{600} of between 0.6 and 0.9 was reached. The cells were then induced with 1 m*M* IPTG, grown for a further 16 h, harvested by centrifugation and frozen.

For purification, the cell paste was defrosted, suspended in buffer *A* (40 m*M* Tris–HCl pH 8.0, 2 m*M* EDTA) and disrupted by three 20 s cycles of sonication at an amplitude of 16 µm. Debris was removed by centrifugation at 40 000*g* for 20 min. The supernatant fraction was applied to a 30 ml column with DEAE-Sepharose Fast Flow (Pharmacia) and the protein was eluted with a 300 ml gradient of NaCl from 0.0 to 0.4 *M* in buffer *A*.

The fractions containing MAL were combined and brought to an ammonium sulfate concentration of 1.4 *M* by addition of 4 *M* ammonium sulfate stock solution. The sample was applied to a 20 ml Butyl-Toyopearl 650S (ToSoh) column and eluted with a 300 ml reverse gradient of ammonium sulfate from 1.4 to 0.0 *M* in buffer *A*. The fractions containing MAL were combined and the protein precipitated again with ammonium sulfate. The pellet was collected by centrifugation for 10 min at 40 000*g* and dissolved in 1.5 ml buffer *A*. Further purification was achieved by gel filtration on a 1.6 × 60 cm Hi-Load Superdex 200 column (Pharmacia) in buffer *B* (50 m*M* K_2PO_4 pH 7.0, 5 m*M* $MgCl_2$). The protein eluted from the column at a position consistent with the proposed dimeric quaternary structure of the enzyme (Asano & Kato, 1994) and the peak fractions were collected and concentrated on a VivaSpin concentrator to 13 mg ml^{-1} as estimated by the method of Bradford (1976). The purity of the protein was estimated using SDS–PAGE (Laemmli, 1970) to be greater than 95% and the yield was approximately 30 mg of protein per litre of cell culture.

2.2. Crystallization

The crystallization of MAL was achieved at 290 K using the hanging-drop method of vapour diffusion, using standard protocols. Crystals with either tetragonal bipyramidal, rectangular plate or rod morphologies were obtained in the same drop after ~72 h using a well solution consisting of 0.1 *M* potassium phosphate buffer pH 8.0, 5 m*M* $MgCl_2$ and 32% ammonium sulfate. For X-ray analysis, the crystals were transferred briefly to a cryoprotectant containing 25% glycerol, 45% ammonium sulfate, 5 m*M* $MgCl_2$ in 0.1 *M* potassium phosphate buffer pH 8.0 before flash-freezing in a stream of nitrogen at 100 K. Test images were collected on a Quantum Q4 CCD detector on station 9.6 at the SRS Daresbury Laboratory and complete data sets were collected in-house on a MAR345 detector with double-mirror focused Cu $K\alpha$ X-rays produced by a Rigaku rotating-anode generator. The rotation images were processed using the *DENZO*/*SCALEPACK* package (Otwinowski & Minor, 1997).

3. Discussion

X-ray analysis of the crystals with the morphology of tetragonal bipyramids showed that they diffracted poorly to only 10 Å; they were not analysed further. Analysis of the other crystals, which grew either as plates or rods, led to the characterization of two distinct crystal forms which could not be distinguished consistently on the basis of morphology. Crystals of form *A* diffract to beyond 2.1 Å at the SRS (Fig. 1*a*) and belong to space group $P4_122$ or $P4_322$, with unit-cell parameters $a = b = 66.0$, $c = 233.1$ Å, $\alpha = \beta = \gamma = 90^\circ$. An in-house data set was collected to 2.85 Å and was 97% complete (96% in the 2.92–2.85 Å resolution shell) with a multiplicity of 2.6, an overall $I/\sigma(I)$ of 15 (4 in the 2.92–2.85 Å resolution shell) and an overall R factor of 0.06 (0.22 in the 2.92–2.85 Å shell). The V_M value of 2.8 Å^3 Da^{-1} for a monomer in the asymmetric unit with 55% solvent content lies within the range given by Matthews (1977). Crystals of form *B* diffract to beyond 1.5 Å at the SRS (Fig. 1*b*) and belong to space group *C*222, with unit-cell parameters

$a = 128.3, b = 237.4, c = 65.8$ Å, $\alpha = \beta = \gamma = 90^\circ$. An in-house data set was collected to 3.2 Å and was 95% complete (93% in the 3.27–3.20 Å resolution shell) with a multiplicity of 3.9, an overall $I/\sigma(I)$ of 16 (5 in the 3.27–3.20 Å resolution shell) and an overall R factor of 0.06 (0.23 in the 3.27–3.20 Å shell). The V_M value of 2.9 Å^3 Da^{-1} for a dimer in the asymmetric unit with 58% solvent content lies within the range given by Matthews (1977). The similarity in unit-cell parameters between crystal forms *A* and *B* suggest that elements of crystal packing in each case may well be related. A full structure determination is now under way and the results should provide important insights into the mechanism of MAL and help underpin the future application of the enzyme in the chiral synthesis of novel amino acids.

We thank the support staff at the Synchrotron Radiation Source at the CCLRC Daresbury Laboratory for assistance with station alignment. This work was supported by the New Energy and Industrial Development Organization, the British Council, the EU, the Wellcome Trust, the Wolfson Foundation and the BBSRC. CWL is supported by a BBSRC industrial case award with SYNGENTA. The Krebs Institute is a designated BBSRC Biomolecular Science Centre and a member of the North of England Structural Biology Centre.

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