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Purification, crystallization and preliminary crystallographic study of an IDS-epimerase from *Agrobacterium tumefaciens* BY6

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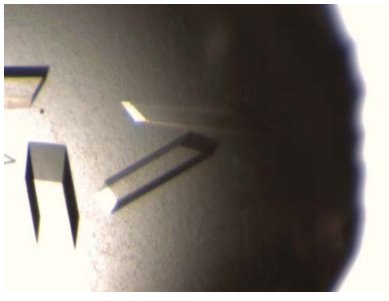
The initial degradation of all stereoisomers of the complexing agent iminodisuccinate (IDS) is enabled by an epimerase in the bacterial strain *Agrobacterium tumefaciens* BY6. This protein was produced in *Escherichia coli*, purified and crystallized by the hanging-drop vapour-diffusion method. Crystals of IDS-epimerase were obtained under several conditions. The best diffracting crystals were grown in 22% PEG 3350, 0.2 *M* $(NH_4)_2SO_4$ and 0.1 *M* bis-Tris propane pH 7.2 at 293 K. These crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters $a = 55.4$, $b = 104.2$, $c = 78.6$ Å, $\beta = 103.3^\circ$, and diffracted to 1.7 Å resolution. They contain two protein molecules per asymmetric unit. In order to solve the structure using the MAD phasing method, crystals of the L-selenomethionine-substituted epimerase were grown in the presence of 20% PEG 3350, 0.2 *M* Na_2SO_4 and 0.1 *M* bis-Tris propane pH 8.5.

1. Introduction

The bacterial strain *Agrobacterium tumefaciens* BY6 mineralizes all three epimers of the environmentally benign complexing agent iminodisuccinate [IDS; 2-(1,2-dicarboxyethylamino)succinic acid]. Biodegradation is initiated by an epimerase, followed by a carbon–nitrogen lyase. The carbon–nitrogen lyase is stereospecific for C atoms having the *S* configuration. Thus, only the epimerization step enables complete biodegradation of the isomeric mixture (Cokesa *et al.*, 2004).

Recently, the gene encoding the IDS-epimerase (*ite*) was identified and sequenced (GenBank entry DQ094782; Bäuerle *et al.*, 2006). The highest degree of amino-acid sequence identity was found to proteins of unknown function. The amino-acid sequence of the epimerase is not similar (*i.e.* >20% identity) to any protein of known structure deposited in the PDB. However, IDS-epimerase appears to belong to the MmgE/PrpD family (Pfam03972) as revealed by a search in the NCBI Conserved Domain Database. The ubiquitously found conserved domain pattern of this family may reflect similarities in substrate binding, catalytical function or structural motifs, but to date no such relationship has been described.

In this paper, we report the purification, crystallization and initial X-ray characterization of crystals of IDS-epimerase. It is expected that the crystal structure analysis will provide a structural basis for investigations of the catalytic mechanism of the enzyme and may allow deductions of the functional or structural properties of other members of the MmgE/PrpD family.



2. Material and methods

2.1. Cloning, heterologous expression and purification of recombinant epimerase

The ORF encoding IDS-epimerase was amplified from the genomic DNA of *A. tumefaciens* BY6 by the polymerase chain reaction (PCR) and inserted between the *Nde*I and *Hin*dIII sites of pJoe4036 (J. Altenbuchner, Institute of Industrial Genetics, University of Stuttgart) for expression of the full-length protein, corresponding to amino acids 1–446. The insert was commercially sequence-verified by MWG (Ebersberg, Germany). The plasmid was

transformed into *Escherichia coli* strain Rosetta2(DE3)-pLysSRARE2 (Merck KGaA, Darmstadt, Germany).

Transformants were grown at 310 K and 100 rev min^{-1} in 2×TY (tryptone–yeast extract; Ausubel *et al.*, 2001) containing final concentrations of 100 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol. At an OD_{600} of 0.6–0.8, the growth temperature was lowered to 303 K and 1 m*M* isopropyl *β*-D-thiogalactopyranoside (IPTG) was added. Cells were harvested 3 h after induction with IPTG at an OD_{600} of 3.0–3.9, resuspended in 50 m*M* Tris–HCl pH 7.5 with DNase II and disrupted by three passages through a French pressure cell (Aminco, SLM Instruments, Rochester, USA) at 7 MPa. The crude extract was fractionated by 30 min centrifugation at 100 000*g*, 277 K and filtration through 0.22 µm filters.

All purification steps were carried out at 279 K. Cell-free extract was dialysed overnight against 50 m*M* Tris–HCl pH 7.5 and loaded onto a 20 ml Q-Sepharose HR16/10 column (Amersham Biosciences, Uppsala, Sweden). IDS-epimerase was eluted with a linear gradient of 5 m*M* NaCl per millilitre in 50 m*M* Tris–HCl pH 7.5 at a concentration range of 0.12–0.23 *M* NaCl. After addition of 0.85 *M* $(NH_4)_2SO_4$ and 1 m*M* 1,4-dithiothreitol (DTT), pooled fractions were loaded onto an 8 ml Phenyl Superose HR10/10 column (Amersham Biosciences, Uppsala, Sweden) with 25 m*M* Tris–HCl pH 8.5, 1 *M* $(NH_4)_2SO_4$ as the binding buffer. Elution was performed with the following linear gradients: 1.0–0.9 *M* ammonium sulfate in 25 m*M* Tris–HCl pH 8.5, −0.1 *M* ml^{-1}; 0.9–0.6 *M*, −7.5 m*M* ml^{-1}; 0.6–0.25 *M*, −12.5 m*M* ml^{-1}. The epimerase was obtained at 0.73–0.61 *M*. Epimerase-containing fractions were dialyzed overnight against 50 m*M* Tris–HCl pH 8.5, 1 m*M* DTT and loaded onto an 8 ml MonoQ HR10/10 column (Amersham Biosciences, Uppsala, Sweden). A linear gradient of 10 m*M* ml^{-1} NaCl in 50 m*M* Tris–HCl pH 8.5, 1 m*M* DTT was used for elution. IDS-epimerase eluted in the salt range 0.25–0.30 *M*. The sample was concentrated and the buffer was exchanged to 50 m*M* Tris–HCl pH 8.5, 250 m*M* NaCl, 1 m*M* DTT in a 10 ml ultrafiltration cell with an YM10 membrane (Amicon/Millipore, Schwalbach, Germany).

2.2. Production of L-selenomethionine epimerase

L-Selenomethionine (L-SeMet) substituted IDS-epimerase was expressed with an N-terminal His_6 tag in *E. coli* B834(DE3)pLysSRARE2 (Novagen from Merck KGaA, Darmstadt, Germany) possessing *ite* on pET-28a(+) between the *Nde*I and *Hin*dIII restriction sites (pEETN; Bäuerle *et al.*, 2006).

An overnight culture of the transformants in Luria–Bertani medium (LB; Ausubel *et al.*, 1987) with 25 µg ml^{-1} chloramphenicol and 50 µg ml^{-1} kanamycin grown at 310 K and 200 rev min^{-1} was diluted 1:50 into minimal medium composed of M9 (Ausubel *et al.*, 1987), 1 m*M* $MgSO_4$, 0.1 m*M* $CaCl_2$, 0.2%(*w*/*v*) glucose, 2 mg l^{-1} thiamine, 0.001%(*w*/*v*) ferric ammonium citrate, a trace-element solution based on Pfennig & Lippert (1966), 25 µg ml^{-1} chloramphenicol, 50 µg ml^{-1} kanamycin and all 19 standard canonical L-amino acids others than methionine at a final concentration of 40 µg ml^{-1} and was grown under the same conditions. At an OD_{600} of 0.4, L-SeMet (60 µg ml^{-1}) was added and the growth temperature was lowered to 303 K. Addition of 1 m*M* IPTG at an OD_{600} of 0.8 (2 h after L-SeMet addition) induced expression of the recombinant protein. Cells were harvested after 6 h expression at an OD_{600} of 2.2. After resuspension in 50 m*M* Tris–HCl pH 8.0, 300 m*M* NaCl, 20 m*M* *β*-mercaptoethanol (lysis buffer), cells were disrupted by two freeze–thaw cycles followed by 30 min incubation on ice in the presence of DNase I and additional lysozyme as well as by ultrasonic treatment in a sonicator (Misonix, Farmingdale, USA).

The crude extract was centrifuged (30 min, 31 000*g*) and filtered (0.22 µm pore size). The protein was purified using an immobilized metal-affinity chromatography gravity-flow column with 4 ml Ni–NTA agarose resin (Qiagen, Hilden, Germany). The column was washed with six volumes of lysis buffer containing 20 m*M* imidazole and two volumes of lysis buffer containing 50 m*M* imidazole. His_6-tagged protein was eluted with 250 m*M* imidazole in lysis buffer. The buffer was exchanged to 25 m*M* Tris–HCl pH 8.0, 5 m*M* DTT with PD10 columns (Amersham Biosciences, Uppsala, Sweden). The protein solution was concentrated as described for the native protein.

2.3. Analysis of protein samples

Protein concentrations were estimated by the method of Bradford (1976) or on the basis of the absorption at 280 nm using the correlation factor calculated by *PROTEAN* (*DNASTAR* software package, DNASTAR, Madison, USA). Specific activities were measured at room temperature in 25 m*M* Tris–HCl pH 8.5 with a starting concentration of 10 m*M* *R*,*S*-IDS and quantified using ion-pair chromatography as described previously (Cokesa *et al.*, 2004). SDS–PAGE was carried out in a Mini-PROTEAN 3 system (Bio-Rad Laboratories, München, Germany) using standard procedures. SeMet incorporation was verified by electrospray ionization mass spectrometry by the Protein Analysis Center (PAC) of the Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden.

2.4. Crystallization of epimerase

Proteins were crystallized by the vapour-diffusion method. For the determination of initial crystallization conditions, commercial 96-well crystal screens were used. The epimerase crystallized under many conditions in the PEG/Ion Screen (Hampton Research, Aliso Viejo, USA). Several of them were optimized in a finer grid. For the L-SeMet-substituted epimerase, crystallization conditions similar to those of the native protein were found in a PACT Premier Screen (Molecular Dimensions Ltd, Soham, England). The crystal appear-

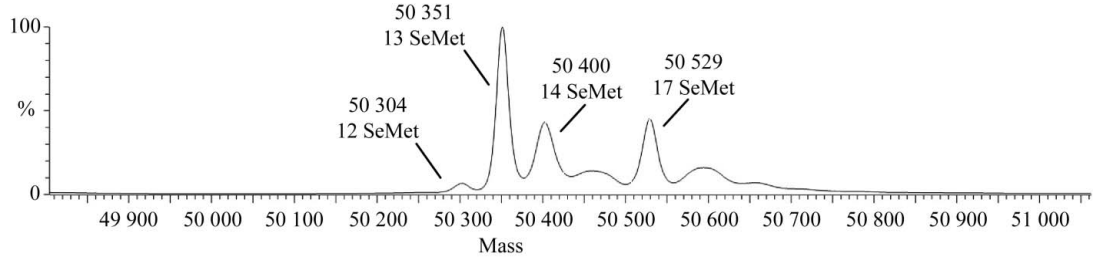


Figure 1
Electrospray ionization mass spectrometry of L-selenomethionine epimerase. Molecular mass (Da) and number of L-selenomethionine residues per subunit are given for each signal.

ance was improved by streak-seeding using a cat whisker. Typically, 1–3.0 µl of protein solution was mixed with the crystallization reservoir solution in a 1:4 to 3:4 ratio by volume, giving a total volume of 2–4 µl. This drop was allowed to equilibrate against 1 ml well solution at 293 K. An optimal drop volume or mixture ratio was not observed.

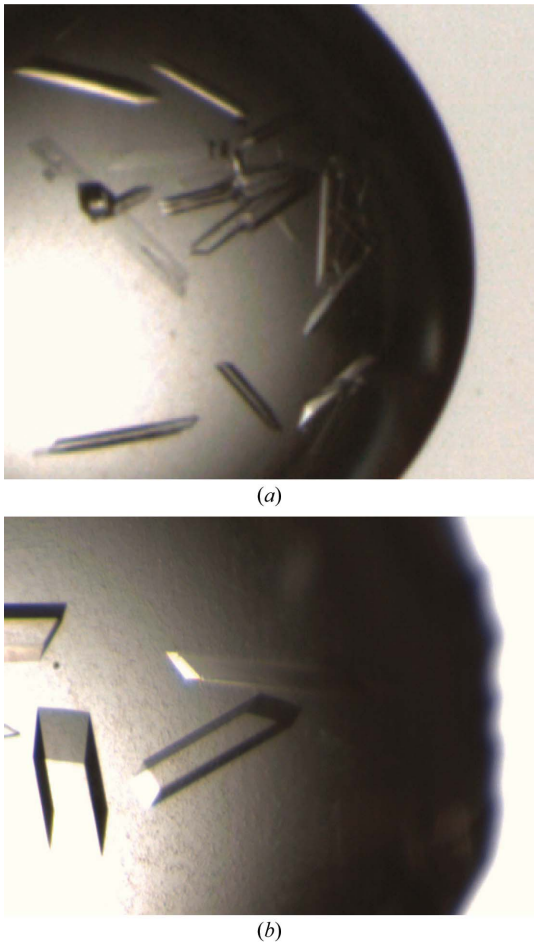


Figure 2
Photograph of typical crystals of IDS-epimerase. (*a*) Native, (*b*) L-selenomethionine derivative.

Table 1
Statistics for data collection.

Values in parentheses are for the highest resolution shell.

X-ray source	I711
Wavelength (Å)	1.0628
Detector type	MAR CCD
Oscillation per image (°)	0.3
Resolution (Å)	23.15–1.70 (1.79–1.70)
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 55.4, b = 104.2, c = 78.6, β = 103.3
No. of unique reflections	92811 (11701)
No. of observed reflections	281433 (32405)
Completeness (%)	97.6 (84.5)
Multiplicity	3.0 (2.8)
$R_{\rm merge}$	0.069 (0.29)
$I/\sigma(I)$	14.3 (3.4)
Mosaicity (°)	0.78
Wilson B factor (Å^2)	14.6

2.5. Data collection and processing

Crystals were quickly washed in reservoir solution diluted in a 1:2 ratio with 50%(*v*/*v*) ethylene glycol (final concentration 25%) as cryoprotectant and frozen in a nitrogen stream for X-ray data collection. X-ray data for native IDS-epimerase were collected at beamline I711 at MAX-II (Lund, Sweden) and were processed using *MOSFLM* (Leslie, 2003). Scaling and reduction of the data were performed using the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

Native and L-SeMet-substituted epimerase were produced in *E. coli* and isolated. 10 mg purified protein was obtained per litre of culture for the native protein and 12 mg per litre of culture for the L-SeMet derivative. SDS–PAGE analyses showed one major band corresponding to the calculated molecular masses of 48 and 50–51 kDa per subunit, respectively. The specific activity was 10 U mg^{-1} for both purifications.

Mass spectrometry of a sample of L-SeMet-substituted epimerase indicated that 12–17 methionine residues had been replaced by L-SeMet, corresponding to 71–100% incorporation (Fig. 1). Native protein, with a calculated molecular mass of 49 732 Da per subunit, was not detected in this sample.

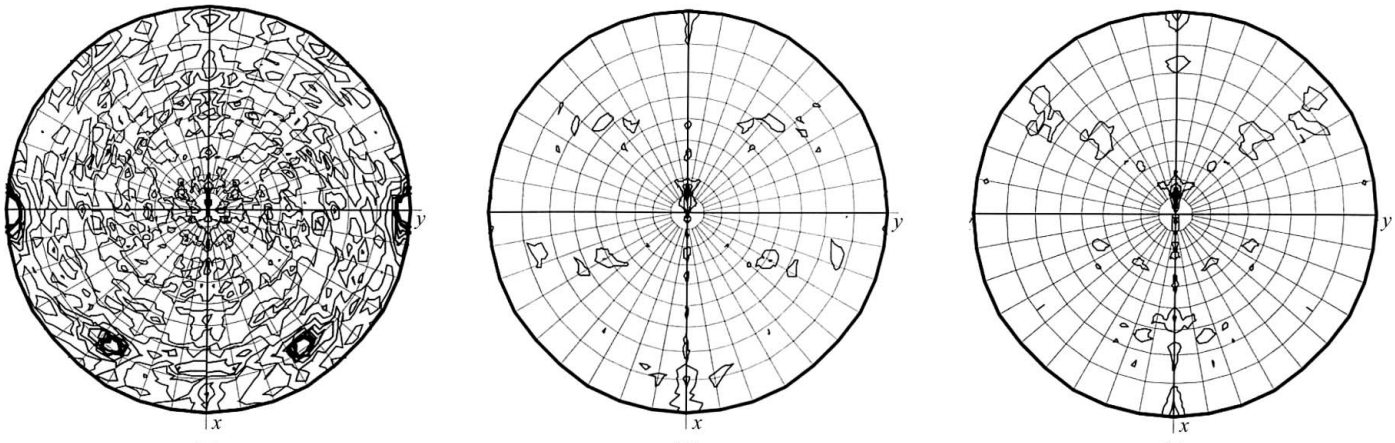


Figure 3
Self-rotation function map for the native epimerase data. The (*a*) $\kappa = 180°$, (*b*) $\kappa = 120°$ and (*c*) $\kappa = 90°$ sections are shown for a self-rotation function calculated with all data in the range 15–3 Å with a radius of integration of 30 Å. The two equivalent peaks in the $\kappa = 180°$ section ($\Theta = 79$, $\varphi = \pm 36°$) are about 45% of the signal for the crystallographic twofold axis and represent a local twofold symmetry axis. There are no peaks in the $\kappa = 90°$ and $\kappa = 120°$ sections above 5% of the origin peak.

Protein solutions of native and L-SeMet epimerase with concentrations of 11 ± 1 mg ml^{-1} were used for final crystallization experiments. The best native epimerase crystals grew with 22%(*w*/*v*) PEG 3350, 0.2 *M* $(NH_4)_2SO_4$ and 0.1 *M* bis-Tris propane pH 7.2 as the reservoir solution. L-SeMet-substituted epimerase was crystallized in the presence of 20%(*w*/*v*) PEG 3350, 0.2 *M* Na_2SO_4, 0.1 *M* bis-Tris propane pH 8.5. Rod-like crystals appeared at 293 K after a few hours and grew in about 1–4 weeks to dimensions of approximately 0.1 × 0.02 × 0.02 mm. Typical crystals are shown in Fig. 2.

Several data sets from native epimerase crystals grown in the presence of different salts were collected. All these crystals belonged to space group $P2_1$, with similar unit-cell parameters. The best crystal of native epimerase, with unit-cell parameters a = 55.4, b = 104.2, c = 78.6 Å, β = 103.3°, diffracted to 1.7 Å resolution (Table 1). Two IDS-epimerase molecules per asymmetric unit were indicated by a Matthews coefficient V_M of 2.2 Å^3 Da^{-1} (corresponding to a solvent content of 44%) and the self-rotation function, which shows the presence of a twofold non-crystallographic axis (Fig. 3).

Crystals of the selenomethionine-substituted enzyme were tested at beamline I911 at MAX-Lab, Lund, Sweden. The crystals are isomorphous to the crystals of the native enzyme and diffract to a resolution of 1.9 Å. They are thus suitable for structure determination by MAD experiments. The crystal structure of the IDS-epimerase is expected to provide a framework for future functional studies.

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