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Crystallization and preliminary crystallographic analysis of D-alanine-D-alanine ligase from *Streptococcus mutans*

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D-Alanine-D-alanine ligase is encoded by the gene *ddl* (SMU_599) in *Streptococcus mutans*. This ligase plays a very important role in cell-wall biosynthesis and may be a potential target for drug design. To study the structure and function of this ligase, the gene *ddl* was amplified from *S. mutans* genomic DNA and cloned into the expression vector pET28a. The protein was expressed in soluble form in *Escherichia coli* strain BL21 (DE3). Homogeneous protein was obtained using a two-step procedure consisting of Ni^{2+}-chelating and size-exclusion chromatography. Purified protein was crystallized and the cube-shaped crystal diffracted to 2.4 Å. The crystal belongs to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 79.50$, $c = 108.97$ Å. There is one molecule per asymmetric unit.

1. Introduction

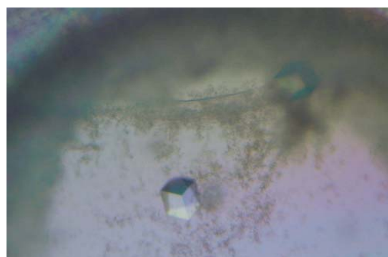
D-Alanine-D-alanine ligase (DDL; EC 6.3.2.4) is a bacterial enzyme involved in cell-wall biosynthesis. It participates in forming UDP-*N*-acetylmuramoyl pentapeptide, the precursor of peptidoglycan (Neuhaus, 1962). These enzymes are proteins of 300–360 amino-acid residues that contain many conserved regions. The enzyme is specific for the D-configuration and can be inhibited by D-cycloserine (Neuhaus, 1962; Neuhaus & Lynch, 1964). To date, the three-dimensional structures of DDLs from *Escherichia coli*, *Thermus caldophilus* and *Staphylococcus aureus* have been reported (Fan *et al.*, 1997, 1995; Lee *et al.*, 2006; Liu *et al.*, 2006). The tertiary structure of DDL is very similar to that of glutathione synthetase (EC 6.3.2.3), despite the low sequence homology between them (Fan *et al.*, 1995). *Streptococcus mutans* is the principal aetiological agent of human dental caries (Loesche, 1986). In *S. mutans*, DDL is encoded by the *ddl* gene and multiple sequence alignment indicates that *S. mutans* DDL has a wide range of sequence identity to the enzyme from other organisms. Since DDL is an important target for drug design, determination of the structure of *S. mutans* DDL will be helpful in the therapy of dental caries and also in understanding the catalytic mechanism of the enzyme.

2. Experimental procedures and results

2.1. Gene cloning and protein expression

The gene of the D-alanine-D-alanine ligase was cloned from the genomic DNA of *S. mutans* by polymerase chain reaction amplification (PCR; Saiki *et al.*, 1988) using the primers 5′-CGCGGATCCATGACTTTTAAAATTTTGACCGA-3′ and 5′-TAGCCATTTCTTCGGTCTATTGAGCTCGCC-3′ containing *Bam*HI and *Xho*I digestion sequences. After digestion by *Bam*HI and *Xho*I at room temperature overnight, the PCR product was ligated into the expression vector pET28a which had been digested with the same enzymes. An N-terminal fusion His_6 tag was added to the gene product with the sequence MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGS. The final vector was verified by DNA sequencing.

The recombinant vector containing the target gene was transformed into *E. coli* strain BL21 (DE3). The transformed cells were



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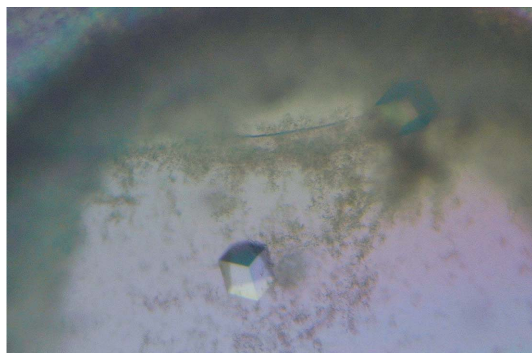

Figure 1
Crystals of *S. mutans* D-alanine-D-alanine ligase with dimensions of 0.25 × 0.25 × 0.25 mm.

grown overnight in 20 ml lysogeny broth (LB) medium containing 50 µg ml^{-1} kanamycin at 310 K. The overnight cultures were then inoculated into 1 l fresh LB medium containing 50 µg ml^{-1} kanamycin and grown at 310 K until an OD_{600} of 0.6–0.8 was reached. The cells were then induced with 1 m*M* isopropyl β-D-thiogalactoside. After growth for a further 6 h at 303 K, the cells were harvested by centrifugation at 6000 rev min^{-1} for 10 min and resuspended in buffer *A* (20 m*M* Tris–HCl, 500 m*M* NaCl pH 7.5).

2.2. Protein purification

The resuspended cells were lysated by sonication on ice and centrifuged at 18 000 rev min^{-1} and 277 K for 2 × 20 min. The supernatant was loaded onto a 5 ml Ni^{2+}-chelating affinity column (HiTrap, GE Healthcare, USA) previously equilibrated with buffer *A*. The column was washed with a linear gradient of imidazole from 0 to 0.5 *M* in buffer *A*. The primary product containing target protein was purified further using a Hiload Superdex 75 column (GE Healthcare, USA) with elution buffer *B* (20 m*M* Tris–HCl, 200 m*M* NaCl pH 7.5). The purity of the target protein was examined by SDS–PAGE during each step. There was only one band visible on the SDS–PAGE gel after size-exclusion purification and it showed the purified protein to have a molecular weight of about 42 kDa, which was in agreement with the predicted molecular weight of 38.84 kDa with an additional 4 kDa fusion part.

2.3. Crystallization

The purified protein from the gel filtration was directly concentrated to 10 mg ml^{-1} in the final elution buffer using a Millipore Centrifugal filter device (Ultra-15, 10 kDa cutoff). Crystallization experiments were carried out at 289 K using the sitting-drop vapour-diffusion method. The crystallization screening kits Crystal Screen, Crystal Screen II, Index and Natrix (Hampton Research, CA, USA) were used as initial screening conditions. 1 µl protein solution was mixed with an equal volume of reservoir solution and equilibrated against 450 µl reservoir solution. Cube-shaped crystals appeared in Natrix condition No. 28, which contained 0.05 *M* magnesium sulfate, 0.05 *M* Na HEPES pH 7.0, 1.6 *M* lithium sulfate. The concentrations of the protein and the precipitant were further optimized. Sufficiently large crystals of diffraction quality were obtained (Fig. 1) when the concentration of the protein was reduced to 5 mg ml^{-1}; the other conditions were same as Natrix condition No. 28.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Resolution (Å)	68.49–2.4 (2.51–2.4)
Completeness (%)	99.78 (100)
R_{sym}† (%)	9.73 (30.58)
Mean $I/\sigma(I)$	5.37 (1.59)
Redundancy	5.98 (5.96)
Space group	$P3_121$ or $P3_221$
Unit-cell parameters (Å)	$a = b = 79.50$, $c = 108.97$
No. of observed reflections	96342
No. of unique reflections	16113
Solvent content (%)	58.39

† $R_{sym} = \sum_h [\sum_i |I(h)_i - \langle I(h) \rangle| / \sum I(h)_i]$, where $I(h)_i$ is the *i*th observation of reflection *h* and $\langle I(h) \rangle$ is the mean intensity of all observations of *h*.

2.4. X-ray diffraction data collection and processing

X-ray diffraction data were collected on a Bruker SMART 6000 CCD detector using Cu *K*α radiation from a Bruker–Nonius FR591 rotating-anode generator operated at 45 kV and 100 mA. The crystal-to-detector distance was set to 6 cm. 400 frames were collected with 0.2° φ oscillation per frame and an exposure time of 60 s per frame. The crystal was flash-cooled directly without any cryoprotectant and maintained at 100 K using a cold nitrogen-gas stream during data collection. Diffraction data were processed using the Bruker *PROTEUM* software suite. The crystals diffracted to a resolution of 2.4 Å and belonged to space group $P3_121$ or $P3_221$, as indicated by systematic absences. The unit-cell parameters are $a = b = 79.50$, $c = 108.97$ Å. Assuming the presence of one molecule per asymmetric unit, the V_M value is 2.58 Å^3 Da^{-1} (Matthews, 1968), corresponding to a solvent content of 58.39%. The data-collection statistics are listed in Table 1.

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