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Crystallization and preliminary X-ray diffraction analysis of shikimate kinase from *Mycobacterium tuberculosis* in complex with MgADP

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Shikimate kinase (SK) from *Mycobacterium tuberculosis* (Mt) was overexpressed in *Escherichia coli*, purified and cocrystallized with MgADP in hanging drops using the vapor-diffusion procedure with PEG 4000 and 2-propanol as precipitants at pH 7.5. The crystal of MtSK–MgADP, which diffracted to 2.2 Å resolution, belonged to space group $P3_221$ or $P3_121$, with unit-cell parameters $a = b = 64.01$, $c = 92.41$ Å. There was one MtSK molecule in the asymmetric unit. Molecular-replacement trials with the crystal structure of SK from *Erwinia chrysanthemi* (PDB code 1shk) and adenylate kinase (PDB code 1ake) as search models were not successful. Heavy-atom derivative screening is in progress.

1. Introduction

The shikimate pathway is a biosynthetic route that converts erythrose-4-phosphate to chorismic acid in seven steps. Chorismic acid is an essential intermediate for the synthesis of aromatic compounds such as aromatic amino acids, *p*-aminobenzoic acid, folate and ubiquinone. The skimate pathway is essential for algae, higher plants, bacteria and fungi, whereas it is absent in mammals (Kishore & Shah, 1988; Haslam, 1993). Existence of the shikimate pathway was recently demonstrated in apicomplexan parasites such as *Plasmodium falciparum* and *Toxoplasma gondii* (Roberts *et al.*, 1998) which causes toxoplasmosis. This makes the enzymes in the pathway very important targets for the development of potentially non-toxic antimicrobial agents (Davies *et al.*, 1994), herbicides (Coggins, 1989) and antiparasite drugs (Ridley, 1998).

It is drawing crucial attention that infectious diseases are the leading causes of death and premature death in the world. *M. tuberculosis* (Mt) is one of the six diseases that cause 90% of deaths associated with infectious diseases, killing more adolescents and adults than any other single infection (World Health Organization, 1999). For the reasons discussed above, shikimate kinase (SK; E.C. 2.7.1.71) from *M. tuberculosis* (MtSK) is an excellent target for the development of novel anti-Mt agents. The three-dimensional structure of MtSK will provide crucial information for elucidating the mechanism of phosphoryl transfer and structure-based design of novel anti-Mt agents. Although the crystal structure of SK from *E. chrysanthemi* (EcSK) has been reported (Krell *et al.*, 1998), it is still crucial to determine the structure of MtSK, as MtSK shares only 35% identity with EcSK and the reported EcSK structure lacks a functionally essential stretch of polypeptide chain (Krell *et al.*, 1998). In this work, we report the crystallization and preliminary X-ray analysis of MtSK.

2. Materials and methods

2.1. Cloning, overexpression and purification

The gene for MtSK was amplified by PCR from *M. tuberculosis* genomic DNA on a cosmid provided by Dr S. T. Cole of the Institut Pasteur (Philipp *et al.*, 1996). The primers for the PCR were 5′-GGA ATT CAT ATG GCA CCC AAA GCG GTT CTC-3′ (forward) and 5′-CG GGA TCC TCA TGT GGC CGC CTC GCT G-3′ (reverse). The PCR product was digested with the restriction enzymes *Nde*I and *Bam*HI and ligated with the expression vector pET-17b previously digested with the same restriction enzymes. The ligation mixture was transformed into the *E. coli* strain BL21(DE3). A clone that overexpressed the SK was selected by SDS–PAGE and designated pET17b-MtSK. The correct coding sequence of the cloned gene was verified by DNA sequencing.

A three-column procedure was developed for the purification of the recombinant MtSK. Briefly, a litre of LB media containing 100 mg ampicillin was inoculated with a single colony of the *E. coli* strain BL21(DE3) containing the expression construct pET17b-MtSK and incubated at 310 K with shaking at ∼200 rev min^{-1} overnight. The cells were harvested by centrifugation and suspended in buffer *A* (10 m*M* Tris–HCl pH 8.3). The bacterial suspension was then sonicated on ice. The resulting lysate was centrifuged for 30 min at ∼27 000*g* at 277 K. The supernatant was loaded onto a DEAE-cellulose DE53 column equilibrated with buffer *A* in a cold room. The column was washed with buffer *A* and the flowthrough was

collected. Ammonium sulfate was added to the flowthrough to 25% saturation and the solution was centrifuged as before. The supernatant was loaded onto a phenyl Sepharose column equilibrated with buffer *B* (20 m*M* MOPS pH 7.0) with ammonium sulfate to 25% saturation. The column was washed with the same buffer and the flowthrough was collected. Ammonium sulfate was added to the flowthrough to 70% saturation and the solution was centrifuged. The pellet was dissolved in buffer *C* (20 m*M* MOPS pH 7.0). After centrifugation, the solution was loaded onto a Sephadex G-75 column equilibrated with buffer *C*. The column was developed with the same buffer. Fractions containing MtSK were identified by SDS–PAGE, concentrated by an Amicon concentrator using a YM10 membrane and lyophilized.

2.2. Crystallization

The protein was further purified before crystallization by gel filtration using a Sephacryl-100 column on a BioCAD/Sprint System. The corresponding fractions were collected, concentrated and dialyzed against 50 m*M* Na HEPES buffer pH 7.5 containing 0.5 *M* NaCl and 5.0 m*M* $MgCl_2$. This preparation was mixed with 5.0 m*M* shikimate acid and 5.0 m*M* ADP and centrifuged before crystallization. The protein concentration was about 7.0 mg ml^{-1}. The crystals were obtained by the hanging-drop vapor-diffusion method. The well solution contained 20% PEG 4000 and 10% 2-propanol in 0.1 *M* Na HEPES buffer pH 7.5 and the drop was a mixture of 1.0 µl of well solution and 4.0 µl of the protein solution.

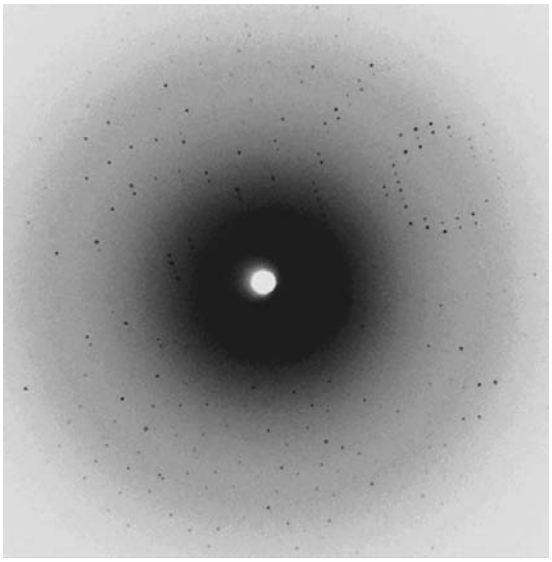

Figure 1
A typical diffraction pattern of the MtSK–MgADP crystal with 1° oscillation range. The crystal diffracts to 2.2 Å resolution.

2.3. Data collection

The data set of MtSK–MgADP was collected from a single crystal (dimensions 0.1 × 0.1 × 0.2 mm) using a MAR345 image-plate system and a Rigaku rotating-anode generator (50 kV, 100 mA) with Cu $K\alpha$ radiation. The cryoprotectant contained 0.5 *M* NaCl, 6% propanol, 12% PEG 4000 and 15% glycerol. The crystal was flash-frozen and maintained at 100 K during the entire data-collection procedure. The oscillation range used was 1.0° and the crystal-to-detector distance was 170 mm.

2.4. Molecular-replacement trials

Molecular-replacement trials were carried out with the program *AMoRe* (Navaza, 1994). The crystal structures of EcSK (PDB code 1shk) and adenylate kinase (PDB code 1ake) were used as search models.

3. Results and discussion

3.1. Purification and crystallization

With 26 basic and 18 acidic residues, MtSK is a rather basic protein, with a calculated pI of 10.6. However, the protein did not bind to cation exchangers such as CM-cellulose. Neither did it bind to anion exchangers such as DEAE-cellulose. The protein was purified with a three-column procedure with a yield of ∼50 mg protein per litre of *E. coli* culture. MtSK was collected in the flowthrough fractions in the first two columns. The purity of MtSK was extremely important for crystal growth. The protein obtained by the three-column procedure was further purified for crystallization with a BioCAD/Sprint System. Crystals were obtained by the hanging-drop vapor-diffusion method. Crystals of the MtSK–MgADP complex appeared in 1–2 weeks at 288 K.

3.2. Data collection and processing

The crystal diffracted to 2.2 Å with relatively low mosaicity (0.34°). Fig. 1 shows a typical X-ray diffraction pattern. 138 raw data images were collected. The data were processed on a Silicon Graphics Indigo2 computer using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Of the 92 641 measured reflections there were 11 559 unique reflections. The overall $R_{\rm merge}$ was 0.093 and the completeness was 100%. The crystal belongs to either $P3_221$ or $P3_121$ space group, with unit-cell parameters $a = b = 64.01$, $c = 92.41$ Å. There is one enzyme molecule in the asymmetric unit. The Matthews constant (Matthews, 1968) was 2.92 Å^3 Da^{-1} and solvent content of the crystal was 58%.

3.3. Structure determination

With the native data of MtSK–MgADP at 2.2 Å resolution, molecular replacement was used to attempt to solve the structure using *AMoRe* (Navaza, 1994). Various search models, including complete and modified structures of EcSK (PDB code 1shk) and adenylate kinase (PDB code 1ake), did not yield any meaningful result. This was most likely to be a consequence of the conformational differences between the search models and the structure under study. Heavy-atom screening is in progress.

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