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Preliminary crystallographic analysis of two hypothetical ribose-5-phosphate isomerases from *Streptococcus mutans*

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Study of the enzymes from sugar metabolic pathways may provide a better understanding of the pathogenesis of the human oral pathogen *Streptococcus mutans*. Bioinformatics, biochemical and crystallization methods were used to characterize and understand the function of two putative ribose-5-phosphate isomerases: SMU1234 and SMU2142. The proteins were cloned and constructed with N-terminal His tags. Protein purification was performed by Ni^{2+}-chelating and size-exclusion chromatography. The crystals of SUM1234 diffracted to 1.9 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters a = 48.97, b = 98.27, c = 101.09 Å, $\alpha = \beta = \gamma = 90^\circ$. The optimized SMU2142 crystals diffracted to 2.7 Å resolution and belonged to space group $P1$, with unit-cell parameters a = 53.7, b = 54.1, c = 86.5 Å, α = 74.2, β = 73.5, $\gamma = 83.7^\circ$. Initial phasing of both proteins was attempted by molecular replacement; the structure of SMU1234 could easily be solved, but no useful results were obtained for SMU2142. Therefore, SeMet-labelled SMU2142 will be prepared for phasing.

1. Introduction

We have been developing a laboratory-affordable structural proteomics platform at Peking University (Su *et al.*, 2006), and one of the whole structural proteome projects that we are working on is that of the human dental pathogen *Streptococcus mutans*. Owing to its very active carbohydrate metabolism, we are particularly interested in the enzymes involved in the sugar metabolic pathways (Loesche, 1986).

Ribose-5-phosphate isomerase (Rpi; EC 5.3.1.6) is a very important enzyme that is involved in the pentose phosphate pathway and in the Calvin cycle of plants (Horecker *et al.*, 1951). It catalyzes the reversible conversion of ribose 5-phosphate to ribulose 5-phosphate. Since phosphosugars are precursors of amino acids, vitamins, nucleotides and cell-wall constituents, Rpi is important in bacterial growth (Sørensen & Hove-Jensen, 1996). Two types of nonhomologous Rpis have been identified to date in nature, designated RpiA and RpiB; both are responsible for the interconversion of ribose 5-phosphate and ribulose 5-phosphate, but the two types of Rpi are structurally distinct enzymes with very different enzyme mechanisms. RpiA is distributed widely from archaea and bacteria to plants and animals. To date, several three-dimensional structures of RpiA from different species have been reported, such as those from *Escherichia coli* (Rangarajan *et al.*, 2002; Zhang *et al.*, 2003), *Pyrococcus horikoshii* (Ishikawa *et al.*, 2002) and *Thermus thermophilus* HB8 (Hamada *et al.*, 2003). RpiB has a relatively narrow distribution compared with RpiA, being found in some bacterial and protozoan genomes. It belongs to the RpiB-LacAB family (Sørensen & Hove-Jensen, 1996).

Both putative RpiA and RpiB have been found in *S. mutans* (Adjić *et al.*, 2002). Using a *BLAST* sequence search, SMU1234 was found to correspond to RpiA, whereas SMU2142 was found to possess a conserved RpiB domain; Fig. 1 shows a structure-based alignment of

RpiBs from six homologous bacterial enzymes. In order to characterize the Rpis and to further study their functional roles in the carbohydrate-metabolism pathways, we have undertaken structure-determination experiments on SMU1234 and SMU2142.

2. Experimental methods and results

2.1. Cloning and expression

The *smu.1234* and *smu.2142* genes were cloned from *S. mutans* strain UA159 genomic DNA by PCR amplification using different primers. The protocols used for cloning, protein expression and purification were the same for both proteins. The PCR product was inserted into vector pET28a (Novagen). An N-terminal His_6 tag was added to the product with the sequence MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGS.

The recombinant vector was transformed into *E. coli* strain BL21 (DE3). The transformed *E. coli* cells were grown in 40 ml Luria–Bertani (LB) medium containing 50 µg ml^{-1} kanamycin at 310 K overnight. The culture was then added to 1000 ml fresh LB medium and grown at 310 K for 3 h. When the OD_{600} reached 0.6, 1 m*M* isopropyl β-D-1-thiogalactopyranoside was added to the culture and growth was continued overnight at 292 K. Finally, the culture was harvested by centrifugation at 1935*g* for 15 min and resuspended in 20 ml buffer *A* (20 m*M* Tris–HCl pH 7.5, 500 m*M* NaCl).

Table 1
Data-collection statistics for *S. mutans* SMU1234 and SMU2142.

Values in parentheses are for the highest resolution shell.

	SMU1234	SMU2142
Resolution (Å)	70.46–1.90 (1.97–1.90)	50.00–2.70 (2.75–2.70)
Completeness (%)	99.6 (97.6)	97.1 (93.2)
R_{merge}† (%)	6.8 (22.2)	3.8 (11.6)
Average $I/\sigma(I)$	14.6 (3.7)	23.3 (6.7)
Space group	$P2_12_12_1$	$P1$
Unit-cell parameters (Å, °)	a = 48.97, b = 98.27, c = 101.09, $\alpha = \beta = \gamma$ = 90	a = 53.7, b = 54.1, c = 86.5, α = 74.2, β = 73.5, γ = 83.7
No. of observed reflections	180075	46660
No. of unique reflections	39061	23870
Molecules per asymmetric unit	2	4
Solvent content (%)	49.49	47.6

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

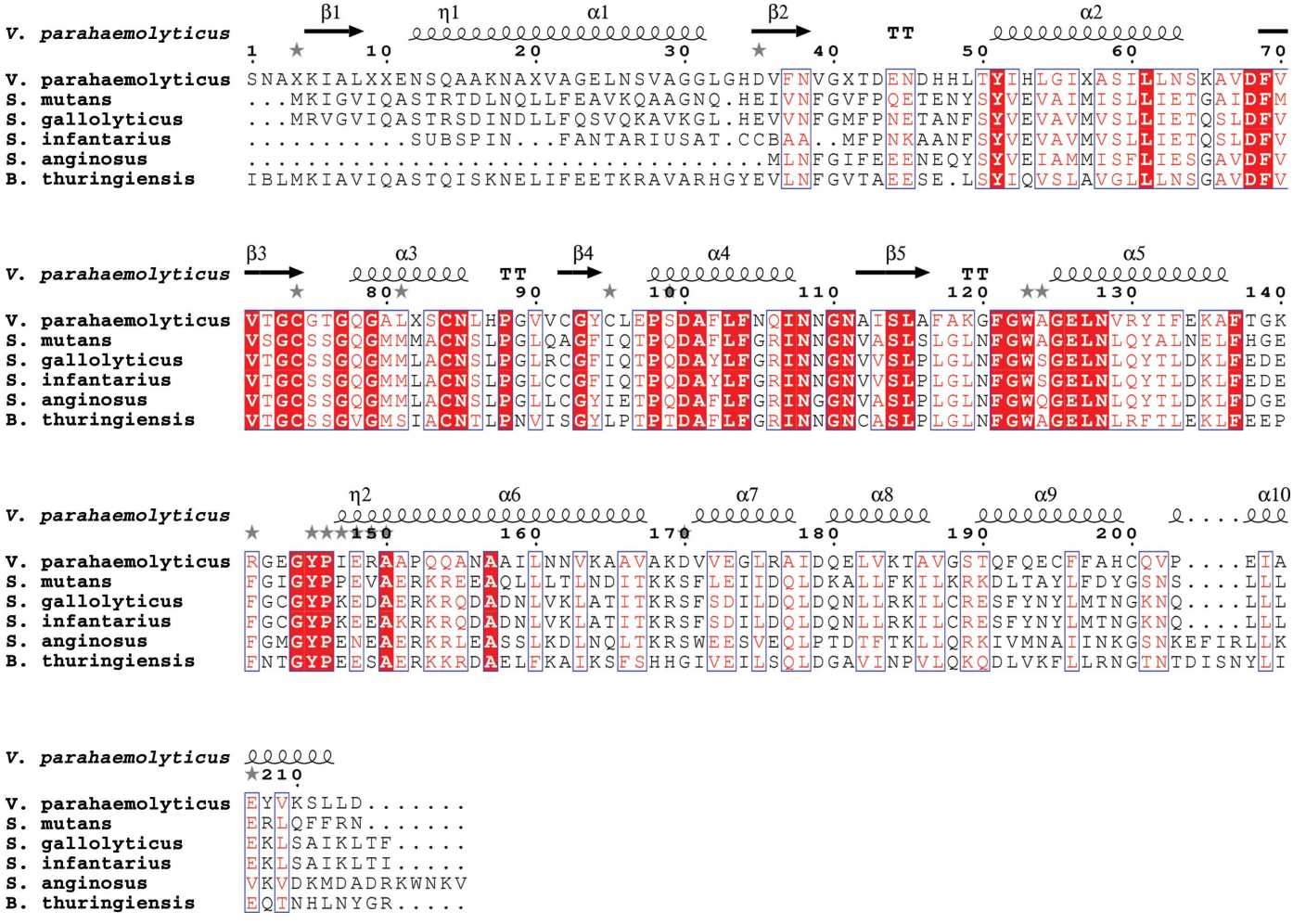
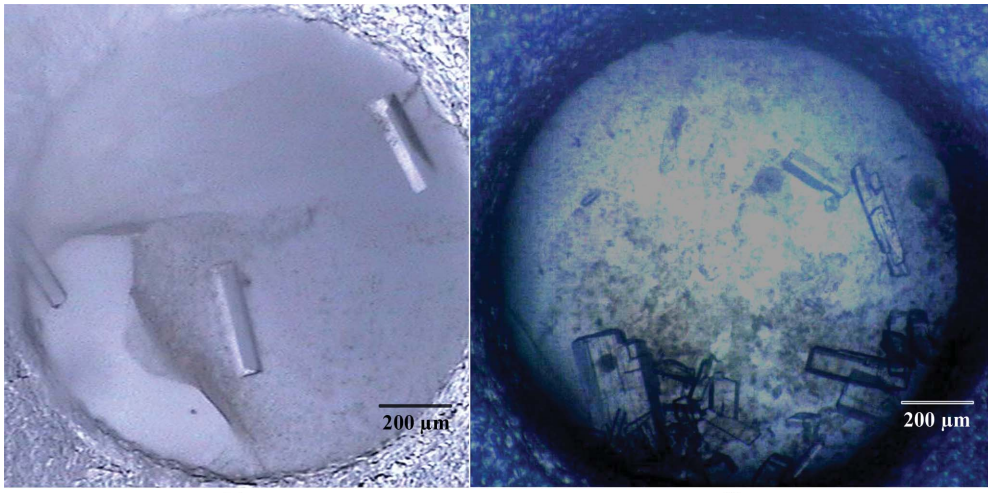


Figure 1
Multiple sequence alignment drawn by *ESPript* 2.2 (Gouet *et al.*, 1999) of RpiB proteins from *S. mutans* (Smu), *Bacillus thuringiensis* (Bth), *S. gallolyticus* (Sga), *Vibrio parahaemolyticus* (Vpa), *S. anginosus* (San) and *S. infantarius* (Sin). Identical residues are shown in red and similar residues are shown in blue boxes. The secondary structures are designated β1–5 and α1–10. All sequences were obtained from the PDB or the NCBI nonredundant protein-sequence database with the following accession codes: *V. parahaemolyticus* RpiB, 3ono; *S. gallolyticus* hypothetical protein GALLO_2238, YP_003431644.1; *S. infantarius* hypothetical protein STRINF_00557, ZP_02919705.1; *S. anginosus* ribose/galactose isomerase, ZP_08525301.1; *B. thuringiensis* galactose-6-phosphate isomerase LacB subunit, ZP_04075471.1; *S. mutans rpiB* gene product, NP_722427.1.



(*a*) (*b*)

Figure 2
(*a*) Crystals of *S. mutans* putative RpiB protein obtained using a 4:1 mixture of 20% PEG 3350, 0.2 *M* ammoinum acetate, 0.1 *M* Tris–HCl pH 8.5 and 25% PEG 3350, 0.2 *M* $MgCl_2$, 0.1 *M* Tris–HCl pH 8.5 (XtalQuest). (*b*) Crystals of SMU1234 obtained using 0.1 *M* HEPES pH 7.5, 0.1 *M* NaCl, 2.0 *M* ammonium sulfate (XtalQuest).

2.2. Protein purification

The resuspended cells were lysed by sonication on ice and centrifuged at 58 545*g* for 1 h. The supernatant was loaded onto a 5 ml Ni^{2+}-chelating affinity column (GE Healthcare, USA) previously equilibrated with buffer *A*. The target protein was eluted with a linearly increasing concentration of imidazole in buffer *A*. The eluted protein was further purified by size-exclusion chromatography using a Superdex S75 column (GE Healthcare) according to its size. SDS–PAGE experiments were used to examine each step of purification. The result showed only one visible band, which corresponded to the size of the target protein (24.57 kDa for SMU1234 and 23.4 kDa for SMU2142).

2.3. Crystallization

Ultrafiltration was used to concentrate the purified protein. Crystallization experiments were performed by sitting-drop vapour diffusion at 298 K. Crystal Screen, Crystal Screen 2, Natrix, Index (Hampton Research, USA), BCR and CAD-BCR (XtalQuest, People's Republic of China) were used in the initial screening stage. 1 µl purified His-tagged protein solution was mixed with an equal amount of reservoir solution and equilibrated against 100 µl reservoir solution. Crystals of SMU1234 suitable for diffraction were obtained within 4 d in the condition 0.1 *M* HEPES pH 7.5, 0.1 *M* NaCl, 2.0 *M* ammonium sulfate (Fig. 2*a*). Crystals of SMU2142 were found in many conditions; the crystal from which the diffraction data were collected was obtained using a 4:1 mixture of 20% PEG 3350, 0.2 *M* ammonium acetate, 0.1 *M* Tris–HCl pH 8.5 and 25% PEG 3350, 0.2 *M* $MgCl_2$, 0.1 *M* Tris–HCl pH 8.5.

2.4. Data collection

Diffraction data for SMU1234 were collected on a Rigaku Saturn 944+ CCD detector using Cu $K\alpha$ radiation from a Rigaku MicroMax-002+ generator operated at 45 kV and 88 mA with Confocal Max-Flux (CMF) optics. The crystal-to-detector distance was 50 mm. 300 frames were collected with 0.5° oscillation and an exposure time of 30 s per frame. The crystal was flash-cooled directly without any cryoprotectant and maintained at 100 K using a cold nitrogen-gas stream during the whole data-collection process. Diffraction data were processed using the *CrystalClear* v.1.4 (*d*TREK*) package (Pflugrath, 1999). Diffraction data-collection and processing statistics are listed in Table 1.

For SMU2142 data collection, a crystal was flash-cooled in liquid nitrogen after immersion in mother liquor with 20%(*v*/*v*) glycerol as an additional cryoprotectant. X-ray diffraction data were collected on a Rigaku Saturn 944+ CCD using Cu $K\alpha$ radiation at a wavelength of 1.5418 Å from a MicroMax-007 HF rotating-anode generator equipped with Osmic VariMax optics and operated at 40 kV and 30 mA. 360 frames were collected with 0.5° oscillation per image at $2\theta = 0°$ with a crystal-to-detector distance of 70 mm at 100 K (Fig. 3). The exposure time for each frame was 60 s. The diffraction data were processed using the *HKL*-2000 software package (Otwinowski & Minor, 1997).

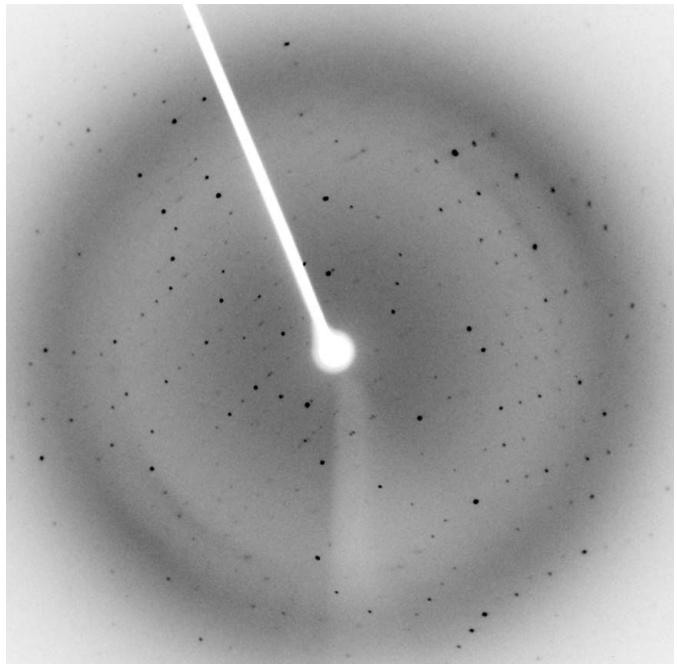


Figure 3
A typical diffraction pattern from an SMU2142 crystal.

2.5. Structure determination

The structure of SMU1234 was determined by molecular replacement using RpiA from *T. thermophilus* (PDB entry 1uj4; 43% identity; Hamada *et al.*, 2003) as the search model. The initial *R* from molecular replacement using *MOLREP* (Murshudov *et al.*, 2011) was 62.5%. After rigid-body refinement, the *R* and R_{free} values were 45.2% and 49.0%, respectively. The crystal structure has been deposited in the Protein Data Bank (PDB entry 3l7o).

The data-collection statistics for SMU2142 are summarized in Table 1. An attempt was made to obtain the initial phase by molecular replacement using RpiB from *Vibrio parahaemolyticus* (PDB entry 3ono; 38% identity; Midwest Center for Structural Genomics, unpbulished work) as a search model, but no useful results were obtained. Preparation of SeMet-labelled protein is under way. There are five Met residues in the SMU2142 sequence.

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