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Intersubunit Salt Bridges of *Bacillus* sp. TB-90 Urate Oxidase

- A Key to Understanding the Mechanism of its Sulfate-Induced Thermal Stabilization -

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1 Introduction

Urate oxidases catalyze the oxidative opening of the urate purine ring to yield 5-hydroxyisourea, CO_2, and H_2O_2. This enzyme is crucial for determining uric acid levels in biological fluids for diagnosis of hyperuricemia, because the highly specific reaction of urate oxidase allows the simple colorimetric detection of uric acid. The thermal, chemical, and long-term stability of urate oxidase plays a critical role in its application to enzymatic analysis and medical treatment for hyperuricemia and gout.

Urate oxidase from *Bacillus* sp. TB-90 (BTUO) is secreted as a tetrameric protein of identical subunits (331 residues per subunit, molecular mass of 37,863.8 Da × 4). [1, 2] With an optimal activity at 45 °C and a transition temperature at which the enzyme retains 50% of its initial activity of 65 °C, BTUO is one of the most thermostable urate oxidases. Calorimetric measurements and size-exclusion chromatographic analyses suggested that sulfate-induced thermal stabilization is related to the binding of a sulfate anion that repressed the dissociation of BTUO tetramers into dimers [3], and such enhancement of thermal stabilization of a urate oxidase has not been seen previously. The crystal structure of the enzyme in the presence of Li_2SO_4 was previously determined at 2.2 Å resolution (PDB ID, 1J2G), and four sulfates per tetramer molecule were bound to the enzyme surface. However, Li_2SO_4 showed an only minor effect on the salt-induced thermal stabilization probably because of the destabilizing effect of lithium ion. In this study, the crystal structure with K_2SO_4, a potent salt in the thermal stabilization as well as Na_2SO_4, was determined at 1.75 Å resolution.

2 Experiment

To obtain high-quality crystals, the 6 (TKHKER) and 13 (MFSDEPDHKGALK) N- and C-terminal residues, respectively, were deleted from wild-type BTUO. The deleted N-terminal region was disordered in the 2.2 Å crystal structure (PDB entry 1J2G), and the C-terminal region was processed in the *Bacillus* but not *E. coli* expression systems. The expressed enzyme was purified as previously described [1]. BTUO crystals were grown by the hanging-drop vapor-diffusion technique, wherein a protein solution (2 μL) was mixed with an equal volume of reservoir solution containing 15% (w/v) PEG 8000, 100 mM Tris-HCl (pH 8.0), 0.07 M K_2SO_4, and 2 mM 8-azaxanthine. A native data set was collected to 1.75 Å resolution using one crystal. The resulting crystal was momentarily soaked in the reservoir solution containing 20% 2-ethoxyethanol, flash-cooled in a 100 K dry nitrogen stream, and then exposed to 1 Å X-ray beam at 100 K.

X-ray diffraction data for the wild-type crystal were collected using an ADSC Quantum 210 CCD camera and synchrotron radiation on beamline NW12A (Photon Factory, Tsukuba, Japan). Individual frames consisted of a 0.5° oscillation angle measured for 5 s at a crystal-to-detector distance of 117.1 mm. The crystal belonged to orthorhombic space group $P2_12_12$ with the following unit cell dimensions: a = 133.57 Å, b = 144.64 Å, and c = 70.79 Å. Intensity data were processed, merged, and scaled with HKL2000. Data collection statistics are listed in Table 1. A 99.8% complete data set from the crystal was processed to 1.75 Å, with an overall R_{merge} of 9.4%, and 1,884,330 total reflections, including 138,568 unique reflections.

Table 1: Data Collection and Refinement Sstatistics

data collection[a]	
space group	$P2_12_12$
unit cell parameters (Å)	a = 133.57, b = 144.64, c = 70.79
resolution range (Å)	30 – 1.75 (1.78–1.75)
total reflections	1,884,330
no. of unique reflections	138,568
completeness (%)	99.8 (100.0)
$R_{merge}(=\Sigma_h\Sigma_i\lvert I_{h,i} - \langle I_h\rangle\rvert / \Sigma_h\Sigma_i\lvert I_{h,i}\rvert)$	0.094 (0.377)
I/σ	56.8 (9.4)
redundancy	13.6 (13.0)
Wilson B factor (Å^2)	27.2
refinement	
refinement resolution (Å)	19.92–1.75
no. of reflections (work/free)	126,254/6,672
R_{work}/R_{free}	0.159 / 0.190
rmsd	
bond length (Å)	0.010
bond angle (°)	1.28
mean B factors (Å^2)	
main chain atoms	18.40
side chain atoms	22.17
ligand atoms	29.39
water atoms	29.91
Ramachandran plot	
Favored region	1,183 (98.3%)
Allowed region	21 (1.7%)

[a]Values in parentheses are for the outer shell.

The structure of BTUO in the complex with 8-azaxanthine has been determined by molecular replacement techniques. The initial phase was solved using the model of PDB entry 1J2G as a search probe. The MR solution was readily obtained and rebuilt using

ARP/wARP. The rebuilt model was then refined against the 1.75-Å data of the crystal using PHENIX. Each round of refinement was alternated with a round of manual rebuilding using COOT, and the refinement progress was monitored by tracking decreases in R_{cryst} and R_{free}. After several rounds of refinement, the electron density from the $|F_o|-|F_c|$ map depicted a clear density for 8-azaxanthine, and then four 8-azaxanthine, two sulfate anions, two K^+ cations, and four chloride anions were included in the tetramer structure. The four subunit molecules in the asymmetric unit could be superimposed upon each other with a root-mean-square deviation (rmsd) for the Cα atoms of < 0.38 Å, so that the refined overall structure was similar from monomer to monomer except at some crystal contact interfaces. Statistics for the refinement are listed in Table 1. The final coordinates were deposited in the Protein Data Bank at entry 3WLV.

3 Results and Discussion

The crystal structure of thermophilic BTUO in the presence of K_2SO_4 was determined in complex with the competitive inhibitor 8-azaxanthine at 1.75 Å resolution. The crystal structure of the tetramer molecule in the asymmetric unit exhibits four identical subunits enclosing a tunnel, with each subunit composed of two tandem tunneling-fold motifs [4] (Fig. 1), which is similar to mesophilic uricase from *Asperigillus flavus* (AFUO, PDB entry 1R51) [5] and that from *Arthrobacter globiformis* (AGUO, PDB entry 1VAY). The sequence of BTUO is 26.3% and 25.6% identical to those of AFUO and AGUO, respectively, and all catalytic residues in the active site are conserved completely. The central tunnel structure is built by stacking of two anti-parallel β-barrels of subunit dimers, and the active site is located at the subunit interface in the dimer β-barrel. Superposition of the 8-azaxanthine-complex of thermophilic BTUO onto that of mesophilic AFUO revealed a rmsd of 1.68 Å for 227 Cα atoms overall, 6-times higher than the averaged rmsd between the four subunit chains of BTUO (0.28 Å). Major structural differences were concentrated in four peripheral loops, and we referred to two characteristic loops at the tetrameric interface as interface loop I (residues 125-145) and interface loop II (residues 277-300). Both loops extend to the neighboring dimer ring and are involved in forming the tetramer structure.

Figure 2 shows the structure around interface loop II of subunit C, which is placed at the interface between subunits A and B. In the N-terminal region of the loop, a bound sulfate anion formed a symmetrical intersubunit salt bridge with the Arg298 residues of subunits A and C, and its structure was well-defined in the electron density map. Arg298 also makes a bifurcated hydrogen bond with Asp280 of subunit A. In the carboxy-terminal region of the loop, the short Lys292-Glu296 strand sof subunit C forms six hydrogen bonds with the antiparallel Phe17-Thr21β-strand of subunit B. As a result, the bound sulfate can lock the tunnel dimer via the interface loop II protruding from another tunnel dimer. The loop contained the highest B-factor region (residues 281-292). The averaged main-chain B-factor of the region was 35.0 $Å^2$, which is about 2-fold higher than that of the overall main chain (18.4 $Å^2$), and suggests that this loop may play a role as a hinge between subunits A and B that could participate in the open-closing motion of the active site cleft at the subunit interface.

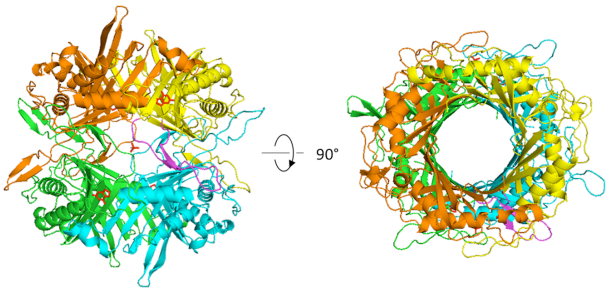

Fig. 1: Tetramer structures of BTUO.

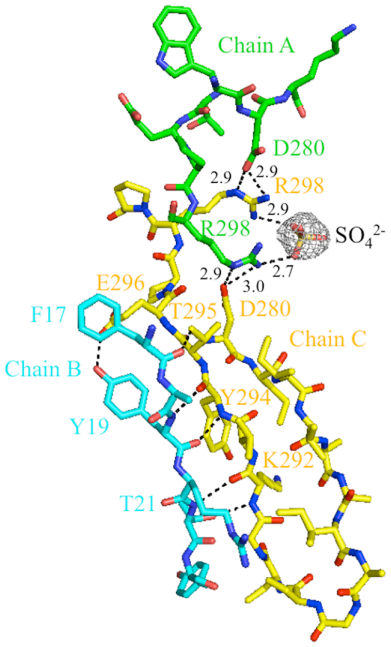

Fig. 2: The structure of interface loop II and the bound sulfate anion.

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