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Engineering SS bridge of Thermostable Mutant of *Bacillus* sp. TB-90 Urate Oxidase

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1 Introduction

Urate oxidase from *Bacillus* sp. TB-90 (BTUO), one of the most thermostable urate oxidases, is secreted as a tetrameric protein of identical subunits.[1, 2] We previously reported that binding of a sulfate anion to the enzyme induced the thermal stabilization, because the bound sulfate formed a salt bridge with two Arg298s in the symmetrical related subunits.[3] To extensively characterize the sulfate-binding site at the subunit interface, site-directed mutagenesis of Arg298 to Cys was done, although the distance between the Cβ atoms of Arg298s seemed to be too long to introduce an engineering disulfide bond. However, this substitution markedly increased the protein melting temperature of the R298C mutant by ~20°C compared with the wild-type one, and then the thermostabilization was canceled by the reduction with dithiothreitol. Here we show the crystal structure of R298C at 2.05 Å resolution, and demonstrate that the symmetrical related Cys298 residues are linked with each other via a disulfide bridge.

2 Experiment

Crystals of the R298C mutant enzyme were grown by the hanging-drop vapour-diffusion technique as previously described.[3] A protein solution (5 ml) was mixed with an equal volume of reservoir solution containing 16%(w/v) polyethylene glycol 8000, 100 mM Tris-HCl pH 8.0, 0.08 M Li_2SO_4 and 1 mM 9-methyluric acid. The crystal was momentarily soaked in the reservoir solution containing 20% polyethylene glycol 400 and was flash-cooled in a 100 K dry nitrogen stream and then exposed to 1 Å X-ray beam. A native data set was collected to 2.05 Å resolution using an ADSC Quantum 315 CCD camera and synchrotron radiation on beam line BL-5A (Photon Factory, Tsukuba, Japan). Individual frames consisted of a 0.5° oscillation angle measured for 10 sec. Intensity data were processed, merged, and scaled with HKL2000. Data collection statistics are given in Table 1.

Table 1: Data Collection and Refinement Statistics

data collection	
space group	$P2_12_12$
unit cell parameters (Å)	a = 131.86, b = 142.58, c = 70.65
resolution range (Å)	50 - 2.05 (2.09 - 2.05)
total reflections	410,560
no. of unique reflections	83,842
completeness (%)	99.8 (99.9)
$R_{merge}(=\Sigma_h\Sigma_i\lvert I_{h,i} - \langle I_h\rangle\rvert / \Sigma_h\Sigma_i\lvert I_{h,i}\rvert)$	0.121 (0.715)
I/σ	20.3 (3.0)
redundancy	4.9 (4.8)
refinement	
refinement resolution (Å)	32.27 - 2.05
R_{work}/R_{free}	0.169 / 0.221
RMSD	
bond length (Å)	0.007
bond angle (°)	0.839
Ramachandran plot	
favored region	98.1%
allowed region	1.9%

The structure of R298C mutant has been solved by molecular replacement techniques using the program PHENIX. The initial phase was solved using the model 3WLV as a search probe. The MR solution was readily obtained and was rebuilt and refined. After several rounds of refinement, the electron density from the omit map depicted a clear density for disulfide bridges. Statistics for refinement are also given in Table 1.

3 Results and Discussion

The crystal structure of R298C was determined in complex with the competitive inhibitor 9-methy uric acid at 2.05 Å resolution. Superposition of the R298C structure onto that of the wild-type enzyme using GASH revealed a rmsd of 0.28 Å for 1187 Cα atoms. Although the differences of overall structures were negligible, the electron densities around residues Pro287-Glu290 were completely lost in the middle of interfce loop II of R298C mutant.

A clear electron density for a left-handed disulfide bridge at the subunit interface was depicted from the σ_A omit map. The disulfide bond formation shortened the distance between the Cβ atoms of Cys298s to be 4.54 Å, 0.9 Å shorter than that of Arg298s of the wild-type enzyme. Instead, the averaged Cβ-Sγ-Sγ angle of 126.4° was abnormally large, and the averaged χ2 torsion angle (Cα-Cβ-Sγ-Sγ) was 5.5°, so that a steric hindrance arose between the Cα-Cβ and the Sγ-Sγ bonds. Further studies on the thermal stabilization is in progress.

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References

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