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Crystallization and preliminary X-ray crystallographic analysis of native and selenomethionyl recombinant tabtoxin-resistance protein complexed with acetyl-coenzyme A

Hongzhen He, Yi Ding, Zhenbo Cao, Yu Shao, Mark Bartlam, Hong Tang, Fan Jiang, Yiwei Liu, Jinyuan Liu, Nanming Zhao and Zihe Rao*

Laboratory of Structural Biology, School of Life Science and Engineering, Tsinghua University, Beijing 100084, People's Republic of China

Correspondence e-mail: raozh@xtal.tsinghua.edu.cn

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Tabtoxin-resistance protein (TTR), an acetyltransferase from *Pseudomonas syringae* pv. tabaci, was overexpressed in *Eschericha coli* M15 and the TTR fusion protein complexed with acetyl-coenzyme A (AcCoA) was purified and crystallized. Diffraction data were collected to 3.0 Å resolution in-house and the crystal was found to belong to space group $P2_1$, with unit-cell parameters a = 47.6, b = 66.6, c = 53.5 Å, β = 104.3°. Furthermore, a selenomethionine (SeMet) TTR fusion protein derivative was overexpressed in the same expression system and its complex with AcCoA was purified in a reductive environment. The SeMet TTR derivative crystallized in two forms: the first was identical to that observed for native crystals and the second belonged to space group $C2$, with unit-cell parameters a = 101.7, b = 45.6, c = 84.2 Å, β = 105.8°. Data from the $P2_1$ crystal form were collected in-house to 2.3 Å resolution. Subsequently, three different wavelength data sets of the $C2$ crystal form to 1.55 Å resolution were collected at the Advanced Photon Source at Argonne National Laboratory.

1. Introduction

Tabtoxinine-β-lactam (TβL; Stewart, 1971), a toxin produced by *P. syringae* pv. tabaci, is strongly antimicrobial and causes wildfire of tobacco by irreversibly inhibiting the target enzyme glutamine synthetase (GS; Sinden & Durbin, 1968). The pathogen, however, retains a significant amount of glutamine synthetase activity during the toxin production, while TβL inactivates the GS from the pathogen *in vitro* (Thomas & Durbin, 1985). One of the mechanisms of self protection of the pathogen from TβL is that in the pathogen there exists a tabtoxin-resistance gene coding for the enzyme tabtoxin-resistance protein (TTR; SWISS-PROT ID TTR_PSESY), which comprises 177 amino acids and detoxifies TβL as an acetyltransferase (Anzai *et al.*, 1989; Marek & Dickson, 1987; Batchvarova *et al.*, 1998). A tabtoxin resistance gene has also been cloned (Liu *et al.*, 1994) from the strain of *P. syringae* pv. tabaci epidemic in China and encodes a protein with the same amino-acid sequence as TTR. In addition, to TTR fusion protein (containing 177 amino-acid residues of TTR and a molecular tag of six histidine, four arginine and one lysine residues at its N-terminus), a high-level expression vector (PTTR10) has been constructed (Ye & Liu, 2001).

A search of the non-redundant sequence database using the program *PSI-BLAST* shows that TTR shares very low sequence homology with proteins of experimentally determined structure. Therefore, the structure of TTR may represent a new fold. In view of this and in order to reveal the molecular mechanism for its detoxification of TβL, we tried to determine the structure of TTR by X-ray crystallography. Low sequence homology and the five methionine residues (Met) contained in TTR make multiwavelength anomalous diffraction (MAD) the preferred method for TTR phasing. In this paper, the expression, purification, crystallization and preliminary X-ray crystallographic analysis of natural and selenomethionyl recombinant tabtoxin-resistance protein complexed naturally with AcCoA are reported.

2. Materials and methods

2.1. Expression, purification and analysis

Expression strain *E. coli* M15 and expression plasmid PQE30 containing the nucleotides which encode a TTR fusion protein (TTR with a molecular tag of six histidine residues, four arginine residues and one lysine residue at its N-terminus) were used in the expression of native TTR as well as the SeMet TTR derivative.

Native TTR fusion protein was expressed and purified essentially as described by Ye & Liu (2001) except for some improvements to increase the yields by introducing DEAE Sepharose Fast Flow anion-exchange column chromatography as the first purification step

and the Ni-NTA column being washed with MCAC-10 (25 m*M* Tris–HCl pH 8.0, 10 m*M* imidazole, 500 m*M* NaCl, 10% glycerol, 0.1% Triton, 10 m*M* β-mercaptoethanol, 1 m*M* PMSF) instead of MCAC-50 (25 m*M* Tris–HCl pH 8.0, 50 m*M* imidazole, 500 m*M* NaCl, 10% glycerol, 0.1% Triton, 10 m*M* β-mercaptoethanol, 1 m*M* PMSF) in Ni-NTA affinity column chromatography.

The SeMet TTR derivative was prepared as described previously (He, Shao *et al.*, 2001). In brief, the derivative was expressed by *E. coli* M15 cultured in the minimal media M9 containing L-SeMet and another six amino acids, lysine, threonine, phenylalanine, leucine, isoleucine and valine, which inhibited Met biosynthesis of the expression strain. The purification was performed by Ni-NTA affinity followed by Mono Q anion-exchange column chromatography in a reducimg environment provided by 10 m*M* β-mercaptoethanol or 5 m*M* DTT.

SDS–PAGE was performed on a Biorad System and mass-spectrometry analysis was performed on a MALDI-TOF (matrix-assisted laser desorption/ionization time of flight) mass spectrometer and an ESI (electrospray ionization) Quadrupole mass spectrometer.

2.2. Crystallization

The purified native TTR fusion protein was dialyzed against 25 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 10 m*M* β-mercaptoethanol, 0.2 m*M* EDTA, 1 m*M* PMSF, concentrated to 5–40 mg ml^{-1} and then filter-sterilized to remove any particulate matter. Crystallization trials were set up in Linbro multiwell tissue-culture plates using the hanging-drop vapour-diffusion method. Preliminary crystallization conditions were established using Hampton Research Crystal Screen kits I and II (Jancarik *et al.*, 1991) at 291 K, followed by a refinement of the conditions through the variation of protein concentration, precipitant, temperature, pH, drop volume or by the addition of detergents.

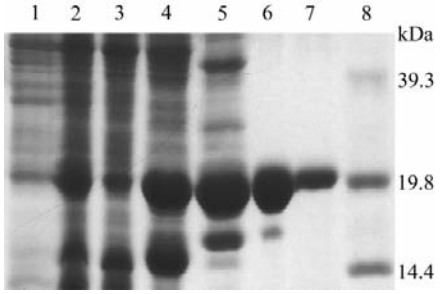

Figure 1
SDS–PAGE (12%). Lane 1, crude cell extract before induction; lane 2, crude cell extract after induction; lane 3, clarified cell extract; lane 4, fraction after DEAE-Sepharose chromatography; lane 5, fraction after Ni-NTA affinity chromatography; lane 6, peak I of the fraction after Resource Q chromatography; lane 7, peak II of the fraction after Resource Q chromatography; lane 8, protein molecular-weight markers, indicated on the right.

The purified SeMet TTR derivative was dialyzed against 25 m*M* Tris–HCl pH 8.0, 150 m*M* NaCl, 5 m*M* DTT, 0.2 m*M* EDTA and 1 m*M* PMSF, concentrated to 20–25 mg ml^{-1} and then filter-sterilized to remove any particulate matter. Crystallization trials were set up based on the conditions used for native TTR.

2.3. Data collection and processing

Data were collected in-house at 115 K using a MAR Research image plate and a 4.8 kW Rigaku rotating-anode generator producing Cu $K\alpha$ radiation of wavelength 1.5418 Å. Multiwavelength anomalous dispersion (MAD) data were collected on beamline 19-ID under cryoconditions at the Advanced Photon Source at Argonne National Laboratory. Crystals were frozen in the crystallization buffer without additional cryoprotectant.

Data processing was performed using the program *DENZO* and data sets were scaled and merged using *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. Expression and purification

Native TTR fusion protein was over-expressed to a high level (Fig. 1, lane 2), more than 60% of which was soluble (Fig. 1, lane 3). Purification of TTR fusion protein required DEAE anion-exchange (Fig. 1, lane 4), Ni-NTA affinity (Fig. 1, lane 5) and Resource Q anion-exchange column chromatography. Two peaks (Fig. 1, lanes 6 and 7) appeared during the purification by Resource Q. The yield from 1 l of *E. coli* culture was approximately 30 mg of pure TTR fusion protein (Fig. 1, lane 7).

For the SeMet TTR derivative, an expression yield similar to native TTR was gained despite the much slower growth of *E. coli* M15 in minimal medium M9 with toxic SeMet. The product was purified to an estimated homogeneity of above 95% by Ni-NTA affinity and Mono-Q anion-exchange chromatography. The yield from 1 l of *E. coli* culture was approximately 5 mg.

According to the mass-spectrometry analysis, the MW of the SeMet TTR derivative is approximately 280 Da larger than that of native TTR, which is close to the theoretical difference value of 235 Da between five Se atoms and five S atoms. The additional increase in molecular weight is likely to arise from oxidation of S atoms, apart from the substitution of Se for S.

3.2. Crystallization

The samples from two peaks (Fig. 1, lanes 6 and 7) were used in the crystallization trials, with the result that crystals appeared only in the sample corresponding to lane 7. From the preliminary screening of crystallization conditions, prism-like crystals (Fig. 2*a*) exhibiting very weak X-ray diffraction were obtained with reagent 22 of Crystal Screen kit I. The reagent comprises 100 m*M* Tris–HCl pH 8.5, 32% PEG 4000 and 0.2 *M* sodium acetate. Further refinement showed that concentrations of protein, precipitant and pH were important factors for crystallization. Only prism-like crystals which were difficult to reproduce and unsuitable for X-ray diffraction appeared at protein concentrations lower than 26 mg ml^{-1}, pH > 8.5 or PEG 4000 concentrations below 30%, with no crystal observed for pH $\leq$ 7.0. Exorbitant concentrations of protein resulted in the crystals forming twins or clusters (Fig. 2*b*), which made it difficult to isolate a single crystal for the diffraction experiments. Finally, the best crystals (Fig. 2*c*), diffracting to 3.0 Å resolution, were obtained 3 d after setting up a hanging drop with 1 µl of a 34–40 mg ml^{-1} protein solution, 1 µl of reservoir solution containing 32–36% PEG 4000, 0.18–0.22 *M* sodium acetate, 100 m*M* Tris–HCl buffer pH 8.0.

Two crystal forms of SeMet TTR derivative were obtained using similar conditions to those for native TTR, except for a lower protein concentration of 25 mg ml^{-1}. The

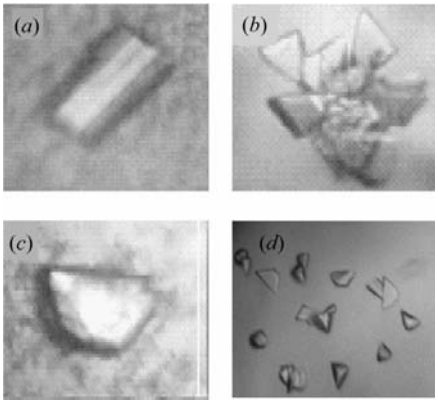

Figure 2
Photomicrographs of crystals of TTR complexed with AcCoA. (*a*) Prism-like crystal, (*b*) clustered crystals, (*c*) crystal diffracting to 3.0 Å, (*d*) crystals of SeMet TTR derivative.

optimum reservoir solution condition, 100 m*M* Tris–HCl pH 8.0, 36% PEG 4000, 0.15–0.21 *M* sodium acetate at 291 K yielded crystals (Fig. 2*d*) which were very compact and stable, as demonstrated by their high-resolution X-ray diffraction to 1.4 Å after storing for about 20 d at 291 K and several days at room temperature. The two crystal forms are indistinguishable in shape.

3.3. Data collection and preliminary X-ray crystallographic analysis

From a native TTR crystal, diffraction data to 3.0 Å resolution were collected in-house. The crystal belongs to the space group $P2_1$, with unit-cell parameters $a = 47.6$, $b = 66.6$, $c = 53.5$ Å, $\beta = 104.3^\circ$. There are two molecules in the asymmetric unit and the solvent content is about 42.6%.

Statistics for data collection of SeMet TTR derivative crystals are shown in Table 1.

MAD data were collected from a single SeMet TTR derivative crystal of form I, with a space group of *C*2 and unit-cell parameters $a = 101.7$, $b = 45.6$, $c = 84.2$ Å, $\beta = 105.8^\circ$, at the peak ($\lambda_1 = 0.9791$ Å), inflection ($\lambda_2 = 0.9793$ Å) and remote ($\lambda_3 = 0.9639$ Å) wavelengths to 1.4 Å resolution. All but the N-terminal Met residue of TTR of the ten selenium sites in the asymmetric unit were located, based on which the structure of TTR complexed with AcCoA at 1.55 Å resolution was determined (He, Ding *et al.*, 2001). The structure of TTR belongs to the acyl-CoA *N*-acyltransferase fold (Nat fold). From a SeMet TTR derivative crystal of form II, diffraction data to 2.3 Å were collected in-house. The parameters, space group $P2_1$ and unit-cell parameters $a = 47.7$, $b = 66.6$, $c = 53.6$ Å, $\beta = 103.7^\circ$, are very similar to those of the native TTR crystal. The structure of TTR complexed with AcCoA from crystal form II was determined by the molecular-replacement method using the structure from crystal form I as a search model.

Table 1
Statistics for data collection.

Values in parentheses are for the highest resolution shell.

Crystal	Form I	Form II
Space group	*C*2	$P2_1$
Unit-cell parameters		
a (Å)	101.7	47.7
b (Å)	45.6	66.6
c (Å)	84.2	53.6
β (°)	105.8	103.7
Wavelength (Å)		1.5418
Peak	0.9791	
Inflection	0.9793	
Remote	0.9639	
Resolution (Å)		30–2.3
Peak	99–1.50	
Inflection	99–1.50	
Remote	99–1.55	
Completeness (%)		99.4 (98.7)
Peak	95.3 (99.1)	
Inflection	90.1 (89.0)	
Remote	92.0 (77.8)	
Mean redundancy		4.7 (4.6)
Peak	3.0 (3.0)	
Inflection	5.4 (3.3)	
Remote	5.5 (2.5)	
$R_{\rm merge}$ (*I*)†		0.056 (0.132)
Peak	0.078 (0.267)	
Inflection	0.072 (0.425)	
Remote	0.071 (0.590)	
Mean $I/\sigma(I)$		18.6 (12.4)
Peak	10.5 (3.3)	
Inflection	11.2 (2.5)	
Remote	10.8 (1.7)	

† $R_{\rm merge}(I)$ is defined as $\sum |I - \langle I \rangle| / \sum I$.

We assume that TTR protein could also have been found crystallizing in two crystal forms if a large number of native crystals had been analyzed by X-ray diffraction, for the crystal morphology is indistinguishable to the two forms.

The binding of AcCoA to TTR was unexpected, as no AcCoA was added during purification and crystallization. The AcCoA must have bound TTR naturally *in vivo*.

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