Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Cloning, crystallization and preliminary X-ray studies of XC2981 from *Xanthomonas campestris*, a putative CutA1 protein involved in copper-ion homeostasis

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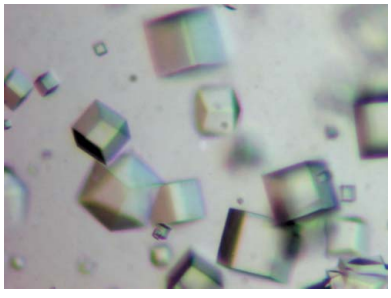
Received 23 July 2006
Accepted 28 September 2006

Divalent metal ions play key roles in all living organisms, serving as cofactors for many proteins involved in a variety of electron-transfer activities. However, copper ions are highly toxic when an excessive amount is accumulated in a cell. CutA1 is a protein found in all kingdoms of life that is believed to participate in copper-ion tolerance in *Escherichia coli*, although its specific function remains unknown. Several crystal structures of multimeric CutA1 with different rotation angles and degrees of interaction between trimer interfaces have been reported. Here, the cloning, expression, crystallization and preliminary X-ray analysis of XC2981, a possible CutA1 protein present in the plant pathogen *Xanthomonas campestris*, are reported. The XC2981 crystals diffracted to a resolution of 2.6 Å. They are cubic and belong to space group *I*23, with unit-cell parameters $a = b = c = 130.73$ Å.

1. Introduction

XC2981 (gi|21111511) from the plant pathogen *Xanthomonas campestris* pv. *campestris* strain 17 (Xcc) is classified as belonging to the CutA1 divalent ion-tolerance protein superfamily in the Pfam database (Bateman *et al.*, 2000). It contains 110 amino acids and shares 34.8, 31.8, 30.0 and 37.3% identity with similar proteins from the bacteria *Escherichia coli*, *Thermotoga maritima*, *Aquifex aeolicus* and *Deinococcus radiodurans* and 36.4, 30.6 and 28.2% identity with similar proteins from the archaea *Pyrococcus horikoshii*, *Archaeoglobus fulgidus* and *Thermoplasma volcanium*, respectively. Molecular-genetics studies on the *E. coli* homologue suggested that some mutations in the *cutA* locus can lead to copper sensitivity caused by its increased uptake (Fong *et al.*, 1995). Although copper is an essential metal ion in all organisms, it is also highly toxic when an excessive amount is accumulated in the cell. This is best demonstrated by the finding that several mammalian neurological pathologies, such as Menke's and Wilson's diseases, Alzheimer's disease, prion diseases and Creutzfeld–Jacob syndrome, can be correlated with the malfunction of copper-binding proteins (Bush, 2000). The homeostasis mechanism of regulated copper intake is thus an important issue that deserves comprehensive studies (Rosenzweig, 2001; Banci & Rosato, 2003).

Recently, several quaternary structures of the CutA1 monomer have been reported. That from *T. maritima* (Savchenko *et al.*, 2004) was found to adopt a trimeric structure, while those from *E. coli* and rat formed a dimer of trimers (Arnesano *et al.*, 2003) and that from *P. horikoshii* consisted of a multimer of trimers (Tanaka *et al.*, 2004). Interestingly, differing rotation angles were found between the two trimer subunits when the CutA1 proteins assembled into a hexamer; in the *E. coli* protein one trimer is rotated by 60° with respect to the other around the axis perpendicular to the trimer planes, while in rat protein only a 25° rotation was observed (Arnesano *et al.*, 2003). Additionally, significantly different hydrophobic and electrostatic interactions were observed between these trimer interfaces (Arnesano *et al.*, 2003). The CutA1 copper-binding proteins may thus adopt a variety of quaternary structures depending on differences in the primary sequences or in environmental conditions. In this manuscript, we describe the cloning, expression, crystallization and



preliminary X-ray analysis of XC2981 from Xcc. While a major trimeric species was found in a denatured SDS–PAGE analysis, we also detected significant amounts of tetramer and some hexamer (Fig. 1), suggesting that XC2981 may possibly form a hexamer in solution.

2. Materials and methods

2.1. Cloning, expression and purification

The XC2981 gene fragment was PCR-amplified directly from a local Xcc genome (*X. campestris* pv. *campestris* strain 17) with a forward 5′-TACTTCCAATCCAATGCTATGAGCGCCTTCTCT-CTC primer and a backward 5′-TTATCCACTTCCAATGTCAG-GATTCCTTGCGGGTTT primer. A ligation-independent cloning (LIC) approach (Aslanidis & de Jong, 1990) was carried out to obtain the desired construct according to a previously published protocol (Wu *et al.*, 2005). The final construct codes for an N-terminal His_6 tag, a 17-amino-acid linker and the XC2981 target protein (110 amino acids) under the control of a T7 promoter. Transformed *E. coli* BL21 (DE3) host cells were grown in LB medium at 310 K until an OD_{600} of 0.8 was attained. Overexpression of the His_6-tagged target protein was induced by the addition of 0.5 m*M* IPTG at 310 K for 4.5 h. The cells were harvested, resuspended in equilibration buffer (20 m*M* Tris–HCl, 80 m*M* NaCl pH 8.0) and lysed using a microfluidizer (Microfluidics). Most tagged target proteins were present in the soluble fraction (Fig. 1). After centrifugation, the target protein was purified by immobilized metal-affinity chromatography (IMAC) on a nickel column (Sigma), which was eluted with 20 m*M* Tris pH 8.0, 80 m*M* NaCl and a gradient of 50–300 m*M* imidazole. The fractions containing XC2981 were monitored by SDS–PAGE, recombined and dialyzed repeatedly against 50 m*M* Na_2HPO_4 pH 8.0, 10% glycerol and 500 m*M* NaCl. After buffer exchange, the His_6 tag and linker were cleaved from XC2981 by TEV (tobacco etch virus) protease at 288 K for 24 h. The purified protein was dialyzed against 20 m*M* Tris pH 8.0 and 80 m*M* NaCl several times. For crystallization, XC2981 was further purified on an anion-exchange column (AKTA, Pharmacia Inc.). The fractions eluted with 20 m*M* Tris pH 8.0, 1 *M* NaCl were combined and dialyzed against 20 m*M* Tris pH 8.0 and 80 m*M* NaCl. The final target protein (110 amino acids) has greater than 99% purity (Fig. 1) and contains only an extra tripeptide (SNA) at the N-terminal end. The overexpression and purification of XC2981 was monitored by SDS–PAGE as shown in Fig. 1.

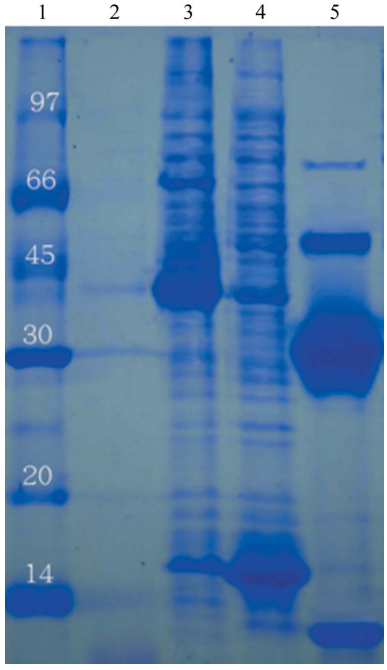


Figure 1
SDS–PAGE monitoring of the overexpression and purification of XC2981. Lane 1, molecular-weight markers in kDa; lane 2, whole cell lysate before IPTG induction; lane 3, whole cell lysate after IPTG induction; lane 4, whole cell lysate after IPTG induction with optional extra SDS; lane 5, purified XC2981 after TEV cleavage without optional extra SDS. It is clear that bands corresponding to monomers (12 kDa), trimers (36 kDa), tetramers (48 kDa) and even hexamers (72 kDa) are present in this gel in the absence of optional extra SDS (lane 5), indicating that XC2981 forms a stable trimer and possibly a hexamer in solution.

Table 1
Data-collection statistics for XC2981.

Values in parentheses are for the highest resolution shell.

Space group	*I*23
Unit-cell parameters (Å)	$a = b = c = 130.70$
Temperature (K)	100
Wavelength (Å)	0.963927
Resolution range (Å)	30.0–2.6 (2.69–2.60)
Mosaicity (°)	0.5
Unique reflections	59537 (1869)
Redundancy	6 (6)
Completeness (%)	100 (100)
R_{merge} (%)	7.0 (23.2)
Mean $I/\sigma(I)$	8.4 (7.1)
Solvent content (%)	51

2.2. Crystallization

For crystallization, the protein was concentrated to 28 mg ml^{-1} in 20 m*M* Tris pH 8.0 and 80 m*M* NaCl using an Amicon Ultra-10 (Millipore). Screening for crystallization conditions was performed using sitting-drop vapour diffusion in 96-well plates (Hampton Research) at 293 K by mixing 0.5 µl protein solution with 0.5 µl reagent solution. Initial screens included the Hampton sparse-matrix Crystal Screens 1 and 2, a systematic PEG–pH screen and the PEG/Ion Screen and were performed using a Gilson C240 crystallization workstation. Cube-shaped crystals appeared in 3 d from a reservoir solution comprising 0.1 *M* CAPS buffer pH 10, 2.2 *M* $(NH_4)_2SO_4$, 0.2 *M* Li_2SO_4.. Crystals suitable for diffraction experiments were grown by mixing 1.5 µl protein solution with 1.5 µl reagent solution at 298 K and reached maximum dimensions of 0.15 × 0.15 × 0.1 mm after 5 d (Fig. 2).

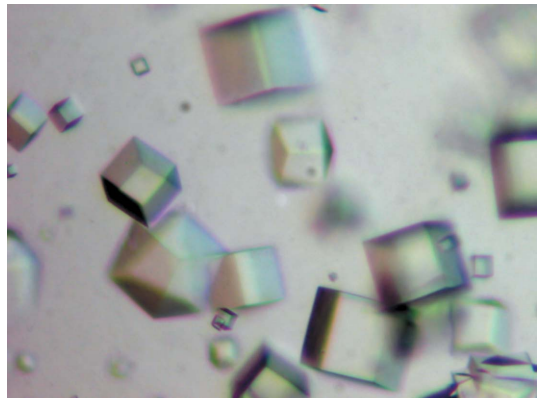


Figure 2
Cube-shaped crystals of XC2981 from Xcc grown by the hanging-drop vapour-diffusion method. The crystallization condition used was 0.1 *M* CAPS buffer pH 10, 2.2 *M* $(NH_4)_2SO_4$, 0.2 *M* Li_2SO_4. The average dimensions of these crystals were all approximately 0.15 × 0.15 × 0.1 mm.

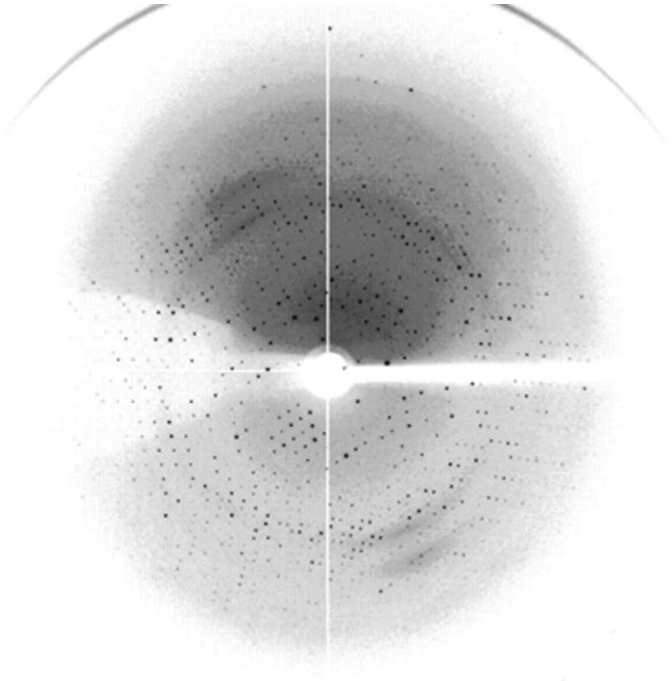


Figure 3
Diffraction pattern of XC2981 collected at NSRRC beamline 13B1 from a flash-frozen crystal in reservoir cryoprotectant. The exposure time was 15 s, with an oscillation range of 1° and a crystal-to-detector distance of 200 mm.

2.3. Data collection

Crystals were soaked in mother liquor and then flash-cooled at 100 K in a stream of cold nitrogen. X-ray diffraction data were collected using the National Synchrotron Radiation Research Center (NSRRC) beamline 13B1, Taiwan. A 2.6 Å resolution native data set was obtained. The data were indexed and integrated using the *HKL*-2000 software (Otwinowski & Minor, 1997), giving a data set that had 100% completeness with an overall $R_{\rm merge}$ of 7.0% on intensities. The crystals belong to the cubic space group $I23$. The data-collection statistics are summarized in Table 1 and an X-ray diffraction image collected at the NSRRC facility is shown in Fig. 3.

3. Results and discussion

The XC2981 gene consists of 333 bp coding for 110 amino-acid residues. The isoelectric point was calculated to be 5.25. Purified XC2981 showed a mixture of monomers (12 kDa), trimers (36 kDa), tetramers (48 kDa) and hexamers (72 kDa) on SDS–PAGE (Fig. 1), indicating that the protein may possibly adopt a quaternary structure consisting of a hexamer as in *E. coli* CutA1 or rat CutA1 (Arnesano *et al.*, 2003). The 'dimer of trimers' structure of *Pho*CutA from *P. horikoshii* (Tanaka *et al.*, 2004) is different from the structures of *E. coli* CutA1 and rat CutA1. The former packs side by side, bridged by copper ions into a multimer (Tanaka *et al.*, 2004), while in the latter structures monomers stack onto each other to form a hexamer that is mainly stabilized by hydrophobic interactions (Arnesano *et al.*, 2003). XC2981 contains two Cys residues (Cys13 and Cys34), but not the C*XX*C motif known to bind copper ions in metallochaperones (Banci & Rosato, 2003; Tanaka *et al.*, 2004; Arnesano *et al.*, 2006). The potential metal-binding features of CutA1 are therefore different from those of metallochaperones. Interestingly, in *Pho*CutA the copper ion was found to be positioned at the trimer interface and coordinated to Asp48 O^{δ}, Lys49 C═O and water O atoms from each respective trimer subunit (Tanaka *et al.*, 2004). It would thus be interesting to see whether or not the quaternary structure of XC2981 is similar to those previously observed.

The high-resolution diffraction data obtained from the native crystals establishes their suitability for X-ray structural analysis (Fig. 3). Molecular replacement using the *AMoRe* program (Navaza, 1994) will first be performed to attempt to obtain protein phases using the deposited coordinates of PDB entries 1naq or 1j2v as the search model. If this approach is unsuccessful, the single-wavelength anomalous diffraction (SAD; Wang, 1985; Dauter, 2002) or the multiwavelength anomalous diffraction (MAD) method (Hendrickson & Ogata, 1997; Terwilliger & Berendzen, 1999) will be applied using selenomethionine-substituted protein to solve the protein phases. Although XC2981 contains only one methionine, it may still be feasible to solve its structure using SAD or MAD because of its short length (110 amino acids).

This work was supported by an Academic Excellence Pursuit grant from the Ministry of Education and the National Science Council, Taiwan to S-HC. We thank the Core Facilities for Protein X-ray Crystallography at the Academia Sinica, Taiwan, at the National Synchrotron Radiation Research Center, Taiwan and at the SPring-8 Synchrotron facility in Japan for assistance during X-ray data collection. The National Synchrotron Radiation Research Center is a user facility supported by the National Science Council, Taiwan and the Protein Crystallography Facility is supported by the National Research Program for Genomic Medicine, Taiwan.

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