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Overexpression, purification, crystallization and crystallographic analysis of CopK of *Cupriavidus metallidurans*

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CopK of *Cupriavidus metallidurans* is a 93-amino-acid protein whose mature form (73 amino acids) has been purified and crystallized by the hanging-drop vapour-diffusion method in 100 m*M* citrate pH 3.5, 200 m*M* Li_2SO_4, 20%(*w*/*v*) glycerol, 13%(*w*/*v*) PEG 8000. Crystals display orthorhombic symmetry, with unit-cell parameters a = 57.53, b = 128.65, c = 49.77 Å, and diffract to 2.2 Å resolution using synchrotron radiation.

1. Introduction

Cupriavidus metallidurans CH34 (Vandamme & Coenye, 2004) [formerly known as *Wautersia metallidurans* (Vaneechoutte *et al.*, 2004), *Ralstonia metallidurans* (Goris *et al.*, 2001) and *Alcaligenes eutropha* (Mergeay *et al.*, 1985)] is a β-proteobacteria found in the late 1970s in sediments in the decantation basin of a zinc factory near Liege in Belgium (Mergeay *et al.*, 1978). This bacterium carries resistance genes to Zn, Co, Cd, Ni, Hg, Pb and Cu on its plasmids pMOL30 (233 kbp) and pMOL28 (171 kbp) (Taghavi *et al.*, 1997). The copper resistance is conferred by the *copRSABCD* chromosomic genes and also by the larger *copTKSRABCDIFGLH* locus present on the pMOL30 plasmid. Recent proteomic studies of the copper resistance led to the discovery of the CopK protein (Mergeay *et al.*, 2003).

2. Material and methods

2.1. Cloning and overexpression

Plasmidic DNA from *C. metallidurans* was prepared as described by Kado & Liu (1981) and used as template to amplify the *copK* gene (Genbank CAI11334) by PCR technology. The following pair of oligonucleotides were used as primers: 5′-CATATGAAACAAAA-ACTGATGGTCGGAGCC-3′ and 5′-GAATTCATCAGCCCCCTT-CGCTGTGGCCCTTCCTGAGGGC-3′. The amplification protocol consisted of 5 min at 367.15 K followed by 30 cycles of 0.5 min at 367.15 K, 0.5 min at 323.15 K and 1 min at 345.15 K, followed by 10 min at 345.15 K. The amplified fragment was directly cloned into pCR2.1 vector using the Original TA Cloning Kit (Invitrogen). After digestion with *Nde*I and *Eco*RI enzymes, the fragment corresponding to the *copK* gene was then ligated into the inducible expression vector pET30b (Novagen) and called pIRMWcopK1.

The resulting protein, identified by DNA sequencing as the CopK protein, was overexpressed in *Escherichia coli* BL21(DE3)pLysS cells (Novagen).

2.2. Enzyme purification

Bacterial pellets were obtained from cultures of recombinant *E. coli* (1 l) in rich medium [1%(*w*/*v*) Bacto-tryptone (Difco), 0.5%(*w*/*v*) yeast extract (Difco), 0.1%(*w*/*v*) glucose, 0.5%(*w*/*v*) NaCl, 0.03%(*w*/*v*) KH_2PO_4, 0.07%(*w*/*v*) K_2HPO_4] supplemented with 30 µg ml^{-1} kanamycin and induced at an OD_{660} of 0.6 with 1 m*M* IPTG for 5 h at 310 K. The pellets were thawed in 50 ml 50 m*M* Tris–HCl buffer pH 8.0 and disrupted by sonication for 10 min at 20% amplitude in a sonicator (model Vibra Cell 75041; Bioblock). The suspension was centrifugated at 15 000*g* for 10 min.

The supernatant fluid was applied onto a copper IMAC (chelating Sepharose supplied by Amersham Pharmacia) column pre-equilibrated with the same buffer.

Bound protein was eluted with a linear gradient of 0–0.5 *M* imidazole in 50 m*M* Tris–HCl buffer pH 8.0. Fractions containing CopK protein were pooled after analysis by SDS–PAGE electrophoresis (Phast System Pharmacia) and dialyzed against 50 m*M* Tris–HCl buffer pH 8.0 using an Amicon PM3 concentration membrane. The sample was loaded onto a ResourceQ column (Pharmacia) pre-equilibrated with 50 m*M* Tris–HCl buffer pH 8.0. CopK did not bind to the ion-exchange resin, but contaminants were retained on it. The washed fraction (containing pure CopK) was then concentrated to 10 mg ml^{-1} on an Amicon PM3 membrane.

Selenomethionyl protein was also produced following the growth conditions described in Doublié (1997). The selenomethionyl protein was purified as described for the native protein.

The purified protein samples (Fig. 1) were concentrated to about 10 mg ml^{-1}, as estimated by UV absorption (calculated extinction coefficient 1280 M^{-1} cm^{-1}), flash-frozen in liquid nitrogen and then kept at 193 K. The yield of the CopK protein was 40 mg per litre of culture for the native enzyme and 10 mg per litre for the selenomethionyl protein. Both purified proteins were characterized by mass spectrometry and Edman degradation analysis. These analyses showed cleavage of the 20-amino-acid N-terminal periplasmic signal. Similar results were also observed from a culture of *C. metallidurans* CH34 grown in the presence of copper (Mergeay *et al.*, 2003).

2.3. Crystallization

Crystallization trials were performed by the hanging-drop vapour-diffusion method (McPherson, 1982) using 24-well tissue-culture VDX plates (Hampton Research) at 293 K. Each drop was prepared by mixing 2 µl protein solution with the same volume of reservoir solution. The drops were suspended over 0.6 ml reservoir solution. A protein concentration in the drop of 5 mg ml^{-1} was determined by measurement of the optical density at 280 nm. Preliminary crystallization conditions were tested using the Crystal Screen and Crystal Screen Cryo kits (Hampton Research). Tiny crystals appeared in the presence of sulfate as precipitant and citrate buffer. Crystallization conditions were refined by systematic variations of the lithium and ammonium sulfate concentrations at varying pH values (3.5–4.5). Only one crystal was obtained, using the conditions 100 m*M* citrate buffer pH 3.5, 200 m*M* Li_2SO_4, 20%(*v*/*v*) glycerol, after 5 d. No crystals of the selenomethionyl protein have been obtained so far.

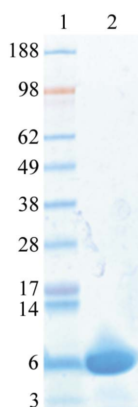

Figure 1
Polyacrylamide-gel electrophoresis of the purified CopK under denaturing conditions. Lane 1, molecular-weight markers (kDa; Invitrogen). Lane 2, purified protein.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.97985
Resolution (Å)	99–2.07 (2.10–2.07)
No. of measured reflections	77641
No. of unique reflections	24675
Multiplicity	3.15
$I/\sigma(I)$	11.7 (3.9)
Completeness (%)	96.5 (98.6)
R_{merge} (%)	8.1 (27.4)
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 57.53, b = 128.65, c = 49.77

No crystals appeared under the conditions described for the wild-type enzyme. Tiny crystals were obtained in MES buffer pH 6.0, 0.8 *M* ammonium sulfate or in HEPES buffer pH 7.0, 3.2 *M* ammonium sulfate, but the crystals were unstable. Seeding did not yield better crystals. Crystallographic analysis could not be performed on the SeMet protein.

3. Data collection and analysis

Preliminary diffraction data were collected on a MAR345 imaging-plate system from MAR Resarch equipped with Osmic optics using an FR591 rotating-anode generator (Cu *Kα*).

A fluorescence scan at the copper edge was performed on a flash-frozen crystal at beamline BM30 at ESRF (Grenoble). The scan did not reveal the presence of copper and therefore MAD phasing was not feasible. A complete diffraction data set was collected from the same crystal using a MAR CCD detector at a single wavelength (0.97985 Å). The crystal diffracted to 2.2 Å. The distance between the crystal and the detector was set to 150 mm. Data were processed with the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and the results are summarized in Table 1. The overall completeness is 96.5% in the resolution range 99.0–2.07 Å, with an overall R_{merge} value of 8.1%.

4. Structure analysis

Based on sequence analysis, no homologous proteins are available in the PDB and therefore molecular replacement could not be applied. The absence of copper from the crystal excluded the application of the MAD technique to the crystals obtained using the described protocol. The absence of copper from the crystal could be a consequence of the chelating role of the citrate buffer during crystallization. This could also suggest that affinity for the metal is not very high, according to mass-spectrometry results (unpublished results).

Efforts to grow suitable crystals of the SeMet protein have so far been unsuccessful. In this context, we are now working on the production of suitably labelled protein in order to study the fold by NMR. In parallel, we are testing other crystallization conditions in the hope of obtaining crystals of either the native or SeMet-containing protein. Furthermore, heavy-atom soaks will be performed in order to solve the structure by MIR if the NMR studies do not produce sufficient structural data. The use of soaking with halides prior to data collection could also be tried.

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