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Overproduction, purification and preliminary X-ray diffraction analysis of CzcE from *Cupriavidus metallidurans* CH34

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CzcE is encoded by the *czc* determinant that allows *Cupriavidus metallidurans* CH34 to modulate its internal concentrations of cobalt, zinc and cadmium. This periplasmic protein was overproduced in its mature form in *Escherichia coli* and purified in two steps. After preliminary screening of crystallization conditions using a robot, well diffracting crystals were obtained using the hanging-drop vapour-diffusion method. Crystals diffracted to 1.96 Å using synchrotron radiation. They belonged to the monoclinic space group C2, with unit-cell parameters $a = 105.54$, $b = 29.68$, $c = 71.10$ Å. The asymmetric unit is expected to contain a dimer, in agreement with the quaternary structure deduced from gel-filtration experiments.

1. Introduction

The tripartite cation-efflux pump CzcCBA is the most important heavy-metal defence in *Cupriavidus metallidurans* CH34. It modulates the internal concentration of cobalt, zinc and cadmium and thus is a key system for the survival of the strain in conditions where there is an excess of these heavy metals (Nies *et al.*, 1987; Legatzki *et al.*, 2003). The genome of *C. metallidurans* CH34 is composed of two megareplicons (a megaplasmid and a chromosome) and two large plasmids: pMol28 (171 kbp) and pMol30 (234 kbp). The *czc* determinant is borne on pMol30 and comprises a total of nine genes with organization *czcINCBADRSE* (Grosse *et al.*, 2004). The regulation of this determinant is quite complex and occurs *via* the concerted action of several different promoters, which are probably regulated as a function of the periplasmic and cytoplasmic concentrations of the various metals. For instance, CzcE is postulated to be a periplasmic metal-cation sensor (Grosse *et al.*, 2004) and transcription of *czcE* from its own promoter is dependent on the presence of zinc (van der Lelie *et al.*, 1997). On the basis of the results of a *czcE-phoA* fusion and of metal-affinity chromatography, preliminary characterization of CzcE led to the conclusion that it is a periplasm-located metal-binding protein (Grosse *et al.*, 2004).

2. Materials and methods

2.1. Cloning, overexpression and purification of CzcE

The DNA fragment expected to encode the CzcE protein devoid of signal peptide was amplified by PCR with suitable restriction sites for subcloning in the expression vector pET30a. The sense primer 5′-ATGTAT*CATATG***TTGGAAATGACCGGGTTG** contains the nucleotides (in bold) coding for an N-terminal amino-acid sequence starting with Leu24 (LEMTGL). The overhanging sequence includes an *Nde*I site (italic) carrying the initiator codon ATG. The antisense primer 5′-ATGTAT*CTCGAG*TCA**GCCACCTGTGAAGAGGTC** was designed to incorporate an *Xho*I site (italic) and a stop codon corresponding to the end of the sequence of CzcE. The nucleotides in bold are complementary to the corresponding coding sequence of the mature form of CzcE. The authenticity of the DNA insert generated by PCR was confirmed by sequencing. The resulting expression plasmid pET-CzcE was used to transform the T7 RNA polymerase-containing host *Escherichia coli* BL21(DE3). Expression of CzcE was

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Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.933
Space group	$C2$
Unit-cell parameters (Å, °)	a = 105.54, b = 29.68, c = 71.10, β = 113.68
Resolution (Å)	48.3–1.96 (2.06–1.96)
No. of measured reflections	25336 (2161)
No. of unique reflections	11285 (1274)
Completeness	76.4 (60.1)
$I/\sigma(I)$	12.5 (4.9)
$R_{\rm merge}$† (%)	5.7 (15.7)

† $R_{\rm merge} = \sum_{\bf h}\sum_m |I_m({\bf h}) - \langle I({\bf h})\rangle| / \sum_{\bf h}\sum_m I_m({\bf h})$.

performed essentially as previously described for CopH (Sendra *et al.*, 2006). Briefly, cells were grown at 310 K in LB medium until the culture reached an absorbance of 0.8 at 600 nm. CzcE expression was induced by addition of 0.4 m*M* isopropyl β-D-1-thiogalactopyranoside and cells were then grown overnight at 293 K before being harvested by centrifugation. The cell pellet was suspended in a buffer composed of 50 m*M* HEPES pH 8.0 and 200 m*M* NaCl (buffer *A*) and lysed by sonic oscillation. The total protein extract was recovered by centrifugation for 90 min at 45 000 rev min^{-1} in a Ti-70 rotor (Beckman) and was used for further purification. Residual nucleic acids were eliminated by 2% streptomycin sulfate precipitation. Ammonium sulfate was then used to precipitate soluble proteins and CzcE was found in the pellet obtained at 50% final saturation. Precipitated proteins were dissolved in a minimal volume of buffer *A*. The protein solution was subjected to filtration on a Superdex-75 column (GE Healthcare) previously equilibrated with the same buffer. Elution was run at 0.8 ml min^{-1} and 1 ml fractions were collected. Fractions were assayed for protein (absorbance at 280 nm) and for the presence of CzcE (SDS–PAGE analysis). CzcE-containing fractions were pooled and concentrated by ultrafiltration using a Diaflo cell equipped with a YM-10 membrane (Amicon Co.). The yield for a typical purification procedure is about 70 mg pure CzcE from 1 l LB medium. The protein preparation was concentrated to about 20 mg ml^{-1}, aliquoted and stored at 193 K for further use.

Protein concentration was determined using bovine serum albumin as a standard and the Micro BCA protein assay (Pierce). The denatured molecular weight of CzcE was estimated by 0.1% SDS/15% polyacrylamide gel electrophoresis (Laemmli, 1970; Fig. 1) and its native molecular weight was ascertained by elution of a calibrated Superdex-75 filtration column. The pure protein eluted in a peak corresponding to 20 kDa, suggesting that CzcE is a dimer in solution. N-terminal sequencing of the pure protein gave MLEMTGLK, indicating that the initial methionine was still present in this form of CzcE. The experimental molecular weight deduced from MALDI–TOF characterization, 11 560 Da, fits perfectly with the expected theoretical molecular weight of mature CzcE including an initial methionine.

2.2. Crystallization

Crystallization conditions were screened using the sitting-drop vapour-diffusion method at 293 K and a series of screening kits from Hampton Research using a Tecan Genesis RSP 100 robot. Drops were prepared by mixing 1 µl reservoir solution with 1 µl of CzcE at a concentration of 20 mg ml^{-1} in 50 m*M* HEPES pH 8.0, 200 m*M* NaCl. Crystals appeared after one week in various conditions, all of which contained 30% PEG 4000 and 0.1 *M* Tris–HCl pH 8.5. Conditions were optimized manually using the hanging-drop vapour-diffusion method in VDX plates from Hampton Research (well capacity 3.5 ml, area per well 2 cm^2). The best crystals (Fig. 2) appeared in approximately two weeks using 2 µl protein at 20 mg ml^{-1} in the buffer described above mixed with 2 µl of a reservoir solution composed of 30% PEG 4000, 0.1 *M* Tris, 50 m*M* pH 8.5, 30 m*M* $CaCl_2$ and 500 m*M* ammonium chloride and equilibrated against 500 µl reservoir solution.

2.3. X-ray diffraction

A crystal (100 × 40 × 20 µm) was harvested in a cryoloop, soaked in Paratone for cryoprotection and frozen in liquid nitrogen. A diffraction data set (Table 1) was collected at a single wavelength (0.933 Å) using an ADSC Q-4R detector at the ID14-2 ESRF synchrotron beamline (Grenoble). The crystal-to-detector distance was set to 191.5 mm. The oscillation range per image was 0.5° for a total angular range of 90°, which was limited by the available beamtime. Data were processed using *XDS* (Kabsch, 1988), *SCALA* and *TRUNCATE* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). The monoclinic space group *C*2 was assigned (Table 1).

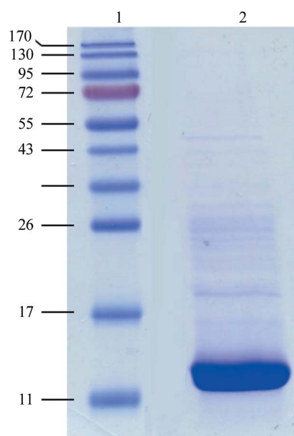

Figure 1
SDS–PAGE of purified CzcE. Lane 1, molecular-weight markers (kDa). Lane 2, 15 µg CzcE.

Figure 2
Native crystals of CzcE.

3. Results and discussion

The excellent overproduction of the mature form of CzcE from *C. metallidurans* CH34, *i.e.* the protein devoid of its periplasm-addressing sequence, allowed the use of a simple and efficient two-step purification protocol. A very selective ammonium sulfate precipitation followed by gel filtration gave a pure protein preparation (Fig. 1) with a very good yield. As CzcE is probably a metal-binding protein (Grosse *et al.*, 2004), no tag, and especially no His tag, was introduced into the sequence. Crystals of CzcE (Fig. 2) were obtained in approximately two weeks using the conditions described above. Owing to the low symmetry of the monoclinic *C*2 space group, the data completeness is only 76.4% (Table 1). The solvent content was estimated by the program *TRUNCATE* to be 32.2% for two molecules in the asymmetric unit and 66.1% for one molecule. This corresponds to Matthews coefficients of 2.2 and 4.4 $Å^3 Da^{-1}$, respectively (Matthews, 1968). Considering the good quality of the diffraction obtained from a quite small crystal, the lower solvent content is assumed. CzcE possesses two paralogues in the genome of *C. metallidurans* CH34 (Grosse *et al.*, 2004). One of these paralogues is CopH, which is part of the *cop* cluster involved in copper homeostasis (Monchy *et al.*, 2006). CopH has also been recently characterized as a periplasm-located protein able to bind two copper equivalents with high affinity (Sendra *et al.*, 2006). However, CopH is not yet structurally characterized and thus cannot be used for molecular replacement.

Preliminary data and homology with CopH suggest that the function of CzcE should be to bind heavy metals. The next step is thus to characterize the metal-binding properties of CzcE and to prepare derivative crystals. The mature form of CzcE contains six methionine residues (including the N-terminal methionine). The production of a SeMet derivative could be an alternative method to solve the structure of the apoprotein.

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