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Crystallization and preliminary X-ray analysis of Mlc from *Escherichia coli*

Mlc is a prokaryotic transcriptional repressor controlling the expression of a number of genes encoding enzymes of the *Escherichia coli* phosphotransferase system (PTS), *ptsG* and *manXYZ*, the specific enzyme II for glucose and mannose PTS transporters, as well as *malT*, the gene of the global activator of the *mal* regulon. The *mlc* gene has been cloned into a pQE vector and recombinant protein with the point mutation R52H was expressed and purified as the selenomethionine-labelled derivative. Crystallization of SeMet-Mlc R52H was carried out using the vapour-diffusion method. The crystals belong to the monoclinic space group *C2*, with unit-cell parameters $a = 235.95$, $b = 74.71$, $c = 154.95$ Å, $\beta = 129.15^\circ$, and diffract to 2.9 Å resolution.

1. Introduction

Mlc was originally cloned onto a multicopy plasmid which rendered *Escherichia coli* cells more versatile to the use of glucose in complex media so that larger colonies could form ('makes large colonies', Mlc; Hosono *et al.*, 1995). It was found that the *mlc* gene product slows down glucose uptake so that the acetate produced in glycolysis can be more efficiently dissipated before it inhibits cell growth (Hosono *et al.*, 1995). Mlc is a member of the ROK family (repressors, ORFs and kinases; Titgemeyer *et al.*, 1994; Hansen *et al.*, 2002) and acts as a transcriptional repressor for a variety of sugar-metabolizing enzymes and transport systems (Decker *et al.*, 1998; Plumbridge, 1998, 1999, 2001, 2002; Böhm & Boos, 2004). The major target for Mlc regulation is the phosphotransferase (PTS) dependent transport of glucose (Potsma *et al.*, 1993). Mlc is known to control the expression of the genes for the general components of PTS I (enzyme I and HPr) and PTS II (EIICB^{Glc} for glucose transport), as well as the genes for the mannose transporter (EIABCD^{Man}) (Plumbridge, 2002). In addition to these proteins, the central transcriptional activator of the maltose regulon, MalT, is also subject to transcriptional control by Mlc (Decker *et al.*, 1998). The maltose regulon consists of ten genes encoding enzymes involved in the uptake and the metabolism of maltose and maltodextrins (Boos & Shuman, 1998). The particular feature that makes Mlc an attractive subject of study is its mode of regulation. In the absence of external glucose, EIICB^{Glc} is phosphorylated and Mlc does not interact with it (Lee *et al.*, 2000; Tanaka *et al.*, 2000). Under these conditions, Mlc acts as a transcriptional repressor by binding to its target promoter sites. As soon as glucose is present in the external medium it is transported into the cell by the PTS. During this step EIICB^{Glc} transfers its phosphate to glucose and thereby becomes dephosphorylated. The dephosphorylated state of EIICB^{Glc} is recognized by Mlc. Upon binding to EIICB^{Glc}, Mlc releases its specific promoter sites, thereby enabling the production of PTS proteins in the cell (Lee *et al.*, 2000; Nam *et al.*, 2001). Seitz *et al.* (2003) showed that several mutations within EIICB^{Glc} eliminate the binding of Mlc to EIICB^{Glc}. Studies using Mlc mutants suggest that both its N-terminal and C-terminal regions are involved in the interaction with EIICB^{Glc} (Seitz *et al.*, 2003; Tanaka *et al.*, 2004). Recently, it has been demonstrated that membrane association of Mlc alone causes derepression of Mlc specific promoters (Seitz *et al.*, 2003; Tanaka *et al.*, 2004).

Here, we describe the expression, purification and crystallization of SeMet-Mlc from *E. coli* with the point mutation R52H.

2. Materials and methods

2.1. Expression and purification

For routine work with recombinant DNA, established protocols were used (Sambrook *et al.*, 1989). For the construction of the Mlc expression plasmid, the *mlc* gene of 1.22 kbp was amplified from the plasmid DNA pSL104 (Lee *et al.*, 2000) using the oligonucleotides A (5'-cat gcc atg gtt gct gaa aac cag cct ggg-3') with an *Nco*I restriction site and B (5'-ggg aag ctt tta acc ctg caa cag acg-3') with a *Hind*III restriction site. After digestion with *Nco*I and *Hind*III (New England Biolabs), the PCR fragment was ligated into pQE60 (Qiagen), resulting in pQE60*mlc*. The inserted *mlc* gene was sequenced commercially. *E. coli* BL21 (DE3) cells (Novagen) were then transformed with pQE60*mlc*. The 44.3 kDa Mlc protein containing ten methionine residues in 406 amino-acid residues was expressed with the selenomethionine-substitution method (Doublé, 1997) in minimal medium M63 supplemented with 50 mg L-selenomethionine (Acros Organics), 40 mg hypoxanthine, 40 mg uracil, 20 mg biotin, 20 mg nicotinamide, 2 mg riboflavin, 20 mg thiamine, 0.25 g MgSO₄, 2 g glucose, 0.1 g ampicillin (Roth) and 40 mg of all amino acids except methionine. The cells were grown to an optical density (A_{600}) of 0.6 at 310 K and the culture was again supplemented with the following amino acids (values are given for 1 l of culture): 100 mg L-lysine, L-phenylalanine and L-threonine, 50 mg L-isoleucine, L-leucine and L-valine and 60 mg L-selenomethionine. 15 min later, expression of SeMet-Mlc was induced with 0.3 mM IPTG (isopropyl- β -D-thiogalactopyranoside; Fermentas). Overexpression of SeMet-Mlc was continued for 20 h at 310 K after induction. Cells were then harvested at 18 500g at 277 K. The cell pellet was resuspended in 25 ml buffer A (10 mM glycine pH 9.5, 50 mM NaCl) and the cells were broken with a French press in two cycles at 138 MPa. The lysate was spun down for 20 min at 18 500g and the supernatant was then spun down again for 30 min at 100 000g. The final supernatant was loaded onto a 15 ml Q-Sepharose column (Amersham Biosciences). SeMet-Mlc was eluted from the column with a linear gradient of buffer B (10 mM glycine pH 9.5, 500 mM NaCl) within five column volumes. SeMet-Mlc eluted at about 40% buffer B. The purity of the elution fractions was tested on 12.5% SDS-PAGE and estimated to

be about 95%. Samples containing SeMet-Mlc were pooled, dialyzed in 2 l buffer A overnight at 277 K in dialysis tubing with 12 kDa cutoff (Serva). The protein solution was then concentrated to about 45 mg ml⁻¹ through a 30 kDa membrane (Vivaspin). The final yield was about 25 mg SeMet-Mlc per litre of culture. Chemicals were purchased from Fluka unless otherwise stated.

2.2. Crystallization and data collection

Crystallization screening was performed using Crystal Screens I (Jancarik & Kim, 1991) and II (Hampton Research), Wizard Screens I and II (Emerald BioSystems), Stura Footprint Screens I and II (Molecular Dimensions) and JB screen No. 10 (Jena Biosciences) at 291 K in 96-well sitting-drop plates (Douglas Instruments) with 100 μ l reservoir solution and a drop size of 1.5 μ l protein solution (with a protein concentration of 45 mg ml⁻¹) plus 1.5 μ l reservoir solution. Crystals grew within two weeks in JB screen No. 10/14 (1.6 M MgSO₄, 100 mM MES pH 6.5) from precipitate (Fig. 1) and were reproduced using the hanging-drop vapour-diffusion method in 24-well plates (Hampton Research) using 1 ml reservoir solution and a protein drop consisting of 2 μ l protein and 2 μ l reservoir solution. Prior to data collection, single crystals were soaked in three drops of cryosolution for 1 min each and were then quickly transferred into liquid nitrogen. The cryosolutions consisted of a 1:1 mixture of the reservoir buffer and the protein buffer A with increasing amounts of glycerol [5, 15 and 25% (v/v), respectively]. The protein content of single crystals washed three times in cryosolution was analyzed by 15% SDS-PAGE and Western blotting using anti-Mlc antibodies (data not shown). To solve the structure of Mlc by multiple anomalous dispersion, three data sets were collected from a single crystal of SeMet-Mlc in the order peak, inflection and remote high at 100 K using a MAR CCD detector (MAR Research) on beamline X06SA of the Swiss Light Source (SLS), Villigen, Switzerland to a resolution of 2.9 Å (Fig. 2). Owing to the radiation-sensitivity of the SeMet-Mlc crystal, we collected each data set from a different part of the crystal.

Figure 1
A single SeMet-Mlc crystal of dimensions 150 × 100 × 50 μ m. For growth conditions, see §2.

Figure 2
X-ray diffraction pattern of a single SeMet-Mlc crystal measured at the Swiss Light Source, Villigen, Switzerland. Image edge: 2.6 Å.

Table 1

Crystal data and X-ray data-collection statistics for a single SeMet-Mlc crystal.

Values in parentheses refer to the highest resolution shell.

	Peak	Infection	Remote high
Wavelength (Å)	0.97637	0.97866	0.97780
Rotation range (°)	180	180	180
Resolution (Å)	∞–2.9 (3.0–2.9)	∞–3.0 (3.1–3.0)	∞–3.3 (3.4–3.3)
Space group	C2		
Unit-cell parameters (Å, °)	$a = 235.95$, $b = 74.71$, $c = 154.95$, $\beta = 129.15$		
Observed reflections	174722	157372	118860
Unique reflections†	89905	80926	61032
Completeness (%)	98.5 (99.7)	98.1 (99.6)	98.3 (99.3)
Average $I/\sigma(I)$	9.9 (2.3)	9.5 (2.1)	9.3 (2.2)
R_{merge} (%)	5.4 (36.6)	5.8 (41.3)	6.3 (38.8)
$R_{\text{meas}}^{\ddagger}$ (%)	7.5 (50.4)	8.0 (56.7)	8.8 (53.6)

† R_{meas} , the redundancy-independent R_{merge} , was calculated according to Diederichs & Karplus (1997). ‡ Bijvoet pairs were not merged for the unique data set statistics.

After data collection, the *mlc* gene carried by the *E. coli* strain BL21(DE3) used for protein expression was sequenced again and the point mutation R52H was found.

3. Results and discussion

In this report, we describe the crystallization and preliminary X-ray analysis of Mlc from *E. coli*. As a search of the Protein Data Bank (PDB; Berman *et al.*, 2000) did not result in sequences sharing a high similarity with Mlc from *E. coli*, molecular replacement was therefore not possible. We have expressed and purified Mlc with the accidental point mutation R52H in *E. coli* BL21(DE3) cells as a selenomethionine derivative. This mutation allows Mlc to be expressed at considerably higher concentrations than the wild type (data not shown) and to form larger colonies than cells expressing wild-type Mlc. We therefore think that the R52H mutant Mlc was accidentally selected.

Crystals grown within a few days (Fig. 1) did not diffract at our in-house X-ray source at first. A year later, single crystals from the same crystallization plate diffracted to 4 Å at our in-house X-ray source and to 2.9 Å at the Swiss Light Source, Villigen, Switzerland (Fig. 2). The reason for this significant improvement in crystal quality is not known. Raw data were processed using *XDS* (Kabsch, 1993) and the space group of a single SeMet-Mlc crystal was determined to be C2 (Table 1), with most likely four molecules per asymmetric unit as estimated from the Matthews coefficient $V_M = 3.0 \text{ Å}^3 \text{ Da}^{-1}$ and the solvent content of 58.5%. Our data is therefore consistent with previous results showing Mlc to form tetramers in solution (Seitz *et al.*, 2003; Nam *et al.*, 2001).

To date, five structures of ROK-family members have been published in the Protein Data Bank, all of which are glucokinases with only a very low sequence similarity to Mlc from *E. coli* as indicated by sequence alignment (Lalign server, data not shown). Currently, Mlc is the only repressor of the ROK family to be crystallized successfully. Owing to its diverse regulatory functions and its special mechanism enabling it to 'shuttle' between several operon sites and the membrane-bound EIICB^{Glc} without any low-molecular-weight inducer, Mlc represents a particularly interesting candidate for structural analysis.

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