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Crystallization and preliminary X-ray crystallographic analysis of the putative NADP(H)-dependent oxidoreductase YncB from *Vibrio vulnificus*

The *yncB* gene product from *Vibrio vulnificus*, which belongs to the medium-chain dehydrogenase/reductase (MDR) superfamily, was crystallized using the microbatch crystallization method at 295 K. Diffraction data sets were collected using synchrotron radiation. Crystals of selenomethionine-substituted YncB protein belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 90.52$, $b = 91.56$, $c = 104.79$ Å. Assuming the presence of two molecules in the asymmetric unit, the solvent content was estimated to be about 57%. Crystals of the YncB–NADP(H) complex belonged to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 90.14$, $c = 105.61$ Å. Assuming the presence of one molecule in the asymmetric unit, the solvent content was estimated to be about 56.42%.

1. Introduction

The medium-chain dehydrogenases/reductases (MDRs) constitute a large enzyme superfamily with close to 15 000 members in the databases and with a typical pairwise sequence identity of around 25%. MDR members are found in all kingdoms of life and exhibit a multitude of enzyme activities (Persson *et al.*, 1994, 2008; Jörnvall *et al.*, 1999; Hedlund *et al.*, 2010). Based upon sequence similarity, the MDR proteins can be grouped into eight large families with hundreds of members and about 1000 smaller families with ten or fewer members (Persson *et al.*, 2008). The eight most widespread families are represented by alcohol dehydrogenases (ADH), cinnamyl alcohol dehydrogenases (CAD), yeast alcohol dehydrogenases (YADH), mitochondrial response proteins (MRF), polyol dehydrogenases (PDH), quinone oxidoreductases (QOR), acyl-CoA reductases (ACR) and leukotriene B₄ dehydrogenases (LTD) (Nordling *et al.*, 2002; Riveros-Rosas *et al.*, 2003).

All MDR enzymes utilize NAD(H) or NADP(H) as a cofactor and catalyze oxidation or reduction reactions. Half of the eight large MDR families are comprised of MDRs that contain one or two Zn²⁺ ions (the CAD, PDH, ADH and YADH families), while the other half are comprised of non-Zn²⁺ MDRs (the QOR, MRF, LTD and ACR families). In MDR proteins containing one Zn²⁺ ion the Zn²⁺ ion is located at the catalytic site, while in MDRs containing two Zn²⁺ ions the second Zn²⁺ ion plays a noncatalytic structural role (Hedlund *et al.*, 2010). In bacteria non-Zn²⁺ MDRs are more frequent than Zn²⁺-containing MDRs, while the opposite is true for eukaryotic MDRs. Many MDR families have now been characterized, and a clear correlation between Zn²⁺ content and coenzyme preference has been observed. Most Zn²⁺-containing MDR proteins prefer NAD(H) as a cofactor and generally act as dehydrogenases. In contrast, non-Zn²⁺ MDR proteins exhibit a preference for NADP(H) and consequently act as reductases (Edwards *et al.*, 1996).

MDR proteins typically consist of N- and C-terminal domains. The cofactor-binding C-terminal domain adopts the ubiquitous Rossmann fold consisting of an often six-stranded parallel β -sheet sandwiched by α -helices on each side. The substrate-binding N-terminal domain has a core of antiparallel β -strands and surface-positioned α -helices and shows distant homology to the GroES structure (Taneja & Mande, 1999). The active site is a cleft at the interface between the

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two domains containing the nicotinamide moiety of the cofactor (Shimomura *et al.*, 2003; Eklund & Ramaswamy, 2008).

Despite the expansion of the MDR superfamily through completed or ongoing genome-sequencing projects, most of the new members are putative gene products and are poorly characterized. Therefore, biochemical and physiological characterization of the members of the MDR superfamily is a challenge. The curcumin-converting enzyme CurA has recently been purified and characterized from *Escherichia coli* in human faeces. Curcumin, which is derived from rhizomes, is a natural antioxidant and is widely used in pharmacology and therapeutics. The ingested curcumin is transformed into multi-hydrated forms (dihydrated, tetrahydrated, hexahydrated and octahydrated curcumin) by indigenous microorganisms. CurA showed NADPH-dependent dihydrocurcumin/tetrahydrocurcumin-producing activity using curcumin as a substrate and belonged to the non-Zn²⁺ MDRs. Because tetrahydrocurcumin has more potent anti-inflammatory (Mukhopadhyay *et al.*, 1982), antidiabetic, antihyperlipidaemic (Pari & Murugan, 2007) and antioxidant activities (Osawa *et al.*, 1995; Sugiyama *et al.*, 1996) than curcumin, elucidation of the enzyme activity of CurA provided new insights into curcumin metabolism (Hassaninasab *et al.*, 2011) and the biological functions of MDR-superfamily proteins. Although the enzymatic activity of CurA has been verified, the mechanisms of its dual substrate (curcumin and dihydrocurcumin) recognition and C=C bond reduction remain unknown. Structural information provides an invaluable platform for enzyme-mechanism studies. CurA shows a significant match to YncB, which is designated as an NAD(P)-binding oxidoreductase in the database for *E. coli* (Hassaninasab *et al.*, 2011). CurA orthologues also exist in various pathogenic bacteria such as *E. coli*, *Shigella*, *Salmonella*, *Klebsiella* and *Vibrio* strains. As a first step towards the structure determination of curcumin-converting enzymes, we selected the CurA orthologue from *V. vulnificus*, YncB, as a target protein. YncB is a putative NAD(P)-binding oxidoreductase that belongs to the LTD-type MDR superfamily. YncB is a 37.9 kDa protein that contains 343 amino acids and shows 68% sequence identity to CurA. Here, we report the overexpression, purification, crystallization and preliminary X-ray crystallographic analyses of YncB and the YncB–NADP(H) complex.

2. Materials and methods

2.1. Expression and purification of the *yncB* gene product

The *yncB* gene (GenBank accession No. 10168803) was amplified by polymerase chain reaction using *V. vulnificus* MO6-24/O genomic DNA as a template (Park *et al.*, 2011). The gene was inserted downstream of the T7 promoter of the expression plasmid pET-24a (Novagen, Madison, Wisconsin, USA) and the resulting construct expressed residues 2–343 of the YncB protein with an in-frame noncleavable N-terminal His₆ tag (MHHHHHH). After verifying the DNA sequence, the plasmid DNA was transformed into *E. coli* strain Rosetta (DE3) pLys (Stratagene, La Jolla, California, USA). The cells were grown to an OD₆₀₀ of approximately 0.5 in Luria–Bertani medium containing 50 µg ml^{−1} kanamycin (Duchefa) at 310 K and expression was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; Duchefa). After 12 h induction at 295 K, the cells were harvested and resuspended in 20 mM Tris–HCl pH 7.5 (Duchefa) containing 500 mM sodium chloride and 5 mM imidazole. The cells were disrupted by sonication and the cell debris was discarded by centrifugation at 20 000g for 30 min at 277 K. The resulting supernatant was loaded onto a nickel–nitrilotriacetic acid (Ni–NTA) column (Qiagen). The column was washed with a buffer

Table 1

Crystal information, data-collection and phasing statistics.

Values in parentheses are for the highest resolution shell.

	SeMet YncB	YncB–NADP(H)
Beamline	MXII, Australian Synchrotron	MXII, Australian Synchrotron
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 4 ₂ 2 ₁ 2 or <i>P</i> 4 ₃ 2 ₁ 2
Unit-cell parameters (Å)	<i>a</i> = 90.52, <i>b</i> = 91.56, <i>c</i> = 104.79	<i>a</i> = <i>b</i> = 90.14, <i>c</i> = 105.61
Wavelength (Å)	0.9791	0.9537
Resolution (Å)	19.91–1.85 (1.96–1.85)	19.81–1.81 (1.91–1.81)
Completeness (>0σ) (%)	99.4 (96.5)	99.4 (96.1)
<i>R</i> _{merge} † (%)	1.3 (16.2)	1.5 (33.8)
Average <i>I</i> /σ(<i>I</i>)	12.5 (1.6)	13.0 (0.6)
Unique reflections	73918 (10339)	39992 (5529)
Multiplicity	14.2 (11.6)	11.5 (9.1)

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of reflection hkl and $\langle I(hkl) \rangle$ is the mean intensity of replicate measurements.

consisting of 20 mM Tris–HCl pH 7.5, 500 mM NaCl, 30 mM imidazole. YncB was eluted with the same buffer containing 500 mM imidazole. The protein fraction partially purified using the Ni–NTA column was dialysed into 20 mM Tris–HCl pH 7.5, 1 mM dithiothreitol and loaded onto a column packed with 30 ml Q-Sepharose resin (GE Healthcare). This column was then washed with a gradient to 20 mM Tris–HCl pH 7.5, 1.0 M NaCl, 1 mM dithiothreitol (DTT). The eluted fraction containing YncB was concentrated and subsequently loaded onto a Superdex 75 HR 16/60 column (GE Healthcare) pre-equilibrated with a buffer consisting of 20 mM Tris–HCl pH 7.5, 300 mM NaCl, 1 mM DTT (buffer A). The YncB protein eluted at ~35.4 min at a flow rate of 1.5 ml min^{−1}. The purified YncB in buffer A was concentrated to approximately 15 mg ml^{−1} for crystallization. For SeMet labelling of YncB, the *E. coli met*[−] auxotrophic strain B834 (DE3) (Novagen) was used as a host for plasmid transformation. Cells were grown to an OD₆₀₀ of approximately 0.5 in M9 medium containing 50 mg l^{−1} SeMet and 50 µg ml^{−1} kanamycin at 310 K and expression was induced by 1 mM IPTG at 295 K. After 12 h, the cells were harvested. SeMet YncB was purified using the same procedure as used for native YncB. The purified SeMet YncB in buffer A was concentrated to approximately 15 mg ml^{−1} for crystallization.

2.2. Crystallization and X-ray data collection

The YncB protein was crystallized and optimized by the microbatch crystallization method at 295 K. Small drops composed of 1 µl

Figure 1
Crystals of YncB protein.

Figure 2
Sequence alignment of *V. vulnificus* YncB (VvYncB), *E. coli* CurA (EcCurA) and LTB₄ 12-HD/PGR (1V3U).

protein solution and an equal volume of crystallization reagent were pipetted under a layer of a 1:1 mixture of silicone oil and paraffin oil in 96-well Impact plates (Greiner Bio-One) or 72-well HLA plates (Nunc). Screening for crystallization conditions was performed with all available screening kits from Hampton Research, Axygen Biosciences and Emerald BioSystems. Initial crystals (Fig. 1) were grown in a precipitant solution consisting of 0.2 M NaCl, 0.1 M HEPES pH 7.5, 20% (w/v) polyethylene glycol (PEG) 8000, 5% 2-methyl-2,4-pentenediol (MPD) (condition No. E10 of CP-CUSTOM-SGC from Axygen Biosciences). Octahedron-shaped crystals were produced in 3 d. SeMet YncB crystals were produced using the same precipitation solution as used for the native YncB crystals. To form the YncB–NADP(H) complex, 5 mM NADP(H) was added to native YncB and the complex solution was mixed with the same precipitation solution as used for native YncB. The YncB–NADP(H) complex crystals were produced in 3 d.

For data collection, crystals of SeMet YncB and of the YncB–NADP(H) complex were mounted using a 50 µm MicroMount polymer loop (MiTeGen, Ithaca, New York, USA) and cooled to 100 K using a Cryostream cooler (Oxford Cryosystems) after a brief soak in a cryoprotectant solution consisting of 20% MPD, 0.2 M NaCl, 0.1 M MES pH 6.5, 20% (w/v) polyethylene glycol (PEG) 8000. A 1.85 Å resolution data set for SeMet YncB and a 1.81 Å resolution data set for the YncB–NADP(H) complex were collected using an ADSC Quantum 315r CCD on beamline MXII at the Australian Synchrotron, Melbourne, Australia. The exposure time to synchrotron radiation was 1 s. 360 and 180 frames of 1° oscillation were measured for the SeMet YncB and YncB–NADP(H) complex crystals, respectively, with the crystal-to-detector distance set to 280 mm. Diffraction data were processed and scaled using *XDS* and *XSCALE* (Kabsch, 2010). The data-collection statistics are given in Table 1.

3. Results and discussion

The SeMet YncB crystal belonged to the primitive orthorhombic space group *P*₂₁₂₁, with unit-cell parameters *a* = 90.52, *b* = 91.56, *c* = 104.79 Å. The crystal volume per unit molecular weight (*V*_M)

was about 2.86 Å³ Da^{−1}, with a solvent content of 57% by volume (Matthews, 1968), when the asymmetric unit was assumed to contain two molecules. The YncB–NADP(H) complex crystal belonged to the primitive tetragonal space group *P*₄₁₂₁2 or *P*₄₃₂₁2, with unit-cell parameters *a* = *b* = 90.14, *c* = 105.61 Å. The crystal volume per unit molecular weight (*V*_M) was about 2.82 Å³ Da^{−1}, with a solvent content of 56.42% by volume, when the asymmetric unit was assumed to contain one molecule (Table 1). The best-matching three-dimensional structure obtained by sequence-based analysis of the Protein Data Bank (PDB) was that of leukotriene B₄ 12-hydroxydehydrogenase/15-oxo-prostaglandin 13-reductase (LTB₄ 12-HD/PGR) from guinea pig (Berman *et al.*, 2000; Hori *et al.*, 2004), which is a member of the zinc-independent MDR family. The deduced amino-acid sequence shows that YncB has the LTD-specific sequence pattern, D-x-(YF)-x-(DE)-N-V-G-(GS)-x₃-(DEN), from Asp218 to Asp230 (²¹⁸DIYFENVGGKVFD²³⁰) and a glycine-rich motif (¹⁵⁵AATGAVG¹⁶¹) that is shared by many oxidoreductases (AxxGxxG) and is known to participate in the binding of the pyrophosphate group of NAD(P)⁺ or NADPH. However, the putative active site of YncB differs from that common to LTD-family members: ⁴⁷SLDPYMRGRMSDAKSYA⁶³ compared with SCDYM(GR)KEEV-TM(NG)-I(QEK) (Fig. 2; Nordling *et al.*, 2002). Because of the failure of all attempts at molecular replacement with *MOLREP* (Vagin & Teplov, 2010) using the structure of LTB₄ 12-HD/PGR (PDB entry 1v3u; Hori *et al.*, 2004) as a search model, we turned to the SeMet YncB data for structure solution. The three-dimensional structures of YncB and its cofactor/substrate complexes will provide insight into the diversification of the structures and functions of the MDR-superfamily proteins.

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