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Cloning, purification, crystallization and preliminary X-ray analysis of two low-molecular-weight protein tyrosine phosphatases from *Vibrio cholerae*

Low-molecular-weight protein tyrosine phosphatases (LMWPTPs) are small cytoplasmic enzymes of molecular weight ~ 18 kDa that belong to the large family of protein tyrosine phosphatases (PTPs). Despite their wide distribution in both prokaryotes and eukaryotes, their exact biological role in bacterial systems is not yet clear. Two low-molecular-weight protein tyrosine phosphatases (VcLMWPTP-1 and VcLMWPTP-2) from the Gram-negative bacterium *Vibrio cholerae* have been cloned, overexpressed, purified by Ni^{2+} -NTA affinity chromatography followed by gel filtration and used for crystallization. Crystals of VcLMWPTP-1 were grown in the presence of ammonium sulfate and glycerol and diffracted to a resolution of 1.6 Å. VcLMWPTP-2 crystals were grown in PEG 4000 and diffracted to a resolution of 2.7 Å. Analysis of the diffraction data showed that the VcLMWPTP-1 crystals had symmetry consistent with space group $P3_1$ and that the VcLMWPTP-2 crystals had the symmetry of space group $C2$. Assuming the presence of four molecules in the asymmetric unit, the Matthews coefficient for the VcLMWPTP-1 crystals was estimated to be $1.97 \text{ Å}^3 \text{ Da}^{-1}$, corresponding to a solvent content of 37.4%. The corresponding values for the VcLMWPTP-2 crystals, assuming the presence of two molecules in the asymmetric unit, were $2.77 \text{ Å}^3 \text{ Da}^{-1}$ and 55.62%, respectively.

1. Introduction

Protein phosphorylation and dephosphorylation are involved in the regulation of many cellular processes such as cell growth, differentiation and metabolism (DeVinney *et al.*, 2000; Hunter, 1995; Mustelin *et al.*, 2005). The formation and hydrolysis of phosphate esters in proteins play key roles in signal transduction, through which external environmental stimuli are converted into internal cellular responses (Mustelin *et al.*, 2005; Neel & Tonks, 1997). Protein kinases and protein phosphatases are among many enzymes that catalyze such reversible reactions in a highly precise manner to control cellular activities. Defective or incorrect regulation of such systems result in loss of cell viability; thus, these proteins are ideal targets for drug design (Zhang, 2001; Hunter, 2000).

Protein tyrosine phosphatases (PTPs) belong to the protein phosphatase superfamily and catalyze the hydrolysis of phosphate esters on tyrosine residues in proteins. Based on their activity towards different phosphorylated amino acids, PTPs can be divided into two families: one class is exclusively active towards phosphorylated Tyr residues and the other acts on both phosphorylated Ser/Thr and Tyr residues in proteins. Low-molecular-weight PTPs fall into the first category (Ramponi & Stefani, 1997). The members of this family of proteins share very low sequence identities amongst themselves apart from the signature motif in the relatively flexible loop at the active site, CX_4CR , where X can be any amino acid. This loop is responsible for binding and hydrolyzing phosphorylated tyrosine residues and is thus known as the protein tyrosine phosphate-binding loop or P-loop (Zhang *et al.*, 1995).

Low-molecular-weight protein tyrosine phosphatases (LMWPTPs) are widely distributed in prokaryotes and eukaryotes (Kennelly & Potts, 1999; Cozzone *et al.*, 2004) and play important roles in many biological processes. The reaction mechanism of eukaryotic LMWPTPs has been structurally, thermodynamically and kinetically characterized (Ramponi & Stefani, 1997). However, LMWPTPs from bacterial sources have been less explored in terms of their structure and function. *Vibrio cholerae* 0395 contains two LMWPTPs (accession Nos. A5F2Q3 and A5F3O7, hereafter termed VcLMWPTP-1 and VcLMWPTP-2, respectively). They have weak sequence identity ($\sim 30\%$), although their active-site signature motif CXGNXCR(S)P and the DPY loop, which play key roles in the hydrolysis of phosphorylated tyrosine, are conserved. However, the amino-acid residues that are located around the P-loop and the DPY loop differ significantly. It is believed that the residues around the P-loop are responsible for modulating substrate recognition, while the residues around the DPY loop are important in catalyzing the dephosphorylation reaction. Therefore, it seems that the physiological targets of these two LMWPTPs are quite different and that these two enzymes should have different dephosphorylation mechanisms. The primary sequences of these two LMWPTPs, especially VcLMWPTP-2, do not produce significant matches with other LMWPTP structures reported in the PDB, suggesting that the three-dimensional structures of these two PTPs may show distinct features that have not been observed in other LMWPTP structures reported to date. Furthermore, no literature is available on the substrate recognition, catalytic mechanism and kinetic parameters of these two PTPs. Importantly, the physiological substrates of these two LMWPTPs are not known and identification of these will definitely shed light on the functional roles of these PTPs in *V. cholerae*. The three-dimensional structures of these two proteins will also be useful to obtain insights into their catalytic functions at the atomic level. Moreover, knowledge of the structure and function of these phosphatases might be of use in drug design against this bacterial pathogen. Here, we report the cloning, overexpression, purification, crystallization and preliminary structural analysis of VcLMWPTP-1 and VcLMWPTP-2 at resolutions of 1.6 and 2.67 Å, respectively.

2. Materials and methods

2.1. Cloning and expression

The genes encoding VcLMWPTP-1 (155 amino acids) and VcLMWPTP-2 (166 amino acids) (accession Nos. A5F2Q3 and A5F307, respectively) were amplified from *V. cholerae* 0395 genomic DNA using the polymerase chain reaction with the following primers: for VcLMWPTP-1, forward primer 5'-CCAGCATATGCAGAAGGTACTCGTGGTGTGC-3' and reverse primer 5'-CGGGATCCTTAATGCTGGCCTTGCTGTTTATAG-3'; for VcLMWPTP-2, forward primer 5'-GCCGCCATATGAAGGTTAAAGGTTTATCAG-3' and reverse primer 5'-CGGGATCCTTATTGAGATAAA-TTTTCGTTGCACGC-3'. The primers were synthesized (NeuPro-Cell) with adaptor sites (shown in *italics*) and restriction-enzyme (*Nde*I and *Bam*HI) sites (shown in **bold**). Chromosomal DNA of *V. cholerae* strain O395 was used as a template to amplify the regions encoding VcLMWPTP-1 and VcLMWPTP-2. The purified PCR products were cloned into the *Bam*HI and *Nde*I sites of the expression vector pET-28a(+), which adds six consecutive histidines to the N-terminus of the desired protein followed by a thrombin cleavage site. Sequence-verified recombinant DNA was transformed into *Escherichia coli* strain BL21 (DE3) and subsequently selected on kanamycin plates for protein expression. The cells were grown in

Luria–Bertani medium containing 30 $\mu\text{g ml}^{-1}$ kanamycin at 310 K for 1 h at a shaker speed of 175 rev min^{-1} followed by a further 1 h at 289 K and 175 rev min^{-1} . The expression of recombinant protein was induced with 0.1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and continued for 18 h at 289 K at a shaker speed of 100 rev min^{-1} .

Figure 1

The homogeneity of the purified VcLMWPTP-1 and VcLMWPTP-2 proteins was checked by 12% SDS–PAGE. Lane 1, VcLMWPTP-1; lane 2, molecular-mass markers (labelled in kDa); lane 3, VcLMWPTP-2.

(a)

(b)

Figure 2

(a) Crystals of VcLMWPTP-1 grown in the presence of ammonium sulfate pH 5 at 293 K. The maximum dimensions of the crystals were $0.4 \times 0.4 \times 0.3$ mm. (b) Crystals of VcLMWPTP-2 grown at 293 K ($0.4 \times 0.3 \times 0.2$ mm) appeared when 5% PEG 6000, 8% MPD pH 5.0 was used as a precipitant.

2.2. Purification

The cells were harvested by centrifugation at 4000g for 20 min at 277 K. The cell pellet was resuspended in lysis buffer (buffer A; 50 mM HEPES pH 7.0, 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and lysozyme) and the cells were disrupted by sonication. The crude lysate was centrifuged at 12 000g for 40 min at 277 K. The supernatant was loaded onto an Ni²⁺-NTA column previously equilibrated with buffer A and subsequently washed with buffer A containing 5 and 10 mM imidazole. The protein was eluted using a gradient to 150 mM imidazole in buffer A. The 6×His tag was cleaved using restriction-grade thrombin (Novagen) and final purification of the protein from contaminating proteins, thrombin and cleaved 6×His tag was achieved by gel filtration using an S-100 (GE Healthcare) column pre-equilibrated with buffer B (50 mM HEPES pH 7.0, 300 mM NaCl, 0.5 mM DTT). The proteins thus purified were used for crystallization. The homogeneity of the purified protein was determined by SDS-PAGE using 15%(v/v) polyacrylamide gel (Fig. 1). The concentrations of both proteins were determined using the Bradford assay.

2.3. Crystallization of VcLMWPTP-1 and VcLMWPTP-2

For crystallization, thrombin-cleaved VcLMWPTP-1 (in a buffer consisting of 50 mM MOPS pH 7.6, 300 mM NaCl) and VcLMWPTP-2 (in a buffer consisting of 50 mM HEPES pH 7.0, 300 mM NaCl, 0.5 mM DTT) were concentrated to 6 mg ml⁻¹ using an Amicon ultracentrifugation unit (molecular-weight cutoff 10 000). Crystallization was performed by the hanging-drop vapour-diffusion method in 24-well crystallization trays (Hampton Research, Laguna Niguel, California, USA). Grid Screen Ammonium Sulfate, Grid Screen PEG 6000, Crystal Screen and Crystal Screen 2 from Hampton Research (Jancarik & Kim, 1991) were used to explore the initial crystallization conditions. 2 µl protein solution was mixed with 2 µl precipitant solution, inverted over a reservoir containing 600 µl precipitant

Table 1
Data-collection and processing parameters for VcLMWPTP-1 and VcLMWPTP-2 crystals.

Values in parentheses are for the outermost resolution shell.

	VcLMWPTP-1	VcLMWPTP-2
Space group	<i>P</i> 3 ₁	<i>C</i> 2
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 87.47, <i>c</i> = 73.85, α = β = 90.0, γ = 120.0	<i>a</i> = 121.38, <i>b</i> = 45.25, <i>c</i> = 88.56, α = γ = 90, β = 121.08
Resolution (Å)	1.6	2.67
Molecules per asymmetric unit	4	2
Matthews coefficient <i>V</i> _M (Å ³ Da ⁻¹)	2.26	2.77
Solvent content (%)	46	56
Total No. of reflections	158204 (22198)	36313 (4216)
No. of unique reflections	82757 (12178)	10945 (1286)
Mosaicity (°)	0.54	1.33
Completeness (%)	99.2 (99.3)	92.1 (74.9)
<i>R</i> _{merge} [†] (%)	0.047 (0.268)	0.043 (0.183)
⟨ <i>I</i> /σ(<i>I</i>)⟩	12.1 (3.0)	19.6 (6.1)

[†] *R*_{merge} = ∑_{*hkl*} ∑_{*i*} |*I*_{*i*}(*hkl*) − ⟨*I*(*hkl*)⟩| / ∑_{*hkl*} ∑_{*i*} *I*_{*i*}(*hkl*), where *I*_{*i*}(*hkl*) is the observed intensity of the *i*th measurement of reflection *hkl* and ⟨*I*(*hkl*)⟩ is the mean intensity of reflection *hkl* calculated after scaling.

solution and maintained at both 277 and 293 K. VcLMWPTP-1 crystallized in 2.4 M ammonium sulfate, 0.1 M citric acid pH 5.0, 2% glycerol at 277 K (Fig. 2*a*) and VcLMWPTP-2 crystallized in 0.2 M ammonium sulfate, 30%(w/v) PEG 8K at 293 K (Fig. 2*b*).

2.4. Data collection and processing

Crystals of VcLMWPTP-1 and VcLMWPTP-2 were looped out from the crystallization drops using a 20 µm nylon loop and flash-cooled in a stream of nitrogen (Oxford Cryosystems) at 100 K. A diffraction data set was collected on an in-house MAR Research image-plate detector of diameter 345 mm using Cu *K*α radiation generated by a Bruker–Nonius FR591 rotating-anode generator equipped with Osmic MaxFlux confocal optics and operated at 50 kV

Figure 3
(*a*) X-ray diffraction image of a VcLMWPTP-1 crystal; the edge of the detector corresponds to a resolution of 1.6 Å. (*b*) X-ray diffraction image of a VcLMWPTP-2 crystal, which diffracted to a resolution of 2.67 Å.

and 65 mA. X-ray diffraction data were collected to a resolution of 1.6 Å from VcLMWPTP-1 crystals (Fig. 3*a*) and to a resolution of 2.67 Å from VcLMWPTP-2 crystals (Fig. 3*b*). Data were processed and scaled using *iMOSFLM* (Battye *et al.*, 2011). Data-collection and processing statistics are given in Table 1.

3. Results and discussion

Both VcLMWPTPs were successfully purified for crystallization and biochemical assays. They share very low amino-acid sequence identity with other bacterial LMWPTPs of known structure. The sequence identity between VcLMWPTP-1 and VcLMWPTP-2 is also low (30%) and their sequences are distinctly different around the P-loop and the DPY loop, implying that these two PTPs are not just two redundant versions of the same protein and that they probably target different physiological substrates for catalysis. Therefore, we crystallized both of these proteins and collected diffraction data. The VcLMWPTP-1 crystals diffracted to 1.6 Å resolution and produced excellent-quality diffraction data (Table 1) with symmetry consistent with space group *P*3₁. Packing considerations based on the molecular mass of 17 kDa indicated the presence of four molecules in the asymmetric unit, corresponding to a Matthews coefficient V_M (Matthews, 1968) of 2.26 Å³ Da⁻¹ and a solvent content of 46%. The VcLMWPTP-2 crystals only diffracted to a resolution of 2.67 Å with moderate data quality (Table 1) and with *C*2 space-group symmetry. Packing considerations indicated a Matthews coefficient of 2.77 Å³ Da⁻¹, which corresponds to a solvent content of 56% considering two molecules of VcLMWPTP-2 (molecular mass of ~18 kDa) in the asymmetric unit.

A BLAST (Altschul *et al.*, 1990) search for a homologous structure showed that the amino-acid sequence of VcLMWPTP-1 possesses the highest identity (43%) to that of protein tyrosine phosphatase from *Entamoeba histolytica* (PDB entry 3ido; Seattle Structural Genomics

Table 2
Improvement of *Phaser* statistics.

	RFZ	TFZ	PAK	LLG
Molecule 1	3.1	5.0	0	27
Molecule 2	3.5	8.4	1	85
Molecule 3	3.6	10.9	3	159
Molecule 4	3.8	10.3	8	247

Center for Infectious Disease, unpublished work) followed by human low-molecular-weight phosphotyrosyl phosphatase (40% identity; PDB entry 5pnt; Zhang *et al.*, 1998). VcLMWPTP-2 has the highest identity (29%) to protein tyrosine phosphatase from *E. histolytica* (PDB entry 3ido). Although the structure of *E. histolytica* protein tyrosine phosphatase (PDB entry 3ido) showed marginally better identity than human LMWPTP (PDB entry 5pnt), human LMWPTP gave slightly better results during molecular-replacement calculations for VcLMWPTP-1. Before proceeding with molecular-replacement calculations, waters and a long loop preceding the DPY loop were deleted from the coordinates and mismatched residues were truncated to Ala. Using this truncated model, *Phaser* (McCoy *et al.*, 2007) placed four molecules in the asymmetric unit with RFZ = 3.8, TFZ = 10.3 and LLG = 247 (Table 2). This model was then subjected to several cycles of rigid-body refinement, which removed the clashes between the molecules; the orientations of the four molecules in the asymmetric unit after rigid-body refinement are shown in Fig. 4(*a*). An electron-density map calculated using the molecular-replacement solution thus obtained showed continuous electron density, and refinement of the structure is in progress to obtain the correct structure. The packing of the VcLMWPTP-1 molecules in the crystal clearly indicates threefold symmetry and large solvent channels (Fig. 4*b*).

Initial molecular-replacement trials to solve the structure of VcLMWPTP-2 did not produce any clear-cut solution. This might be because of the low sequence identity (29%) of the search models used

Figure 4

(*a*) Arrangement of four VcLMWPTP-1 molecules in the asymmetric unit showing no intermolecular clashes. The four molecules are shown in four different colours. (*b*) Arrangement of the VcLMWPTP-1 molecules in the crystal showing the threefold symmetry and the large solvent channels.

for molecular replacement. The moderate quality of the diffraction data of VcLMWPTP-2 (Table 1) might also be a reason why a clear solution was not obtained, especially when using models with low sequence identity. At present, we are trying to obtain the phases of VcLMWPTP-2 experimentally.

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