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Atomic resolution crystal structure of *Vc*LMWPTP-1 from *Vibrio cholerae* O395: Insights into a novel mode of dimerization in the low molecular weight protein tyrosine phosphatase family ☆

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ABSTRACT

Low molecular weight protein tyrosine phosphatase (LMWPTP) is a group of phosphotyrosine phosphatase ubiquitously found in a wide range of organisms ranging from bacteria to mammals. Dimerization in the LMWPTP family has been reported earlier which follows a common mechanism involving active site residues leading to an enzymatically inactive species. Here we report a novel form of dimerization in a LMWPTP from *Vibrio cholera* 0395 (*Vc*LMWPTP-1). Studies in solution reveal the existence of the dimer in solution while kinetic study depicts the active form of the enzyme. This indicates that the mode of dimerization in *Vc*LMWPTP-1 is different from others where active site residues are not involved in the process. A high resolution (1.45 Å) crystal structure of *Vc*LMWPTP-1 confirms a different mode of dimerization where the active site is catalytically accessible as evident by a tightly bound substrate mimicking ligand, MOPS at the active site pocket. Although being a member of a prokaryotic protein family, *Vc*LMWPTP-1 structure resembles very closely to LMWPTP from a eukaryote, *Entamoeba histolytica*. It also delineates the diverse surface properties around the active site of the enzyme.

1. Introduction

Reversible protein phosphorylation is an important event involved in intracellular signal transduction pathways in response to triggering factors which regulate crucial metabolic activities in cell. The net result of incorporation of phosphate groups in proteins is diverse and this probably acts as a key step in cellular regulation [1]. The overall content of phosphotyrosine in cells is reciprocally controlled by protein tyrosine kinases (PTKs) which specifically phosphorylate tyrosines in proteins [2]. An abundant member of this class of proteins is the low molecular weight protein tyrosine phosphatases (LMWPTPs) ubiquitously found in all organisms ranging from prokaryotes to higher eukaryotes. LMWPTPs belong to class II PTPs which act on tyrosine phosphorylated proteins, low molecular weight aryl phosphates and natural and synthetic acyl phosphates [3].

Several structures of LMWPTP from eukaryotic organism such as human – HCPTPA (PDB: 5PNT) [4], bovine – BPTPA (PDB: 1Z12) [5], mouse – RPTPA (PDB: 2P4U) [6], yeast – LTP1 (PDB: 1D1P) [7] and protozoan parasite *Entamoeba histolytica* – EhPtp (PDB: 3IDO) [8] are available. From the prokaryotic counterpart, structures of LMWPTP from gram-positive eubacteria *Staphylococcus aureus* – PtpA (PDB: 3ROF) [9], gram-negative eubacteria *Thermus thermophilus* – TT1001 (PDB: 2CWD), gram-positive proteobacteria *Bacillus subtilis* – YwlE (PDB: 4ETI) [10], gram-negative proteobacteria *Escherichia coli* – Wzb (PDB: 2WJA) and pathogenic *Mycobacterium tuberculosis* MPtpA (PDB: 1U2Q) [11] are available. It has been reported that the self association of mammalian LMWPTP (viz. *Bos taurus* LMWPTP, BPTP) produces inactive oligomers that are in equilibrium with its active monomers [12]. Among the prokaryotic LMWPTPs weak oligomerization has been found to exist in YwlE from *B. subtilis*, PtpB from the Gram-negative bacterium *Salmonella aureus* and *E. coli* Wzb. However the mode of dimerization is similar in both the cases and takes place through the direct involvements of the active site residues and the tyrosines of the DPY-loop, leading to a catalytically inactive species [13].

Abbreviations: LMWPTP, low molecular weight protein tyrosine phosphatases; FPLC, fast protein liquid chromatography; MOPS, 3-(N-morpholino) propanesulfonic acid.

☆ Protein Data Bank: Coordinates and structure factor files have been deposited with the accession code 4LRQ.

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Here we report a novel mode of dimerization of *Vc*LMWPTP-1, a 17.9 kDa (155 amino acids) protein from *Vibrio cholerae* O395. Fast protein liquid chromatography and glutaraldehyde crosslinking reveal the existence of the dimeric species of the protein in solution. Kinetic studies of *Vc*LMWPTP-1 using para-nitro phenyl phosphate (pNPP) as the substrate depicts that the protein is active, suggesting that the mode of dimerization in *Vc*LMWPTP-1 is different from other LMWPTPs. In an attempt to investigate the molecular mechanism of this form of dimerization, we solved a high resolution crystal structure (1.45 Å) of *Vc*LMWPTP-1 which depicts a mode of dimerization markedly different from those reported for other dimeric LMWPTPs. The extensive dimerization interface area of *Vc*LMWPTP-1 is about double than that of other mode of dimerization and active site residues are not seen to be involved in the oligomerization. The active site of each monomer is totally accessible to the substrate which is evident from the crystal structure where the active site of each monomer is occupied by a tightly bound MOPS molecule in a substrate like manner. Comparison of the *Vc*LMWPTP-1 structure and surface properties with similar structures in the PDB illuminates its structural convergence with LMWPTP from a eukaryote, *E. histolytica*.

2. Materials and methods

2.1. Cloning, expression and purification

*Vc*LMWPTP-1 protein was cloned, over expressed and purified as mentioned earlier [14]. Active site mutant of *Vc*LMWPTP-1, C8S was cloned using two-step PCR method and inserted after the start codon of pET24b(+). The mutant was overexpressed as C-terminal His-tag and was purified in similar way of the wild type.

2.2. Determination of oligomers using FPLC

*Vc*LMWPTP-1 (0.85 mg/ml, 1.75 mg/ml, 2.58 mg/ml and 4.25 mg/ml) in three different 50 mM Tris buffer, each at pH 7.6 containing 150 mM, 300 mM and 500 mM NaCl was fractionated by a Sephacryl S-100 (Amersham Biosciences) column (46 × 1.6 cm) at 0.9 MPa, pre-equilibrated with respective buffers and precalibrated with a protein mixture containing Lysozyme (MW14.3 kDa) Ovalbumin (MW 36.0 kDa) and Bovine serum albumin (MW 66.45 kDa) at room temperature. Fractions were collected at a flow rate of 0.4 ml per minute using an ÄKTAPrime chromatographic system. The elution profile was determined by monitoring the absorbance at 280 nm.

2.3. Crystallization, data collection and structure solution

Crystallization and data collection of *Vc*LMWPTP-1 was reported earlier [14].For phasing the 1.45 Å data of the crystal, the coordinates of *E. histolytica* (PDB: 3IDO) [8] was used for molecular replacement with Phaser [15] in CCP4 [16] with V_m of 2.18 (solvent content 43.63%), final TFZ = 13.3 and LLG = 157. Model building was done with Coot [17] and refinement was carried out with Phenix refine [18] with twin law -k,-h,-l. TLS refinement was performed during the final stages of refinement [19].

2.4. Structural analysis

Average B-factors for each residue were calculated using B average in CCP4 [20]. PISA webserver [21] and PIC server [22] were used for analysis of the structure and oligomeric state. The oligomeric state and other structural of the protein was analyzed using the. Sequence alignment of *Vc*LMWPTP-1 with other lmwptps was done using ClustalO [23,24]. Figures were prepared using Pymol (http://www.pymol.org). The surface electrostatic potential was mapped using Chimera [25] [−10 kT/e (red) to +10 kT/e (blue)].

2.5. Enzyme kinetics

Kinetic parameters were calculated for *Vc*LMWPTP-1 using *p*-nitrophenyl phosphate (pNPP) as the substrate as described [26]. Briefly, pNPP at a concentration range of 1–40 mM was treated with 100 μM *Vc*LMWPTP-1 and C8S mutant and quenched with 1 N NaOH after 10 min. The absorbance of the product, *p*-nitrophenol, thus formed is measured at 405 nm. The amount of *p*-nitrophenol was calculated from the standard curve of *p*-nitrophenol. For standard curve, stock solution of *p*-nitrophenol was diluted with 0.05 N NaOH and the absorbance of the samples was measured at 405 nm. To check the effect of temperature on the enzymatic activity samples were incubated at 5 °C interval in the water-bath prior to check the absorbance.

2.6. Glutaraldehyde crosslinking to capture the dimeric state in solution

For crosslinking assays, 2.3% freshly prepared solution of glutaraldehyde was added to a reaction mixture of 100 μl containing about 50 μg of the protein in 50 mM MOPS, pH 7.6, 300 mM NaCl and the reaction carried out at room temperature (25 °C). Samples were collected at 15 s, 30 s, 1 min and then up to 5 min at an interval of 1 min and quenched by addition of 10 μl of 1 M Tris–HCl, pH 8.0. Cross-linked proteins were analyzed through 15% SDS–PAGE.

3. Results and discussion

3.1. VcLMWPTP-1 forms dimer in solution

*Vc*LMWPTP-1 elutes as a single peak in fast protein liquid chromatography (FPLC) (Fig. 1A) and calculations based upon standard curve shows that this species is the dimeric form of *Vc*LMWPTP-1 (Fig.1B and C). Moreover, presence of the dimeric form is observed irrespective of protein or salt (NaCl) concentration. Further confirmation of the dimeric form was performed through glutaraldehyde crosslinking where a gradual increase in the dimeric band with time could be seen in 15% SDS–PAGE (Fig. 1F).

3.2. Phosphatase activity of VcLMWPTP-1

Enzyme kinetic assays for *Vc*LMWPTP-1 were performed at pH 4.8 and pH 7.6 at 25 °C using *p* nitrophenyl phosphate as a substrate (Materials and Methods). The determined K_m value under these conditions was 2.07 ± 0.2 mM and 2.03 ± 0.4 mM respectively (Fig. 1D), which is consistent with the K_m values reported for other LMWPTPs like PtpA and PtpB (1.2 and 1.5 mM) [27]. This leads us to propose that the respective active site of the dimer is accessible to the substrate. So the dimer formed in case of *Vc*LMWPTP-1 does not involve active site residues as commonly found in previously reported inactive dimeric species of LMWPTPs and pH variation has no effect on its catalytic activity. The enzyme shows optimum activity at 25 °C and the activity decreases with increasing temperature leading to complete loss of activity at 55 °C (Fig. 1E). Mutating the active site Cys8 to Ser (C8S) results in complete loss of enzymatic activity (Fig. 1E inset) as reported for other LMWPTPS [28].

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