Archives of Biochemistry and Biophysics 585 (2015) 32–38

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Crystal structure and tartrate inhibition of *Legionella pneumophila* histidine acid phosphatase

Richa Dhatwalia [a, 1], Harkewal Singh [a, 2], Thomas J. Reilly [b, c], John J. Tanner [a, d, *]

[a] Department of Chemistry, University of Missouri-Columbia, Columbia, MO 65211, USA
[b] Veterinary Medical Diagnostic Laboratory, University of Missouri-Columbia, Columbia, MO 65211, USA
[c] Department of Veterinary Pathobiology, University of Missouri-Columbia, Columbia, MO 65211, USA
[d] Department of Biochemistry, University of Missouri-Columbia, Columbia, MO 65211, USA

ARTICLE INFO

Article history:
Received 8 July 2015
Received in revised form
3 September 2015
Accepted 12 September 2015
Available online 14 September 2015

Keywords:
X-ray crystallography
Histidine acid phosphatase
Histidine phosphatase superfamily
Substrate recognition

ABSTRACT

Histidine acid phosphatases (HAPs) utilize a nucleophilic histidine residue to catalyze the transfer of a phosphoryl group from phosphomonoesters to water. HAPs function as protein phosphatases and pain suppressors in mammals, are essential for *Giardia lamblia* excystation, and contribute to virulence of the category A pathogen *Francisella tularensis*. Herein we report the first crystal structure and steady-state kinetics measurements of the HAP from *Legionella pneumophila* (LpHAP), also known as *Legionella* major acid phosphatase. The structure of LpHAP complexed with the inhibitor L(+)-tartrate was determined at 2.0 Å resolution. Kinetics assays show that L(+)-tartrate is a 50-fold more potent inhibitor of LpHAP than of other HAPs. Electrostatic potential calculations provide insight into the basis for the enhanced tartrate potency: the tartrate pocket of LpHAP is more positive than other HAPs because of the absence of an ion pair partner for the second Arg of the conserved RHGXRXP HAP signature sequence. The structure also reveals that LpHAP has an atypically expansive active site entrance and lacks the nucleotide substrate base clamp found in other HAPs. These features imply that nucleoside monophosphates may not be preferred substrates. Kinetics measurements confirm that AMP is a relatively inefficient *in vitro* substrate of LpHAP.

1. Introduction

The histidine phosphatase superfamily comprises phosphoryl transfer enzymes that share a common catalytic core, featuring a nucleophilic histidine that is phosphorylated during the catalytic cycle [1]. The superfamily has two main branches. Branch 1 includes several functionally diverse enzymes, such as cofactor-dependent phosphoglycerate mutases and a variety of phosphatases. Branch 2 contains mostly phytases and histidine acid phosphatases (HAPs). The latter is the subject of this report.

HAPs catalyze phosphoryl transfer from phosphomonoesters to water optimally at acidic pH. The accepted mechanism begins with attack by the conserved histidine on the substrate P atom forming a phosphohistidine intermediate and liberating the alcohol of the phosphomonoester substrate. A conserved Asp residue facilitates this step of the mechanism by protonating the leaving group. In the second step, hydrolysis of the phosphohistidine produces inorganic phosphate and regenerates the enzyme for another round of catalysis.

Only three HAPs have been characterized biochemically and structurally. Pioneering work by Van Etten and coworkers using mainly human prostatic acid phosphatase (hPAP) identified key active site residues and established the catalytic mechanism [2–6]. Crystal structures of rat PAP (84% identical to hPAP) [7–9] and hPAP [10–12] revealed the fold, domain architecture, and active site structure. More recently, we determined several structures of a bacterial HAP from the category A pathogen *Francisella tularensis* (FtHAP), including the structure of a substrate-trapping mutant of FtHAP complexed with 3′-AMP [13]. In the substrate-trapping

Abbreviations used: HAP, histidine acid phosphatase; LpHAP, *Legionella pneumophila* histidine acid phosphatase; FtHAP, *Francisella tularensis* histidine acid phosphatase; hPAP, human prostatic acid phosphatase; *p*NPP, *p*-nitrophenyl phosphate.

* Corresponding author. Department of Biochemistry, University of Missouri-Columbia, Columbia, MO 65211, USA.
E-mail address: tannerjj@missouri.edu (J.J. Tanner).
[1] Present address: Sigma Aldrich Corporation, 3500 Dekalb St., St. Louis, MO 63118, USA.
[2] Present address: Sigma Aldrich Corporation, 2909 Laclede Ave, St. Louis, MO 63103, USA.

http://dx.doi.org/10.1016/j.abb.2015.09.010
0003-9861/

mutant, the conserved Asp that protonates the leaving group has been mutated to Ala (D261A). The structure of D261A complexed with 3′-AMP (PDB 3IT3) revealed a hydrophobic clamp that binds the nucleotide base of the substrate.

The biological roles of HAPs are diverse and continue to emerge. Early work suggested that the cellular form of hPAP functions as a protein tyrosine phosphatase, with potential substrates including c-ErbB-2 [14] and the epidermal growth factor receptor [15]. More recently, the transmembrane isoform of hPAP has been shown to suppress pain by dephosphorylating extracellular 5′-AMP to adenosine [16,17]. The HAP Api m 3 is the major allergen of honeybee venom [18,19]. In *Giardia lamblia*, dephosphorylation of cyst wall proteins by the lysosomal HAP known as AcPh is required for excystation, the process by which trophozoites emerge from cysts ingested by the host [20]. FtHAP and other acid phosphatases are thought to contribute to the virulence of *F. tularensis*, and a mutant strain of *Francisella* lacking functional genes for FtHAP and three other acid phosphatases showed promising protective capacity as a single-dose live vaccine [21]. Although the *in vivo* substrates of bacterial HAPs are unknown, our studies of FtHAP implicated small molecule phosphomonoesters rather than phosphoproteins as potential substrates [13].

The discovery that hPAP is a pain suppressor has renewed interest in studying HAPs [16,17,22–26]. Studies show that hPAP markedly reduces sensitivity to painful stimuli (antinociception) and is eight times more potent than morphine. The antinociceptive function of hPAP is due to its ability to catalyze the dephosphorylation of 5′-AMP to adenosine, which activates A_1-adenosine receptors in the dorsal spinal cord. These studies have led to the idea of using recombinant HAPs as a treatment for chronic pain, such as injection of enzymes at acupuncture points ("PAPupuncture") [23].

To gain additional molecular information for HAPs, we targeted the HAP from *Legionella pneumophila* (LpHAP) for crystal structure determination. *L. pneumophila* is a Gram-negative, intracellular pathogen of freshwater protozoa and human alveolar macrophages. In the latter context, *L. pneumophila* is the etiologic agent of Legionnaires' disease [27]. Also known as the major acid phosphatase, LpHAP is a 39 kDa enzyme that is secreted in a *pilD*-dependent process [28]. LpHAP shares 29% global sequence identity with hPAP and 39% identity with FtHAP. Herein we report the 2.0 Å resolution structure of LpHAP complexed with the inhibitor L(+)-tartrate, along with measurements of L(+)-tartrate inhibition and kinetic parameters for adenosine monophosphate substrates.

2. Experimental procedures

2.1. Cloning, expression, and purification

The gene for LpHAP (NCBI RefSeq WP_027265797.1) was cloned from genomic DNA into pET-20b using NcoI and XhoI restriction sites. The cloning was performed such that the *pelB* leader peptide of pET20b replaced the natural N-terminal export signal peptide. The expressed protein contains an N-terminal hexahistidine tag.

LpHAP was expressed using a modified autoinduction method [29]. Briefly, the cells were grown in BL21(AI) at 37 °C for ~3h, then 0.2% arabinose was added and the temperature was reduced to 18 °C. The cells were harvested after 28 h and frozen at −80 °C until further use.

The protein was purified using immobilized metal (Ni^{2+}) affinity chromatography and anion exchange chromatography as follows. Frozen cells were thawed and ruptured using sonication. The cell debris was removed by centrifuging the lysate at 16,500 rpm (SS-34 rotor) for 60 min at 4 °C. The clarified supernatant was loaded onto Ni^{2+}-charged HisTrap (GE Healthcare Life Sciences) column equilibrated with 20 mM phosphate and 150 mM NaCl at pH 7.0 (Buffer A). The column was washed with buffer A supplemented with 20 mM imidazole; LpHAP was eluted with buffer A supplemented with 300 mM imidazole. Fractions were pooled and dialyzed against 50 mM Tris, 50 mM NaCl, and 1 mM EDTA at pH 7.0 (Buffer B) and loaded onto a HiTrap Q anion exchange column (GE Healthcare Life Sciences) pre-equilibrated with Buffer B. The protein was eluted with linear 0–1 M NaCl gradient over 25 column volumes. Based on acid phosphatase activity and SDS-PAGE analysis, fractions were pooled and dialyzed against 50 mM Tris, 50 mM NaCl, and 1 mM EDTA at pH 7.5. The dialyzed protein was concentrated to 8 mg/mL using centrifugal devices having a molecular weight cutoff of 10 kDa (Millipore Amicon Ultra). The protein concentration was estimated using the bicinchoninic acid assay (Pierce kit). Typically this procedure produced approximately 2 mg of 99% pure protein per liter of culture.

2.2. Crystallization

Crystallization trials were performed using vapor diffusion in 24-well sitting drop trays at 298 K. The protein stock solution contained LpHAP at 8.0 mg/mL in 50 mM Tris, 50 mM NaCl, and 1 mM EDTA at pH 7.5. Drops were formed by mixing 1.5 μL each of the protein and reservoir solutions. Crystal screening using commercially available kits (Hampton Index, Crystal Screens 1 and 2, and Emerald Wizards 1, 2, and 3) resulted in plate-shaped crystals obtained in 10% (w/v) PEG 8000, 0.1 M imidazole pH 8.0, and 0.2 M calcium acetate. These crystals diffracted weakly to 3.5 Å resolution. The screens were repeated using enzyme that had been incubated with 10 mM of the inhibitor L(+)-tartrate. A 100 mM stock solution of L(+)-tartrate was prepared in the buffer into which the protein had been dialyzed, and 20 μL of this stock solution was added to 180 μL of the 8 mg/mL protein solution. These experiments produced crystals shaped like tetragonal bipyramids using a reservoir solution of 20% (w/v) PEG 3350 and 0.2 M sodium acetate at pH 4.5. The crystals were cryoprotected with 25% (w/v) PEG 3350, 0.2 M sodium acetate pH 4.5, and 25% (v/v) PEG 200. The crystals were picked up with Hampton nylon loops and plunged into liquid N_2.

2.3. X-ray diffraction, data collection, and refinement

Crystals of LpHAP complexed with L(+)-tartrate were analyzed at Advanced Photon Source beamline 24-ID-C using a Quantum 315 detector, where they diffracted to 2.0 Å resolution. The space group is *C*2 with the unit cell dimensions listed in Table 1. The asymmetric unit contains eight HAP protomers arranged as four dimers. Using the method of Matthews, the solvent content is estimated to be 54% with V_m of 2.7 $Å^3$/Da [30]. We note that our crystal form is different from the ones reported recently for LpHAP [31]. The data were processed with HKL [32]. Intensities were converted to amplitudes using the French and Wilson [33] method as implemented in Truncate via CCP4i [34]. Data processing statistics are listed in Table 1.

The structure of LpHAP was solved by molecular replacement using MOLREP [35] with a search model derived from the coordinates of FtHAP (PDB entry 3IT1). The initial solution from MOLREP was used to initiate automated model building in Phenix.Autobuild [36]. The structure was completed via several iterative rounds of modeling building in COOT [37,38] and refinement in PHENIX [39]. The *B*-factor model consisted of an isotropic *B*-factor for each atom and TLS refinement with eight groups (one group per chain). Water molecules were modeled into strong F_o-F_c peaks. The $2F_o-F_c$ density was inspected after refinement, and water molecules with weak or non-spherical $2F_o-F_c$ density were deleted. Non-crystallographic symmetry (NCS) restraints were not used because of the relatively high resolution of the data set (2.0 Å). As a