Structure of Francisella tularensis AcpA

PROTOTYPE OF A UNIQUE SUPERFAMILY OF ACID PHOSPHATASES AND PHOSPHOLIPASES C

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AcpA is a respiratory burst-inhibiting acid phosphatase from the Centers for Disease Control and Prevention Category A bioterrorism agent Francisella tularensis and prototype of a superfAMILY of acid phosphatases and phospholipases C. We report the 1.75-Å resolution crystal structure of AcpA complexed with the inhibitor orthovanadate, which is the first structure of any F. tularensis protein and the first for any member of this superfamily. The core domain is a twisted 8-stranded β-sheet flanked by three α-helices on either side, with the active site located above the carboxyl-terminal edge of the β-sheet. This architecture is unique among acid phosphatases and resembles that of alkaline phosphatase. Unexpectedly, the active site features a serine nucleophile and metal ion with octahedral coordination. Structure-based sequence analysis of the AcpA superfamily predicts that the hydroxyl nucleophile and metal center are also present in AcpA-like phospholipases C. These results imply a phospholipase C catalytic mechanism that is radically different from that of zinc metallophospholipases.

Francisella tularensis is a highly infectious intracellular bacterial pathogen and the cause of tularemia (1). The organism can be isolated from numerous rodent hosts and arthropod vectors, readily grown in broth culture, and mechanically aerosolized. It is one of the most infectious pathogenic agents known, requiring fewer than ten organisms to establish infection (1). Inhalation of aerosolized F. tularensis can result in pneumatic tularemia (2), which has a case fatality rate of up to 30% if untreated (1). The U.S. Centers for Disease Control and Prevention considers F. tularensis to be a Category A bioterrorism agent, which has led to renewed interest in identifying genes and pathways that underlie virulence to facilitate development of new antimicrobial drugs and vaccines (1, 3, 4).

Acid phosphatase A from F. tularensis (AcpA)2 is a highly expressed 57-kDa polyspecific periplasmic acid phosphatase (ACP) (5). AcpA hydrolyzes a variety of substrates, including p-nitrophenylphosphate (pNPP), p-nitrophenylphosphorylcholine (pNPC), peptides containing phosphotyrosine, inositol phosphates, AMP, ATP, fructose 1,6-bisphosphate, glucose and fructose 6-phosphates, NADP+*, and ribose 5-phosphate (5, 6). The enzyme is inhibited by the metal oxynions orthovanadate, molybdate, and tungstate. Based on amino acid sequence analysis, AcpA is distinct from histidine ACPs (7) and purple ACPs (8), as well as class A, B, and C bacterial nonspecific ACPs (9).

Purified AcpA inhibits the respiratory burst of stimulated neutrophils, which suggests that AcpA helps the pathogen elude the host oxidative defense system during the initial stages of macrophage infection (5). Furthermore, proteomics studies have shown that AcpA is expressed at a higher level in virulent F. tularensis strains compared with the nonvirulent vaccine strain (10). Most recently, it has been shown that a mutant strain of F. tularensis subspecies novicida lacking a functional acpA gene is less virulent in mice than the wild-type strain due to a defect in phagosomal escape.3 Thus, AcpA appears to be important for survival of the microbe at two critical junctures of infection: colonization and intracellular survival.

Amino acid sequence alignments show that AcpA belongs to a superfAMILY of bacterial enzymes that includes ACPs and phospholipases C (PLCs) from a variety of microbial pathogens, including Pseudomonas aeruginosa, Mycobacterium tuberculo-
sis, Bordetella pertussis, and several Burkholderia species (11). AcpA is the only characterized enzyme from the ACP branch of the superfamily. PLCs from this superfAMILY are important virulence factors in P. aeruginosa (12) and M. tuberculosis (13) infections, with the hemolytic PLC from P. aeruginosa (PlcH) being the best characterized example from the PLC branch of the superfamily (14). PlcH is particularly interesting, because it

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The atomic coordinates and structure factors (code 2D1G) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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Structure of F. tularensis AcpA

is a multifunctional enzyme that displays sphingomyelin synthase activity in addition to PLC activity (15). PLCs of the AcpA/PlcH superfamily share no sequence homology with the well studied zinc metallophospholipases Clostridium perfringens α-toxin and Bacillus cereus phosphatidylcholine-prefering PLC (16), which suggests that PLCs of the AcpA/PlcH superfamily have a novel, and as yet uncharacterized, catalytic mechanism. To gain insights into the structural basis of the catalytic activity of enzymes of the AcpA/PlcH superfamily, we have determined the crystal structure of AcpA bound to the competitive inhibitor orthovanadate.

EXPERIMENTAL PROCEDURES

Crystallization and X-ray Diffraction Data Collection—Expression of recombinant F. tularensis AcpA in Escherichia coli, protein purification, and the growth of three different crystal forms were described previously (17). Structure determination utilized crystal form III, which was obtained by incubating the enzyme with the competitive inhibitor sodium orthovanadate (Na3VO4, 5 mM) prior to crystallization and using polyethylene glycol 1500 as the precipitating agent (17). These crystals have space group C2221 with unit cell dimensions a = 112.1 Å, b = 144.4 Å, c = 123.9 Å, two molecules per asymmetric unit, and 43% solvent content.

The derivative used for phasing was produced by soaking an AcpA/orthovanadate crystal in 40 mM Sm(C2H3O2)3 for 10 min. Diffraction data extending to 2.4-Å resolution were collected from the Sm derivative at Advanced Photon Source beamline 19-ID using λ = 1.6531 Å, which corresponds to an energy between the L-I and L-II absorption edges of Sm. Data processing was done with HKL2000 (18). Anomalous difference Patterson maps showed several strong features on the u = 0 Harker section.

The data set used for phase extension and refinement calculations at 1.75-Å resolution was collected from an AcpA/orthovanadate crystal at Advanced Light Source beamline 8.3.1. A second data set, which was used for anomalous difference Fourier analysis of the active site metal center, was collected from another AcpA/orthovanadate crystal at beamline 8.3.1. This data set was collected at low energy (λ = 1.74 Å) to enhance the anomalous signal of the metal ion. Both data sets were processed with HKL2000 (18). See Table 1 for a summary of data processing statistics.

Phasing and Refinement Calculations—The structure was solved using single wavelength anomalous diffraction phasing. SnB (19) was used to identify a 10-atom anomalous constellation for the Sm derivative, which was input to SHARP (20) for single wavelength anomalous diffraction phase calculations and solvent flattening. The resulting SHARP phases had a figure of merit of 0.84 for reflections to 2.4-Å resolution. An electron density map calculated from the SHARP phases clearly

### Table 1

Data collection and refinement statistics

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<tr>
<th></th>
<th>Orthovanadate (high energy)</th>
<th>Orthovanadate (low energy)</th>
<th>Sm derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (Å)</td>
<td>1.1271</td>
<td>1.740</td>
<td>1.6531</td>
</tr>
<tr>
<td>Space group</td>
<td>C222</td>
<td>C222</td>
<td>C222</td>
</tr>
<tr>
<td>Unit cell dimensions (Å)</td>
<td>a = 112.1, b = 144.4, c = 123.9</td>
<td>a = 111.2, b = 142.6, c = 125.6</td>
<td>a = 112.2, b = 144.2, c = 123.9</td>
</tr>
<tr>
<td>Diffraction resolution (Å)</td>
<td>50-1.75 (1.81-1.75)</td>
<td>50-2.20 (2.28-2.20)</td>
<td>50-2.40 (2.48-2.40)</td>
</tr>
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<td>No. of observations</td>
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<td>295,644</td>
<td>572,236</td>
</tr>
<tr>
<td>No. of unique reflections</td>
<td>98,137</td>
<td>51,195</td>
<td>39,618</td>
</tr>
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<td>5.8 (4.9)</td>
<td>14.4 (13.4)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>97.2 (94.9)</td>
<td>99.9 (99.9)</td>
<td>99.9 (99.9)</td>
</tr>
<tr>
<td>Average I/σ</td>
<td>26.6 (2.2)</td>
<td>16.8 (3.4)</td>
<td>38.6 (23.8)</td>
</tr>
<tr>
<td>Rsym (I)</td>
<td>0.047 (0.357)</td>
<td>0.098 (0.443)</td>
<td>0.065 (0.118)</td>
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<tr>
<td>No. of non-hydrogen atoms</td>
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<tr>
<td>No. of residues in chain A</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>No. of residues in chain B</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>550</td>
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<tr>
<td>Rcryst</td>
<td>0.198 (0.244)</td>
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<tr>
<td>Rfree</td>
<td>0.231 (0.291)</td>
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</tr>
</tbody>
</table>

a A 5% random test set.

b Compared to the Engh and Huber force field (50).

TheRamachandran plot was generated with RAMPAGE (51).
showed features resembling protein secondary structural elements. A partial backbone tracing consisting of a few α-helices and β-strands was obtained with the automated model building program MAID (21). The MAID tracing was used to determine the noncrystallographic symmetry transformation relating the two protein molecules in the asymmetric unit. In preparation for noncrystallographic symmetry averaging, the programs MAMA (22) and CNS (23) were used to create a mask that covered one of the molecules in the asymmetric unit. The SHARP phases were then improved and extended to 1.75-Å resolution with 2-fold noncrystallographic symmetry averaging and solvent flipping in CNS. The 1.75-Å resolution density-modified phases were input to ARP/wARP (24) for automated electron density map interpretation. The best model from ARP/wARP included the backbone for 97% of the expected residues in the asymmetric unit and 83% of the expected side chains. The model was improved with several rounds of model building in COOT (25) followed by refinement with REFMAC5 (26). Refinement statistics are listed in Table 1.

The asymmetric unit includes 953 amino acid residues belonging to two AcpA molecules (chains labeled A and B). The following sections of the polypeptide chains are disordered: A1–A4, A15–A18, A490–A498, B1–B5, B12–B18, B129–B137, and B490–B494. The root mean square difference between chains A and B is 0.27 Å for Cα atoms and 0.55 Å for all atoms, which indicates that the two chains have nearly identical conformations. Each AcpA molecule contains one orthovanadate ion (HVO₄²⁻) and one metal ion bound in the active site. The metal ion was modeled as Ca²⁺ for purposes of crystallographic refinement but appears with atom name X1 and residue name UNK in the coordinate file deposited in the Protein Data Bank (PDB (27)) to indicate that the identity of the metal is unknown at this time. The solvent structure includes 550 ordered water molecules and 4 bound polyethylene glycol fragments. There is also a decavanadate ion (V₁₀O₂₈⁻) bound in a crystal contact region, where it interacts with the carboxyl-terminal histidine affinity tag of one of the AcpA molecules. Coordinates and structure factor amplitudes have been deposited in the PDB under accession code 2D1G.

**Site-directed Mutagenesis and Activity Assays**—AcpA mutant Ser-175 → Ala was generated using the QuikChange mutagenesis kit (Stratagene), and the mutation was verified by DNA sequencing. The mutant enzyme was expressed and purified using methods employed for AcpA (17). SDS-PAGE analysis showed that Ser-175 → Ala had the expected molecular weight, and Western blots using rabbit anti-AcpA polyclonal and anti-His tag antibodies were positive. Enzymatic activities of AcpA and Ser-175 → Ala were measured using a discontinuous colorimetric assay with pNPP and pNPPC as substrates (6).

**RESULTS**

**Overall Structure of AcpA**—The structure of AcpA comprises three domains and has approximate dimensions of 60 Å × 48 Å × 66 Å. The core domain is a highly twisted, 8-stranded β-sheet flanked by three α-helices on either side (Fig. 1A). The strand order of the β-sheet is 12, 2, 11, 10, 1, 9, 3, then 8, with all but strand 11 in parallel (Fig. 2). There are two smaller domains located above the carboxyl-terminal edge of the 8-stranded β-sheet. One of these small domains consists of residues 47–147 and features four short α-helices (labeled A–D) connected by rather long loops (Fig. 1, blue domain). This domain has a disulfide bond linking Cys-102 and Cys-138 (Figs. 1A and 2).
Structure of F. tularensis AcpA

2). As discussed below, this domain forms part of the dimer interface. The other small domain (residues 258–283) consists of a pair of 2-stranded anti-parallel β-sheets (β4–β7), which resembles a flap (Fig. 1, orange domain), and there is a disulfide bond that links Cys-269 of this domain to Cys-216 of the β-sheet core domain.

Purified AcpA forms an apparent dimer according to analytical ultracentrifugation and gel-filtration chromatography data (6). The two proteins chosen for the asymmetric unit (Fig. 3A) form the largest intermolecular surface between any two proteins in the crystal lattice, based on analysis with PISA (28). This interface buries 2398 Å² of surface area, whereas the next largest interface buries only 932 Å² of surface area. Furthermore, this interface had the highest possible PISA complexation significance score (1.0), compared with 0 for all other possible interfaces. It is concluded that the pair of protein molecules in the asymmetric unit represents the AcpA dimer in solution.

The small helical domain (residues 47–147) and the β1 face of the β-sheet core domain form the dimer interface (Fig. 3A). Secondary structural elements involved in dimerization include αB and its adjacent loops (residues 73–87), the loop following αC (residues 116–119), a 10-residue section of the loop connecting β10 and β11 (394–404), β12 and its adjacent loops (residues 425–433), and residues in a loop near the carboxyl terminus (residues 459–466). Together, these residues form a flat surface (Fig. 3B) that spans 40 Å in one direction and 30 Å in the other.

The dimer interface is highly hydrophilic, and hydrogen bonding appears to play a major role in dimer stability. There are 14 direct intersubunit hydrogen bonds (Table 2) but no ion pairs. Hydrogen-bonding side chains in the interface include Asn-74, Thr-79, Gln-81, Asn-116, Gln-401, Asp-404, and Tyr-428. Note that the intersubunit hydrogen bonds display 2-fold symmetry (Table 2). In addition, there are 16 interfacial water molecules that mediate 20 intersubunit hydrogen bonds (Fig. 3C). As with the intersubunit hydrogen bonds, the 16

FIGURE 2. Secondary structure topology diagram of AcpA. α-Helices are shown as rectangles labeled A–J, and β-strands are shown as arrows numbered 1–12. Cys residues are represented in yellow with disulfides bridges shown as dashed lines. The green boxes denote active site residues, with red numbering for residues coordinating to the bound metal and blue numbering for residues interacting with the vanadate inhibitor.

FIGURE 3. Dimeric structure of AcpA. A, the AcpA dimer is drawn in ribbon representation and covered with a semi-transparent molecular surface. The surfaces are colored blue for one subunit and yellow for the other subunit. The orientation of the yellow subunit is nearly identical to that of Fig. 1A. For each subunit, the coloring scheme of the ribbon is the same as that used in Fig. 1. The red spheres represent interfacial water molecules. The arrow denotes the non-crystallographic molecular 2-fold symmetry axis. B, this is the same as panel A except that the yellow subunit has been removed to show the flatness of the dimer interface. C, this is the same as panel A except that the blue subunit has been removed and the yellow subunit has been rotated 90° so that the interfacial surface points toward the viewer. Note the 2-fold symmetry in the constellation of interfacial water molecules.
bridging water molecules obey the 2-fold symmetry of the dimer (Fig. 3C). Although hydrogen bonding is prominent in the interface, a few nonpolar residues contribute significant surface area to the interface. For example, Leu-82 packs against Leu-119, whereas Leu-433 from one subunit packs against Leu-433 of the opposite subunit at the centroid of the dimer.

**Active Site Architecture and Implications for Catalytic Mechanism**—The location of the active site was clearly indicated by a strong electron density feature corresponding to the bound orthovanadate inhibitor (Fig. 4A). The active site is located above the carboxyl-terminal edge of the 8-stranded $\beta$-sheet near $\beta1$ and $\alpha F$ (Fig. 1A). The inhibitor binds in one end of a 12-Å long trough, which is located in a broad, shallow depression formed by residues from all three domains (Fig. 1B). Note that four water molecules are bound in the trough (Fig. 1B). The shape of the trough suggests that it may be involved in binding the leaving group of the substrate. This idea was tested by modeling $p$NPP in the active site. We found that the nitrophenyl group of $p$NPP fits edgewise into the trough (water removed) without causing steric clash.

Surprisingly, there is a metal ion bound in the active site, based on the observation of a very strong electron density feature that could not be assigned to the protein, inhibitor, or solvent (Fig. 4A). Four lines of evidence suggest that this feature represents a metal ion. First, it is surrounded by an octahedral array of six oxygen ligands (Fig. 4, A and B): Glu-43, Asn-44, Ser-175, Asp-386, Asp-387, and the vanadate inhibitor. Three of the six coordinating ligands are carboxyl groups, which is suggestive of a bound metal ion with a charge of at least +2.

**TABLE 2**

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<th>Hydrogen bond partners</th>
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<th>Chain A</th>
<th>Chain B</th>
<th>Chain A</th>
<th>Chain B</th>
<th>Chain A</th>
<th>Chain B</th>
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<td></td>
<td>Asp-404 (O$^3$)</td>
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<td>Asp-404 (O$^3$)</td>
<td>3.0</td>
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<td>Asp-404 (O$^3$)</td>
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<td>Asn-74 (N$^2$)</td>
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<td>Val-429 (O)</td>
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**FIGURE 4.** Active site of AcpA. A, stereographic drawing of the AcpA metal center covered by two electron density maps. The cyan cage represents a simulated annealing $\alpha$-weighted $mF_o - DF_c$ electron density map (3 $\sigma$). The metal ion, orthovanadate, and surrounding residues within 3.9 Å were omitted prior to simulated annealing refinement and map calculation. The red cage represents an anomalous difference Fourier map (3.5 $\sigma$) calculated with phases from the final model and anomalous differences from the low energy orthovanadate data set. The orthovanadate inhibitor is shown in magenta/red, and the metal ion is colored yellow. Protein residues appear in white. B, stereographic drawing of the AcpA active site highlighting protein-inhibitor electrostatic interactions (dashed lines). The orthovanadate inhibitor is shown in magenta/red, and the metal ion is colored yellow. Protein residues appear in white. C, schematic diagram of the active site.
Structure of *F. tularensis* AcpA

Electron density maps were analyzed to gain insights into the elemental identity of the bound metal ion. The anomalous difference Fourier peak corresponding to the metal ion was much stronger than that of the vanadate (Fig. 4A, red cage), which implies that the metal ion is a stronger anomalous scatterer than the V atom of the inhibitor. This result is consistent with the active site metal ion being a first row transition metal. Also, several simulated annealing refinements were performed against the 1.75-Å data set with different metal ions modeled in the active site. The resulting difference electron density maps (σ_A-weighted mFo - DFo) suggested that the metal ion has at least the number of electrons of Ca^{2+} and is more likely a first row transition metal ion. Therefore, the metal ion was conservatively modeled as Ca^{2+} in the current structure pending further biochemical and analytical studies of the metal content of AcpA.

The orthovanadate inhibitor exhibits distorted trigonal bipyramidal geometry and is bound by six side chains and the metal ion (Fig. 4, B and C). The inhibitor axial oxygen atom interacts with His-106 and His-350, whereas the equatorial oxygen atoms bind to Asn-44, His-287, His-288, Asp-208, His-350, and the metal ion. Asp-208 appears to share a proton with the inhibitor.

The location of Ser-175 relative to the inhibitor and metal ion suggests that it plays the role of nucleophile that attacks the substrate P atom. The hydroxyl oxygen atom of Ser-175 is 1.8 Å from the metal ion and 2.2 Å from the inhibitor V atom (Fig. 4C). Ser-175 appears to be in an ideal location for backside nucleophilic attack at the substrate P atom. Thus, the active site structure strongly suggests that Ser-175 is the enzyme nucleophile, and the role of the metal ion is to activate Ser-175 for nucleophilic attack (Fig. 5A, step 2). This hypothesis implies formation of a covalent Ser-175-phosphoryl intermediate during catalysis (Fig. 5A, step 2).

We engineered the Ser-175 → Ala mutant to test the importance of this residue for catalysis. The mutant exhibited no detectable activity using either pNPP or pNPPC as the substrate even at enzyme concentrations >0.01 mM and substrate concentrations up to 20 mM. Thus, Ser-175 plays an essential role in catalysis, which is consistent with our hypothesis that it is the enzyme nucleophile.

Hydrolysis of the Ser-175-phosphoryl intermediate (Fig. 5A, step 3) presumably requires a general base to activate a water molecule. Residues that bind the inhibitor and that are located on the solvent side of the active site are possible candidates for this role. Asp-208 is, perhaps, the most likely candidate, because aspartic acid residues serve as the general base in other phosphatases, such as protein tyrosine phosphatase (30), and the carboxyl of Asp-208 forms a hydrogen bond (2.8 Å) with a water molecule (Wat-285) in our structure.

**Comparison to Other Protein Structures**—To understand the relationship of AcpA to other phosphatases, we searched the PDB for structural homologs of AcpA using the program DALI (31). Surprisingly, the closest homolog was not a phosphatase but was human arylsulfatase A (ASA, PDB code 1AUK, DALI Z-score = 16), followed by phosphoglycerate mutase (PDB code 1EJJ, Z = 15), phosphonoacetate hydrolase (PDB code 1EI6, Z = 10), and *E. coli* alkaline phosphatase (AlkP, PDB code.
His-288 overlap nearly perfectly with the analogous to His-125 and His-229, respectively, of ASA (Fig. 6). For example, His-287 and His-350 of AcpA are structurally similar to the nucleophilic oxygen atom, and the inhibitor/phosphoryl. Recognition with three carboxyl groups, an asparagine side chain, the metal ion and AlkP ZnII occupy analogous locations in their respective structures (Fig. 6B). In both enzymes, the metal ion has octahedral coordination with three carboxyl groups, an asparagine side chain, the nucleophilic oxygen atom, and the inhibitor/phosphoryl. Recognition of the substrate phosphoryl is also similar in the two enzymes. For example, His-287 and His-350 of AcpA are structurally analogous to His-125 and His-229, respectively, of ASA (Fig. 6B). Moreover, imidazole nitrogen atoms of AcpA His-106 and His-288 overlap nearly perfectly with the e-amino groups of ASA Lys-302 and Lys-123 (Fig. 6B). The only major difference between the two active sites, besides the nucleophile, appears to be Asp-208 of AcpA, which is replaced by Val-91 in ASA.

Like AcpA, AlkP has a Ser nucleophile (34), but AlkP binds three metal ions: two Zn$^{2+}$ (ZnI and ZnII) and Mg$^{2+}$. The AcpA metal ion and AlkP ZnII occupy analogous locations in their respective structures, although ZnII has tetrahedral coordination and the AcpA metal ion has octahedral coordination (Fig. 6C). Despite the difference in coordination geometry, Glu-43 and Asp-386 of AcpA are analogous to Asp-51 and Asp-369 of AlkP. There are also similarities between the two enzymes in terms of binding the substrate phosphoryl group. For example, AcpA His-106 is analogous to AlkP His-412, whereas the side chains of AcpA His-287 and AlkP Arg-166 occupy similar locations in their respective structures (Fig. 6C). One notable difference between the two enzymes is that AlkP does not have an acidic residue equivalent to AcpA Asp-208.

Conservation of Active Site Residues in the AcpA/PlcH Superfamily—Analysis of available sequence databases using BLAST (35) shows that close homologs of AcpA are present in other bacteria, including Burkholderia mallei, Corynebacterium jeikeium, and Bradyrhizobium japonicum. These proteins have 520–648 residues and share 38–46% global amino acid sequence identity with AcpA. The 10 residues that contact vanadate or the metal ion in AcpA are identically conserved in these proteins: Glu-43, Asn-44, His-106, Ser-175, Asp-208, His-287, His-288, His-350, Asp-386, and Asp-387. The only major differences between the two enzymes, besides the nucleophile, appear to be Asp-208 of AcpA, which is replaced by Val-91 in ASA.

Like AcpA, AlkP has a Ser nucleophile (34), but AlkP binds three metal ions: two Zn$^{2+}$ (ZnI and ZnII) and Mg$^{2+}$. The AcpA metal ion and AlkP ZnII occupy analogous locations in their respective structures, although ZnII has tetrahedral coordination and the AcpA metal ion has octahedral coordination (Fig. 6C).
The AcpA structure also provides insights into the active site architectures of enzymes from the PLC branch of the AcpA/PlcH superfamily. PlcH has 730 residues, and the amino-termina
two-thirds of the enzyme shares 23% amino acid sequence identity with AcpA. The AcpA structure, however, suggests that
the sequence homology between AcpA and PlcH is much stronger within the active site. For example, five of the ten
AcpA active site residues (Glu-43, Asn-44, His-106, His-350, and Asp-386) are identically conserved in PlcH (Fig. 7).
Moreover, PlcH residue Thr-178 aligns with AcpA nucleophile Ser-175, and PlcH residue Glu-358 aligns with AcpA metal-binding
residue Asp-387 (Fig. 7). In addition, ion pair residues Asp-393 and Arg-414 of AcpA are also present in the PlcH sequence
(Asp-364 and Arg-401). All nine of these conserved residues are also present in close homologs of PlcH (Fig. 7, lower nine pro-
tein sequences). Thus, enzymes in the PLC branch of the AcpA/PlcH superfamily likely retain the essential hydroxyl nucleo-
phile and octahedral metal-binding site of AcpA.

DISCUSSION

The AcpA Family of Phosphatases—The structure reported here shows that AcpA from F. tularensis is distinct from other
ACP s in terms of overall fold and active site architecture and that AcpA shares a common α/β core with enzymes in the AlkP
superfamily. A major result from our work is the discovery that AcpA is a Ser-based metallophosphatase. This result was unex-
pected because AcpA had been predicted to be a Cys-based phosphatase based on a putative catalytic motif in residues
216–224 (CX3KSG). Furthermore, there were no reports in the literature showing that metal ion is required for activity. The
AcpA structure thus provides the framework for experiments that will establish new paradigms for the AcpA family of ACPs.

The structural similarity between AcpA and AlkP enzymes sheds new light on the catalytic mechanism of AcpA. For example,
the mechanism of AlkP involves two consecutive in-line nucleo-
philic attacks at the phosphorous, which results in retention of the phosphorus at the P center (36–39). Zn11 activates Ser-102 for the
first nucleophilic attack, and Zn1 activates a water molecule for the second attack. We propose that AcpA follows an AlkP-like
mechanism with AcpA Ser-175 serving as nucleophile, the AcpA metal ion playing the role of AlkP Zn11, and a protein side chain, possibly Asp-208, substituting for AlkP Zn11.

Interestingly, although the active site structures of ASA and AcpA are very similar (Fig. 6B), it is unlikely that they share a
common catalytic mechanism. The ASA mechanism proceeds through a gem-diol intermediate formed by reaction of water with
FGly-69 (Fig. 5B, step 1). One of the hydroxyl groups of the gem-
diol is activated for nucleophilic attack at the substrate S atom (Fig. 5B, step 2). Proton transfer from the other hydroxyl to His-125
facilitates release of the product sulfate (Fig. 5B, step 3). Because the nucleophilic oxygen atom leaves with the product, the reaction
occurs with overall inversion of configuration of the sulfate (33, 40). Formation of a gem-diol intermediate from AcpA Ser-175 is
chemically unfavorable. Thus it appears that AcpA and ASA have quite different catalytic mechanisms despite having nearly identi-
metal ion-binding sites, similar constellations of substrate-
bindings and, a common protein fold.

Catalytic Mechanism of AcpA-like PLCS—The AcpA struc-
ture also provides new insights into the catalytic mechanism of
PLCs of the AcpA/PlcH superfamily. As discussed above, structure-
base sequence analysis suggests that these PLCS have a hydroxyl nucleophile (e.g. Thr-178 in PlcH) coupled to an octa-
heiral metal center in the active site. Involvement of a threo-
nine nucleophile implies a double-displacement catalytic
mechanism for PlcH and related PLCs in which a covalent intermediate is formed between the nucleophilic threonine
and the phosphoryl head group of the substrate. This predicted mechanism is radically different from that of zinc metallophos-
pholipases C. perfringens α-toxin and B. cereus phosphatidyl-
choline-prefering PLC, which utilize a single nucleophilic
attack on the phosphodiester substrate by an activated water
molecule without formation of a covalent intermediate (41). The AcpA structure provides a basis for designing experiments to
test this proposed mechanism.

Role of AcpA in Virulence of F. tularensis—F. tularensis is a faculta-
тивыe intracellular pathogen whose primary target of infection is the macrophage (1). An essential aspect of virulence is the ability of F. tularensis to escape phagosomal containment,
which leads to over 1000-fold replication of the pathogen in the cytoplasm and eventual apoptosis of the infected macrophage.
Several proteins are thought to contribute to intramacrophage growth and survival of F. tularensis, including putative trans-
criptional regulators MglA and MglB (42, 43), phosphatases
such as AcpA, and proteins with unknown functions IglC (44,
45) and ET0918 (46).

A current challenge is to understand the role of AcpA in intramacrophage survival. Our structure-based sequence analy-
ysis shows that essential elements of the AcpA active site are
shared by PlcH-like PLCS, in particular, the hydroxyl nucleo-
philic and mononuclear metal center. This structural similarity
raises the possibility that AcpA exhibits PLC activity and suggests
a new hypothesis that AcpA facilitates phagosomal escape
by hydrolyzing phospholipids of the phagosomal inner mem-
brane. Interestingly, AcpA efficiently hydrolyzes the phos-
pholipid-like substrate pNPPC (6). However, this is not necessarily
an accurate indicator of bona fide PLC activity, because pNPPC
lacks a hydrocarbon tail. We note that purified AcpA does not
exhibit lecithinase activity on egg yolk agar, nor does it lyse red
blood cells (data not shown). Thus, additional studies are
needed to determine whether AcpA exhibits true PLC activity.
Structure of F. tularensis AcpA

A second hypothesis about the role of AcpA in virulence is that AcpA might affect host signaling pathways by dephosphorylation of host proteins, inositol phosphates, or phosphoinositides, the latter being critically important for phagosome formation (47) and respiratory burst activation (48). The wide and relatively flat surface surrounding the active site (Fig. 1B) is compatible with AcpA docking to a protein substrate.

Finally, it is possible that AcpA functions in a phosphate retrieval system that is activated upon phagosomal containment. AcpA would be an effective phosphate scavenger because of its broad substrate specificity and high abundance. Central to all three hypotheses is the question of whether expression of AcpA is controlled by MglA/B, as has been suggested by Baron and Nano (42). The AcpA structure provides a framework for exploring these hypotheses and for designing AcpA and PchI inhibitors.

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