Characterization and Sequencing of a Respiratory Burst-inhibiting Acid Phosphatase from Francisella tularensis

(Received for publication, October 16, 1995, and in revised form, January 16, 1996)

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Acid phosphatases (Acp) of intracellular pathogens have recently been implicated as virulence factors that enhance intracellular survival through suppression of the respiratory burst. We describe here the identification, purification, characterization, and sequencing of a novel burst-inhibiting acid phosphatase from the facultative intracellular bacterium, Francisella tularensis. Similar to other the burst-inhibiting Acps, F. tularensis Acp (AcpA) is tartrate-resistant and has broad substrate specificity. The AcpA enzyme is unique, however, in that it is easily released from the bacterial cell in soluble form, is a basic enzyme, suppresses the respiratory burst of not only fMet-Leu-Phe but also phorbol 12-myristate 13-acetate-stimulated neutrophils and does not fit into any of the three currently recognized classes of acid phosphatase. We also report the complete nucleotide sequence of the gene acpA, encoding AcpA, and the deduced primary structure of its encoded polypeptide. Comparative sequence analyses of AcpA is discussed. To our knowledge, this is the first report describing the cloning and sequencing of a burst-inhibiting acid phosphatase.

Acid phosphatases (EC 3.1.3.2) are a ubiquitous class of enzymes that catalyze the hydrolysis of phosphomonoesters at an acidic pH. In addition to mobilization of phosphate, some members of this class of enzymes perform many essential biological functions including regulation of metabolism, energy conversion, and signal transduction. These enzymes have been identified and characterized from many eukaryotic and prokaryotic sources and comprise several distinct subgroups based on substrate specificity, molecular weight, and sensitivity to known inhibitors.

In the past decade, a new emphasis has been placed on understanding the role acid phosphatases may play in microbial pathogenesis. Comprehensive studies of acid phosphatases purified from Leishmania donovani (1) and Legionella micdade (2) suggest that members of a class of tartrate-resistant, nonspecific acid phosphatases (TRAPs) may play a crucial role in the survival of intracellular pathogens within a host’s phagocytic cells. An exciting discovery in these studies was that TRAPs purified from these organisms suppressed the respiratory burst of activated human neutrophils (3, 4). Although information is now becoming available about some of the enzymatic, biochemical, and biophysical properties of the burst-inhibiting TRAPs, unequivocal proof of the role of these enzymes as virulence factors in vivo has yet to be obtained. Progress toward this goal is currently limited by the lack of protein or gene sequence information and the absence of isogenic TRAP mutants.

Francisella tularensis is the etiologic agent of the potentially fatal human disease tularemia and is capable of survival and multiplication within a host’s professional phagocytes as well as nonphagocytic cells (5, 6). Although many studies have been conducted into the host’s immune response to Francisella infection, until recently relatively little attention has been focused on biochemical characterization of purified macromolecules which may function as virulence factors in these organisms (7). In initial studies, we found a particular strain of F. tularensis (ATCC 6223, B38) to be enriched in acid phosphatase activity. The Acp specific activity in this strain was greater than previously reported for any other bacterial or protozoan organism. It was also easily solubilized in the absence of detergents allowing relatively large amounts of enzyme to be purified to apparent homogeneity. We describe here the identification, purification, and characterization of some of the unique properties of this burst-inhibiting acid phosphatase (AcpA) as well as its complete primary structure derived from cloning and nucleotide sequencing of the AcpA gene (acpa).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Materials—F. tularensis strains (ATCC 6223 and 29684) were purchased from American Type Culture Collection (Rockville, MD), and strain NBDR 101 LVS was obtained from The National Drug Company (Philadelphia, PA). Francisella novicida was purchased from the ATCC (15482). Strains of Mycobacteria were provided by Dr. John Urbance (University of Illinois, Urbana, IL). Bacteriological media including Bacto Cystine Heart agar (CHA) and IsoVitalex were obtained from Difco (Detroit, MI). All other chemicals, unless stated otherwise, were purchased from Sigma and were of the highest purity available. Chromatography resins were purified

1 The abbreviations used are: TRAP, L-(+)-tartrate-resistant acid phosphatase; acpA, acid phosphatase encoding gene; BSA, bovine serum albumin; CHA, Cystine Heart agar; fMLP, N-formyl-methionyl

N-leucyl-phenylalanine; AcpA, Francisella tularensis acid phosphatase; HPLC, high pressure liquid chromatography; MES, 2 (N-morpholino)ethanesulfonic acid; MUP, 4-methylumbelliferylphosphate; PMA, phorbol 12-myristate 13-acetate; PAG, polyacrylamide gel electrophoresis; PLC, phospholipase C; FPLC, fast protein liquid chromatography; IP3, inositol 1,4,5-trisphosphate; PTPase, peptide tyrosine phosphatase; PIP, phosphatidylinositol phosphate; CHAPS, 3-(3-cholamidopropyldimethylammonio)-1-propanesulfonic acid.

§ Supported in part by a United States Department of Agricultural Sciences National Needs Graduate Fellowship Program Grant 87-GRAD-9-0088. This work is in partial fulfillment for the degree Doctor of Philosophy in the Dept. of Pathobiology, College of Veterinary Medicine, University of Illinois, Urbana, IL. Bacteriological media including Bacto Cystine Heart agar (CHA) and IsoVitalex were obtained from Difco (Detroit, MI). All other chemicals, unless stated otherwise, were purchased from Sigma and were of the highest purity available. Chromatography resins were purified.
chased from Pharmacia Biotech Inc. Protein electrophoresis reagents and amylomaltase were obtained from Bio-Rad Laboratories. SDS-PAGE molecular weight standards were obtained from Integrated Separation Systems (Hyde Park, MA) or NOVEX (San Francisco, CA). Heteropoly-molate complexes were gifts from Dr. Robert Glew (University of New Mexico School of Medicine, Albuquerque, NM).

F. tularensis—F. tularensis ATCC 9410 was grown in 0.3 ml aliquots to a 2.0 × 10^7 cfu/ml concentration in 10 ml of buffer A (50 mM sodium phosphate buffer, pH 6.0).

Screening of F. tularensis Hydrolase Activities—Bacterial cultures from CHA were resuspended to a protein concentration of 1 mg/ml, 200-μl aliquots were added to api-ZYM® strips (bioMérieux Ville, Inc., Hazelwood, MO), the strips were incubated for 12 h at 37 °C and then analyzed for detection of hydrolase activities according to the manufacturer’s instructions.

Enzyme Assays—Acp activity was measured fluorometrically using an Amino-Bowman spectrofluorophotometer. The 0.3 mM standard assay mixture contained 0.2 mM sodium acetate buffer, pH 6.0, 1.0 mM 4-methylumbelliferyl phosphate (MUP), and varying amounts of enzyme. The mixtures were incubated at 37 °C for 15 min and 1.2 ml of 0.5 mM glycine, pH 10, was added to stop the reaction. Under these conditions, enzyme activity was linear with the amount of enzyme added. During kinetic experiments, enzyme activity was linear with time for at least 60 min. Only initial rates (slopes within the first 15 min) were used for calculation of enzyme activity and associated kinetic parameters. One unit of enzyme activity is defined as the amount of enzyme required to convert 1 nmol of substrate to product per h. Assays to determine the pH optimum were performed using either 0.2 mM MES or 0.2 mM HEPES as the buffer, and the final substrate concentration was 1.0 mM. Determination of the Michaelis-Menten constant for MUP and tyrosine phosphate was performed using 0.06 unit of AcpA and a wide range (K_m/10 to 5 K_m) of each substrate. Replicates of five were tested at each concentration. Data were analyzed using the least squares regression computer program (8) graciously supplied by Dr Stephen P. J. Brooks, Carleton University, Ottawa, Canada. Phosphatase C (PLC) activity was measured by monitoring the hydrolysis of p-nitrophenylphosphorylcholine as described previously (9).

Substrate Specificity Assays—Substrate specificity was determined by measuring the release of inorganic phosphate from phosphonomonoesters (including MUP) using the method of Lanza et al. (10). This assay was also used for the determination of the pH optimum of AcpA for phosphonomonoesters other than MUP. Phosphatidylinositol phosphates were assayed in the presence of 1.0% Triton X-100.

Peptide-tyrosine Phosphatase Activity of AcpA—A synthetic peptide p60 (TEPQpYQPGE) containing a single phosphorylated tyrosine was synthesized by the University of Illinois Genetic Engineering facility. The synthetic peptide was used to prepare an antibody against the tyrosine phosphorylated peptide, and this antibody was used to detect tyrosine phosphorylated proteins in Western blots.

Purification of Anti-Acp Antibodies—Monospecific anti-Acp antibodies (IgG) were purified from anti-Acp-antisera by affinity chromatography. Polyclonal antibody preparations were used for the detection of tyrosine phosphorylated proteins in Western blots.

Measurement of Respiratory Burst in Neutrophils—Respiratory burst activity of isolated porcine neutrophils was measured in the presence and absence of AcpA by following the production of superoxide using modifications of a previously described method (16). Briefly, the superoxide dismutase-inhibitable reduction of ferricytochrome c at 550 nm was continuously measured at 37 °C using either a Bedmar DU-50 spectrophotometer or an Amino dual-beam recording spectrophotom-
Acp specific activity of strain 29684 Acp specific activity was only 100 units/mg. The activity in strain NDBR101 was 550 to 3089 units/mg, whereas *Legionella micdadei* times that of *Francisella tularensis* reported for a bacterial or protozoan acid phosphatase. Incompletely in excess of 18,000 units/mg (13,000 to 30,000) and *Coxiella burnetii* that of the protozoan parasite, *Legionella micdadei*. The activity of *Francisella novicida* was 1729 units/mg. *Francisella tularensis* LVS NDBR 101 was 3087 units/mg. *Mycobacterium seagal* 3628 units/mg. *Coxiella burnetii* PRS Q177 3840 units/mg. *Leishmania donovani* 8360 units/mg. *Francisella tularensis* B-38 (ATCC 6223) 18138 units/mg.

**TABLE I** Comparison of acid phosphatase activities among various microorganisms

| Strain                      | Acp specific activity |
|-----------------------------|-----------------------|
| Francisella tularensis (ATCC 29684) | 100                   |
| Emeria vermiformis           | 135*                  |
| Mycobacteria chelonae         | 204                   |
| Escherichia coli Y1090       | 372                   |
| Mycobacterium thune          | 388                   |
| Salmonella typhimurium SB137 | 532                   |
| Legiendia micdadei           | 640*                  |
| Escherichia coli K300        | 791                   |
| Francisella novicida         | 1729                  |
| Francisella tularensis LVS NDBR 101 | 3087               |
| Mycobacterium seagal         | 3628                  |
| Coxiella burnetii PRS Q177   | 3840*                 |
| Leishmania donovani          | 8360*                 |
| Francisella tularensis B-38 (ATCC 6223) | 18138               |

*Values obtained from the literature (see text).

**TABLE II** Summary of the purification of AcpA

| Purification step | Total activity (units × 10³) | Total protein (mg) | Specific activity (units × 10³/mg) | Purification | Yield |
|-------------------|------------------------------|--------------------|-------------------------------|--------------|-------|
| Whole cells       | 4.06                         | 3036               | 1.34                          | 1 (100)      |       |
| Super I           | 4.41                         | 1062               | 4.15                          | 3 (109)      |       |
| Super II          | 3.94                         | 801                | 4.92                          | 6 (97)       |       |
| S-Sepharose       | 3.83                         | 88.6               | 43.23                         | 22 (94)      |       |
| Sephadex G-100    | 3.36                         | 6.1                | 551                           | 411 (83)     |       |
| Superdex 75       | 1.05                         | 1.1                | 955                           | 713 (26)     |       |

Acid phosphatase was measured using MUP as substrate as described under "Experimental Procedures." 1 unit = 1 nmol of MUP hydrolyzed.

**RESULTS**

Detection of Acid Phosphatase Activity in *F. tularensis—* Acid phosphatase specific activities varied markedly between species of *Francisella* and among strains of *F. tularensis.* *F. tularensis* strain 6223 displayed the highest specific activity. It was generally in excess of 18,000 units/mg (13,000 to 30,000) and represents, to our knowledge, the highest specific activity ever reported for a bacterial or protozoan acid phosphatase. In comparison to other Acp-enriched intracellular pathogens (Table I), *F. tularensis* strain 6223 Acp specific activity is greater than 10 times that of *L. micdadei* (28), more than 4 times that of *Coxiella burnetii* strain PRS Q177 strain (29), and about twice that of the protozoan parasite, *L. donovani* (3). Acp specific activity in strain NDBR 101 was 550 to 3089 units/mg, whereas strain 29684 Acp specific activity was only 100 units/mg. The Acp specific activity of *F. novicida* was approximately 1700 units/mg.

The rather wide variation in acid phosphatase specific activity among members of the *Francisella* genus may correlate with the passage history of individual strains. During experiments aimed at optimizing expression of Acp, we observed a large decrease in Acp specific activity upon repeated passage of strain 6223 on CHA (data not shown). A loss of almost 90% (8–10-fold reduction) of the starting Acp specific activity was seen following 9 passages. The reduction was most likely not due to irreversible accumulation of reversible inhibitors since washing the cells in physiological saline followed by extraction of Acp failed to increase Acp specific activity, and mixing of extracts from passaged cultures with purified Acp did not result in the inhibition of the activity of the purified enzyme. Furthermore, detection of AcpA by Western blot analysis indicated a marked reduction in anti-AcpA reactive material following 9 passages as compared to that found in initial cultures (data not shown).

Therefore, single passage *F. tularensis* (6223) was selected as the source for enzyme purification.

**AcpA Purification—** In initial attempts to solubilize the enzyme, we found at least 70% of the phosphatase activity could be extracted with 1 M NaCl alone; including sodium cholate in the extraction buffer resulted in complete solubilization of the enzyme. Furthermore, essentially no difference in total AcpA activity was observed in the extracted material compared to the activity exhibited by intact bacteria (Table I). All of the enzymatic activity detected in intact bacteria was solubilized by the cholate NaCl extraction buffer and remained in the supernatant following extensive dialysis and two centrifugations (200,000 × g, 1.5 h). The soluble AcpA was completely retained during loading at pH 6 on cation exchange resins S-Sepharose and Mono S and eluted as a single peak of activity between 0.17
M and 0.26 M NaCl (Fig. 1A). AcpA eluted in the breakthrough volume, however, during attempted anion exchange chromatography on either Q-Sepharose or Mono Q at pH 7.3. The material recovered from cation exchange chromatography was enriched 32-fold in acid phosphatase activity and contained 94% of the starting activity. Gel filtration chromatography through Sephadex G-100 superfine (Fig. 1B) resulted in an additional 13-fold increase in specific activity with 83% recovery of the applied activity. Final purification of the enzyme was achieved by gel filtration FPLC (Fig. 1C). This step resulted in a further 1.7-fold increase in specific activity with 26% of the sample recovered in a single protein peak coincident with AcpA activity. The apparently low recovery from the FPLC column is explained by the conservative pooling of AcpA active fractions as described under “Experimental Procedures.” The actual recovery was approximately 75%, but only the two fractions containing the highest AcpA activity were pooled for further analyses. Overall, AcpA was purified 713-fold over that in intact bacteria (Table II). The purification behavior of AcpA from strains 6223, NDBR 101, and 29684 and the results of comparative molecular weight (Fig. 2A) and immunoreactivity with rabbit anti-Ft (6223) AcpA IgG (Fig. 2B) suggested the enzyme is very similar in all strains of \textit{F. tularensis}.

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The enzyme activity chromatographed as a single entity throughout.

**Fig. 1.** Purification steps of \textit{F. tularensis} acid phosphatase. AcpA activity (●) and protein concentration (○) as described under “Experimental Procedures.” Twenty-one 6.0-ml fractions (38–58) found to contain AcpA activity eluted between 0.17 and 0.26 M NaCl. B, Sephadex G-100 Superfine chromatography of pooled and concentrated AcpA from S-Sepharose (5.3 ml, 6.7 mg/ml protein). Application and elution of AcpA to this gel filtration resin was performed as described under “Experimental Procedures.” C, Superdex 75 HR 10/30 FPLC chromatography of a 0.3-ml aliquot of pooled Acp activity from Sephadex G-100. D, SDS-PAGE separation of samples from the purification procedure.

**Fig. 2.** SDS-PAGE and Western blot analyses of acid phosphatase from three strains of \textit{F. tularensis}. A, Novex standards, as described for Fig. 1 (lane 1), 30 µg of extracted proteins from \textit{F. tularensis} strains NDBR 101, 29684, and 6223 (lanes 2–4), and 8 µg of purified acid phosphatase from these same strains (lanes 5–7) were subjected to SDS-PAGE and stained with Coomassie Blue R-250. B, Western blot analysis of blotted acid phosphatases from \textit{F. tularensis} strains NDBR 101, 29684, and 6223 (lanes 1–3) using rabbit anti AcpA (6223) IgG.

**Fig. 3.** Evaluation of AcpA purity by radiiodination of pooled fractions from Superdex 75 chromatography. Ten µg of pooled AcpA from Superdex 75 gel filtration chromatography was iodinated as described under “Experimental Procedures” and subjected to SDS-PAGE and autoradiography. The position of molecular weight markers are shown on the far left of the autoradiograph. The 5 lanes to the right of the markers (lane 1) contain 2, 4, 6, 8, and 10 µl, respectively, of the 1.5-ml void volume from the desalting column. Molecular weight standards are: β-galactosidase (116,000), phosphorylase b (95,000), BSA (68,000), glutamic dehydrogenase (55,000), carbonic anhydrase (29,000), and lysozyme (14,000).
all purification steps suggesting that multiple acid phosphatases may not exist in *F. tularensis* in contrast to the results reported for some other facultative intracellular organisms (1, 2).

AcpA Purity and Molecular Weight—The purity of AcpA was assessed in several experiments. 1) SDS-PAGE of samples obtained throughout the purification procedure demonstrate the presence of an ~57-kDa protein which was continuously enriched as the purification proceeded and electrophoresed as a single Coomassie Blue or silver (data not shown)-stained band following recovery from the final FPLC gel filtration step (Fig. 1D). 2) In an effort to visualize minor protein contaminants or those which may be refractory to staining, the purified AcpA fraction (Superdex 75 fraction) was radioiodinated, subjected to SDS-PAGE, and the 125I-labeled proteins were visualized by autoradiography. A single major band was seen on autoradiographs as increasing amounts of the radiolabeled AcpA fraction were applied to the SDS-PAGE gel (Fig. 3). This band, comprising 98% of the total signal as measured by quantitative densitometry, had a molecular weight of approximately 57,000. 3) N-terminal amino acid sequence analysis through the first 20 amino acids revealed the presence of a single threonine residue at the N terminus of the sequence (TDVNKNPNDYGTLVKIEQK).

The molecular mass of the purified enzyme was determined by gel filtration chromatography, SDS-PAGE, and matrix-assisted laser desorption time of flight MS. Superdex 75 FPLC gel filtration chromatography gave a partition coefficient for AcpA of 0.09 (Fig. 4A). This value was compared to the regression

![Fig. 4. Estimation of the molecular weight of AcpA. A, regression line(●) of the log molecular weight of the gel filtration standards versus their respective partition coefficients: BSA (67,000 $K_v$ = 0.035), ovalbumin (43,000 $K_v$ = 0.165), chymotrypsin (25,000 $K_v$ = 0.324), and RNase A (13,700 $K_v$ = 0.501); Elution position and partition coefficient of AcpA are indicated by arrow. B, regression line of log molecular weight standards (Fig. 1) versus electrophoretic mobility. Mobility and estimated molecular weight of AcpA are indicated by the arrow. C, matrix-assisted laser desorption time of flight profile of purified AcpA. $M^+ = m/z$ 55759.4. Matrix, sinapinic acid; laser wavelength, 337 nm.](http://www.jbc.org/)

![Fig. 5. Determination of pH optimum. A–D, purified AcpA was incubated with 1 mM indicated substrate in either 0.2 M MES (●, $p_K_a$ 6.10) or 0.2 M HEPES (●, $p_K_a$ 7.48) at varying pH values. Acp activity was determined by the Lanzetta assay for inorganic phosphate as described under "Experimental Procedures." Data are plotted as percent of optimal activity for each substrate. A, 5'-AMP; B, Glc-6-PO$_4$; C, MUP; D, tyrosine phosphate.](http://www.jbc.org/)
line generated from the four molecular weight standards, and the \( K_a \) corresponded to an apparent molecular weight of 56,000. A similar value, 57,000, was obtained with SDS-PAGE (Fig. 4B) using both reducing and nonreducing conditions (data not shown). Finally, mass spectrometry of AcpA indicated a singly charged species at 55,759 atomic mass units with a mass accuracy of 0.1% (Fig. 4C).

**Table III**

Substrate specificity of F. tularensis acid phosphatase

All substrates were tested at a final concentration of 1 mM except phosvitin and yeast mannan (10 mg/ml). Inositol phosphates and phosphorylinostr phosphates were used at concentrations of 40 \( \mu \)M in 1% Triton X-100. All measurements were made in quadruplicate and in at least two separate experiments.

| Substrate                     | Relative activity (%)
|-------------------------------|----------------------
| MUP                          | 100                  |
| O-Phospho-o-tyrosine          | 102                  |
| Phenylphosphate               | 98                   |
| Inositol 1-phosphate          | 93                   |
| AMP                           | 89                   |
| ATP                           | 89                   |
| p-Nitrophenyl phosphatase     | 89                   |
| Mannose 6-phosphate           | 85                   |
| Phosphoenolpyruvate           | 82                   |
| Phospho-serine                | 79                   |
| Fructose 1,6-bisphosphate     | 76                   |
| Glucose 6-phosphate           | 76                   |
| \( \beta \)-NADP              | 63                   |
| Fructose 6-phosphate          | 60                   |
| Pyridoxal phosphosphate       | 58                   |
| Ribose 5-phosphate            | 56                   |
| O-Phospho-o-threonine         | 51                   |
| Inositol 4-phosphate          | 28                   |
| Inositol 1,4,5-trisphosphate  | 15                   |
| Inositol cyclic phosphate     | 3                    |
| PIP                           | <1                   |
| PIP2                         | <1                   |
| Phytic acid                   | <1                   |
| Yeast mannan                  | <1                   |
| Phosvitin                    | <1                   |
| Cysteamine phosphate          | ND*                  |

* ND, none detected.

**Table IV**

Effects of various compounds on F. tularensis acid phosphatase

Acid phosphatase activity was tested in the presence and absence of each of the indicated inhibitors as described under “Experimental Procedures.” Each inhibitor, solubilized in \( \text{dH}_2\text{O} \), was tested for effect on pH of the assay before use as an inhibitor.

| Inhibitor                  | \( IC_{50} \) | Max concentration tested |
|----------------------------|--------------|--------------------------|
| Mercury chloride           | 0.5 \( \mu \)M | 5 \( \mu \)M              |
| Mb complex 84              | 5 \( \mu \)M  | 10 \( \mu \)M              |
| Ferrous sulfate            | 22 \( \mu \)M  | 150 \( \mu \)M             |
| Arsenic acid               | 36 \( \mu \)M  | 250 \( \mu \)M             |
| Hydroxymercaprolphenylsulfonate | 40 \( \mu \)M  | 160 \( \mu \)M             |
| Zinc chloride              | 50 \( \mu \)M  | 100 \( \mu \)M             |
| Cupric sulfate             | 56 \( \mu \)M  | 56 \( \mu \)M              |
| Ammonium molybdate         | 90 \( \mu \)M  | 100 \( \mu \)M             |
| Sodium vanadate            | 162 \( \mu \)M | 162 \( \mu \)M             |
| Silver nitrate             | 200 \( \mu \)M | 1 \( \mu \)m               |
| Sodium phosphate           | 1,800 \( \mu \)M | 15 \( \mu \)M              |
| Sodium dithionate          | 14,900 \( \mu \)M | 25 \( \mu \)M             |
| Dithiocolchitol            | 25,000 \( \mu \)M | 25 \( \mu \)M             |
| CHAPS                      | NI*          | 0.1%                     |
| Triton X-100, 114          | NI*          | 1%                       |
| Okadaic acid               | NI*          | 1 \( \mu \)M              |
| EDTA, EGTA                 | NI*          | 20 \( \mu \)M             |
| Sodium fluoride            | NI*          | 20 \( \mu \)M             |
| L- (+) Sodium tartrate     | NI*          | 50 \( \mu \)M             |
| Threonine                  | NI*          | 10 \( \mu \)M             |
| Serine                     | NI*          | 10 \( \mu \)M             |
| Glycerol                   | NI*          | 1.4 \( \mu \)M            |

* NI, no inhibition.

either side of this optimum. The pH optimum was independent of phosphomonoester substrates assayed including adenosine monophosphate (Fig. 5A), glucose 6-phosphate (Fig. 5B), tyrosine phosphate (Fig. 5D), and phosphorylated tyrosine residue.
of p60\textsuperscript{src} (Fig. 7C, inset). When the purified AcpA was subjected to isoelectric focusing (pH 3–12), a single peak of activity was found at pH 9.2 (Fig. 6). The basic pI of this enzyme is consistent with its fractionation behavior during ion exchange chromatography (Fig. 1A).

Substrate Specificity—AcpA has a broad in vitro substrate specificity (Table III). Sixteen of the 26 phosphonomonooesters tested were hydrolyzed at greater than 50% of the rate of MUP. The most active physiological substrates included tyrosine phosphate, AMP, ATP, and mannose 6-phosphate. Of the inositol phosphates tested, the monophosphates were preferred substrates. Inositol 1-monophosphate was hydrolyzed at near the same rate as MUP while inositol 4-phosphate was hydrolyzed at only 28% of the rate of MUP. Inositol 1,4,5-trisphosphate (IP\textsubscript{3}) was also recognized as a substrate, although it was hydrolyzed at only 15% of the rate of MUP. Inositol cyclic phosphate was the most slowly hydrolyzed substrate and may be a consequence of its cyclic nature. In contrast to some of the inositol phosphates which were good substrates for AcpA, phosphatidyl- and phosphatidylinositol phosphate derivatives, PIP and PIP\textsubscript{2}, were poor substrates. In general, these studies showed that small phospho-monooesters were more easily hydrolyzed than larger or multiply phosphorylated compounds. For example, yeast mannann, phosvitin, and phytic acid were not recognized as substrates by AcpA. The acidic pH optimum of AcpA, and, more importantly, its inability to hydrolyze the thiophosphate substrate, cysteamine phosphate, which is an alkaline phosphatase-specific substrate, is consistent with the designation of AcpA as an acid phosphatase.

Determination of Kinetic Parameters and Peptide-tyrosine Phosphatase (PTPase) Activity of AcpA.—The K\textsubscript{m} of AcpA for MUP and tyrosine phosphate was estimated to be 0.25 mM and 0.27 mM, respectively (Fig. 7) at pH 6.0. In addition to tyrosine phosphate activity, AcpA displayed readily measurable PTPase activity. The K\textsubscript{m} of the monophosphorylated peptide p60\textsuperscript{src} (determined by the release of inorganic phosphate) was 0.34 mM. The V\textsubscript{max} values were 9.6 \times 10\textsuperscript{3}, 8.0 \times 10\textsuperscript{3}, and 6.7 \times 10\textsuperscript{3} nmol of P\textsubscript{i} released per h per mg of enzyme for MUP, tyrosine phosphate, and p60\textsuperscript{src}, respectively (Fig. 7, B and D).

Effect of Inhibitors—To further characterize and classify this new AcpA, we measured the effects of acid phosphatase inhibitors. As shown in Table IV, the enzyme is not inhibited by L-(+)-sodium tartrate, sodium fluoride, okadaic acid, divalent cation chelators (EDTA, EGTA), or detergents (CHAPS, Triton X-100, Triton X-114). However, the enzyme was sensitive to the early transition metal oxyniars such as molybdate and vanadate. As is true of the acid phosphatases described for other intracellular pathogens (2, 28), this enzyme was sensitive to the heteropolyoxymolybdate complex E\textsubscript{3}. Monofunctional sulfhydryl group reagents such as mercury and silver inhibited the enzyme by 50% at 0.5 \muM and 290 \muM, respectively. Hydroxymercuriphenylsulfonate, a potent inhibitor of bovine liver acid phosphatase (30) inhibited AcpA by 50% at a concentration of 50 \muM. Zinc was also found to be an inhibitor of the enzyme; 50 \muM ZnCl\textsubscript{2} inhibited AcpA activity by 50%. Inorganic phosphate was found to be a competitive inhibitor with a K\textsubscript{i} of approximately 50 \muM (data not shown). Glycerol, serine, and threonine had no inhibitory effect.

AcpA-mediated Inhibition of the Respiratory Burst in Porcine Neutrophils.—In preliminary experiments, we found that a high speed supernatant from a crude F. tularensis extract containing an intense, heat-labile acid phosphatase activity was capable of dose-dependent inhibition of fMLP-activated porcine neutrophils. When this supernatant was subjected to gel filtration chromatography, the AcpA and respiratory burst inhibitory activities eluted coincidentally. To determine if AcpA was responsible for burst inhibition, porcine neutrophils were treated with the purified enzyme prior to activation with either fMLP or PMA. Under these conditions, AcpA caused a dose-dependent inhibition of the respiratory burst when added to either fMLP- or PMA-activated porcine neutrophils (Figs. 8, A and B). The inhibition was also seen when AcpA was added following PMA or fMLP addition but required larger amounts of enzyme, monitoring superoxide formation for longer times, and was seen only after a lag of 2–3 min following addition of AcpA except when the highest amounts of AcpA were used (data not shown). A greater inhibitory effect was obtained by preincubation of the neutrophils with AcpA prior to activation (Fig. 8C). Maximum burst inhibition was seen following preincubation for 15 min at 37 °C. Heat-inactivated AcpA had no effect on the rate of superoxide formation in activated neutrophils (Fig. 8A). We also did not detect catalase or superoxide dismutase activities in AcpA (data not shown), and AcpA had...
no inhibitory effect on the rate of xanthine oxidase-catalyzed generation of superoxide (data not shown). Thus, it is unlikely this enzyme affects the respiratory burst indirectly through electron scavenging.

Nucleotide Sequencing and Deduced Primary Structure of acpA—To further characterize the structure and function of AcpA, we cloned and sequenced the AcpA structural gene (acrA). A nondegenerate oligonucleotide was prepared and used to screen an F. tularensis ATCC29684 and subsequently an F. novicida genomic library (see “Experimental Procedures”).

The complete acrA nucleotide sequence and derived primary structure is shown in Fig. 9. The first 21-amino acid sequence of the open reading frame, prior to the N-terminal Thr residue of AcpA, contains many of the functional elements of a standard Gram-negative signal peptide (31). The next 20-amino acid deduced sequence is identical with the N-terminal sequence (TDVNNSKQHGTKLRLAQELN) determined by Edman degradation of the purified AcpA. Furthermore, the deduced sequence (YPQDYGQYTS) at position 228–240 was identical with the CNBr peptide sequence prepared from AcpA. The molecular weight of the signal peptide cleaved AcpA predicted from the nucleotide sequence (55,593) is in close agreement with the molecular weight of AcpA (55,759) determined by mass spectrometry.

Comparative sequence analyses (Blast, National Center for Biotechnology Information) indicate acpA has no overall sequence similarity to other known acid phosphatases, but it is partially similar to bacterial phosphatidylcholine phospholipases (PLC-N and PLC-H) identified in Pseudomonas aeruginosa (32, 33). The amino acid sequence of PLC-N is 40% homologous to PLC-H (33). The majority of this homology lies within the amino two-thirds of the protein’s sequence while the remaining one-third shows very little homology. AcpA shows an overall sequence identity of 16% to either PLC-N or PLC-H. For comparison, the sequence alignment of AcpA to PLC-N is shown in Fig. 10. Considering both identical and conserved amino acid residues, AcpA shows an overall sequence similarity to PLC-N of 51%.
The specific activity of AcpA, although comparable to that of a commercial Clostridium phospholipase, was 3000 times lower than the $I_{50}$ values for either Leishmania or Legionella acid phosphatases (1, 2).

The recognized classes of acid phosphatases include high and low molecular weight acid phosphatases, some protein phosphatases specific for phosphoserine or phosphothreonine and purple acid phosphatases (35). The purple acid phosphatases are readily distinguished from other acid phosphatases by their purple color in solution, which is due to the presence of a binuclear iron center or iron-zinc center (36). AcpA is not purple in solution, and preliminary x-ray diffraction and proton accelerator studies of AcpA crystals did not indicate the presence of any metal cofactors.\footnote{E. Garman, personal communication.} Results from our inhibitor studies also suggest the enzyme is not a serine/threonine-specific protein phosphatase. This class of protein phosphatases, consisting of groups 1, 2A, 2B, and 2C, is either acutely sensitive to okadaic acid or has an absolute requirement for divalent cations (37, 38). AcpA is resistant to okadaic acid and retains full activity in 20 mM EDTA.

AcpA also does not fit into either the high or low molecular weight class of acid phosphatases. High molecular weight acid phosphatases differ in several respects from their low molecular weight counterparts. A comparison of the class-distinctive properties of the high and low molecular weight Acps to those of AcpA is shown in Table V. According to its molecular weight, AcpA should be classified as a high molecular weight Acp. However, it has broad substrate specificity and is resistant to tartrate and fluoride, which are common inhibitors of high molecular weight acid phosphatases.

Although AcpA was shown to have PTPase activity but it did not possess an unambiguous phosphate binding loop signature sequence, (H/V)C(X)_{3,4}(I/V)G/A/P, present in Yop51 and more than 40 other PTPases (39). We did find a possible phosphate binding loop motif (H/V)C(X)_{4}GK(T/S) in which the critical arginine residue found in all PTPases is replaced by a lysine and this may explain why AcpA still retains PTPase activity. P-loop motifs found conserved in GTP- and ATP-binding proteins also have the general sequence G(\ldots)K(T/S) in which a lysine residue is conserved in all cases (40). It is tempting to speculate that AcpA has a diverged cysteine active site, phosphate binding loop in which an arginine has been conservatively replaced by a lysine. The lack of a consensus PTPase P-loop, however, precludes its classification as a PTPase.

Inhibition of AcpA activity by monofunctional sulfhydryl inhibitors including mercuric ions, silver, and hydroxymercuriphosphorylsulfonate suggests this enzyme may possess a cysteine active site and may therefore be classified as a "low molecular weight" acid phosphatase despite its high molecular weight. This is not without precedent since a cysteine active site, low molecular weight TRAP that has high molecular mass (35 kDa), has been described (41).

Interestingly, comparative nucleotide sequence analyses revealed partial homology to known phosphatidylincholine phospholipases (PLC) of P. aeruginosa but failed to reveal homology to any known acid phosphatase and did not detect the presence of any known acid phosphatase, protein-tyrosine phosphatase, or phospholipase signature motifs. In preliminary experiments, we were able to detect phospholipase C activity in the purified AcpA when assayed using a synthetic substrate, p-nitrophenylphosphorylcholine, at pH 7.0 but not at pH 6.0, the pH optimum for phosphomonooesterase activity. The phospholipase C specific activity of AcpA, although comparable to that of a commercial Clostridium phospholipase, was 3000 times lower of the activity of AcpA at concentrations that are 100 and 1000 times lower than the $I_{50}$ values for either Leishmania or Legionella acid phosphatases (1, 2).

The physical and chemical properties of AcpA indicate this enzyme is unique not only among burst-inhibiting acid phosphatases but also among acid phosphatases in general. AcpA, in contrast to burst-inhibiting Acps (1, 2, 29), is easily released from the bacterial cell in soluble form, is a basic enzyme, and suppresses the respiratory burst of not only fMLP but also PMA-stimulated neutrophils. AcpA is also much more sensitive to inhibition by molybdate compounds than other burst-inhibiting Acps. As shown in Table IV, these compounds inhibit 50%
both construction and use of isogenic Acp-negative mutant strains in identification of these enzymes as virulence factors must await further studies using natural substrates.

PLC. Unequivocal demonstration of PLC activity of AcpC must await further studies using natural substrates. There is no unequivocal proof that any of the burst-inhibiting Acp gains entry or accessibility to PIP2 or IP3 (1). Whether AcpA's burst-inhibiting activity is relevant to the pathogenicity of F. tularensis remains to be determined. Until now, there has been no nucleotide sequence information reported for any burst-inhibiting Acp. The results of cloning and sequencing of the AcpA gene reported here should help in the design of experiments aimed at elucidating the physiological function of AcpA and to directly test its role, if any, in F. tularensis virulence.

Acknowledgments—We would like to thank Dr. J. im C. Williams of the Food and Drug Administration for our first samples of F. tularensis. We are also grateful to Dr. Graeme Laver (The Australian National University) for growing AcpA crystals and Dr. Elspeth Garman (University of Oxford) for her preliminary AcpA x-ray diffraction and proton acceleration studies. We would also like to acknowledge the University of Illinois Biotechnology and Mass Spectrometry Laboratories for their efforts in obtaining the N-terminal sequences and the matrix-assisted laser desorption mass spectrometry determined molecular mass of AcpA. We also thank Dr. Saul Roseman, The Johns Hopkins University, for his many helpful suggestions in the preparation of this manuscript.

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Characterization and Sequencing of a Respiratory Burst-inhibiting Acid Phosphatase from *Francisella tularensis*

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*J. Biol. Chem.* 1996, 271:10973-10983.

doi: 10.1074/jbc.271.18.10973

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