Identification of organelles in bacteria similar to acidocalcisomes of unicellular eukaryotes*

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* This work was supported in part by a National Institutes of Health grant (AI23259) to RD.

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The abbreviations used are: polyP, polyphosphate; PPI, pyrophosphate, H+-PPase, proton pyrophosphatase.
Acidocalcisomes are acidic calcium storage compartments described in several unicellular eukaryotes, including trypanosomatid and apicomplexan parasites, algae, and slime molds. In this work, we report that the volutin granules of *Agrobacterium tumefaciens* possess properties similar to the acidocalcisomes. Transmission electron microscopy revealed that each intracellular granule was surrounded by a membrane. X-ray microanalysis of the volutin granules showed large amounts of phosphorus, magnesium, potassium, and calcium. Calcium in the volutin granules increased when the bacteria were incubated at high extracellular calcium concentration. Immunofluorescence and immunoelectron microscopy, using antisera raised against peptide sequences conserved in the *A. tumefaciens* proton pyrophosphatase (H+-PPase), indicated localization in intracellular vacuoles. Purification of the volutin granules using iodixanol density gradients indicated a preferential localization of the pyrophosphatase activity in addition to high concentrations of phosphate, pyrophosphate, short and long chain polyphosphate, but lack of markers of the plasma membrane. The pyrophosphatase activity was potassium-insensitive, and inhibited by the pyrophosphate analogs amynomethylenediphosphonate and imidodiphosphate, and by dicyclohexylcarbo-diimide, and the thiol reagent N-ethylmaleimide. Polyphosphate was also localized to the volutin granules by 4′,6′-diamino-2-phenylindole staining. The organelles were acidic as demonstrated by staining with Lysosensor blue DND-167, a dye especially used to detect very acidic compartments in cells, and cycloprodigiosin, a compound isolated from a marine bacterium that has been shown to uncouple H+-PPase activity acting as a chloride/proton symport. The results suggest
that acidocalcisomes arose before the prokaryotic and eukaryotic lineages diverged.
Intracellular membranous structures have been reported in several bacterial species (1-5). One group is formed by the abundant membrane-bound vesicles of photosynthetically grown cells of several purple bacteria, like *Rodospirillum rubrum* and *Rhodopseudomonas spheroides* (4). These vesicles are usually designated chromatophore membranes because of their similarity to particulate fractions known as chromatophores that could perform photophosphorylation *in vitro*, and that are obtained after sonication of these bacteria (4). Cyanobacteria possess sac-like membranes (thylakoids) with the same function (5). Another group of intracellular membranous structures is formed by a number of inclusions such as the poly-β-hydroxybutyrate-containing granules of several bacteria, the sulfur globules of the *Thiorhodaceae*, the gas vacuoles of aquatic bacteria, the chlorophyl-containing *Chlorobium* vesicles, the ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco)-rich carboxysomes of cyanobacteria, nitrifying bacteria, and thiobacilli, and the related polyhedral bodies or enterosomes of *Salmonella enterica* (1-3, 5). These are not considered to be equivalent to the organelles found in the eukaryotes (2). They usually consist, with the exception of carboxysomes and perhaps enterosomes, of a material lacking enzymatic function. They are surrounded by a nonunit membrane apparently composed entirely of protein (1, 2). Other, more recently described intracytoplasmic membranes found in *Planctomycetales*, are the pirellulosome, which surrounds the cell DNA in *Pirellula* species, and the anamoxosome of *Candidatus* species (6). The anamoxosome is a dedicated intracytoplasmic compartment where the anamox process (anaerobic ammonium oxidation) takes place (6, 7). All these membranous structures
are different from the mesosomes described initially in Gram positive bacteria and that were shown to arise as a fixation artifact (8). In addition to all these membrane-bound compartments, a number of apparently non-membrane bound inclusions are also present in several bacterial species, such as the multi-L-arginyl-poly(L-aspartic acid)-containing cyanophycin granules of cyanobacteria, the polyglucoside granules containing polymers of glucose, and the volutin granules (1, 2, 5).

Volutin or metachromatic granules were the first subcellular entities to be recognized in bacteria (9). Their metachromatic nature is due to the reaction of the polyphosphate (polyP) present in the granules with certain basic dyes, such as toluidine blue, which induces a characteristic shift in their absorption spectrum to a higher wavelength (10). They have been described as devoid of internal structure or limiting membrane (11), and have also been found in unicellular eukaryotes (12-16). In recent years volutin granules of unicellular eukaryotes such as *Chlamydomonas reinhardtii* (17), *Dictyostelium discoideum* (18), and a number of human pathogens including malaria parasites, *Toxoplasma gondii*, and trypanosomatids (19), were found to be surrounded by a membrane with a number of pumps and exchangers and were named acidocalcisomes. Acidocalcisomes are characterized by their acidic nature, their high electron density, and their high concentration of calcium, magnesium, and other elements in addition to pyrophosphate (PP_i) and polyP. It has been postulated that acidocalcisomes may have important roles as energy stores, and in intracellular pH, calcium and osmotic regulation (19).

Since volutin granules in bacteria and unicellular eukaryotes are morphologically
similar, we investigated whether the bacterial volutin granules are also surrounded by a membrane and have similar characteristics to the acidocalcisomes. We chose for our initial studies the $\alpha$-protoplast Agrobacterium tumefaciens, which is known to possess a $\text{H}^+$ translocating pyrophosphatase (H+-PPase) (20), a marker for acidocalcisomes in unicellular eukaryotes (19). In this report, we describe the isolation and biochemical properties of volutin granules of A. tumefaciens and demonstrate that, as the acidocalcisomes, they are surrounded by a membrane, are acidic due to the presence of the H+-PPase in their membrane, are rich in PP$_i$ and polyP, and are able to accumulate calcium and other elements. Acidocalcisomes are therefore the first organelles described in prokaryotes that are also present in eukaryotes.

**EXPERIMENTAL PROCEDURES**

*Cell cultures*- A. tumefaciens (strain C58) was provided by Stephen K. Farrand (University of Illinois at Urbana-Champaign). Cells were grown in liquid Luria Bertani (LB) medium at 28 °C with agitation. Cells were cultured overnight and harvested at the stationary phase.

*Isolation of volutin granules*- Bacteria were collected by centrifugation, and incubated with a solution containing 0.5 M NaCl, 20 mM EDTA, 50 mM Tris-Cl, pH 8.0, and 1% N-lauroyl-sarcosine, to disrupt the bacterial capsule, for 20 min at room temperature. Bacteria were then centrifuged at 3,900 x g and the pellet was resuspended
in lysis buffer (125 mM sucrose, 50 mM KCl, 4 mM MgCl\(_2\), 10 mM EDTA, 20 mM K-Hepes, 5 mM dithiothreitol, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 µM pepstatin, 10 µM leupeptin, 10 µM trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane, and 10 µM N\(\alpha\)-tosyl-L-lysine chloromethyl ketone, pH 7.2) containing 2 mg/ml lysozyme and incubated on ice for 30 min. Benzonase\registered (Novagen) (1 µl/ml) was added and bacteria were passed through a French press (SLM-Amico, Spectometric Instruments) two times at 1,000 psi. The lysate was incubated on ice under agitation for 1 h with an equal volume of 0.5 x silica (silicon dioxide) and 0.5 x silicon carbide solution to remove fragments of DNA and RNA. The lysate was then centrifuged at 1,000 x g for 5 min, and washed two times under the same conditions. The supernatant fractions were combined and centrifuged for 10 min at 14,500 x g. The pellet was resuspended in 2 ml of lysis buffer with the aid of a 22-gauge needle. The suspension was diluted 1:1 in OptiPrep (60% iodixanol), and applied as the 30% step of a discontinuous gradient of Optiprep, with 4-ml steps of 24, 28, 30, 35 and 40% iodixanol, diluted in lysis buffer. The gradient was centrifuged at 50,000 x g in a Beckman SW 28 rotor for 60 min. The volutin granule fraction pelleted at the bottom of the tube and was resuspended in lysis buffer. Gradient fractions and markers were assayed as previously described (21).

**Analytical methods and immunoblotting**-Bacteria were washed once with Dulbecco’s PBS and PP\(_i\), long chain or short chain polyP were extracted as described before (22). Pyrophosphatase activity was assayed by measuring released phosphate
using the EnzChek phosphate assay as described before (21, 23). The apparent $K_m$ for PP$_i$ was calculated by using a computerized nonlinear regression program (Sigma Plot 1.0, Jandel Scientific) using the Hill equation. Protein determination was done with the Coomassie Blue protein assay reagent from Bio-Rad. Proteins were separated by SDS-PAGE, using 10% gels, and blotted onto nitrocellulose using a Bio-Rad Transblot apparatus by standard techniques. Subsequent processing steps were done in Dulbecco’s PBS containing 0.1% Tween 20. Blots were blocked for 1 h in 5% non-fat dry milk, washed three times, and incubated with polyclonal antibody against *Trypanosoma cruzi* H$^+$-PPase (24) (1:5,000) for 1 h at room temperature. Blots were then washed three times, incubated for 1 h with horseradish peroxidase-labeled anti-mouse IgG (1:20,000), washed three times, and processed for chemiluminescence detection as per manufacturer’s (Amersham Pharmacia Biotech) instructions. Molecular weights were calculated using prestained molecular weight markers.

**Immunofluorescence microscopy**—For subcellular localization of H$^+$-PPase bacteria were washed with Dulbecco’s PBS and fixed in 4% freshly prepared formaldehyde for 10 min at room temperature and 50 min at 4 °C, attached to poly L-lysine treated glass slides, and permeabilized with 0.2 % NP40 in PBS for 5 min. Samples were blocked for 1 h with PBS containing 3% BSA, 1% cold fish gelatin, and 50 mM NH$_4$Cl, and first incubated for 1 h at room temperature with the polyclonal antibody against the *T. cruzi* H$^+$-PPase (24) diluted 1: 20 in 1% cold fish gelatin. Bacteria were subsequently
incubated for 60 min at room temperature with rhodamine-conjugated secondary antibody diluted 1:100 in PBS plus 1% cold fish gelatin. Coverslips were mounted in glass slides with Vectashield® media and sealed. Images were collected with an Olympus laser scanning confocal microscope or an Olympus BX-60 fluorescence microscope.

For polyP localization, bacteria were washed twice with Dulbecco’s PBS and resuspended in the same buffer and fixed for 30 min with 4% formaldehyde. 45 µl of this suspension was incubated at room temperature with 10 µg/ml DAPI. After 10 min, the samples were mounted on a slide and observed with the fluorescence microscope.

For localization of LysoSensor blue DND-167 (9,10-bis(N-morpholinomethyl)anthracene), bacteria were centrifuged and resuspended in pre-warmed (28 °C) LB medium containing 1 µM LysoSensor. Bacteria were incubated for 1 h at 28 °C, centrifuged and resuspended in fresh pre-warmed LB medium. Bacteria were mounted on a slide and observed with the fluorescence microscope using UV excitation. For cycloprodigiosin detection, bacteria were centrifuged, resuspended in Dulbecco’s PBS containing 100 nM cycloprodigiosin and incubated for 30 min. Bacteria were mounted on a slide and observed with the fluorescence microscope using a red emission filter. Bacteria resuspended in Dulbecco’s PBS or LB medium, but without cycloprodigiosin or LysoSensor, respectively, were used as controls.

Electron microscopy and X-ray microanalysis-For routine electron microscopy, bacteria were washed with Dulbecco’s PBS and fixed for 1 h with 2.5% grade II
glutaraldehyde, 4% freshly prepared formaldehyde, 0.03% CaCl\textsubscript{2}, and 0.03% picric acid, in 0.1 M cacodylate buffer, pH 7.2. Bacteria were post-fixed with OsO\textsubscript{4} for 45 min, and then for 15 min with potassium ferricyanide, washed and treated with 2% uranyl acetate for 30 min. Subsequently, samples were dehydrated by successive incubations of 6 min with increasing concentrations of ethanol (10%, 25%, 50%, 75%, 95% and 100%) at room temperature. Epoxy embedding was carried out by resuspending the sample once in 1:1 ethanol/acetonitrile, twice in 100% acetonitrile; then 30 min in 1:1 Epoxy/acetonitrile, 1.5 h in 3:1 Epoxy/acetonitrile, and 4 h in 100% Epoxy. Embedded samples were polymerized for 20 h at 85 °C. Epoxy blocks were ultrathin-sectioned, sections were deposited on 300 mesh copper grids and grids were stained with uranyl acetate for 30 min and triple lead stain (lead citrate, lead nitrate and lead acetate) for 1 min.

For immunocytochemistry, bacteria were washed with Dulbecco’s PBS, fixed for 1 h at 4 °C in a solution containing 0.5% grade I glutaraldehyde, 4% freshly prepared formaldehyde, 1% picric acid, in 0.1 M cacodylate buffer, pH 7.2. Fixed bacteria were washed with Dulbecco’s PBS and dehydrated by successive incubations of 6 min with increasing concentrations of ethanol (10%, 25%, 50%, 75%, 95%, 100% and 100%) at ~20 °C. Samples were embedded in Unicryl at 4 °C by incubation with 1:1 ethanol/Unicryl for 1 h and 100% Unicryl for 1, 16 and 8 h. Embedded samples were polymerized under UV irradiation at ~20 °C for 48 h. Thin sections were collected on 300 mesh nickel grids and blocked for 30 min with PBS containing 0.1% Tween 20 and 0.5% cold fish gelatin (PBS-
TW-FG). Grids were incubated for 3 h with a mouse monoclonal antibody against *T. cruzi* H+-PPase (24) diluted 1:10 in PBS-TW-FG. After washing in PBS-TW-FG, grids were incubated for 1 h with a 5 nm gold-conjugate goat anti-mouse antibody. Subsequently, grids were washed with PBS and then in distilled water, stained with uranyl acetate and lead citrate. Routine and immunocytochemistry samples were observed in a Hitachi H 600 electron microscope.

For imaging whole bacteria and electron-dense vacuole fractions the preparations were washed in 0.25 M sucrose and a 5-µl sample was placed on a Formvar-coated 200-mesh copper grid, allowed to adhere for 10 min at room temperature, blotted dry and observed directly with a Hitachi H 600 transmission electron microscope operating at 100 kV (18). Energy-dispersive X-ray analysis was done at the Electron Microscopy Center, Southern Illinois University (Carbondale, IL). Specimen grids were examined in a Hitachi H-7100FA transmission electron microscope at an accelerating voltage of 50 kV. Fine probe sizes were adjusted to cover the electron-dense vacuoles (or a similar area of the background), and X-rays were collected for 100 sec by utilizing a thin-window (Norvar) detector. Analysis was performed by using a Noran Voyager III analyzer with a standardless analysis identification program.

RESULTS

Volutin granules are recognized by their high electron-density when the cells are observed by transmission electron microscopy without fixation and staining (5). *A.*
*tumefaciens* typically shows a large granule towards one of the cellular poles (Fig. 1A, arrows), and additional smaller granules in different regions of the cells (Fig. 1A, arrowheads). The large granules have a diameter of about $210 \pm 18$ nm. X-ray microanalysis was performed on these granules (Fig. 1B). The spectrum shown was the one that yielded the most counts in 100 s (out of 10 spectra obtained from granules of different bacteria), but all other spectra taken from volutin granules were qualitatively similar: counts for phosphorus were about 4-fold greater than counts for magnesium, which were about the same as counts for potassium. Counts for calcium were much lower. Counts for oxygen and phosphorus were about the same. Peaks for phosphorus, magnesium, potassium, and calcium were not present in spectra taken from the background (Fig. 1C). Peaks for copper and in part for carbon and oxygen, arise from the grid.

Examination of cells in thin sections showed a round vacuole of about 210 nm in one of the poles of the cell, corresponding to the location of the large granules in Fig. 1A. An electron-dense inclusion partially (Fig. 1D, and 1E, arrows), or completely (Fig. 1E, arrowhead), occupied its interior. Each intracellular granule appeared to be surrounded by a membrane (Fig. 1E and 1F, arrow and arrowhead, respectively). The determination of the membrane was made difficult because of the so-called "phase" or "out of focus" effect often accompanying electron-dense particles of bacteria visualized by transmission electron microscopy (25). A better identification of the surrounding membrane was achieved with isolated volutin granules. As has been observed in acidocalcisomes of unicellular eukaryotes (19), a ring of electron-dense material was
observed below the membrane of the isolated volutin granules (Figs. 1G, and 1H).

To purify the volutin granules and investigate their chemical and enzymatic content we adapted the purification procedure used for the isolation of acidocalcisomes from *T. cruzi* (21). The utility of the method was assessed by assaying marker enzymes (Fig. 2). The yield of pyrophosphatase activity (a marker of acidocalcisomes) in the volutin granule fraction (fractions 12) was 80% (Fig. 2A), whereas the yield of protein in the same fraction was only 3% (Fig. 2H), a 27-fold purification. Plasma membrane (marked by succinate cytochrome *c* reductase) was not enriched in this fraction (Fig. 2B). The volutin granule fraction (fraction 12) contained around 20% of the total amount of PP$_i$ (Fig. 2D), and short chain polyP (Fig. 2E), and more than 35% of the total amount of P$_i$ (Fig. 2C), and long chain polyP (Fig. 2F). Levels of PP$_i$ of 274 ± 77 nmol/mg protein, and of polyP of 315 ± 79 and 217 ± 19 nmol/mg protein (in terms of P$_i$ residues) in chains of less than 50 residues long and in chains of about 700-800 residues long, respectively, were found in total extracts of *A. tumefaciens*.

Electron microscopy of the volutin granule fraction (fractions 12), by observation of air dried samples (Fig. 3A), showed the presence of granules with the same appearance as the granules observed in preparations of whole cells (Fig. 1A). When submitted to the electron beam, changes in their internal structure led to the appearance of a sponge-like structure, which has been described before in acidocalcisomes of *T. cruzi* (26), *Trypanosoma brucei* (27), and *C. reinhardtii* (17). The results of X-ray microanalysis of the isolated granules were similar to those of whole cells except that
proportionally less potassium and calcium were detected (Fig. 3B), probably due to ionic changes occurring during the fractionation procedure. This phenomenon has also been described after isolation of acidocalcisomes of unicellular eukaryotes (17-19).

To investigate whether *A. tumefaciens* volutin granules are similar to acidocalcisomes of unicellular eukaryotes (19), we used fluorescence microscopy and X-ray microanalysis methods to detect their ability to accumulate H⁺ and calcium and their polyP content. Fig. 3D shows that incubation of whole bacteria with LysoSensor Blue DND-167, a dye especially used to detect very acidic compartments in cells (28), stained granules located at one pole of the cells (arrowheads), at the same position where the large volutin granules were located by direct transmission electron microscopy (Fig. 1A). Cycloprodigiosin is a compound isolated from a marine bacterium that has been shown to uncouple H⁺-PPase activity acting as a chloride/proton symport (29), and that has been shown to stain acidocalcisomes of *T. cruzi* (21). Fig. 3E (arrowheads) shows that cycloprodigiosin stained granules located at the same position as those stained by LysoSensor (Fig. 3E, arrowheads). 4’,6-diamino-2-phenylindole (DAPI) has been shown to shift its maximum emission fluorescence from 426 to 525 nm in the presence of polyP, this change being specific for polyP and not produced by PP₁ or other anions (22, 30). We observed localization of DAPI-reactive material in the granules located at one pole of the cell (arrowheads in Fig. 3F). The medium used to grow *A. tumefaciens* for the previous experiments is calcium-deficient. This might explain the lower counts for calcium in the X-ray microanalysis of
the volutin granules (Fig. 1B) as compared to acidocalcisomes (19). We therefore
cultivated the bacteria in the presence of 100 mM CaCl₂ for 16 h before preparing them
for X-ray microanalysis. Fig. 3G shows a dramatic increase in the counts for calcium
and no counts for magnesium and potassium in the volutin granules of these cells. The
presence of these elements was not detected in spectra taken from the background (Fig.
3H) and demonstrates the ability of volutin granules to accumulate calcium.

Acidocalcisomes are characterized by the presence of a membrane-bound H⁺-
PPase that maintains their acidity (19). The pyrophosphatase activity detected in the
volutin granule fraction of *A. tumefaciens* (Fig. 2A), as measured by inorganic
phosphate detection (23), was 0.69 ± 0.03 µmol pyrophosphate consumed/min x mg
protein (means ± S.E. of results from 3 separate experiments) and was totally inhibited
by 20 µM aminomethylenediphosphonate (AMDP), a specific inhibitor of H⁺-PPases.
As expected for a type 2 H⁺-PPase (31), the enzyme was potassium-insensitive (data
not shown). The dependence of the initial rate of hydrolysis on pyrophosphate
concentration in *A. tumefaciens* volutin granule fraction is shown in Fig. 4A. Activity
was maximal at about 20 µM pyrophosphate with an apparent Kₘ of 5.5 µM. Fig. 4B
shows the effect of pH on the initial rate of pyrophosphate hydrolysis in *A. tumefaciens*
volutin granule fraction. Activity was optimal at pH 7.5-8.0. Pyrophosphate hydrolysis
of the volutin granule fraction was inhibited, in a dose-dependent manner, by
pyrophosphate analogs aminomethylenediphosphonate (AMDP) (Fig. 4C) and
imidodiphosphate (IDP) (Fig. 4D). Fig. 4 also shows that potassium fluoride (Fig. 4E),
dicyclohexylcarbo-diimide (DCCD) (Fig. 4F), and the thiol reagent N-ethylmaleimide (NEM) (Fig. 4G), agents known to inhibit the H\(^+\)-PPases from plants (32), trypanosomatids (27, 33), and apicomplexan parasites (24, 34) were also effective in inhibiting the *A. tumefaciens* pyrophosphatase activity in a dose-dependent manner.

To confirm that the pyrophosphatase in the volutin granules was a H\(^+\)-PPase, we investigated its localization by immunocytochemistry using an antibody (24) against a peptide of *T. cruzi* H\(^+\)-PPase (amino acids 735-761) (33) that is conserved (Fig. 5A) in the C-terminal region of the *A. tumefaciens* sequence available in GenBank (accession number AAL42186; amino acids 641-669). This antibody showed cross-reactivity with a band of 72 kDa present in *A. tumefaciens* volutin granule fraction (Fig. 5B, left lane). No background staining was observed when pre-immune serum was used as a control (Fig. 5B, right lane). Immunofluorescence microscopy using these antibodies resulted in staining of granules located at one pole of the cells (Fig. 5C, arrowheads, and inset), at the same position where the large volutin granules were located by direct transmission electron microscopy (Fig. 1A) or by LysoSensor (Fig. 3D), cycloprodigiosin A (Fig 3E), and DAPI (Fig. 3F) staining. These results are in agreement with the co-localization of polyP and the pyrophosphatase in the volutin granules as assayed biochemically (Fig. 2). In order to analyze in more detail the structures labeled with the anti-H\(^+\)-PPase antibody, immunoelectron microscopy was performed on thin sections of bacteria embedded in Unicryl hydrophilic resin. The results obtained confirmed labeling in vacuoles with an 'empty' appearance located at one pole of the cell (Fig. 5D, arrows).
We report that volutin granules of *A. tumefaciens* are discrete intracellular acidic compartments rich in P$_i$, PP$_i$, polyP, magnesium, potassium, and calcium that possess an enclosing membrane with a calcium-accumulating activity, and a pyrophosphatase to maintain their acidity. *A. tumefaciens* volutin granules provide evidence for the existence of an organelle common to both prokaryotes and eukaryotes. The structural and biochemical resemblance of volutin granules of *A. tumefaciens* with eukaryotic acidocalcisomes suggests potential functional similarities.

H+-PPases (31, 35), as volutin granules (11, 19), are widely distributed phylogenetically. Heterologous expression studies have documented the attribution of K$^+$ requirement to a particular sequence. Enzymes from the archeon *Pyrobaculum aerophilum* (36), the plant *Arabidopsis thaliana* (AVP2) (37), and the bacterium *R. rubrum* (38) are capable of PP$_i$ hydrolysis in the absence of K$^+$ whereas those from the plants *A. thaliana* (AVP1) (39) and *Vigna radiata* (40), the bacteria *Thermatoga maritima* (41) and *Carboxydothermus hydrogenoformans* (31), and the trypanosomatid *T. cruzi* (33) require millimolar concentrations of K$^+$ for activity (31). Based on phylogenetic analyses of a large set of H+-PPase sequences, it was suggested that K$^+$-dependent and K$^+$-independent enzymes form two independently evolving groups (35, 41). A comparison of the amino acid conservation patterns in K$^+$-dependent and K$^+$-
independent H\textsuperscript{+}-PPases revealed only two sequence positions where a residue conserved in one type is absent from the equivalent position in all the sequences from the other type (31). K\textsuperscript{+}-independent H\textsuperscript{+}-PPases (or type 2 H\textsuperscript{+}-PPases) possess conserved Lys and Thr in two sequence positions that are absent in K\textsuperscript{+}-dependent H\textsuperscript{+}-PPases (or type 1 H\textsuperscript{+}-PPases) and substitution of Lys for Ala in C. \textit{hydrogenofomans} H\textsuperscript{+}-PPase is sufficient to confer K\textsuperscript{+}-independence to both PP\textsubscript{i} hydrolysis and PP\textsubscript{i}-energized H\textsuperscript{+}-translocation (31). It was suggested that this is due to the substitution of K\textsuperscript{+} with the NH\textsubscript{3}\textsuperscript{+} group of Lys (31). In agreement with these conclusions, we found that the \textit{A. tumefaciens} H\textsuperscript{+}-PPase, whose sequence has Lys and Thr in the positions investigated (31), is K\textsuperscript{+}-insensitive. Only another proteobacterial H\textsuperscript{+}-PPase has been biochemically characterized, the enzyme from \textit{R. rubrum} (38, 42, 43). As this enzyme, the H\textsuperscript{+}-PPase from \textit{A. tumefaciens} was sensitive to inhibition by aminomethylenediphosphonate, dicyclohexylcarbo-diimide, and N-ethylmaleimide, and has low sensitivity to the soluble pyrophosphatase inhibitor fluoride (Fig. 4).

Having an homolog enzyme in a similar organelle is a strong argument of the common origin of the two organelles. Many of the organisms described to possess this enzyme (31, 35) are also known to possess volutin granules or acidocalcisomes, such as bacteria of the genera \textit{Rhodospirillum}, \textit{Rhodopseudomonas}, \textit{Chlorobium}, and \textit{Chromatium} (4, 11), the apicomplexan parasites \textit{P. falciparum} (34) and \textit{T. gondii} (24), the trypanosomatids \textit{T. cruzi}, \textit{T. brucei} and \textit{Leishmania} spp. (19), the ciliate \textit{Tetrahymena}
pyriformis (14), the slime mold D. discoideum (18), and the green alga C. reinhardtii (17).

Evidence for the presence of the H+-PPase in acidocalcisomes of several of these organisms has been published (17-19, 24, 34) but definitive evidence is still lacking for others. Further work is also needed to investigate the evolutionary relationship of acidocalcisomes to other organelles with some similarities in morphology and composition, like the plant and yeast vacuoles, and the secretory granules of higher eukaryotes, such as the dense granules of platelets, the chromaffin granules of the adrenal gland, the atrial natriuretic factor granules of the heart, and many others, which are also acidic and contain calcium (44), and are often referred to as lysosome-related organelles or secretory lysosomes (45).

In conclusion, volutin granules or acidocalcisomes are widely distributed in organisms from different phylogenetic groups. The elucidation of the function of acidocalcisomes in eukaryotic organisms and in their bacterial counterparts may have significant and widespread implications on important issues, such as pathogenesis and adaptive mechanisms to changing environments as well as in the dynamics of the evolutionary process of prokaryotic and eukaryotic organisms. The origin of eukaryotic organelles is a matter of extensive debate, although the principle of endosymbiosis has been advanced as one of its possible mechanisms. Whether this organelle originated previously to the basal bifurcation of the universal tree of life (46), and was maintained in both prokaryotes and eukaryotes deserves further investigation.

Acknowledgments-We thank Arthur Kornberg for E. coli CA38 pTrcPPX1, Hajime
Hirata for cycloprodigosin, Michael Martin for AMDP, John Bozzola and Steve Schmitt for help with the X-ray microanalysis, Stephen K. Farrand for the C58 A. *tumefaciens* strain, Yinping Qin and Shuhong Luo for technical advice, and David A. Scott for critically reading the manuscript.

REFERENCES


FIGURE LEGENDS

FIG. 1. Electron microscopy and X-ray microanalysis of whole A. tumefaciens. (A) Visualization of volutin granules in whole unfixed cells allowed to adhere to a Formvar- and carbon-coated grid and then observed in the transmission electron microscope. A large granule appears located at one pole of the cells (arrows) and smaller granules of varying sizes appear distributed in the cytosol (arrowheads). Bar: 5 µm. (B) X-ray microanalysis spectrum of volutin granules in whole cells. (C) X-ray microanalysis spectrum of background. (D-H) Electron micrographs of intact bacteria (D-F) and volutin granule fractions (G, H). Cells and fractions were fixed as described in Experimental Procedures. Arrows show vacuoles, some containing an electron-dense material in the periphery (D, E), some completely occupied by electron-dense material (E, arrowhead) and some apparently empty (F, arrowhead). Fractions show almost empty vacuoles containing an electron dense material in their periphery (G, H). A membrane is clearly seen enclosing the vacuoles (arrow in E, arrowheads in F, G and H). Bars: 0.1 µm.

FIG. 2. Distribution of different markers from A. tumefaciens on iodixanol gradients. Pyrophosphatase activity (A) is concentrated in a distinct dense fraction (fraction 12). This distribution was compared with that of the established plasma membrane marker, succinate cytochrome c reductase (B). A lower amount of pyrophosphatase activity co-localized with the plasma membrane marker. Closed diamonds in (H) indicates protein distribution in the different fractions and density distribution is shown in (G). P$_1$ (C), PP$_1$ (D) short (SC, E) and long chain (LC, F) polyPs are also concentrated in fraction 12 (volutin granule fraction).

FIG. 3. Electron microscopy (A), X-ray microanalysis (B, C) of the volutin granule fraction, and staining of volutin granules with LysoSensor (D), cycloprodigiosin (E), and DAPI (F). (A)
Direct observation of iodixanol fraction 12. Scale bar = 0.1 µm. (B) X-ray microanalysis spectrum of volutin granules in fraction 12. (C) X-ray microanalysis spectrum of background of fraction 12 preparation. (D-F) Cells were incubated with LysoSensor (D), cycloprodigiosin (E) or DAPI (negative image) (F) as described under Experimental Procedures and observed by fluorescence microscopy. Note the staining located at one pole of the cells (arrowheads). Bars = 0.5 µm. (G) X-ray microanalysis spectrum of volutin granules of bacteria incubated with 100 mM CaCl₂ for 16 h. (H) X-ray microanalysis spectrum of background of the same preparation.

FIG. 4. Initial rate of pyrophosphate hydrolysis as a function of pyrophosphate concentration (A), and medium pH (B), and effect of inhibitors (C-G). Aliquots of volutin granule fraction were added to a standard reaction mixture (18) (A and B) in the presence of increasing concentrations of pyrophosphate (A) or incubated in the standard reaction mixture adjusted to different pH values (18) (B). Error bars indicate SE of means from at least 3 separate experiments. Aliquots of volutin granules were added to the standard reaction mixture in the presence of increasing concentrations of AMDP (C), IDP (D), KF (E), DCCD (F) or N-ethylmaleimide (G). Percentual of inhibition compared to the control in the absence of inhibitors (100%) is indicated. Control activities were 0.69 ± 0.03 µmol PP_i consumed/min x mg protein. Error bars indicate S.E. of mean values from at least 3 separate experiments.

FIG. 5. Western blot analysis, immunofluorescence analysis, and immunoelectron microscopy of H⁺-PPase in A. tumefaciens. (A) CLUSTAL W alignment of the C-terminal region of putative H⁺-PPases from A. tumefaciens (GenBank accession number AAL42186); and T. cruzi (AF159881). Homologous residues are in yellow, conserved residues are in blue. (B) Detection of the H⁺-PPase by immunoblot, using polyclonal antibody against the T. cruzi H⁺-PPase. A. tumefaciens proteins (14 µg) were separated by SDS-PAGE and transferred to nitrocellulose. Left lane, immunoblot probed with antibody against the H⁺-PPase recognized a polypeptide of apparent molecular mass of 72 kDa. Right lane shows immunoblot probed with pre-
immune serum. (C) Confocal immunofluorescence analysis of H⁺-PPase in *A. tumefaciens*. Arrowheads show labeling at one pole of the cells. Confocal fluorescence image was overlaid on bright field image of the same cells. Inset shows greater magnification of one of the cells by fluorescence microscopy. Bars = 1 µm. (D) Immune electron microscopy of the cells. Labeling of a cytoplasmic vacuole (vg) is evident (arrowheads). Bar = 40 nm.
Seufferheld et al., Fig. 2
A. tumefaciens

641SGGAWDNALKKSFDGFI

T. cruzi

735NTGGAWDNALKYIEKGRLDKN

B

PPase

C

72 kDa

D

vg
Identification of organelles in bacteria similar to acidocalcisomes of unicellular eukaryotes

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J. Biol. Chem. published online June 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M304548200

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