Acidocalcisomes are acidic, calcium storage compartments with a H⁺ pump located in their membrane that have been described in several unicellular eukaryotes, including trypanosomatid and apicomplexan parasites, algae, and slime molds, and have also been found in the bacterium Agrobacterium tumefaciens. In this work, we report that the H⁺-pyrophosphatase (H⁻⁻PPase) of *Rhodospirillum rubrum*, the first enzyme of this type that was identified and thought to be localized only to chromatophore membranes, is predominantly located in acidocalcisomes. The identification of the acidocalcisomes of *R. rubrum* was carried out by using transmission electron microscopy, x-ray microanalysis, and immunofluorescence microscopy. Purification of acidocalcisomes using iodixanol gradients indicated co-localization of the H⁻⁻PPase with pyrophosphate (PPi) and short and long chain polyphosphates (polyPs) but a lack of markers of the plasma membrane. polyP was also present in the chromatophores (10, 11). H⁻⁻PPases are also present in the chromatophores (10, 11). H⁻⁻PPases may also be present in the plasma membranes of some plant cells (12, 13), as well as unicellular eukaryotes (14). The H⁻⁻PPase from the phototrophic bacterium *Rhodospirillum rubrum* was the first H⁻⁻PPase discovered (10, 11). This enzyme is unique in that it catalyzes not only the hydrolysis of PPi but also the synthesis of PPi in the light (15). Synthesis is driven by the energy derived from the electrochemical H⁺ gradient generated across the membrane of the chromatophores during illumination (15).

Acidocalcisomes have recently been found (9) to be morphologically similar to the volutin granules described in bacteria (16). Volutin or metachromatic granules were the first subcellular entities to be recognized in bacteria (16, 17). Because *R. rubrum* is known to possess volutin granules that accumulate PPi under illumination (18), we investigated whether the H⁻⁻PPase was also present in these organelles. In this report, we describe the isolation and biochemical properties of the acidocalcisomes of *R. rubrum* and show that, as with the acidocalcisomes of *A. tumefaciens* (9), they are surrounded by a membrane, are acidic because of the presence of the H⁻⁻PPase in their membrane, are rich in PPi and polyP, and are able to accumulate calcium and other elements. The number of acidocalcisomes as well as the amount of PPi and polyP significantly increase when the bacteria are grown in light. We also demonstrate that the H⁻⁻PPase is predominantly located in the acidocalcisomes of *R. rubrum*.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures—** *R. rubrum* cells (strain Esamarch, Molisch ATCC 17031) were obtained from the American Type Culture Collection. The cells were grown in liquid Sistrom succinate medium (19) with agitation (160 rpm) in the dark or anaerobically in the light (an intensity of 80 μmol photons/m² × s⁻¹) at 30 °C. The cells were cultured for 4 days and harvested at the stationary phase.

**Chemicals—** Dulbecco’s PBS and reagents for marker enzyme assays were purchased from Sigma. Silicon carbide (400 mesh) was bought from Aldrich. Iodixanol (40% solution; OptiPrep; Nycomed) was obtained from Invitrogen. Benzonase® was from Novagen (Wisconsin, MD). Cycloprodigiosin was a gift from Prof. Hajime Hirata (Himeji Institute of Technology, Hyogo, Japan). Polyonal antibodies raised against a keyhole limpet hemocyanin-conjugated synthetic peptide corresponding to the hydrophilic loop XII (antibody PABHK or 326) of plant V-H⁻⁻PPase (20) were kindly provided by Prof. Philip Rea (University of Pennsylvania, Philadelphia, PA). Aminomethylendiphosphonate (AMDP) was synthesized by Michael Martin (Department of Chemistry, University of Illinois at Urbana-Champaign). Monoclonal antibody...
against a keyhole limpet hemocyanin-conjugated synthetic peptide corresponding to the hydrophilic loop XII of Trypanosoma cruzi H⁺-PPase (21) was prepared at the University of Illinois Biotechnology Center. Molecular weight markers and Coomassie Blue protein assay reagent were from Bio-Rad. EnzChek phosphate assay kit and LysoSensor blue DND-167 (9,10-bis (N-morpholinomethyl) anthracene) were from Molecular Probes (Eugene, OR). Prof. Arthur Kornberg (Stanford University School of Medicine, Stanford, CA), kindly provided Escherichia coli strain CA38 pTrcPPX1. Prof. Mary Lynne Perille Collins (University of Wisconsin, Milwaukee, WI) provided a polyclonal antibody raised against crude membranes of phototrophic R. rubrum cells. All other reagents were of analytical grade.

Isolation of Acidocalcisomes—Bacteria were collected by centrifugation at 3,900 × g, and the pellet was resuspended in lysis buffer (125 mM sucrose, 50 mM KCl, 4 mM MgCl₂, 0.5 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-tosyl-l-lysine chloromethyl ketone, pH 7.2) containing 2 mg/ml lysozyme. Benzonase® (1 μl/ml) was added, and the bacteria then passed through a French press (SLM-Aminco, Spectrometric Instruments) twice at 1,000 p.s.i. The lysate was incubated on ice under agitation for 1 h with an equal volume of silica/silicon carbide (1:1) to remove DNA and RNA fragments. The lysate was then centrifuged at 50,000 × g in a Beckman SW 28 rotor for 60 min. The acidocalcisome fraction pelleted at the bottom of the tube and was resuspended in lysis buffer. Gradient fractions and markers were assayed as previously described (23).

Analytical Methods and Immunoblotting—Bacteria were washed once with Dulbeco’s PBS, and then PPi and long chain and short chain polyPs were extracted as described previously (24). Pyrophosphatase activity was assayed by measuring released phosphate using the EnzChek phosphate assay (23, 25). The apparent Km for PPi was calculated by using a nonlinear regression program (Sigma Plot 1.0, Jandel Scientific) using the Hill equation. Protein determination was carried out by using the Coomassie Blue protein assay reagent from Bio-Rad. The proteins were separated by SDS-PAGE using 10% gels and then blotted onto nitrocellulose using a Bio-Rad Transblot apparatus. Subsequent processing steps were performed in Dulbeco’s PBS containing 0.1% Tween 20. The blots were blocked for 1 h in 5% nonfat dry milk, washed three times, and then incubated with polyclonal antibody 326 against Arabidopsis H⁺-PPase (20) (1:1,000) for 1 h at room temperature. The blots were then washed three times, incubated for 1 h with horseradish peroxidase-labeled anti-rabbit IgG (1:20,000), washed three times, and processed for chemiluminescence detection as per the manufacturer’s instructions (Amersham Biosciences). Molecular weights were calculated using prestained molecular weight markers.

Immunofluorescence Microscopy—For subcellular localization of H⁺-pyrophosphatase in Rhodospirillum rubrum, visualization of acidocalcisomes in whole unfixed cells allowed to adhere to a Formvar- and carbon-coated grid and then observed in the transmission electron microscope. Large granules appear located at bending sites and smaller granules of varying sizes appear distributed in the cytosol (arrows). Bar, 0.5 μm. B, x-ray microanalysis of acidocalcisomes in whole cells. C, x-ray microanalysis of bacteria grown in calcium-rich medium. D, x-ray microanalysis of the background.
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% Nonidet P-40 in PBS for 10 min. The samples were blocked for 1 h with PBS containing 3% bovine serum albumin, 1% cold fish gelatin, and 50 mM NH₄Cl and were first incubated for 1 h at room temperature with the polyclonal antibody against the Arabidopsis thaliana H⁺-PPase (20) or monoclonal antibody against T. cruzi H⁺-PPase (21), diluted 1:50 (polyclonal) or 1:100 (monoclonal) in 1% cold fish gelatin. Bacteria were subsequently incubated for 60 min at room temperature with fluorescein-conjugated secondary antibody diluted 1:200 in PBS plus 1% cold fish gelatin. Coverslips were mounted on glass slides with Vectashield® media and sealed. The 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 using the fluorescence microscope (9).

For localization of LysoSensor blue DND-167, bacteria were centrifuged and resuspended in prewarmed (30 °C) Sistrom medium containing 1 μM LysoSensor. Bacteria were incubated for 1 h at 30 °C, centrifuged, and resuspended in fresh prewarmed Sistrom 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 Sistrom medium, but without cycloprodigiosin or LysoSensor, respectively, were used as controls.

For co-localization studies of chromatophore proteins and H⁺-PPase, polyclonal antibodies against crude membranes of phototrophic R. rubrum cells were adsorbed with R. rubrum grown aerobically in the dark,
to remove nonspecific antibodies (22). Cells grown aerobically were fixed with 4% formaldehyde for 1 h followed by permeabilization with 0.3% Triton X-100 in PBS for 10 min and then washed twice with PBS. The cells were then incubated with the antibodies against crude membranes for 45 min at 37 °C and centrifuged, and the antibody that was not adsorbed was collected from the supernatant. For co-localization studies, the cells were incubated 1 h with antibodies diluted 1:20 and with a mouse monoclonal antibody against T. cruzi H'-PPase, diluted 1:50. The cells were subsequently incubated for 60 min at room temperature with rabbit fluorescein-conjugated secondary antibody diluted 1:100 and with mouse rhodamine-conjugated secondary antibody diluted 1:200, with plus 1% cold fish gelatin. Control preparations were incubated with preimmune serum or without the primary antibody.

Three-dimensional Confocal Immunofluorescence Microscopy Reconstruction Analysis of H'-PPase Staining in R. rubrum—Volumetric renderings through a representative bacterial cell were compiled using the average projection ray tracing algorithm (26, 27) in the Olympus Fluoview software suite. Further image processing was conducted using the ImageJ software tools and Application Programming Interface originally developed by Wayne Rasband at the National Institutes of Health (rsb.info.nih.gov/ij/). The voxel signal intensities were normalized (28) to enable easier discrimination of relative pixel intensities, and a median filter (28, 29) was applied to correct image artifacts and a median filter (28, 29) was applied to correct image artifacts. Images were then transferred to a representative bacterial cell were compiled using the average projection ray tracing algorithm (26, 27) in the Olympus Fluoview software suite. Further image processing was conducted using the ImageJ software tools and Application Programming Interface originally developed by Wayne Rasband at the National Institutes of Health (rsb.info.nih.gov/ij/). The voxel signal intensities were normalized (28) to enable easier discrimination of relative pixel intensities, and a median filter (28, 29) was applied to correct image artifacts and a median filter (28, 29) was applied to correct image artifacts.

| Growth conditions | PP$_i$ (µmol/g protein) | Short chain polyP (µmol/g protein) | Long chain polyP (µmol/g protein) |
|-------------------|-------------------------|-----------------------------------|----------------------------------|
| Dark              | 127 ± 0.5$^a$           | 241 ± 0.6                         | 448 ± 0.3                        |
| Light             | 534 ± 0.2               | 356 ± 0.18                        | 508 ± 0.12                       |

$^a$ The values are the means ± S.D. (n = 3).

For imaging whole bacteria, 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 600 transmission electron microscope operating at 100 kV (9). Energy-dispersive x-ray analysis was done at the Electron Microscopy Center of Southern Illinois University (Carbondale, IL). The spectra shown are the ones that yielded the most counts in 100 s (of 10 spectra obtained from granules of different bacteria in each preparation), but all other spectra taken from aicidocalciosomes of the same preparation were qualitatively similar. 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 s by utilizing a thin window (Norvar) detector. Analysis was performed by using a Noran Voyager III analyzer with a standardless analysis identification program.

For immunocytochemistry, bacteria were washed twice with buffer A (116 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO$_4$, 50 mM Hepes, pH 7.2) and then extracted with ice-cold 0.5 M HClO$_4$ (2 ml/g wet weight cells). After 30 min of incubation on ice, the extracts were centrifuged at 3,000 × g for 5 min. The supernatants were neutralized by the addition of 0.72 M KOH to pH 8. All of the extracts contained 10% D$_2$O (v/v) to provide a field frequency lock.

NMR Spectroscopy—Phosphorus NMR spectra were acquired at 303.6 MHz using a Varian INOVA NMR spectrometer equipped with a 17.6 Tesla Oxford Instruments magnet. For perchloric acid extracts, 16,384 transients were collected at room temperature using 25-µs (90°) pulse excitation, 20-kHz spectral width, 32,768 data points, and a 5-s recycle time. Inverse-gated proton decoupling was used to remove nuclear Overhauser effect and J-coupling effects. The chemical shifts of all $^{31}$P spectra were referenced to 0 ppm using an 85% phosphoric acid external reference (33). The specific assignments of individual resonances were initially based on published chemical shifts and $^{31}$P-$^{31}$P scalar couplings (33). NMR spectra were processed using the VNMR.
RESULTS

Acidocalcisomes are recognizable by their strong electron density when observed by electron microscopy in unfixed and unstained whole cell mounts (6). Using this technique, R. rubrum grown in the dark showed both large and small granules, in different locations of the cells (Fig. 1A, arrows). The large granules have a diameter of ~205 ± 24 nm. X-ray microanalyses were performed on these granules, and a representative spectrum (Fig. 1B) shows that the counts for phosphorus were ~3-fold greater than the counts for magnesium, which were approximately the same as the counts for potassium. The counts for oxygen and phosphorus were approximately the same. The medium used to grow R. rubrum in the previous experiments was calcium-deficient, which might explain the lack of detectable levels of calcium in the x-ray microanalyses of the acidocalcisomes (Fig. 1B). To test this idea, we therefore next cultivated bacteria in the presence of 100 mM CaCl₂ for 4 h before preparing them for x-ray microanalysis. Fig. 1C shows that there is a dramatic increase in the counts for calcium as well as a decrease in the counts for magnesium and potassium in the acidocalcisomes of these cells. The presence of these elements was not detected in spectra taken from the background (Fig. 1D) and demonstrates the ability of acidocalcisomes to accumulate calcium. Peaks for copper and in part for carbon arise from the grid.

Examination of cells in thin sections showed round vacuoles of ~200 nm in cells grown both in the light (Fig. 2A, arrowhead) and in the dark (Fig. 2B, arrowhead), clearly different from the chromatophore membranes detected in cells grown in the light (Fig. 2A). As is characteristic of the morphology of acidocalcisomes (6), an electron-dense ring was observed surrounding the apparently empty vacuoles (Fig. 2A, arrowhead). Each intracellular vacuole appeared to be surrounded by a membrane (Fig. 2B, arrowhead). R. rubrum acidocalcisomes (Fig. 2C) showed a sponge-like appearance that is also typical of acidocalcisomes in different organisms (6–9).

We purified the acidocalcisomes following a purification procedure used for the isolation of acidocalcisomes from A. tumefaciens (9). The distribution of different phosphorus-containing compounds and a plasma membrane marker was compared with that of the established marker for acidocalcisomes, H⁺-PPase (Fig. 3). The aminomethylendiphosphonate-sensitive pyrophosphatase activity peaks in two different regions along the gradient. Observation of these fractions by electron microscopy showed that the only fraction containing acidocalcisomes was fraction 12. Fraction 6 was rich in cell ghosts and membranes. The succinate cytochrome c reductase, used as a plasma membrane marker, peaks together with the second peak, having pyrophosphatase activity.

R. rubrum contains considerable levels of long and short chain polyP, as measured by determining degradation of polyP with recombinant yeast exopolyphosphatase (24), as well as PP₇₀ (Table I). The acidocalcisome fraction (fraction 12) contained significant amounts (20 and 35%, respectively) of short and long chain polyP, together with considerable amounts of P₇₀ and PP₇₀ (25 and 30%, respectively). These results suggest preferential acidocalcisomal localization of these compounds.

Fig. 4 shows the 303.6 MHz (1H decoupled) ³¹P NMR spectra of R. rubrum grown under light (Fig. 4A) and dark (Fig. 4B) conditions. Resonance assignments for these spectra are given in Table II. The dominant peak in all spectra is inorganic phosphate (peak A). The insets of Fig. 4 are the upfield regions of the spectra (2.5 to ~22.5 ppm) magnified 20X relative to the overall spectra. The region between ~4 and ~6.5 ppm contains peaks for the terminal phosphates of nucleotide di- and triphosphates (peaks D and C, respectively) in addition to a peak for the terminal phosphates of polynucleotides (peak B) and a peak for pyrophosphate (peak E). The region from ~9 to ~11 ppm contains peaks for the α-phosphates of nucleotide di- and triphosphate (peaks F and G, respectively) as well as peaks for NAD (peak H). The most upfield parts of the spectra contain peaks from the β-phosphate of nucleotide triphosphate (peak J) and peaks for the β-phosphates of tri- and tetrapolyphosphates (peak I). The main difference between the bacteria grown under light and dark conditions is that those grown in the dark contain less nucleotide triphosphates (peak J), pyrophosphate (peak E), and polynucleotides (peaks B and I).

To confirm the acidocalcisomal localization of polyP, we detected it using DAPI, which as reported previously, can be used
for the cytochemical detection of this inorganic polymer (24). Fig. 5A shows labeling of acidocalcisomes with DAPI. As occurs with acidocalcisomes from *A. tumefaciens* (9), and other organisms (6), the organelles from *R. rubrum* could also accumulate dyes used to detect acidic compartments, such as Lysosensor Blue DND-167 (Fig. 5B) and cycloprodigiosin (Fig. 5C). Cycloprodigiosin is a compound isolated from a marine bacterium that has been shown to uncouple H⁺/H11001-PPase activity acting as a chloride/proton symport (34) and has been shown to stain acidocalcisomes of *T. cruzi* (23). Based on the positive staining with Lysosensor blue DND-167, which has been used to detect very acidic intracellular compartments (35), we can estimate a very low pH in *R. rubrum* acidocalcisomes (pH ~5–5.5).

Acidification of acidocalcisomes takes place in most organisms by the action of a membrane-bound H⁺-PPase (6). The pyrophosphatase activity detected in the acidocalcisome fraction of *R. rubrum* (Fig. 6A), as measured by inorganic phosphate detection (25), was 0.73 ± 0.04 μmol of pyrophosphate consumed/min × mg protein (means ± S.E. of results from three separate experiments) and was partially (60%) inhibited by 20–50 μM AMDP, a specific inhibitor of H⁺-PPases (36). The lack of complete inhibition suggests the presence of another pyrophosphatase activity in the acidocalcisome fraction. In this regard, it has recently been reported that *Trypanosoma brucei*
Acidocalcisomes possess an inorganic pyrophosphatase, in addition to the H⁺-PPase (37). The presence of a soluble pyrophosphatase in *R. rubrum* has also been reported (38). The dependence of the initial rate of hydrolysis on pyrophosphate concentration in *R. rubrum* acidocalcisome fractions is shown in Fig. 6B. Activity was maximal at ~60 μM pyrophosphate with an apparent *Kₘ* of 17.8 μM. Fig. 6C shows the effect of pH on the initial rate of pyrophosphate hydrolysis in the *R. rubrum* acidocalcisome fraction. Activity was optimal at pH 7.5–8.0, and potassium-insensitive as reported previously (39). Pyrophosphate hydrolysis of the acidocalcisome fraction was inhibited, in a dose-dependent manner, by the pyrophosphate analog IDP (Fig. 6D). Fig. 6 also shows that dicyclohexylcarbodiimide (DCCD) (Fig. 6E) and the thiol reagent N-ethylmaleimide (Fig. 6F) were also effective in inhibiting the *R. rubrum* pyrophosphatase activity in a dose-dependent manner, as has been reported before in other studies of this enzyme (39). Taken together, these results indicate that the pyrophosphatase activity identified in acidocalcisomes has similar characteristics to the H⁺-PPase activity detected previously in *R. rubrum*.

We also investigated the localization of the H⁺-PPase by immunocytochemistry using an antibody (20) against a peptide of *A. thaliana* H⁺-PPase (326) that is conserved in the C-terminal region of the *R. rubrum* sequence available in GenBank™ (accession number AAC38615; Fig. 7A). This antibody showed cross-reactivity with a band of 65 kDa present in the *R. rubrum* acidocalcisome fraction (Fig. 7B). No background staining was observed when preimmune serum was used as a control (data not shown). Immunofluorescence microscopy using these antibodies resulted in staining of acidocalcisomes.
(Fig. 7, C–E, arrows and arrowhead), at the same position where the organelles were located by direct transmission electron microscopy (Fig. 1A) or by LysoSensor (Fig. 5B), cyclopro- digiosin A (Fig. 5C), and DAPI (Fig. 5A) staining. These results are in agreement with the co-localization of polyP and the pyrophosphatase in the acidocalcisomes as assayed biochemically (Fig. 3). Immunoelectron microscopy confirmed labeling in vacuoles with an “empty” appearance (Fig. 7F, arrowheads). Volumetric rendering of the image shown in Fig. 7C showed the predominant acidocalcisome localization of the H^+—PPase (see movie in the supplemental material).

When R. rubrum cells were grown in the light (Fig. 8B), there were more acidocalcisomes detected by staining with monoclonal antibodies against the T. cruzi H^+—PPase than found in cells grown in the dark (Fig. 8A). There were 3.3 ± 0.2 acidocalcisomes/cell under light conditions and 2.1 ± 0.1 acidocalcisomes/cell under dark conditions (n = 49; p < 0.05). Subcellular fractionation of R. rubrum grown in the light showed a similar distribution of P_0, PP, and short and long chain polyP (Fig. 9) as shown in cells grown in the dark (Fig. 3), except that lower percentages of PP, and short chain polyP were detected in the acidocalcisomal fractions (fraction 15 in these gradients).

R. rubrum H^+—PPase was originally detected in chromatophores obtained after sonication and differential centrifuga-
tion. However, immunolocalization studies to determine its localization in the chromatophore membranes have not been reported previously. We therefore investigated whether the H^+—PPase co-localized with the photosynthetic apparatus of R. rubrum that is known to be located in the plasma and chromatophore membranes of the bacterium. As a marker of the photosynthetic apparatus, we incubated bacteria grown in the dark with antibodies developed against whole bacteria grown in the light to adsorb nonspecific antibodies (22) and then used the supernatants as specific antibodies against the photosynthetic apparatus in co-localization studies. Fig. 10A shows that antibodies against the H^+—PPase stain predominantly the acidocalcisomes with a diffuse staining of the cytosol. An increase in the concentration of the antibody did not increase the staining of the plasma membrane. Antibodies against the photosynthetic apparatus stain mainly the plasma membrane and regions adjacent to it (Fig. 10B). A yellowish color in the plasma membrane of overlays suggested some co-localization, although most structures showed distinct staining (Fig. 10C).

**DISCUSSION**

The H^+—PP synthase/H^+—PPase was first described in chromatophores from R. rubrum (10, 11) where it was shown to
catalyze light-induced formation of PPi from Pi, or the reverse hydrolysis of PPi to Pi. Proton movement, which was induced by light in chromatophores (40), was shown by addition of PPi in the dark to be linked also to the H+-PPase (41).

Chromatophores are a particular fraction obtained after sonication of several purple bacteria (42). Their counterparts in the intact bacteria are known as chromatophore membranes. The apparent continuity between the chromatophore and plasma membranes has led to the hypothesis that chromatophore membranes are formed by invagination of the plasma membrane (22), and this has been supported by freeze etching studies in _R. rubrum_ (43). Interestingly, freeze etching studies of _R. rubrum_ (44) have also revealed the presence of large membrane-bound vesicles very similar in size to the acidocalcisomes described in this work (Fig. 2). Because the H+-PPase was found in chromatophores, it was argued that the enzyme physiologically pumps protons across the plasma membrane and that the plasma membrane is its usual environment (15, 38). Our results indicate that the H+-PPase of _R. rubrum_ is predominantly located in an intracellular compartment, similar to the acidocalcisome of _A. tumefaciens_ (9) and unicellular eukaryotes (6), where it might be responsible for its acidification. These results also provide further evidence for the presence of acidocalcisomes, defined as acidic organelles rich in PPi, polyP, calcium, and other elements, in another prokaryote distantantly related to _A. tumefaciens_. Although our results provide strong evidence for a predominant acidocalisomal location of the H+-PPase, previous biochemical experiments have shown that the H+-PPase is able to synthesize PPi when an electrochemical H+ gradient is formed across the chromatophore membrane upon illumination (10, 15), providing support for an additional plasma membrane/chromatophore membrane location. This is a similar situation to what has been described in eukaryotes, where the H+-PPase is present in several locations, such as, for example, vacuolar and plasma membranes of pea cotyledons (13) and contractile vacuole (7, 46) and acidocalcisomes (7) of _C. reinhardtii_. However, the H+-PPase could be used as a marker of acidocalcisomes, not because is absent in other locations but because it is highly concentrated in them (6).

The results presented in this work are also in agreement with a recent report (47) that showed a higher expression of the H+-PPase in cells grown anaerobically in the light than in cells grown in the dark in aerobic cultures not subjected to intense agitation.

Because _R. rubrum_ apparently possesses only one gene for the H+-PPase (48), our results invalidate the distinction between vacuolar (V-H+-PPase) and nonvacuolar (H+-PPase) enzymes based solely on their localization in vacuoles (tonoplasts and acidocalcisomes) or bacterial plasma membranes (49). A more appropriate classification based on phylogenetic analyses of a large set of H+-PPase sequences separates H+-PPases into K+-dependent and K+-independent (39, 50–52) forms. _R. rubrum_ and _A. tumefaciens_ (9) H+-PPases are K+-independent enzymes, and as with the enzyme from _A. tumefaciens_ (9), the H+-PPase from _R. rubrum_ is also sensitive to inhibition by aminomethylendiphosphonate, imidodiphosphate, DCCD, and N-ethylmaleimide but has only a low sensitivity to the soluble PPase inhibitor, fluoride (Fig. 5 and Ref. 39).

It has been reported that in _rbrum_ PP, can be as high as 43 nm in illuminated cells and that most of this PP is in the form of granules (18, 45). In agreement with these results, we detected larger quantities of PP, and short chain polyP (Table 1) and a larger number of acidocalcisomes (Fig. 8) in illuminated cells.

In conclusion, the H+-PPase of _R. rubrum_ is located not only in the plasma membrane where it can function in PPi synthesis but also in the acidocalcisomes where it can act as a proton pump resulting in their acidification.
H⁺-pyrophosphatase in Rhodospirillum rubrum

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