

Purification and Characterization of the Ca^{2+} -ATPase of *Flavobacterium odoratum**

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The P-type Ca^{2+} -ATPase from *Flavobacterium odoratum* has been purified to homogeneity and characterized. Inside-out membrane vesicles were extracted with C_{12}E_8 , followed by ammonium sulfate fractionation, centrifugation through two successive 32–48% glycerol gradients, and DE52 ion exchange chromatography. The purified Ca^{2+} -ATPase consists of a single polypeptide. It migrates electrophoretically with an apparent molecular mass of 60,000 Da, consistent with the phosphorylation pattern originally reported in membrane vesicles. This single polypeptide is functional and capable of calcium-dependent vanadate-sensitive ATP hydrolysis and of forward and reverse phosphorylation. Maximum hydrolysis activity occurs at pH 8.0, with a specific activity of $\sim 75 \mu\text{mol}$ of ATP hydrolyzed $\text{min}^{-1} \text{mg}^{-1}$ protein. The purified Ca^{2+} -ATPase has an apparent K_m for calcium of $1.5 \mu\text{M}$ and for ATP of $90 \mu\text{M}$. Vanadate strongly inhibits the activity with an IC_{50} of $0.6 \mu\text{M}$. The prokaryotic Ca^{2+} -ATPase is insensitive to the SR Ca^{2+} -ATPase inhibitors fluorescein isothiocyanate, thapsigargin, and cyclopiazonic acid. It is rapidly phosphorylated by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in a calcium-dependent vanadate-inhibited manner and can be phosphorylated by P_i in both the presence and absence of calcium.

In eukaryotic cells, the utilization of calcium in signal transduction requires tight regulation of free calcium concentration. Maintenance of low calcium is achieved by both ATPases and antiporters. The Ca^{2+} -ATPase of sarcoplasmic reticulum has been studied in the greatest detail and becomes the model for the analysis of structure-function relationships in ion transport. However, for the vast majority of bacterial species examined, Ca^{2+} transport is carried out solely via secondary transport, by $\text{Ca}^{2+}/\text{H}^+$ or $\text{Ca}^{2+}/\text{Na}^+$ antiporters (1, 2). To date, ATP-driven vanadate-sensitive calcium accumulation has been identified only in (a) three species of Gram-positive bacteria, *Enterococcus* (3–5), (b) the Cyanobacterial species *Anabaena variabilis* and *Synechococcus* sp. (6, 7), and (c) the Gram-negative bacteria *Flavobacterium odoratum* (8, 9). In the former cases, either low activity or failure to stably extract from the membrane has precluded purification and further study of the ATPases. However, the abundance of stable detergent-extractable Ca^{2+} -ATPase activity in *F. odoratum* has allowed it to be the most completely characterized of the prokaryotic Ca^{2+} -ATPases (10). Its K_m for Ca^{2+} and ATP and IC_{50} for vanadate

are very similar to that of the sarcoplasmic reticulum Ca^{2+} -ATPase (8). The enzyme is rapidly phosphorylated by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in a calcium-dependent manner. Both the forward and reverse reaction cycle intermediates have been examined. Although the prokaryotic pump is functionally similar to its eukaryotic counterpart, the forward limb of its reaction cycle is mechanistically distinct. Unlike the eukaryotic P-type ATPases, the forward reaction of the prokaryotic Ca^{2+} -ATPase appears to be ordered, and the high affinity Ca^{2+} binding site is not accessible until ATP binds (9, 10).

Clearly, comparison of the molecular mechanism of the well characterized eukaryotic P-type ATPases should provide extremely valuable information concerning both evolution and function. Only two prokaryotic P-type ATPases have been purified to homogeneity. The best characterized is the high affinity potassium pump (Kdp-ATPase) of *Escherichia coli* (11). The Kdp-ATPase consists of three subunits of 60,000, 72,000, and 20,000 Da (12, 13). Preliminary evidence suggests that the functional complex is oligomeric with respect to the subunits ($\text{A}_2\text{B}_2\text{C}_2$), however it is subunit B that bears homology to the eukaryotic ATPase-conserved sequences and that demonstrates a phosphorylated intermediate. The second purified pump is a K^+ -ATPase that has been isolated from the Gram-negative bacterium *Enterococcus hirae* as a single polypeptide with an M_r of 78,000 (14).

In this report, we describe the purification of the *F. odoratum* Ca^{2+} -ATPase to homogeneity. By monitoring calcium-dependent ATP turnover and the formation of an alkaline-labile phosphointermediate, a purification factor of 1800 was achieved, using glycerol gradient centrifugations and column chromatography. The ATPase is a single polypeptide with an apparent M_r of 60,000. The isolated enzyme is very stable and capable of forming an alkaline-labile phosphointermediate in the presence of either ATP or inorganic P_i . It demonstrates kinetics of hydrolysis similar to the SR Ca^{2+} -ATPase and is the smallest P-type ATPase as described to date.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were obtained from Sigma (reagent grade) except as noted. C_{12}E_8 and DCCD were obtained from CalBiochem. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (6000 Ci/mmol), $^{45}\text{CaCl}_2$ (136 Ci/mmol), and $[\text{P}^{32}]\text{orthophosphate}$ (9120 Ci/mmol) were obtained from DuPont NEN. All ATP solutions were made up at pH 7.0, aliquoted, and stored at -20°C as 100 mM stocks and discarded after first thawing. Pyruvate kinase/lactate dehydrogenase mixture (450 units/ml) was obtained from Boehringer Mannheim. Buffers for K_m determinations were made up in high pressure liquid chromatography-grade water (Fisher).

Bacterial Strains and Growth Conditions

F. odoratum strain (ATCC 29979) was obtained from ATCC and was grown aerobically in Luria Broth (15) at 37°C and harvested at mid-log phase.

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Purification of the Ca^{2+} -ATPase

Preparation of Membrane Vesicles—Membrane vesicles were prepared by resuspending mid-log cells at an approximate concentration of 1 gram of bacteria, wet weight, per ml of ice-cold buffer A (20 mM MOPS,¹ pH 7.5, 100 mM KCl, 0.5 mM MgCl_2 , 1 mM dithiothreitol) to which was added 100 μM phenylmethylsulfonyl fluoride and 1.0 $\mu\text{g}/\text{ml}$ DNase. Cells were broken by passage through an Aminco French pressure cell at 20,000 psi producing high pressure vesicles, and unbroken cells and debris were pelleted by centrifugation at $27,000 \times g$ for 30 min. The supernatant was removed, and membrane vesicles were recovered by ultracentrifugation at $200,000 \times g$ for 90 min. The pellet was resuspended in buffer A to a concentration of approximately 20 mg of protein/ml and rapidly frozen in liquid nitrogen.

Solubilization and Ammonium Sulfate Fractionation—High pressure membrane vesicles were rapidly thawed and then extracted with 2% C_{12}E_8 on ice for 60 min and then centrifuged at $360,000 \times g$ for 60 min at 4°C . The supernatant was precipitated with increasing concentrations of ammonium sulfate. Ammonium sulfate was added to bring to a concentration of 35% of saturation, incubated on ice for 20 min, then centrifuged $10,000 \times g$ for 20 min (all ammonium sulfate concentrations given as % of saturation). The supernatant was carefully removed without disturbing the floating precipitate, and then ammonium sulfate was added to 55% saturation. After centrifugation at $10,000 \times g$ for 20 min, the calcium-dependent ATPase activity was recovered in the supernatant. Ammonium sulfate was again added to a final concentration of 65% saturation, and the solution was centrifuged at $10,000 \times g$ for 20 min. The supernatant, which contained the Ca^{2+} -ATPase, was carefully pipetted into a fresh centrifuge tube and brought to 90% ammonium sulfate and centrifuged ($10,000 \times g$ for 20 min). The 90% precipitate was carefully separated and resuspended in buffer A at 2 mg of protein/ml and dialyzed overnight against 1 liter of ice-cold buffer A. Calcium-dependent activity was present in the 65–90% pellet with essentially all of the calcium-independent activity remaining in the 35–55% and 55–65% ammonium sulfate cuts.

Glycerol Density Gradients—Aliquots of the resuspended, dialyzed 65–90% pellet were layered over a linear gradient of 32–48% (v/v) glycerol in buffer A and centrifuged at $180,000 \times g$ for 8 h at 4°C . 1-ml fractions were collected from the bottom of the tube and assayed for protein content and ATPase activity. The peak fractions were concentrated and dialyzed against buffer A with a Centricon 30 (Amicon) small volume desalting concentrator and layered on a second 32–48% (v/v) glycerol gradient in buffer A and centrifuged as for the first gradient. 1-ml fractions were again collected and assayed for ATPase activity and protein concentration.

DE52 Chromatography—The active fractions were pooled and applied to a Whatman DE52 anion exchange column (1.5×10 cm) equilibrated with buffer A. The column was then washed with five column volumes of buffer A. Bound protein was eluted with a step gradient of KCl in buffer A starting with 100 mM KCl for two column volumes followed by 150, 200, and 250 mM KCl elution steps. The Ca^{2+} -ATPase was eluted with 200 mM KCl. Aliquots of the fractions were assayed for calcium-dependent vanadate-sensitive ATPase activity and protein concentration, subject to SDS-PAGE (16), and then silver stained. Fractions with highest activity were pooled, concentrated and dialyzed using a Centricon 30 (Amicon) concentrator, and stored at -70°C without any appreciable loss of activity for at least a year.

ATP Hydrolysis—ATPase hydrolysis for all assays except nucleotide specificity was performed as described (17) with the following modifications. Membrane vesicles, partially purified fraction or the purified enzyme fraction were incubated in 45 μl of buffer A with either 0.5 mM CaCl_2 (calcium buffer) or 2 mM EGTA (EGTA buffer) for 10 min. The reaction was initiated with 5 μl of 1 mM $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (final concentration 100 μM at 100–200 cpm/pmol). After 10 s, the reaction was terminated by the addition of 150 μl of 30% trichloroacetic acid, 1 mM K_2HPO_4 and placed on ice. Inorganic phosphate (P_i) was extracted by the addition of 400 μl of 20 mM ammonium molybdate and 400 μl of isopropyl acetate. The sample was vortexed for 7 s and centrifuged at $2000 \times g$ for 15 s. One-third of the upper portion (organic phase) was carefully removed, and the radioactivity was measured by liquid scintillation counting. Blanks were assayed as described above except that no protein was added. Addition of trichloroacetic acid to vesicles prior to addition of ATP yielded P_i values equal to the blanks. Blank values were subtracted from the total counts, and total hydrolysis was calculated using

the specific activity of the ATP mixture. Calcium-dependent hydrolysis was taken as the difference between the hydrolysis rate obtained in calcium buffer minus the rate obtained in EGTA buffer after both are corrected for the blank. A time course for hydrolysis (assayed from 1 s to 20 min) indicated that the reaction rate was linear through 10 s; therefore, this value was used for all subsequent studies.

The apparent K_m for Ca^{2+} and ATP and the IC_{50} for vanadate were determined for the purified Ca^{2+} -ATPase. For the determination of the K_m for calcium, the free calcium concentration in the reaction buffer was adjusted using calcium-EGTA mixtures according to the affinity constants calculated by Fabiato and Fabiato (18). Hydrolysis at each concentration was assayed in triplicate, and the experiment was performed three times with independently isolated purified Ca^{2+} -ATPase. All water used in buffers was high pressure liquid chromatography grade. For determination of the K_m for ATP, the final concentration of ATP in the reaction was adjusted by varying the amount of cold ATP used to make the ATP mixture. For each concentration, hydrolysis was assayed in triplicate, and the specific activity of the ATP was calculated and a new set of blanks obtained. The values plotted are the average of three independent experiments. The total ATP hydrolyzed in the assays did not exceed 6% of the total amount of substrate present.

Rapid Mix-Quench Experiments—Millisecond reactions were done with a KinTek Instruments quench-flow apparatus, which uses a step motor to push down on syringes, forcing equal volumes of the reactants to rapidly mix. The mixed reactants then travel through a reaction loop made of narrow tubing. Quench travels through separate tubing. Eventually, the reaction tubing interconnects to the quench tubing where the reaction is stopped. By varying the distance of the reaction loops and the speed of the step motor, time points from 2 ms up to several seconds can be reliably done. Final conditions were 20 mM MOPS, pH 7.0, 100 mM KCl, 1 mM MgSO_4 , 50 μM CaCl_2 , 41.6 ng/ μl purified *F. odoratum* Ca^{2+} -ATPase, and ATP concentrations as noted. Syringe A contained 50 μM CaCl_2 and 83.3 ng/ml purified *F. odoratum* Ca^{2+} -ATPase. Syringe B contained 50 μM CaCl_2 and twice the concentration of ATP as noted. The reaction was quenched with 35% trichloroacetic acid in syringe C at 6 ms for ATP concentration of 1 μM , 4 ms for ATP concentrations between 25 and 50 μM , 3 ms for ATP concentrations between 75 and 150 μM [R₁] AND AT 2 MS FOR ATP CONCENTRATIONS ABOVE 150 μM . All reactions were done at 8°C . The samples were centrifuged at $11,000 \times g$. The pellets were resuspended in loading buffer and analyzed by SDS-PAGE (16). The bands were visualized by autoradiography, cut, and then counted.

 Ca^{2+} Dependence

Coupled Enzyme Technique—ATPase activity was measured using the coupled enzyme assay to determine nucleotide specificity following a modified protocol as described (19). Final reaction concentrations were 0.2 mM NADH, 0.5 mM CaCl_2 , 2 mM phosphoenolpyruvate with 15 units of lactate dehydrogenase, 15 units of pyruvate kinase, and 100 ng of purified protein per ml of buffer A. Nucleotide was added to a final concentration of 0.1 mM. NADH oxidation was monitored spectrophotometrically at 340 nm.

ATP Phosphorylation—Purified protein was incubated in 50 μl of calcium buffer or EGTA buffer on ice for 10 min. Reactions were started by the addition of 5 μl of 100 μM ATP [$\gamma\text{-}^{32}\text{P}$]ATP (1000–2000 cpm/pmol) and terminated 5 s later by the addition of 750 μl of 10% trichloroacetic acid. Samples were centrifuged at $12,000 \times g$ for 5 min, and the pellet was solubilized in 20 μl of Sarkadi loading buffer (20) for 20 min at 65°C and then sonicated. The samples were analyzed by SDS-PAGE, stained with Coomassie Blue, dried, and visualized using a phosphor-imaging system (Molecular Dynamics).

P_i Phosphorylation—Purified protein was incubated in 50 μl of calcium buffer or EGTA buffer at room temperature for 10 min. Reactions were started by the addition of 10 μl of [^{32}P]orthophosphate (2 μM , final concentration, 912 Ci/mM) and terminated 5 s later by the addition of 750 μl of 5% trichloroacetic acid, 5 mM potassium P_i . Samples were centrifuged at $12,000 \times g$ for 5 min, and the pellet was solubilized in 20 μl of Sarkadi loading buffer (20) for 20 min at 65°C and then sonicated. The samples were analyzed as described for ATP phosphorylation.

Protein Assays—Protein concentrations were determined by the method of Bradford (21) as modified by Bio-Rad.

NH_2 -terminal Sequencing—All sequencing was done by Drs. Chang Su and Yu Ching Pan (the Department of Protein Biochemistry, Roche Research Center, Nutley, NJ) and by Midwest Analytical Inc. (St. Louis, MO). Purified protein was either sequenced directly or the purified sample blotted from gels onto polyvinylidene difluoride membranes. After staining with Coomassie Brilliant Blue R-25, the band was cut out

¹ The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; AMP-PNP, 5'-adenylyl- β , γ -imidodiphosphate.

TABLE I
Summary of purification

Step	ATPase activity ^a	Total protein	Yield	Purification factor
	nmol/mg/min	mg	%	
Membranes	23.49	1237.50	100.00	1.00
C_{12}E_8 detergent extraction	69.33	245.60	58.58	2.95
Ammonium sulfate fractionation	729.70	19.20	48.20	31.06
Centrifugation by glycerol gradients	4086.24	1.73	24.32	173.96
DE52 ion exchange chromatography	43,754.86	0.06	9.03	1862.70

^a Activity assay conditions were 0.1 M KCl, 20 mM MOPS (pH 8), 0.5 mM MgCl_2 , 0.1 mM ATP, 0.5 mM CaCl_2 .

and sequenced with a gas phase sequencer (model 47A, ABI, Foster City) with an on-line PDH amino acid analyzer (model 120A ABI).

RESULTS

Inside-out membrane vesicles from *F. odoratum* contain at least three distinct ATPases. Approximately 25% of the ATPase activity is sensitive to DCCD and oligomycin but not vanadate and appears to be from a F_1 -type H^+ -ATPase (8). The remainder of the activity is refractory to inhibition by DCCD and oligomycin but is sensitive to micromolar amounts of vanadate. The calcium-dependent vanadate-sensitive ATPase activity makes up the majority of this activity (greater than 50% of the total membrane ATPase activity). The remaining calcium-independent vanadate-sensitive ATPase activity can be ascribed to one or more other P-type ATPases. Thus, the calcium-dependent ATPase activity can be easily detected, which made it amenable to isolation.

Several detergents were tested for their ability to solubilize the *F. odoratum* Ca^{2+} -ATPase. The non-ionic detergent C_{12}E_8 was best, and detergents such as Triton X-100, deoxycholate, cholate, and octyl glucoside were much less effective. A summary of the purification scheme is shown in Table I. Treatment of membrane vesicles with 2% C_{12}E_8 preferentially solubilizes the calcium-dependent hydrolysis activity, leaving most of the vanadate-sensitive calcium-independent activity and 50% of the DCCD-sensitive F_1 -ATPase activity in the detergent-extracted pellet. The weight ratio of the detergent to protein is optimized at 2% C_{12}E_8 with 18 mg/ml membrane protein. This resulted in the solubilization of 50–70% of the calcium-dependent vanadate-sensitive ATPase with a 2–3-fold purification. After solubilization, proteins in the detergent extract can be sequentially precipitated with increasing amounts of ammonium sulfate. All of the F_1 -ATPase activity and 20% of the calcium-independent P-type ATPase activity is precipitated at 55% ammonium sulfate. At 65% ammonium sulfate saturation, the remainder of the calcium-independent activity is precipitated. The fraction precipitated at 90% saturation contains >95% calcium-dependent activity, which is completely abolished by vanadate. This yields a fraction with a specific activity of 729 nmol of ATP hydrolyzed $\text{min}^{-1} \text{mg}^{-1}$ protein, a 30-fold purification over that in ISO membrane vesicles and represents a little less than 50% of the Ca^{2+} -ATPase activity in the membrane vesicles. This was then layered over a 32–48% glycerol gradient in buffer A containing no additional C_{12}E_8 and centrifuged $180,000 \times g$ for 8 h at 4 °C. The active fractions were pooled, concentrated, dialyzed against buffer A, and then applied to a second 32–48% glycerol gradient and centrifuged as described above. The active fractions were pooled and applied directly to a DE52 column. Elution is achieved with a KCl step gradient of 50 mM steps from 100 to 250 mM. The Ca^{2+} -ATPase activity is eluted upon the addition of 200 mM KCl. The purified Ca^{2+} -ATPase consists of a single polypeptide component of M_r 60,000 that represents at least 95% of the total protein in the final fraction (Fig. 1, lane 4). The NH_2 -terminal amino acid sequence was determined by Yu Ching Pan (Roche Research Center, Nutley, NJ) and Midwest Analytical, Inc. from both the

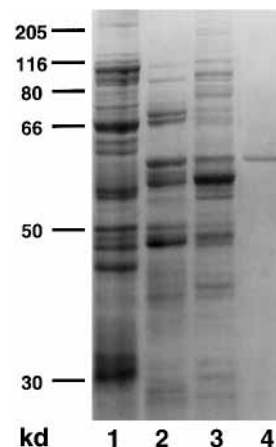


FIG. 1. SDS-PAGE of the active fractions from various purification steps. Active fractions of Ca^{2+} -activated vanadate-sensitive ATPase were dissociated and resolved by SDS-PAGE. The gel was silver stained. Lane 1, 90% ammonium sulfate pellet. Lane 2, first glycerol density gradient fraction. Lane 3, second glycerol density gradient fraction. Lane 4, peak fraction from DE52 chromatography.

purified preparation and the 60,000-Da PAGE isolated band blotted onto polyvinylidene difluoride. This was to ensure that there were no other proteins present that were not detected by Coomassie Brilliant Blue R-25 or silver staining. As expected, both preparations yielded identical sequences and showed there was no significant contamination from other copurified polypeptides. The first 22 residues of the prokaryotic Ca^{2+} -ATPase are NH_2 -SEKLEKPKLVVGLVVDQMRWDY. The absence of an NH_2 -terminal methionine raises the distinct possibility that the prokaryotic pump may have a leader sequence.

The purified Ca^{2+} -ATPase exhibits a maximal rate of ATP hydrolysis at pH 8.0 and is active over a narrow pH range (data not shown). The enzyme loses 50% of its activity at pH 7.0 or 8.5 and is at less than 20% of its maximal rate at pH 6.5. It has virtually no activity at pH 9.0. The best substrate is MgATP with a K_m of 90 μM (Fig. 2) and a V_{max} of $\sim 75 \mu\text{mol min}^{-1} \text{mg}^{-1}$. The kinetics of hydrolysis of the purified Ca^{2+} -ATPase as a function of [ATP] appears to be simple (hyperbolic) and saturable. The V_{max} of the purified prokaryotic enzyme appears to be approximately 10-fold greater than that of the purified SR Ca^{2+} -ATPase (22). There is no detectable modulatory effect of ATP on enzyme activity when examining steady state ATP hydrolysis. Modulatory effects of ATP on the purified calcium pump, if present, might be detectable using rapid-quench flow techniques, which permit the resolution of individual steps in the reaction mechanism. Fig. 3 shows a Hanes-Woolf plot of phosphointermediate ($\text{E} \sim \text{P}$) formation as a function of the concentration of MgATP in the range of 0.5–500 μM . Rates of $\text{E} \sim \text{P}$ formation were calculated at 2-ms time points to reflect true initial velocity at 8 °C and at 50 μM Ca^{2+} where the Ca^{2+} binding sites would be saturated. The single turnover kinetics of $\text{E}_1 \sim \text{P}$ (calcium) formation of the purified prokaryotic Ca^{2+} -ATPase are simple and reveal a single K_m of activation at $\sim 90 \mu\text{M}$ MgATP. Importantly, detergent-solubilized SR Ca^{2+} -

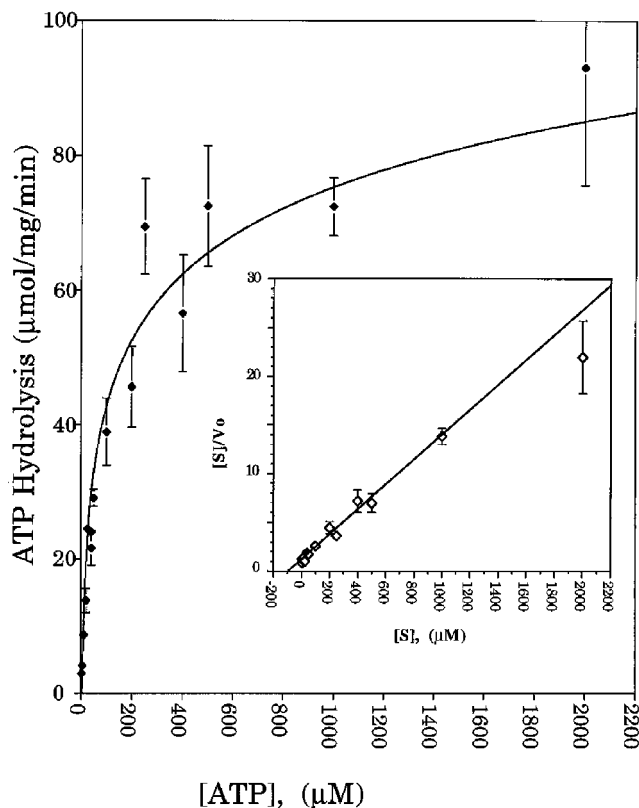


FIG. 2. **Affinity of purified Ca^{2+} -ATPase for ATP.** Initial rates of ATP hydrolysis by the purified Ca^{2+} -ATPase were measured at varying ATP concentrations. Blank values and specific activity were adjusted for each new ATP concentration. Hydrolysis assays were performed in triplicate for each point under initial rate conditions. Total ATP hydrolyzed did not exceed 6% of the initial substrate concentration. Rate was plotted as a function of [ATP], and the direct plot represents an average of three independent experiments, performed on independently isolated enzyme fractions. The data were replotted (inset) as $[S]/V$ versus $[S]$, and the x intercept ($-K_m$) was determined by extrapolation.

ATPase also has simple kinetics with a single K_m of MgATP activation of $100 \mu\text{M}$. It is only the membrane-bound form of the SR Ca^{2+} -ATPase that has two activating phases for ATP, one occurring between 2 and $50 \mu\text{M}$ and the other well above $100 \mu\text{M}$ MgATP (22). Therefore, the kinetics observed for ATP hydrolysis and E-P formation in the purified prokaryotic Ca^{2+} -ATPase are precisely what has been observed for the detergent-solubilized SR Ca^{2+} -ATPase. To determine whether the purified prokaryotic ATPase single K_m for ATP is attributable to a detergent-solubilized state like the SR pump or whether it is due to a more fundamental difference between the two enzymes, we examined the effect of MgATP concentration on the activation of the pump in membrane vesicles. In contrast to what is seen in the purified ATPase, the rate of hydrolysis for the prokaryotic pump in inside-out membrane vesicles is a sigmoid function of MgATP concentration. A break in the Hanes-Woolf plot (Fig. 4) was observed when the MgATP was varied over a wide range (from $0.2 \mu\text{M}$ to 1 mM). One straight line was observed in the range of 0.2 – $30 \mu\text{M}$ giving an apparent K_m at $10 \mu\text{M}$, and a second straight line ($30 \mu\text{M}$ – 1 mM) reveals an apparent K_m at $>400 \mu\text{M}$. The appearance of two K_m values in this range is consistent with what was observed for the SR Ca^{2+} -ATPase (22).

The only other nucleotides hydrolyzed at significant rates by the prokaryotic Ca^{2+} -ATPase are dATP, GTP, UTP, and ITP at rates of 70, 57, 25, and 25%, respectively, of that measured by ATP. ADP, CTP, acetyl phosphate, and AMP-PNP were not hydrolyzed. *P*-nitrophenylphosphate, which is readily hydro-

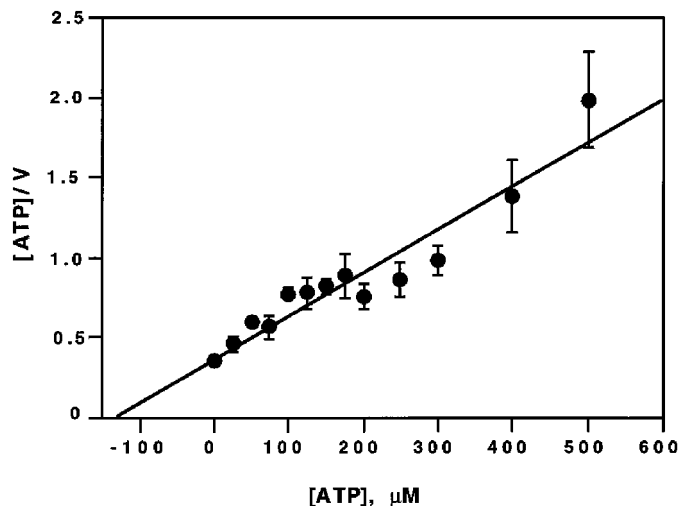


FIG. 3. **E-P formation of purified Ca^{2+} -ATPase as a function of ATP concentration.** Initial rates of E-P formation by the Ca^{2+} -ATPase were examined at 8°C using a three-syringe rapid quench apparatus. Final conditions were 20 mM MOPS (pH 7.0), 100 mM KCl, 1 mM MgSO_4 , $50 \mu\text{M}$ CaCl_2 , $41.7 \text{ ng}/\mu\text{l}$ *F. odoratum* Ca^{2+} -ATPase, and ATP concentrations as indicated. Syringe A contained varying amounts of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ($2000 \text{ cpm}/\text{pmol}$) at twice their final concentration. Syringe B contained $83.3 \text{ ng}/\mu\text{l}$ of Ca^{2+} -ATPase and $100 \mu\text{M}$ CaCl_2 . Syringe C contained 35% trichloroacetic acid quench solution. Other components were present at their final concentrations in all syringes except syringe C. Blank values and specific activity were adjusted for each new ATP concentration. To each quenched reaction, $15 \mu\text{g}$ of bovine serum albumin was added, and the protein was precipitated. The precipitated protein was analyzed by SDS-PAGE. The bands were visualized by autoradiography, cut, and then counted. The data were plotted as $[S]/V$ versus $[S]$, and the x intercept ($-K_m$) was determined by extrapolation.

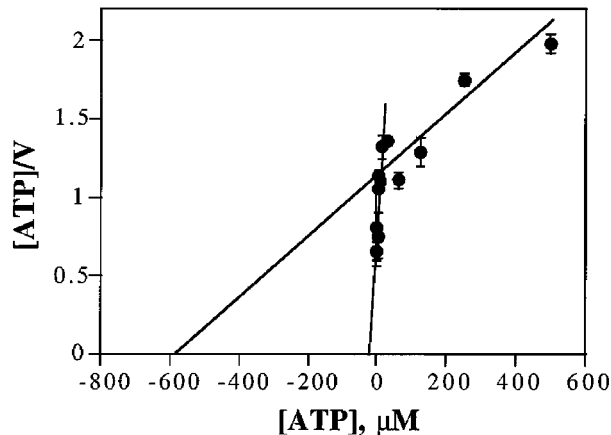


FIG. 4. **Affinity of vesicular Ca^{2+} -ATPase for ATP.** Initial rates of ATP hydrolysis by the Ca^{2+} -ATPase in inside-out membrane vesicles were measured at varying ATP concentrations. Blank values and specific activity were adjusted for each new ATP concentration. Hydrolysis assays were performed in triplicate for each point under initial rate conditions. Total ATP hydrolyzed did not exceed 6% of the initial substrate concentration. The data were plotted as $[S]/V$ versus $[S]$, and the x intercepts ($-K_m$) were determined by extrapolation.

lyzed by the SR Ca^{2+} -ATPase (23), is also hydrolyzed by the prokaryotic Ca^{2+} pump.

Importantly, ATP hydrolysis is absolutely dependent on Mg^{2+} and Ca^{2+} . Fig. 5 shows hydrolysis of ATP by the purified prokaryotic Ca^{2+} -ATPases as a function of $p\text{Ca}$ at an MgATP concentration of $100 \mu\text{M}$. Similar to the SR Ca^{2+} -ATPase, the apparent K_m for calcium is $1.5 \mu\text{M}$, and the plot of ATPase activity at various $p\text{Ca}$ is sigmoidal (Fig. 5). The activation process is characterized by a Hill coefficient of 2.4 (average of four experiments). This is comparable with the $n = \sim 2$ value measured for the SR Ca^{2+} -ATPase (24, 25). Therefore, at low

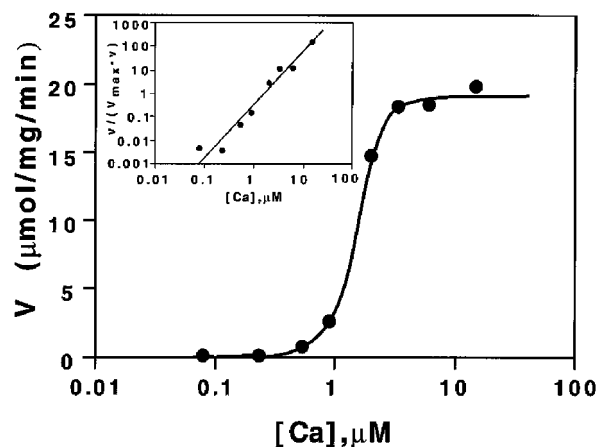


FIG. 5. Calcium dependence of ATP hydrolysis of purified Ca^{2+} -ATPase. Hydrolysis was assayed using calcium-EGTA mixtures to buffer free calcium concentration, according to the program described by Fabiato and Fabiato (18). ATP concentration was maintained at 100 μM for all assays. Hydrolysis assays were performed in triplicate for each point, under initial rate conditions, and activity was calculated as a function of free calcium concentration. The direct plot is an average of three independent experiments, using independently isolated enzyme fractions. The inset contains Hill plots of the calcium titration curve.

concentrations of free calcium the prokaryotic Ca^{2+} -ATPase clearly exhibits positive cooperativity for calcium consistent with the binding of at least two calcium in its activation just like the SR Ca^{2+} -ATPase. The addition of 1 mM Na^+ , Ba^{2+} , or the replacement of 100 mM KCl with 100 mM NaCl or with 100 mM K_2SO_4 had little or no effect on hydrolysis activity (data not shown). The addition of 1 mM Mn^{2+} or Cd^{2+} inhibited activity by about 50%. Lanthanide, a calcium analog and calcium channel inhibitor (26), inhibits hydrolysis. Lanthanum and lanthanide salts have an ionic radius similar to that of calcium and have been reported to complex with ATP to form stable lanthanum phosphointermediates in the SR Ca^{2+} -ATPase, which dramatically inhibits its turnover rate. Significantly, no other divalent or monovalent cation can substitute for calcium. Inesi and colleagues (27) have clearly demonstrated that H^+ serves as a counter ion in the SR Ca^{2+} -ATPase reaction mechanism. Unfortunately, we have been unsuccessful in our attempts to reconstitute the purified *F. odoratum* Ca^{2+} -ATPase and therefore have not yet determined whether it requires a H^+ as a counter ion. Orthovanadate inhibits the purified prokaryotic Ca^{2+} -ATPase in a concentration-dependent manner, with a calculated IC_{50} of 0.6 μM (Fig. 6). This value is much closer to that reported for the sarcolemmal (28) and erythrocyte Ca^{2+} -ATPase of 0.5 μM (29) than the IC_{50} of 10- μM vanadate reported for the SR Ca^{2+} -ATPase (30). The purified Ca^{2+} -ATPase is insensitive to DCCD and *N*-ethylmaleimide. Importantly, it is also insensitive to the SR Ca^{2+} -ATPase inhibitors fluorescein isothiocyanate (31), thapsigargin (32), and cyclopiazonic acid (33).

All P-type ATPases form an acylphosphate with the γ -phosphate of ATP as an intermediate in the reaction cycle. The purified Ca^{2+} -ATPase is phosphorylated by ATP only in the presence of Ca^{2+} , and phosphointermediate formation is inhibited by vanadate (Fig. 7A). Importantly, the phosphointermediate formed from the purified Ca^{2+} -ATPase comigrates with the phosphointermediate formed from *F. odoratum* membrane vesicles (Fig. 7B). We have also examined the reverse phosphorylation of the purified Ca^{2+} -ATPase. We have shown previously that unlike the SR Ca^{2+} -ATPase, the prokaryotic Ca^{2+} -ATPase is phosphorylated by P_i in the presence of Ca^{2+} (10). Fig. 7C demonstrates that E-P formation by P_i in the purified Ca^{2+} -ATPase is identical to what was seen in detergent-solu-

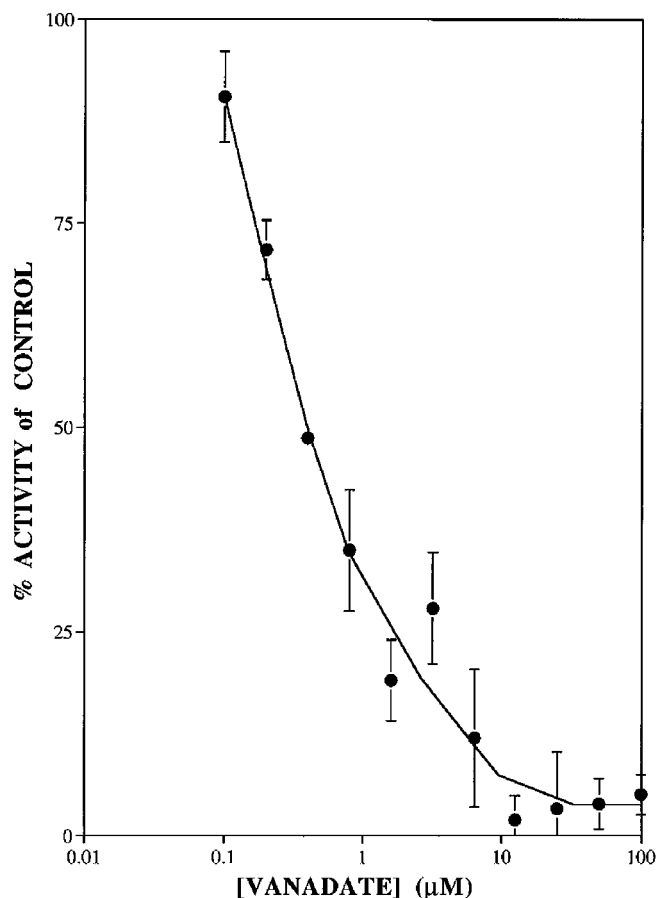


FIG. 6. Inhibition by vanadate. Purified samples were incubated for 10 min in reaction buffer of 100 mM KCl, 20 mM MOPS, pH 7.5, 0.1 mM CaCl_2 , 0.5 mM MgSO_4 and vanadate at concentrations ranging from 0.1 to 100 μM . Hydrolysis assays were performed in triplicate. Inhibition is plotted as % activity versus log [vanadate], with an apparent IC_{50} of 0.6 μM .

bilized Ca^{2+} -ATPase (10). The enzyme is phosphorylated both in the presence and absence of Ca^{2+} , and E-P formation is much greater in the presence of Ca^{2+} . To detect E-P formed in the absence of Ca^{2+} , the level of purified Ca^{2+} -ATPase loaded on the gel was five times greater than that loaded for E-P formation in the presence of Ca^{2+} . Presumably the $\text{E}_2\text{Ca-P}$ intermediate is less labile to hydrolysis than the $\text{E}_2\text{-P}$ intermediate. Importantly, the phosphointermediate formed by P_i in the presence and absence of Ca^{2+} is sensitive to hydroxylamine and inhibited by micromolar vanadate (Fig. 7D). The phosphointermediate formed in the presence of Ca^{2+} turns over rapidly upon chase with a 500-fold excess of cold P_i or ADP (Fig. 7, E and F). The phosphointermediate formed in the absence of calcium also turns over rapidly with excess cold P_i but does not turnover in the presence of excess ADP (data not shown, see Ref. 10).

DISCUSSION

The SR Ca^{2+} -ATPase has been extensively characterized and serves as the structure-function model for the analysis of ATP-catalyzed cation translocation. However, until recently very little was known about the prokaryotic calcium pumps. This work is the first report of the purification of a prokaryotic P-type calcium ATPase. The isolated protein migrated as a single band on SDS-PAGE, with an apparent M_r of 60,000. Calcium-dependent ATP hydrolysis with a V_{max} of $\sim 75 \mu\text{mol min}^{-1} \text{mg}^{-1}$ was fully inhibited by micromolar vanadate, and the formation of an alkaline-labile phosphointermediate was observed in the forward (ATP) and reverse (P_i) limbs of the

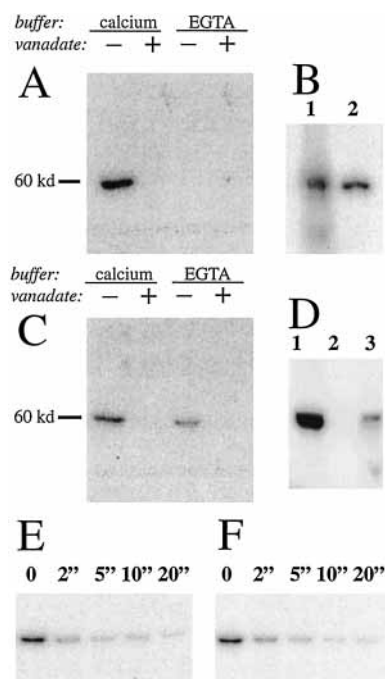


FIG. 7. Phosphorylation of the purified Ca^{2+} -ATPase. Forward phosphorylation (A) was carried out on ice with 0.1 μg of purified protein incubated for 10 min in 0.1 M KCl, 20 mM MOPS, pH 7.5, and 0.5 mM MgCl_2 with the indicated additions of calcium (0.5 mM), EGTA (2 mM), and vanadate (0.1 mM). 20 μM [γ - ^{32}P]ATP (2000 cpm/pmol) was added to initiate the 10-s reaction, which was stopped with 750 μl of 5% trichloroacetic acid/5 mM potassium P_i . The precipitated protein was pelleted, resuspended, and analyzed by SDS-PAGE and fluorography. B, forward phosphorylation was carried out on ice with 75 μg of *F. odoratum* membrane vesicles (lane 1), 0.05 μg of purified Ca^{2+} -ATPase incubated for 10 min in 0.5 mM CaCl_2 , 0.1 M KCl, 20 mM MOPS, pH 7.5, and 0.5 mM MgCl_2 (lane 2). 20 μM [γ - ^{32}P]ATP (2000 cpm/pmol) was added to initiate the 10-s reaction, which was stopped and analyzed for phosphointermediate formation as described above. Reverse phosphorylation (C-F) was carried out on ice with 0.2 μg of purified protein incubated for 10 min in 0.1 M MES, pH 6.5, and 0.5 mM MgCl_2 with the indicated additions of calcium (0.5 mM), EGTA (2 mM), and vanadate (0.1 mM). 2 μM [^{32}P]orthophosphate (912 Ci/mM) was added to initiate the 10-s reaction, which was stopped with 750 μl of 5% trichloroacetic acid, 5 mM potassium P_i . The precipitated protein was pelleted, resuspended, and analyzed by SDS-PAGE and fluorography. In C, reactions were with the indicated additions of calcium (0.5 mM), EGTA (2 mM), and vanadate (0.1 mM). Total protein used in the EGTA reactions was 1 μg . In D, all reactions included 0.5 mM CaCl_2 with 0.1 mM vanadate added to lane 2. In lane 3, the pellet was resuspended in 100 mM MES, pH 6.0, 100 mM hydroxylamine. In the cold chase experiments (E and F), 0.5 mM CaCl_2 was included in all reaction buffers. Reactions were initiated with the addition of 2 μM [^{32}P]orthophosphate for 15 s, at which point 2 mM ADP (E) or 2 mM cold potassium P_i (F) was added for the indicated times (0 is no chase) before the reactions were stopped.

reaction cycle. The K_m values for calcium and ATP and the IC_{50} for vanadate of the purified prokaryotic ATPase are very similar to that of the SR pump. In contrast to the SR ATPase, the prokaryotic Ca^{2+} -ATPase is phosphorylated by P_i in the presence of calcium. This is consistent with what was observed in *F. odoratum* membrane vesicles and partially purified fractions. These data suggest that either the prokaryotic Ca^{2+} -ATPase forward reaction is ordered (10), i.e. the enzyme does not bind calcium at the high affinity site until ATP binds, or that ATP and not Ca^{2+} determines the direction of the reaction cycle. Significantly, the prokaryotic enzyme's apparent M_r is only 55% that of the SR Ca^{2+} -ATPase, making it the smallest P-type ATPase reported to date. The high purification factor and final yield of protein suggests that, though active and stable, the calcium pump is not present in great abundance in the membrane.

The *F. odoratum* calcium pump is constitutively expressed

under most growth conditions (high or low media calcium, pH, or O_2 tension) and, unlike the derepressible Kdp ATPase, does not appear to be subject to proteolytic degradation during purification. The V_{max} for the prokaryotic ATPase is at least 10-fold greater than that reported for the SR calcium pump. The purified enzyme is very stable even at room temperature and shows no appreciable loss of activity even with multiple freeze-thaw cycles. Initial isolation was carried out with the addition of 0.25% C_{12}E_8 and lipids to the buffers for glycerol gradients and DE52 chromatography. Interestingly, we later discovered that no detergents or lipids needed to be added to any of the buffers after the initial 2% C_{12}E_8 solubilization. This suggests that the prokaryotic Ca^{2+} -ATPase has large hydrophilic domains that allow it to remain soluble, active, and stable even in very low concentrations of detergents and phospholipids.

Unfortunately, so far our efforts to reconstitute the purified prokaryotic Ca^{2+} -ATPase into liposomes have not been successful, but we are continuing to look for conditions that will allow us to examine the calcium translocation properties of the purified pump. Our failure to successfully reconstitute the Ca^{2+} -ATPase brings up the possibility that we have isolated the catalytic subunit of a multi-subunit ATPase. Importantly, the phosphointermediate formed from the purified Ca^{2+} -ATPase is the same size as that formed from the membrane vesicles. Therefore, the small size, high level of turnover, and stability in very low detergent concentrations are not due to proteolysis of a larger more hydrophobic form. These data do support the possibility that the 60,000-Da polypeptide is the catalytic subunit of a larger complex and that at least a second transmembrane component may be required for transport. The Na^+, K^+ -ATPase and H^+, K^+ -ATPase require a β subunit for proper assembly and activity, but no homology for the β subunit has been identified for any of the prokaryotic P-type ATPases. The Kdp-ATPase is composed of three subunits, A, B, and C, of 60,000, 72,000, and 20,000 Da (12, 13), respectively. KdpB forms an acylphosphate intermediate and has regions of homology with the eukaryotic P-type ATPases. Genetic evidence demonstrates that both KdpA and KdpC are both required for function. None of the subunits of the Kdp ATPase have been isolated to homogeneity or demonstrated to have partial or complete activity. Importantly, the purified *F. odoratum* Ca^{2+} -ATPase is very active, carrying out calcium-dependent vanadate-sensitive ATP hydrolysis and forward and reverse phosphorylation. Our attempts at reconstitution may have not been successful because of a possible requirement for one or more of the unusual lipids present in the *F. odoratum* membrane (34). We are currently identifying conditions for successful reconstitution and examining the possibility of other subunits required for transport.

The purification of the Ca^{2+} -ATPase will allow us to directly address these and other structure-function questions. Importantly, the purification of the Ca^{2+} -ATPase has given us sequence information that has facilitated its cloning and subsequent functional expression in *E. coli*.² The ability of heterologous expression of mutants of the Ca^{2+} -ATPase, which can be purified for characterization, makes this system especially useful for the analysis of the molecular mechanisms of transport ATPases.

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