Cloning and Expression of the Unique Ca\textsuperscript{2+}-ATPase from Flavobacterium odoratum*

Wendy E. Peiffer, Michael G. Desrosiers, and Donald R. Menick‡

From the Cardiology Division, Department of Medicine, Department of Biochemistry and Molecular Biology, and the Gazes Cardiac Research Institute, Medical University of South Carolina, Charleston, South Carolina 29425-2221

The 60-kDa Ca\textsuperscript{2+}-ATPase from Flavobacterium odoratum is kinetically and mechanistically similar to other P-type ATPases, suggesting its use as a model system for structure-function studies of ion transport. A portion of the gene was amplified by polymerase chain reaction of genomic DNA with degenerate oligonucleotide primers, one based on the N-terminal amino acid sequence of the purified protein and the other based on a consensus sequence for the phosphorylation site of P-type ATPases. This gene fragment was used to screen a λ library of F. odoratum 29979 DNA. Clone "C" is 3.3 kilobases in length and contains one complete and part of a second open reading frame, the first of which encodes a 58-kDa protein containing the exact N-terminal amino acid sequence of the purified protein. We have named this gene cda, for calcium-dependent ATPase. Escherichia coli, transformed with clone C, demonstrates high levels of calcium-dependent and vanadate-sensitive ATP hydrolysis activity, forms a 60-kDa phosphointermediate, and cross-reacts with antibodies to the purified Ca\textsuperscript{2+}-ATPase. The gene has almost no sequence homology to even the highly conserved regions characteristic of P-type ATPases but does possess significant homology to a protein with alkaline phosphatase activity (PhoD), from Zymomonas mobilis. The putative phosphorylation site is a Walker A (P-loop) ATP binding sequence and is modified relative to P-type ATPases, suggesting that the F. odoratum Ca\textsuperscript{2+}-ATPase may represent an ancestral link between the F- and the P-type ATPases or perhaps a new class of ATPases.

Calcium is an important component of the signal transduction process in eukaryotic cells. It is therefore necessary that intracellular calcium levels are kept low so that even small changes in concentration are detectable for signal transduction. In prokaryotes, however, calcium has not been shown to offer the advantage of allowing high level of expression of a protein in its native environment with no interference from endogenous wild-type activity.

We have therefore set out to clone the gene for the Ca\textsuperscript{2+}-ATPase from F. odoratum to analyze the structure and function of this prokaryotic ATPase. The approach employed relies on polymerase chain reaction (PCR) amplification of short regions from genomic DNA using degenerate primers based on both the N-terminal amino acid sequence of the purified protein (12) and highly conserved sequences in other P-type ATPases. The PCR products were then used to probe a genomic library for a full-length clone. The cloned gene, which we have named cda for calcium-dependent ATPase, has been expressed in E. coli and

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†To whom correspondence and reprints should be addressed: Cardiology Division, Medical University of South Carolina, 171 Ashley Ave., Charleston, SC 29425-2221. Tel.: 803-792-3405; Fax: 803-792-7771.

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The abbreviations used are: SERCA, sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}-ATPase; PCR, polymerase chain reaction; DCCD, N,N-dicyclohexylcarbodiimide; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; MOPS, 3-morpholinopropanesulfonic acid.
 Vesicles were solubilized with 2% C12E8 on ice for 60 min and then centrifuged at 36,000 × g for 30 min. Ammonium sulfate was added to 20% of the final volume, and the mixture was incubated for 90 min. The pellet was solubilized and subsequently into EcoRI and HindIII XOLR and XL1 Blue were obtained from Stratagene, while Nova Blue cells (Novagen) and E. coli XET-21 vector was obtained from Novagen.}

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemicals were obtained from Sigma (reagent grade) except where indicated. C_{2}H_{5}OH was obtained from Calbiochem. {γ-32P}ATP (6000 Ci/mmol) was obtained from DuPont NEN. ATP solution was prepared as 100 mM stocks, pH 7.0, aliquoted, stored at −20 °C, and then discarded after one use. Bacterial Strains and Vectors—E. coli Strains and Vectors—F. odoratum strain (ATCC 29979) was obtained from ATCC and grown aerobically in Luria-Bertani medium, pH 7.5, at 37 °C and harvested mid-log phase. E. coli strains XOLR and XL1 Blue were obtained from Stratagene, while Nova Blue and Nova Blue (DE3) E. coli cell lines were obtained from Novagen (Madison, WI); both were grown under the same conditions as F. odoratum. λ ZAP Express cloning vector was obtained from Stratagene, and pET-21 vector was obtained from Novagen. PCR of the Partial Clone—Degenerate oligonucleotides were synthesized to correspond to a portion of the N-terminal amino acid sequence, EKLEKPLV (GAAAA(A/G)TT(A/G)GAAAA(A/G)CC(G/T)AA(A/G)TT), and used to generate a consensus phosphorylation site (GKTFK) (GTGATA/G/GATA/C/CC/GGT/TTATG/GTCGACAG). PCR was performed on a Perkin-Elmer Corp. DNA thermal cycler model N801-0150 with 30 cycles of (94°, 1 min; 51°, 1 min; 72°, 2 min) using Taq polymerase and buffers from Life Technologies, Inc. and deoxynucleotides from Perkin-Elmer. Products were analyzed on a 1% agarose gel in TBE buffer (50 mM Tris, 50 mM borate, 1 mM EDTA, pH 8.3) with 0.1 μg/ml ethidium bromide to visualize the DNA. The PCR reaction product mixtures were purified from excess primer and nucleotides by the Wizard PCR purification kit (Promega). The mixture was ligated into the pT7 blue vector (Novagen) according to the manufacturer’s directions, transforming into E. coli Nova Blue (Novagen), and ligated into the pT7 blue vector (Novagen) according to the manufacturer’s directions, transforming into E. coli Nova Blue (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novagen), and ligated into the pT7 blue vector (Novag...
quences of 11, 21, or 42 amino acids. The EGCG program “sigcleave” analyzed the entire sequence and predicted the presence of a signal sequence that cleaves at the N-terminal serine. The translation start site is likely to be the methionine ester either 22 or 242 since both contain the hydrophobic helical region (from amino acids 213 to 219) typical of signal sequences. Starting from the N-terminal serine, the gene consists of 1581 bp, coding for a protein of 527 amino acids with a molecular mass of 58,805 Da, very close to the 60-kDa size predicted from the protein’s mobility on SDS-PAGE. We therefore suggest that the first open reading frame of clone C encodes the Ca\(^{2+}\)-ATPase protein purified from \textit{F. odoratum}. We propose to call this gene \textit{cda}, for calcium-dependent ATPase.

These second open reading frame continues for 1310 bp to (and probably past) the end of the clone, coding for a protein of at least 49,906 Da (436 amino acids). The GCG program “motifs” revealed homology to the insulinase family of proteins, divalent cation-dependent proteases that process peptides including insulin (17). Comparison of the predicted amino acid sequence to GenBank and PIR protein data bases revealed no significant sequence homology to any known P-type ATPase. In fact, the only conserved sequence from the P-type ATPases that is present in the \textit{F. odoratum} Ca\(^{2+}\)-ATPase is the consensus phosphorylation site used to generate the original partial clone. However, even this sequence, starting at residue 158, is altered relative to the P-type ATPases. The GCG program “motifs” identifies this region as an ATP binding site, commonly known as a Walker A or Ploop motif ((G/A)XXXXGKT)(22). Most ATP binding proteins contain a Walker A sequence; however, the P-type ATPases are an exception, containing instead the highly conserved 7-amino acid sequence DKTGT(I/L)T as part of the ATP binding site. The \textit{F. odoratum} sequence instead contains DGKTGDWIT including the conserved aspartate (Asp-158), which has been implicated in the autophosphorylation of the protein as an integral step in the reaction cycle of P-type ATPases.

Sequence Analysis and Predicted Structure of the Ca\(^{2+}\)-ATPase—Comparison of the \textit{cda} gene’s predicted amino acid sequence to GenBank and PIR protein data bases revealed no significant sequence homology to any known P-type ATPase. In fact, the only conserved sequence from the P-type ATPases that is present in the \textit{F. odoratum} Ca\(^{2+}\)-ATPase is the consensus phosphorylation site used to generate the original partial clone. However, even this sequence, starting at residue 158, is altered relative to the P-type ATPases. The GCG program “motifs” identifies this region as an ATP binding site, commonly known as a Walker A or Ploop motif ((G/A)XXXGKT)(22). Most ATP binding proteins contain a Walker A sequence; however, the P-type ATPases are an exception, containing instead the highly conserved 7-amino acid sequence DKTGT(I/L)T as part of the ATP binding site. The \textit{F. odoratum} sequence instead contains DGKTGDWIT including the conserved aspartate (Asp-158), which has been implicated in the autophosphorylation of the protein as an integral step in the reaction cycle of P-type ATPases.

The \textit{F. odoratum} Ca\(^{2+}\)-ATPase exhibits moderate sequence homology (30%) with an alkaline phosphatase (\textit{phoD}) from \textit{Zymomonas mobilis} (23). Despite weak homology to short regions of several Ca\(^{2+}\)-ATPases, including the \textit{Synechococcus pacificus}, which has been hypothesized to be a Ca\(^{2+}\)-ATPase, the \textit{Z. mobilis} sequence contains none of the highly conserved regions found in other P-type ATPases, including the phosphorylation site.
The Kyte-Doolittle hydropathy plot of the F. odoratum Ca\(^{2+}\)-ATPase (Fig. 2) definitively indicates only one transmembrane helix in the signal sequence that is cleaved off in the isolated protein. This result is confirmed by the program PredictProtein from the EMBL-Heidelberg (24, 25). However, many other regions of the protein are weakly hydrophobic, and the protein partitions with the membrane during cellular disruption (Ref. 1 and data not shown), suggesting that it is a peripheral membrane protein, possibly part of a multi-subunit membrane complex.

Functional Expression of the Ca\(^{2+}\)-ATPase in E. coli—E. coli, which has no endogenous Ca\(^{2+}\)-ATPase activity, was transformed with pBK (no insert) or pBK-C (contains the complete open reading frame for cda and part of the putative insulinase). French-pressed membrane vesicles from X1L Blue cells transformed by pBK-C demonstrated very high levels of calcium-dependent ATP hydrolysis (Fig. 3). The level of activity is severalfold higher, on a per protein basis, than that endogenously expressed in F. odoratum. The K_m for ATP was determined to be 100 \(\mu\)M by ATP hydrolysis assays (data not shown), similar to the 90 \(\mu\)M value found for the purified protein (12). The activity was sensitive to vanadate, a phosphate analog that inhibits all P-type but not F-type ATPases. The 0.8 \(\mu\)M K_v vanadate (data not shown) is also similar to the purified protein. Importantly, vesicles from cells transformed with vector alone (pBK) showed no calcium-dependent ATPase activity. Moreover, upon addition of ATP, vesicles from cells with pBK-C formed a vanadate-sensitive 60-kDa phosphointermediate in the presence of calcium but not EGTA, as observed in F. odoratum membrane vesicles (Fig. 4). No phosphointermediate is observed in vesicles from E. coli transformed with pBK alone.

Western blots of vesicles from pBK-C cells cross-react with antibodies prepared against the purified protein. The plot starts at the first possible initiation methionine with residue 1 corresponding to the N-terminal serine residue (as determined by the purified protein). Positive values represent hydrophobic regions. A potential transmembrane helix is apparent near the N terminus, while there are several other regions of weak hydrophobicity throughout the sequence.

Western blots of vesicles from pBK-C genes expressed in E. coli. Calcium-dependent ATP hydrolysis activity of the 90% fraction from E. coli X1L Blue cells transformed by the vector pBK with (pBK-C) or without (pBK) clone C were compared to F. odoratum on a per protein basis. ATP hydrolysis was carried out with the 90% fraction incubated in 45 \(\mu\)l of buffer A (plus 0.5 \(\mu\)M DCCD) with 0.1 \(\mu\)M CaCl\(_2\) (+ calcium), 2 \(\mu\)M EGTA (− calcium), or 0.1 \(\mu\)M CaCl\(_2\) plus 100 \(\mu\)M vanadate (+ vanadate) for 10 min, and the reaction was initiated with 5 \(\mu\)l of a 1 \(\mu\)M \([\gamma-32\text{P}]\text{ATP mixture (final concentration, 100 \(\mu\)M at 100–200 cpmp). After 10 s, the reaction was terminated by the addition of 150 \(\mu\)l of 30% trichloroacetic acid, 1 \(\mu\)M KHPO\(_4\). Inorganic phosphate was extracted and counted as described (12).

Western blots of vesicles from pBK-C cells cross-react with antibodies prepared against the purified F. odoratum Ca\(^{2+}\)-ATPase, while cells with pBK (no insert) do not show a reaction (Fig. 5). The band labeled by the antibodies migrates at 60 kDa, as does the phosphorylated protein produced by the F. odoratum and the clone.

The pBK vector did not tightly control the expression of the cda gene, and induction with isopropyl-1-thio-\(\beta\)-D-galactopyranoside often resulted in levels of expression that adversely affected cell viability. Therefore, the clone C insert was ligated into the pET-21 vector (pET-C), which more tightly controls expression, allowing us to regulate levels of expression. In addition, the sequence beyond the cda open reading frame of pET-C (including the second open reading frame of clone C) was deleted (pET-cda) so that only the Cda protein would be expressed. Importantly, vesicles from E. coli transformed with pET-cda demonstrated high levels of calcium-dependent, vanadate-sensitive ATP hydrolysis (Fig. 6). The cda gene product also formed a 60-kDa phosphointermediate in the presence of calcium and cross-reacted with our anti-F. odoratum Ca\(^{2+}\)-ATPase antibody (not shown). These data verify that cda encodes the F. odoratum Ca\(^{2+}\)-ATPase. Calcium transport assays were performed with vesicles treated with 0.1 \(\mu\)M DCCD and 0.5 mM N-ethylmaleimide, which inhibited the endogenous Ca\(^{2+}\) antiporters but not the heterologously expressed Ca\(^{2+}\)-ATPase. Unfortunately, we were unable to detect ATP-driven ATP hydrolysis activity of the pBK-C genes expressed in E. coli. Calcium-dependent ATP hydrolysis activity of the 90% fraction from E. coli X1L Blue cells transformed by the vector pBK with (pBK-C) or without (pBK) clone C were compared to F. odoratum on a per protein basis. ATP hydrolysis was carried out with the 90% fraction incubated in 45 \(\mu\)l of buffer A (plus 0.5 \(\mu\)M DCCD) with 0.1 \(\mu\)M CaCl\(_2\) (+ calcium), 2 \(\mu\)M EGTA (− calcium), or 0.1 \(\mu\)M CaCl\(_2\) plus 100 \(\mu\)M vanadate (+ vanadate) for 10 min, and the reaction was initiated with 5 \(\mu\)l of a 1 \(\mu\)M [\(\gamma-32\text{P}\)]ATP mixture (final concentration, 100 \(\mu\)M at 100–200 cpmp). After 10 s, the reaction was terminated by the addition of 150 \(\mu\)l of 30% trichloroacetic acid, 1 \(\mu\)M KHPO\(_4\). Inorganic phosphate was extracted and counted as described (12).

Calcium-dependent ATP hydrolysis activity for the 90% fraction from E. coli XL1 Blue cells transformed by the vector pBK with (pBK-C) or without (pBK) clone C were compared to F. odoratum on a per protein basis. ATP hydrolysis was carried out with the 90% fraction incubated in 45 \(\mu\)l of buffer A (plus 0.5 \(\mu\)M DCCD) with 0.1 \(\mu\)M CaCl\(_2\) (+ calcium), 2 \(\mu\)M EGTA (− calcium), or 0.1 \(\mu\)M CaCl\(_2\) plus 100 \(\mu\)M vanadate (+ vanadate) for 10 min, and the reaction was initiated with 5 \(\mu\)l of a 1 \(\mu\)M [\(\gamma-32\text{P}\)]ATP mixture (final concentration, 100 \(\mu\)M at 100–200 cpmp). After 10 s, the reaction was terminated by the addition of 150 \(\mu\)l of 30% trichloroacetic acid, 1 \(\mu\)M KHPO\(_4\). Inorganic phosphate was extracted and counted as described (12).

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calcium uptake (data not shown). This may be due to insufficient inhibition of the endogenous Ca\(^{2+}\) antiporters, the membrane being leaky to calcium, or the possible requirement of a second transmembrane protein for calcium transport activity.

**DISCUSSION**

A 1581-bp gene from *F. odoratum* has been cloned and sequenced. The gene encodes a protein with the same 60-kDa molecular mass and N-terminal amino acid sequence as the purified Ca\(^{2+}\)-ATPase. The expressed gene product exhibits calcium-dependent and vanadate-sensitive ATP hydrolysis, forms a phosphointermediate in the presence of calcium and ATP, and is immunologically related to the F. odoratum Ca\(^{2+}\)-ATPase. We therefore conclude that the first gene of clone C, which we call *cda* for calcium-dependent ATPase, encodes the Ca\(^{2+}\)-ATPase, that we have previously purified.

This gene, however, appears to code for a protein very different from other P-type ATPases, despite its functional similarities. Most of the highly conserved regions found in all other P-type ATPases are missing, including the TGES transduction domain, the KGAPE fluorescein isothiocyanate binding site, and the MTGDGVNDAPAL ATP binding domain. Only the putative phosphorylation site shares homology with other P-type ATPases, and it is altered relative to them. All other P-type ATPases conserve the 7-amino acid sequence DKTGT-(I/L), in which aspartate is the residue phosphorylated by ATP during the reaction cycle. In contrast, the *F. odoratum* Ca\(^{2+}\)-ATPase contains the sequence DGKTGDWIT, notably different from other P-type ATPases (22). This Walker A or P loop sequence is not found in other than the P-type ATPases (22). This Walker A sequence does not form an aspartylphosphate intermediate as part of their reaction mechanism.

The three-dimensional structure of the *F. odoratum* Ca\(^{2+}\)-ATPase may differ significantly from other P-type ATPases. The Kyte-Doolittle plot predicts only one transmembrane helix, that of the signal sequence, with the remaining protein being hydrophilic or only weakly hydrophobic. This, together with experiments suggesting that at least some of the protein’s activity cannot be removed from inside-out membrane vesicles by high salt wash (data not shown), suggests that the protein is a peripheral, not an integral, membrane protein. It is highly unlikely that a peripheral membrane protein alone could transport calcium across a membrane, and therefore a second, transmembrane, component would be required. The fact that we have been unsuccessful in demonstrating calcium transport by the reconstituted protein and by *E. coli* expressing *cda* supports the theory that the *Cda* protein is part of a multisubunit membrane complex. The subunits of the *F. odoratum* Ca\(^{2+}\)-ATPase may be analogous to the subunits of the plasmid-mediated *E. coli* arsenate transporter (although not a P-type ATPase), in which soluble ArsA is capable of arsenate-dependent ATP hydrolysis (27), but ArsB (transmembrane protein) and ArsC are required for transport (21, 28). Similarly, potassium transport in *E. coli* is mediated by the multisubunit transporters.
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P-type ATPase called Kdp, in which KdpA is postulated to be responsible for K\(^+\) transport (29) while KdpB binds ATP and is phosphorylated (30). In this case, both KdpA and KdpB are transmembrane proteins (6). In contrast to the F. odoratum Ca\(^{2+}\)-ATPase, it is not known whether KdpB alone is active since it has never been isolated to homogeneity.

Although it has no significant homology with P-type ATPase sequences, the F. odoratum Ca\(^{2+}\)-ATPase does show a moderate homology (overall 30\%) to an alkaline phosphatase (phOD) from Z. mobilis (23), which is itself weakly homologous to short regions of several P-type ATPases, in particular Ca\(^{2+}\)-ATPases. The F. odoratum protein is not highly homologous to the Z. mobilis protein in most of those regions. Interestingly, those regions homologous between the Z. mobilis and the Ca\(^{2+}\)-ATPases are not in the most highly conserved regions of P-type ATPases, and the Z. mobilis is missing an obvious phosphorylation site or ATP binding site. The PhoD protein demonstrates high phosphatase activity at alkaline pH using p-nitrophenolphosphate as a substrate, as does the F. odoratum Ca\(^{2+}\)-ATPase (12), as well as other P-type ATPases including SERCA (31, 32). The Z. mobilis PhoD protein is found in the cytosol and does hydrolyze ATP but at rates that are dramatically below that of the F. odoratum Ca\(^{2+}\)-ATPase (23).

The Z. mobilis enzyme has a similar hydropathy profile to the F. odoratum protein in that there is only one predicted transmembrane helix, at the N terminus (it is not clear whether there is a cleaved signal sequence), with weakly hydrophobic regions throughout the remainder of the protein. Given the ease of removal from the membrane, the PhoD protein has been postulated to contain only one transmembrane helix (23). Further experimentation will reveal whether our protein is structurally similar to the PhoD protein whose sequence it conserves, or to the P-type ATPases whose function it conserves.

Perhaps because it is a peripheral and not an integral membrane protein, the F. odoratum Ca\(^{2+}\)-ATPase is easily expressed in E. coli, in contrast to SERCA, which has only been expressed at relatively low levels in mammalian cell cultures (33), and baculovirus-infected insect cells (34), which both contain wild-type Ca\(^{2+}\)-ATPase. Molecular biological techniques have greatly increased our understanding of the molecular mechanism of the P-type ATPase, but unfortunately the level of expression of the ATPase in most eukaryotic expression systems is too limited to allow for extensive biochemical characterization of site-directed mutants. However, functional expression of the F. odoratum Ca\(^{2+}\)-ATPase in E. coli at very high levels will allow for easy purification and biochemical characterization of any site-directed mutant. This makes the F. odoratum Ca\(^{2+}\)-ATPase a very promising system for structure-function studies.

In conclusion, the F. odoratum Ca\(^{2+}\)-ATPase appears to be a highly unique ATPase. Functionally, it behaves like a P-type ATPase, forming an alkaline-labile phosphointermediate, and displaying vanadate-sensitive activity with \(K_m\) values for ATP and Ca\(^{2+}\) similar to those for SERCA. The primary structure, however, is different from the P-type ATPases, containing none of the highly conserved regions that appear to be involved in ATP binding and hydrolysis. The putative phosphorylation site is similar but not identical to that of P-type ATPases, resembling the Walker A/P loop motif found in other ATP binding proteins including V- and F-type ATPases. The F. odoratum Ca\(^{2+}\)-ATPase may possibly represent an ancestral link between the F-type and the P-type ATPases or a new class of ATPases. Further study is anticipated to clarify these structural puzzles and to elucidate how such apparently different structures can accomplish the same functional goals.

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