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FINAL REPORT

Title: Mechanism and Structure of the Plant Plasma Membrane Ca^{2+}-ATPase

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The plasma membrane Ca^{2+}-ATPase couples ATP hydrolysis to the extrusion of Ca^{2+} from the cytoplasm to the cell exterior (apoplast) of plant cells. This process is important for the maintenance of the low cytoplasmic Ca^{2+} concentration (about 0.1 μM) necessary for the function of this divalent cation as a "second messenger" in cellular signal transduction events. When research was initiated with DOE funding, relatively little was known at the biochemical level regarding the plasma membrane Ca^{2+}-ATPase. Only preliminary characterization of the enzyme had been conducted in plasma membrane fractions by measurement of its Ca^{2+} transport activity using $^{45}Ca^{2+}$ and there was also confusion regarding the properties of this enzyme due to several studies concerning the purification of a "plant Ca^{2+}-ATPase" of unknown membrane origin .

The first objective pursued during the current project was the development of an enriched preparation of the red beet plasma membrane Ca^{2+}-ATPase. The goal was to develop a procedure for detergent solubilization of the enzyme from the membrane using detergents, resolution by a method which could be scaled up for batch isolation, and then reconstitution into liposomes to allow characterization of Ca^{2+} transport by the purified enzyme. Of the detergents screened (ex. triton x-100, lubrol wx, deoxycholate, octylglucoside, zwitergent 3-12, zwittergent 3-14, cholate), Genapol, appeared to preferentially solubilize the plasma membrane Ca^{2+}-ATPase to a 100,000 xg supernatant while leaving much, but not all of the plasma membrane H^+-ATPase in the 100,000 xg pellet when used at 0.8% (w/v) and a detergent to protein ratio of about 8 to 1. If the plasma membrane fractions were first washed with 2 mM EGTA (twice) prior to detergent solubilization, the enzyme could be further enriched by fractionation on a calmodulin affinity column. Although alternative techniques for resolution were attempted (gel exclusion chromatagraphy, ion exchange chromatography, glycerol gradient ultracentrifugation), affinity chromatagraphy on calmodulin Sepharose appeared to produce preparations with the highest specific activity. This procedure showed promise as a means to generate sufficient quantities of the enzyme for simple mechanistic studies, and transport assays following reconstitution in liposomes.

Our second objective focused on characterization of the reaction mechanism for the coupling of nucleoside triphosphate hydrolysis to Ca^{2+} transport as mediated by the plasma membrane Ca^{2+}-ATPase. Our initial proposal was to use [γ-^{32}P]-GTP as an alternative substrate for the enzyme. Since the plasma membrane H^+-ATPase is substrate-specific for ATP and the Ca^{2+}-ATPase can use GTP or ITP at levels 50 to 70% of that found for ATP , it was reasoned that the use of [γ-^{32}P]-GTP would allow mechanistic studies to be conducted in plasma membrane fractions where the plasma membrane H^+-ATPase was also present. However, studies with this radiolabeled substrate proved to be unsuccessful due to its utilization by proteins in addition to the Ca^{2+}-ATPase. Likely candidates include G-proteins or other GTPases. Therefore an alternative strategy of using [γ-^{32}P]-ITP was explored. However, one problem was that this radiolabeled substrate was not available commercially. To address this problem, we utilized a procedure for the synthesis of [γ-^{32}P]-ITP involving nucleotide kinase. When the radiolabeled [γ-^{32}P] ITP was used with a red beet plasma membrane fraction, it was possible to observe the formation of a radiolabeled phosphorylated intermediate associated with the Ca^{2+}-ATPase. Formation of the phosphorylated intermediate was Ca^{2+}-dependent and showed the rapid turnover expected for an enzyme reaction intermediate. On

the other hand, phosphoenzyme formation was enhanced by La^{3+} but inhibited by 50 nM erythrosin B. These properties are consistent with the phosphointermediate representing that of the Ca^{2+}-ATPase.

Obtaining structural information on the red beet plasma membrane Ca^{2+}-ATPase at the biochemical level represented the third objective of this project. For this objective several approaches were utilized. Amino acids essential for the catalytic activity of the enzyme were identified using chemical modification by amino acid specific reagents. Using 2,3-butanedione and phenylglyoxal, an arginine residue was identified as being essential for the mechanism of the enzyme. The observation that GTP or ITP could protect against derivitization further confirmed the concept that these nucleoside phosphate compounds represent true alternative substrates for the enzyme. A similar approach using diethylpyrocarbonate and n-ethylmaleimide (NEM) demonstrated the presence of essential histidine and cysteine residues being associated with the enzyme active site. From earlier studies involving measurements of ATP-dependent $^{45}Ca^{2+}$ transport it was shown that inhibition by nanomolar amounts of erythrosin B represented a property of the red beet plasma membrane Ca^{2+}-ATPase, allowing its distinction from the plasma membrane H^{+}-ATPase . Our hypothesis has been that this inhibitor binds to the active site at or near the region where the nucleoside phosphate handle (i.e. adenine, inosine or guanosine) interacts with the protein. For many transport ATPases, this adenine recognition region of the active site typically contains an essential lysine residue that can be covalently derivitized by fluorescein isothiocyanate. To test for this possibility, erythrosin isothiocyanate was utilized as a histidine modification reagent. Treatment of plasma membrane vesicles, and in later studies, an enriched enzyme preparation with erythrosin isothiocyanate did result in inhibition of ATP or ITP driven $^{45}Ca^{2+}$ transport (in vesicles) and Ca^{2+}-dependent ITP hydrolysis (vesicles and enriched enzyme). Although it was expected that derivitization with this compound would show a simple competition with the substrate (i.e. ATP, ITP or GTP), this was not the case and more complicated results were found. Instead it appears as though this reagent may bind at two sites with differing affinity. One site appeared to be the expected active site location (high affinity) while the other appeared to reside outside the active site. Our conclusion was that if used under appropriate conditions (i.e. low concentration) this reagent could serve as a suitable probe for tagging the active site region of the enzyme. Due to the apparent differences in substrate specificity between the plasma membrane Ca^{2+}-ATPase and other transport ATPases, this region of the enzyme would be expected to show sequence differences and likely be unique for this enzyme. Our goal is to then synthesize a radiolabeled version of erythrosin isothiocyanate for tagging this region prior to isolation of radiolabeled peptides for the Ca^{2+}-ATPase active site.

Studies were also conducted to examine the quarternary structure of the red beet plasma membrane Ca^{2+}-ATPase present in the native membrane using radiation inactivation analysis. Frozen plasma membrane fractions were subjected to irradiation with high energy γ-rays from a ^{60}Co source (Neely Nuclear Center, Georgia Institute of Technology). Following irradiation, the samples were thawed and ATP-dependent $^{45}Ca^{2+}$ transport was measured. Residual transport activity associated with plasma membrane vesicles declined in an exponential manner with increasing radiation dose. According to "Target Theory" this data would be consistent with a single molecular size mediating Ca^{2+} transport and analysis of the data suggested a molecular size of 245 kDa. As our studies and those of others have indicated that the catalytic subunit of the red beet plasma membrane Ca^{2+}-ATPase has a molecular weight of about 100 to 120 kDa, these results suggest that this enzyme is present as a dimer in the native membrane. This dimeric structure may be important for the coupling of substrate hydrolysis to Ca^{2+} transport and in the regulation of the enzyme.

Publication of Results

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