Studies on the auto-inhibitory interactions of the plasma membrane Ca^{2+}ATPase

Dissertation (Ph.D. thesis)

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1. ABSTRACT

The plasma membrane calcium pump (PMCA) is the sole Ca^{2+} extrusion system in many cells that is responsible for maintaining the low cytosolic calcium concentration critical to cell function. The role of this protein is to eject Ca^{2+} from the cytosol to the extra cellular space using the energy of ATP. The carboxyl terminus of PMCA contains a high affinity calmodulin-binding sequence that interacts with the catalytic core and inhibits the activity of the pump in resting cells. In activated cells binding of Ca^{2+}-calmodulin to the calmodulin-binding sequence frees the catalytic core from the auto-inhibition and the pump becomes activated. In addition to the changes between the activated and inhibited states, the conformation of the pump also changes between the high (E1) and low (E2) Ca^{2+} affinity states during the reaction cycle.

In this study we utilized limited proteolysis and site directed mutagenesis to monitor the regions involved in the auto-inhibitory interactions of the ubiquitous PMCA4b isoform. We used three different proteases that all cleaved the protein at its carboxyl terminus upstream (caspase-3) or downstream (chymotrypsin and calpain) of the calmodulin-binding sequence. We found that the fragmentation highly depended on the conformational state. When the pump was in the auto-inhibited state and the E1-E2 equilibrium was shifted more towards the E2 conformation (in the absence of Ca^{2+}) the C-terminal region was resistant to proteolysis because of tight intra-molecular interactions. When the equilibrium was shifted more towards the E1 conformation (in the presence of Ca^{2+}) the cleavage sites became more exposed and the pump was partially degraded suggesting that the interaction between the C-terminal region and the catalytic core was weaker. Binding of Ca^{2+}-calmodulin to the binding site made the carboxyl terminus more flexible and the fragmentation more intensive. Our

experiments show that the conformational changes in the C-terminal region induced by Ca^{2+} or by Ca^{2+}-calmodulin extend to the regions both upstream and downstream of the calmodulin-binding sequence. In contrast, replacing Trp^{1093} (a key residue within the calmodulin-binding sequence) to an alanine exposed only proteolytic sites within the calmodulin-binding sequence. We also showed that an aspartate residue (Asp^{1080}, the target of caspase-3 cleavage), although, located outside of the calmodulin-binding sequence greatly contributes to the stabilization of the auto-inhibited conformation of the pump.

7. REFERENCES

Closely linked to the dissertation:

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Other publications:

K.Pászty, Verma,A.K., R.Padányi, Filoteo,A.G., Penniston,J.T., Á.Enyedi: Plasma membrane Ca^{2+}ATPase isoform 4b is cleaved and activated by caspase-3 during the early phase of apoptosis. *Journal of Biological Chemistry* 2002, Vol. 277, No. 9, Issue of March 1, pp. 6822-6829.

Lectures:

R.Padányi, K.Pászty, K.Lór, Verma,A.K., Penniston,J.T., Á.Enyedi: Phosphorylation of the plasma membrane calcium pump isoform 4b with PKC prevents proteolysis by calpain, European Calcium Society VI. European Symposium, France, Paris, abstractbook p51 (2000)

6. SUMMARY

The intra-molecular interactions of hPMCA4b in different conformational states were monitored by limited proteolysis using proteases (calpain, chymotrypsin, caspase-3) that cleave the pump at its carboxyl terminus. In the absence of Ca^{2+} and calmodulin (inhibited state, E2 conformation of the catalytic core) no fragmentation could be observed indicating that the intra-molecular interactions are tight. In the presence of Ca^{2+} (inhibited state, E1 conformation of the catalytic core) the fragmentation became more pronounced suggesting that the intra-molecular interactions are weaker in the E1 conformational state. Binding of Ca^{2+}-calmodulin to the calmodulin-binding site induces large conformational changes so that the proteolytic sites are fully exposed. This change extends to the whole inhibitory region from the „hinge" region to the region downstream of the calmodulin-binding sequence (downstream auto-inhibitory region).

Our data confirm that Trp^{1093} has a key role in the auto-inhibitory interaction of the calmodulin-binding sequence with the catalytic core since the mutation of Trp^{1093} exposed sites otherwise inaccessible for proteolysis. However, in contrast to the more global effect caused by calmodulin binding, this mutation affected only the calmodulin-binding sequence.

We demonstrated that the "hinge" region upstream of the calmodulin-binding sequence plays an essential role in auto-inhibition presumably by stabilizing the interaction between the calmodulin-binding sequence and the catalytic core. The mutation of Asp^{1080} – a residue that is located in this region – increased greatly the basal activity of the enzyme in the absence of calmodulin. Moreover, calmodulin activated the Asp^{1080} mutants faster than the wild type enzyme. These data suggest that the intra-molecular interactions are weaker in the mutants and, therefore, the calmodulin-binding sequence is more accessible to the Ca^{2+}-calmodulin complex. Limited proteolysis experiments demonstrated that the effect of this mutation is also local and did not extend downstream of the "hinge" region.

2. INTRODUCTION

Ca^{2+} ion is a fundamental and ubiquitous factor in signaling systems of cells. The resting intracellular Ca^{2+} concentration is low (~ 0,1 ?M), about 10^5 times lower than that in the extracellular space. Triggered by an external stimulus the intracellular Ca^{2+} level rises transiently to 0,5-1 ?M and activates numerous cellular processes depending on stimulus and cell type (e.g. contraction of muscle, secretion, neurotransmitter secretion). Subsequently, active Ca^{2+} transport mechanisms – the **P**lasma **M**embrane **C**a^{2+}**A**TPase (PMCA), the **S**arco/**E**ndoplasmic **R**eticulum **C**a^{2+}**A**TPase (SERCA) and the Na^+-Ca^{2+} exchanger – remove excess Ca^{2+} from the cytoplasm to restore the resting Ca^{2+} level.

One of the high affinity Ca^{2+} transport systems is the ubiquitously expressed plasma membrane Ca^{2+}ATPase (PMCA), which extrudes Ca^{2+} from the cytosol to the extracellular space. PMCA translocates Ca^{2+} ions across the cell membrane using the energy of ATP hydrolysis. It belongs to the family of P-type ion transporting ATPases characterized by the formation of a high-energy phosphate intermediate during the reaction cycle. Binding of Ca^{2+} and phosphorylation alternate with Ca^{2+} transfer and ATP hydrolysis during the kinetic cycle of these ATPases. The process is coupled with conformational changes resulting in changes of the Ca^{2+}-binding sites from high (E1 conformation) to low (E2 conformation) affinity state.

The topology of PMCA is similar to that of other P-type ATPases: it consists of 10 trans-membrane helices and two cytoplasmic loops. The residues forming the Ca^{2+}-binding sites are located in the trans-membrane region. The major catalytic sites – the ATP binding site and the aspartate residue that forms the acyl-phosphate intermediate – are located in the large cytosolic loop between trans-membrane spans 4 and 5. PMCA differs from other P-type ATPases in having an extended C-terminal regulatory region

that contains a high affinity calmodulin-binding sequence. In the absence of calmodulin this calmodulin-binding sequence interacts with the catalytic core, thus, inhibiting the calcium transport activity of the pump. An increase in intracellular Ca^{2+} concentration is followed by the formation of the Ca^{2+}-calmodulin complex that binds to the calmodulin-binding sequence at the C-terminal region. This binding breaks the interaction between the catalytic core and the C-terminal region thus, the pump becomes activated.

Four different genes encode PMCA and alternative splicing of the primary transcripts produce more than 20 PMCA variants. PMCA1 and PMCA4 are ubiquitous, whereas PMCA2 and PMCA3 are expressed mainly in neural, skeletal muscle and heart cells.

The PMCA isoforms show great diversity in their regulation with Ca^{2+}-calmodulin. They have different basal activities, different affinities for calmodulin, different rates of activation and inactivation. Among the isoforms, PMCA4b has the lowest basal activity and the greatest stimulation by calmodulin. The low basal activity of PMCA4b indicates that the interaction between the carboxyl terminus and the catalytic core is strong. Previous experiments demonstrated that the calmodulin-binding sequence is not sufficient for complete inhibition of hPMCA4b and other regions downstream also contribute to the auto-inhibition.

loosened the binding of the immediate surroundings of Trp^{1093} to the catalytic core, its effect did not extend to the regions upstream and downstream. Our experiments confirm that Trp^{1093} has an essential role in the auto-inhibitory interaction of the calmodulin-binding sequence with the catalytic core.

5.3. The role of an aspartate (Asp^{1080}) residue – located upstream of the calmodulin-binding sequence – in the auto-inhibition of hPMCA4b

Asp^{1080} is located in the „hinge" region between trans-membrane helix 10 and the calmodulin-binding sequence. Previously, we demonstrated that Asp^{1080} is a target for caspase-3 cleavage. Here we showed (section 5.1.) that the fragmentation with caspase-3 depended strongly on the tightness of the intra-molecular interaction. These results suggested that the „hinge" region might also be involved in the auto-inhibitory interaction.
Therefore, we studied the effect of mutation of Asp^{1080} (Asp^{1080}? Ala, Asp^{1080}? Lys Asp^{1080}? Asn mutants) on the activity and activation by calmodulin of hPMCA4b. We showed that all these mutations increased the basal activity of the enzyme, substantially. In addition to the change in the basal activity, the Asp^{1080} mutants all had higher affinity for calmodulin than the wild type enzyme. The increase in affinity was due to an increase in the rate of activation by calmodulin. Our data suggest that the mutations at Asp^{1080} shifted the pump more toward an open conformation (? higher basal activity) and induced a weaker auto-inhibitory interaction between the C-terminal region and the catalytic core. This resulted in an increase in the accessibility of the calmodulin-binding sequence to calmodulin (? increased rate of activation by calmodulin). The limited proteolysis experiments showed that the effects of these mutations were local and did not affect the intra-molecular interactions downstream.

We found that in the absence of Ca^{2+} the pump was resistant to proteolysis whereas in the presence of Ca^{2+} the cut sites were somewhat more exposed. These data suggest that the C-terminal auto-inhibitory region is tightly bound to the catalytic core in the E2 conformation. Based on the three dimensional structure of SERCA we presume that in the E1 configuration the domains of the catalytic core are more separated shielding the carboxyl terminus less effectively thus, exposing more of the proteolytic sites. The most pronounced fragmentation was observed in the presence of Ca^{2+}-calmodulin when the pump was in a fully activated state. This is consistent with the idea that the binding of Ca^{2+}-calmodulin to the calmodulin-binding site induces global structural changes in the interaction between the catalytic core and the C-terminal region.

5.2. The effect of mutating Trp^{1093} – a key residue of the calmodulin-binding sequence – on the intra-molecular interactions of hPMCA4b

Replacing a tryptophan residue in the calmodulin-binding sequence with an alanine (Trp^{1093}? Ala mutant) has been shown to increase the basal activity of the pump and also the rate of activation by calmodulin. These findings suggested less effective intra-molecular interactions in the mutant protein.
In this study we demonstrated that proteolytic sites otherwise inaccessible within the calmodulin-binding sequence became more exposed to proteolysis in the Trp^{1093}? Ala mutant. Our data are consistent with previous findings and show that the interaction between the calmodulin-binding sequence and the catalytic core is weaker in the mutant than in the wild type enzyme. We also showed that digestion at the sites upstream and downstream of the calmodulin-binding sequence was not affected by the mutation. This indicated that the effect was local and specific: although, the mutation

3. SPECIFIC AIMS

The structural basis of the complex regulation typical to PMCAs is the auto-inhibitory interaction between the catalytic core and the C-terminal region. We studied these interactions in the human PMCA4b (hPMCA4b) isoform by proteolysis and site directed mutagenesis. The access of proteases to their sites of cleavage in the C-terminal region was used to monitor conformational changes induced by Ca^{2+} and Ca^{2+}-calmodulin. We also tested the effects of mutations upstream of the calmodulin-binding sequence on the activity of the pump. Our aims were as follows:

- To determine the cleavage sites of proteases (calpain, chymotrypsin, caspase-3) in the C-terminal auto-inhibitory region of hPMCA4b *in situ* in the membrane.
- To investigate the fragmentation of hPMCA4b in different conformational states.
- To study the effects of mutation at Trp^{1093} - a key residue in the calmodulin-binding sequence - on the intra-molecular interactions by limited proteolysis.
- To investigate the effects of mutation at an aspartate residue (Asp^{1080}) - located in the „hinge" region upstream of the calmodulin-binding sequence - on the auto-inhibition.

4. METHODS

Cell culture, transfection and preparation of microsomal membranes: PMCA proteins were expressed in COS-7 cells originated from monkey kidney using Lipofectamine reagent. Subsequently, microsomal membranes from COS-7 cells were prepared.

Proteolytic digestion of PMCA4b with ?-calpain, chymotrypsin or caspase-3: Microsomal membranes from COS-7 cells transfected with the appropriate plasmid DNA were digested at 37 ^{o}C. The proteolytic fragments were detected by immunoblotting.

Gel electrophoresis and electro-transfer: Membrane proteins were separated by SDS poly-acrylamide gel-electrophoresis and electro-blotted to PVDF (poly-vinylidene difluoride) membranes. The blots were immunostained by pan-anti-PMCA antibody, 5F10 or the isoform specific anti-hPMCA4b antibody, JA9. Immunostaining was detected by the ECL (Enhanced Chemiluminescence) technique. The constructs and their fragments were quantified using the Bio-Rad Molecular Imager system and analyzed using the Molecular Analyst and GraphPad Prism softwares.

Ca^{2+} transport assay: Ca^{2+} transport activity was measured by monitoring $^{45}Ca^{2+}$ uptake into COS-7 cell membrane vesicles.

Pre-steady state rate of the activation and inactivation of PMCA: The rate of activation and inactivation of partially purified and reconstituted PMCA proteins was studied by monitoring the ATPase activity of PMCA4b and its mutants using a coupled assay (phosphorolysis of 2-amino-6-mercapto-7-methyl purine ribonucleoside (MESG)). Data were analyzed with the help of GraphPad Prism software (GraphPad Software Inc.).

Structural model of PMCA4b: The sequence of PMCA4b was aligned with that of SERCA1a. The alignment was then processed with the MODELLER 6v2 software, using the SERCA1a structure files (1EUL.pdb, 1IWO.pdb).

5. RESULTS

5.1. Investigation of the intra-molecular interactions of hPMCA4b by limited proteolysis

The Ca^{2+} transport activity of PMCA is modulated by intra-molecular interactions between the catalytic core and the C-terminal region. In our experiments these interactions were monitored using proteases that cleave the enzyme specifically at the C-terminal regulatory region.

5.1.1. *Cleavage sites in the C-terminal region of hPMCA4b*

Three proteases with different substrate specificities (calpain, chymotrypsin, caspase-3) were used to digest hPMCA4b in its natural membrane environment. PMCA4b was expressed in COS-7 cells, and limited proteolysis was performed *in situ* in native COS-7 cell microsomal membranes. The cleavage sites were determined by comparing the electrophoretic mobility of the fragments to that of the intact PMCA4b and C-terminally truncated PMCA4b mutants with known molecular masses.

We found that both calpain and chymotrypsin have one major cut site downstream of the calmodulin-binding sequence, in a region known to be involved in auto-inhibition (downstream auto-inhibitory region). In contrast, caspase-3 cuts the protein upstream of the calmodulin-binding sequence after aspartate 1080.

5.1.2. *Proteolytic degradation of hPMCA4b in different conformations*

The digestion of the pump in the auto-inhibited state was studied both in the absence of Ca^{2+}, when the conformation of the catalytic core was shifted toward the low affinity E2 conformation and in the presence of Ca^{2+}, which favored the high affinity E1 conformation.