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The role of the Ca^{2+} binding ligand Asn879 in the function of the plasma membrane Ca^{2+} pump

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ABSTRACT

Asn879 in the transmembrane segment M6 of the plasma membrane Ca^{2+} pump (PMCA human isoform 4xb) has been proposed to coordinate Ca^{2+} at the transport site through its carboxylate. This idea agrees with the fact that this Asn is conserved in other Ca^{2+}-ATPases but is replaced by Asp, Glu, and other residues in closely related 2P-type ATPases of different ionic specificity. Previous mutagenesis studies have shown that the substitution of Ala for Asn abolishes the activity of the enzyme (Adebayo et al., 1995; Guerini et al., 1996). We have constructed a mutant PMCA in which the Asn879 was substituted by Asp. The mutant protein was expressed in *Saccharomyces cerevisiae*, solubilized and purified by calmodulin affinity chromatography. The Asn879Asp PMCA mutant exhibited about 30% of the wild type Ca^{2+}-dependent ATPase activity and only a minor reduction of the apparent affinity for Ca^{2+}. The decrease in the Ca^{2+}-ATPase of the mutant enzyme was in parallel with the reduction in the amount of phosphoenzyme formed from Ca^{2+} plus ATP. Noteworthy, the mutation nearly eliminated the ability of the enzyme to hydrolyze pNPP which is maximal in the absence of Ca^{2+} revealing a major effect of the mutation on the Ca^{2+}-independent reactions of the transport cycle. At a pH low enough to protonate the Asp carboxylate the pNPPase activity of Asn879Asp increased, suggesting that the binding of protons to Asn879 is essential for the activities catalyzed by E_2-like forms of the enzyme.

1. Introduction

The PMCAs couple the extrusion of Ca^{2+} from the cell with the hydrolysis of ATP [1]. They are members of the P-type family of ion translocating ATPases which form an acylphosphate from ATP and a side chain carboxylate of an aspartic acid as a part of their transport mechanism. In mammals four genes alternative splicing of the primary mRNAs generate a diversity of PMCA isoforms. The best characterized PMCA isoform is hPMCA4xb, the predominant PMCA in human erythrocytes.

Despite some criticisms and limitations, the mechanism of ion transport by the PMCA, like that of the other P-type ATPases, is usually interpreted in terms of the E_1E_2 reaction cycle involving two conformational states of the enzyme known as E_1 and E_2 [2]. According to this model the Ca^{2+}-free enzyme adopts the E_2 conformation and the binding of Ca^{2+} to the transport site promotes the conversion to the E_1 form. E_1 then reacts with ATP leading to the formation of the phosphoenzyme intermediate and the concomitant ion occlusion. The hydrolysis of the phosphoaspartyl-enzyme results in the liberation of the transported Ca^{2+} to the other side of the membranes and the binding of H^+ to the E_2 form which in the PMCA has been proposed to result in the countertransport of one H^+ per Ca^{2+} ion transported [3].

A detailed knowledge of the ionic transport sites of P-ATPases has been a long standing goal. The pioneering work of McLennan and coworkers [4] led to the identification of the amino acid residues involved in the two high affinity Ca^{2+} binding sites of SERCA, the Ca^{2+}-ATPase of the sarcoplasmic reticulum. Based on the results of single mutations of residues within the hydrophobic segments, it was proposed that the Ca^{2+} binding sites of the SERCA were located within the membrane bound portion of the protein. Further mutagenesis studies, and recently the high-resolution structures of SERCA confirmed this hypothesis [5,6]. In the SERCA pump Ca^{2+} binds first to site I which is entirely formed by side-chain oxygen atoms of residues from transmembrane segments M5, M6 and M8 and then to Site II formed by backbone oxygens of M4 and side-chain oxygens from M4 and M6 [6].

Asn879 of the PMCA is a particularly interesting residue because it seems distinctive of P-ATPases that transport Ca^{2+}. While an Asn is conserved at this position of the SERCA type subgroup 2A and PMCA like subgroup 2B of P-ATPases, it is replaced by Asp or Glu in closely related pumps of different ionic specificity as those from subgroup 2C like the Na^+,K^+-ATPase and by hydrophobic residues in those of subgroup 2D.

Abbreviations: PMCA, plasma membrane calcium pump; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PC, L-α-phosphatidylcholine; BE, lipidic extract from bovine brain containing acidic lipids; $C_{12}E_{10}$, polyoxyethylene-10-laurylether

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PMCA Asn879 is homologous to Asn796, one of the Ca^{2+} ligands at site II of SERCA. It has been shown that substitution of Asn796 by Ala or Asp abolishes Ca^{2+}-transport, Ca^{2+}-ATPase and Ca^{2+}-dependent phosphorylation by ATP [4,5,7,8]. At concentrations of Ca^{2+} 100 times higher than those needed for the maximal Ca^{2+}-ATPase of the wild type, the SERCA Asn796Ala mutant reaches about 2% of the activity. On the other hand the formation of the phosphoenzyme by Pi phosphorylation is preserved and is inhibited by Ca^{2+} with an apparent affinity similar to the wild type. These findings are consistent with the idea that Asn796Ala disrupts the Ca^{2+} binding site II and blocks the catalytic activation while still allowing the binding of Ca^{2+} to the other site and the subsequent inhibition of Pi phosphorylation [7,8].

The plasma membrane Ca^{2+} pump in contrast with SERCA has been proposed to handle only one Ca^{2+} per cycle [9,10]. This proposal agrees with the fact that the primary structure of PMCA only retains a strong homology to Ca^{2+} binding site II of SERCA. PMCA mutants of Asn879 expressed in COS cells were reported inactive [9,10] consistently with its proposed role as a ligand for Ca^{2+}. Our recent work using yeasts for the expression of recombinant PMCA has improved our ability to characterize the effects of mutations on the PMCA function using a purified preparation of the enzyme with high specific activity [11]. Here we have investigated further the role of the lateral chain of Asn879 of the PMCA by mutating this residue to Asp which preserves the carbonyl oxygen involved in Ca^{2+} binding. We found that the substitution of Asp for Asn879 reduced but did not abolish the Ca^{2+}-dependent ATPase activity, and the residual activity of the mutant Asn879Asp exhibited near wild type Ca^{2+} dependency. In addition, the mutation nearly eliminated the ability of the enzyme to hydrolyze pNPP in the absence of Ca^{2+}, indicating that a Ca^{2+}-independent reaction of the transport cycle was affected. The low pNPPase activity of the mutant in the absence of Ca^{2+} was stimulated by lowering the pH suggesting that the change Asn879Asp decreases the apparent affinity of the enzyme for the counter transported ion.

2. Materials and methods

2.1. Chemicals

Polyoxyethylene-10-laurylether ($C_{12}E_{10}$), L-α-phosphatidylcholine type XVI-E Sigma from fresh egg yolk, brain extract (BE) Type I Folch Fraction I from bovine brain containing approximately 10% phosphatidylinositol, 50% phosphatidylserine, and other lipids, calmodulin-agarose, calcimycin (A23187), ATP (disodium salt, vanadium-free), SDS, yeast synthetic drop-out media supplement without leucine, yeast nitrogen base without amino acids, dextrose, enzymes and cofactors for the synthesis of [γ-^{32}P]ATP, and all other chemicals were obtained from Sigma. Proteinase K was from Promega. Tryptone and yeast extract were from Difco. [γ-^{32}P]ATP was provided by PerkinElmer Life Sciences (Boston, MA). Salts and reagents were of analytical reagent grade.

2.1.1. Construction of the DNA coding for mutant Asn879Asp

The cDNA coding for mutant Asn879Asp of PMCA human isoform 4xb [12] was obtained by a two-step PCR using Pfu DNA polymerase. For the first PCR primers forward N879D with the sequence 5′-GCAGATGTTGTGGGTTGATCTGATCATG-3′ and reverse h4end 5′-TGAACTGGGC CCTCAAACTG ATGTCTCTAG GCTCTG-3′ along with hPMCA4xb DNA as template were used. Primer h4end contains a restriction site for nuclease *Apa*I at its 3′ position. The amplified fragment was isolated by electrophoresis in a 1% agarose gel, extracted using DNA QIAEX II (QIAGEN), and used in the next PCR step along with primer A 5′-GCCCATGGCCTGTGATGGACT-3′ using the cDNA coding for hPMCA4xb as template. Primer A anneals to the hPMCA4xb DNA upstream of a naturally occurring *Bsp*EI unique site. The amplified fragment was purified, treated with the nucleases *Bsp*EI and *Apa*I, ligated into the pMP-hPMCA4xb vector and sequenced.

2.1.2. Yeast expression, membrane isolation and purification of PMCA

Saccharomyces cerevisiae strain DBY 2062 (MATa his4–619 leu2–3,112) was used for expression of the PMCA as described previously [13]. Yeast cells were transformed with the pMP625 vector containing a Leu^+ marker and the PMAI promoter. The cells were grown at room temperature in the absence of leucine on plates containing 6.7% yeast-nitrogen base without amino acids (YNB), 0.67% complete supplemented medium minus Leu (Leu^-) and 2.2% dextrose.

Total membranes from four liters of yeasts were obtained, solubilized at 4 °C for 10 min by adding 0.5% of $C_{12}E_{10}$ with the addition of 0.1% L-α-phosphatidylcholine type X-E from dried egg yolk (Sigma) and the PMCA protein was purified from the solubilizate by calmodulin-affinity chromatography as described previously [11,13,14], aliquoted and kept in liquid N_2. Before measuring the activity 1 ml of purified PMCA (~20 μg of protein) was added to 250 μl of a previously sonicated mixture of 2.89% of a mixture of acidic lipids (bovine brain extract) and 5.70% of $C_{12}E_{10}$. After 5 min 0.25 g of prewashed wet SM-2 Bio-Beads (Bio-Rad) were added and the mixture was stirred at room temperature for 30 min before the Bio-Beads were separated by filtration and enzyme was directly used for the activity measurements.

2.2. Protein assay

The protein concentration was initially estimated by the method of Bradford [15] using bovine serum albumin as a standard. To achieve a better assessment of the content of PMCA protein in each preparation, the samples were analyzed by SDS-PAGE using bovine serum albumin as a standard, and the intensity of the bands was compared after staining the gels with Coomassie Blue.

2.3. Western blotting and protein staining

SDS electrophoresis and immunoblotting were carried out as previously described [16]. Proteins were electrophoresed on a 7.5% acrylamide gel according to Laemmli [17] and revealed by Coomassie Blue staining, or subsequently electrotransferred onto Millipore Immobilon P membranes. Nonspecific binding was blocked by incubating the membranes overnight at 4 °C in a solution of 160 mM NaCl, 0.05% Tween 20, and 1% non-fat dry milk. The membranes were incubated for 1 h with antiPMCA monoclonal antibodies [18] from ascitic fluid (dilution 1:1000). For staining, biotinylated antimouse immunoglobulin G and avidin-horseradish peroxidase conjugate were used.

2.4. Proteolytic digestion of PMCA

Limited proteolysis of the purified proteins was carried out at 37 °C. The reaction media contained 0.1 μg of PMCA solubilized in 0.17% $C_{12}E_{10}$, 0.08% of BE lipids, 20 mM HEPES-K (pH 7.2 at 37 °C), 100 mM KCl, 4 mM $MgCl_2$, 0.5 mM EGTA, enough $CaCl_2$ for 10 μM free Ca^{2+}. The reaction was initiated by the addition of proteinase K (ratio ATPase/protease of 50:1 by mass) and was arrested at the indicated times (minutes) by the addition of 2.2 μg aprotinine. The samples were suspended in electrophoresis sample buffer and the proteolytic fragments were analyzed by Western Blot.

2.5. Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity was estimated from the release of [^{32}P]P_i from [γ-^{32}P]ATP at 37 °C [19]. The ATPase reaction medium contained 20 mM HEPES-K (pH 7.2 at 37 °C), 100 mM KCl, 4 mM $MgCl_2$, 10 μM of calcimycin, 500 μM EGTA, 3 mM [γ-^{32}P]ATP and enough $CaCl_2$ to give the concentrations of Ca^{2+} indicated in each experiment. The final volume of the ATPase reaction was 0.3 ml. The tubes were transferred to a water bath at 37 °C and the reaction was initiated by adding 50 μl of PMCA (about 2 μg of PMCA protein previously supplemented with

BE lipids as described above). The reaction was terminated by acid denaturation after 60 min. The free Ca^{2+} concentration in reaction medium was calculated using the Maxchelator program [20].

2.6. pNPPase activity

The pNPPase activity was estimated as described [21]. The reaction mixture contained 20 mM HEPES-K (pH 7.20 at 37 °C), 100 mM KCl, 0.5 mM EGTA, 4 mM $MgCl_2$, and 12 mM pNPP. The tubes were transferred to a water bath at 37 °C and the reaction was initiated by adding 50 μl of PMCA (about 2 μg of PMCA protein previously supplemented with BE lipids). The total assay volume was 0.3 ml. The incubation time was 60 min. The (Ca^{2+}-ATP)-dependent pNPPase was measured in an identical media except for the addition of 3 mM ATP and $CaCl_2$ to give the concentrations of free Ca^{2+} indicated in the experiment.

When the pH was varied the reaction media contained in addition 20 mM Mops-imidazol adjusted at each pH. The reaction was stopped by the addition of 1 ml of 1 M NaOH. The tubes were centrifuged at 14,000 rpm in a microcentrifuge for 5 min and the supernatants were monitored for the optical density at 410 nm. Blanks obtained without protein were subtracted from each point. A molar extinction coefficient of 1.78×10^4 M^{-1} cm^{-1} was used to convert optical density in micromoles of p-nitrophenol released.

2.7. Phosphorylation of PMCA

5 μg of purified ATPase in 100 μl of elution buffer was supplemented with 0.57% $C_{12}E_{10}$ and 0.29% of lipids and phosphorylated at 4 °C in 0.25 ml reaction buffer containing 50 μM Tris–HCl pH 7.6 at 4 °C with or without $CaCl_2$ to give a concentration of 100 μM free Ca^{2+}. The reaction was started by the addition of 30 μM [γ32P]-ATP and was stopped after 180 s with 10% ice-cold trichloroacetic acid. After adding 20 μg of bovine serum albumin, the denatured proteins were collected by centrifugation at 20,000×*g* for 10 min, washed once with 5% trichloroacetic acid and 150 mM NaH_2PO_4 and once more with distilled water. The precipitated protein was suspended in sample buffer and separated by acidic SDS-PAGE. The gels were dried and the radioactivity detected using a Storm Molecular Image System.

3. Results

3.1. The Asn879Asp mutant was expressed at a level similar to that of the wild-type enzyme

Microsomal membranes from yeasts expressing the wild type PMCA or the Asn879Asp PMCA mutant were isolated. The immunoblot as shown in Fig. 1A using antiPMCA antibody 5F10 revealed the presence of bands with the expected migration for the PMCA of about the same intensity. The yeast membranes were solubilized with detergent $C_{12}E_{10}$ and the PMCA protein was purified by calmodulin affinity chromatography. Fig. 1B shows a Coomassie Brilliant Blue stained SDS-PAGE gel containing different amounts of purified proteins. A similar yield of Asn879Asp and wild type was obtained after purification. Both proteins showed, in addition to the band corresponding to the full-length PMCAs, lower amounts of a faster migrating fragment which probably resulted from the cleavage of the autoinhibitory C-terminal segment by the action of endogenous proteases.

3.2. The Asn879Asp and wild-type proteins showed similar patterns of proteolytic degradation

In general native proteins are relatively resistant to full degradation by proteases as a consequence of their tight domain folding. In contrast disorganized proteins are usually degraded faster by lower concentrations of proteases. The global structure of the Asn879Asp protein was compared with the wild-type by examining the susceptibility to digestion by proteinase K. Fig. 2 shows that at short times of proteolysis both the full-length wild-type and the mutant proteins were cleaved to a main product of about 126 kDa and smaller amounts of a fragment of 138 kDa. As the digestion advanced, similar fragments of about 90, 76, 64 and 52 kDa were produced from the wild-type and mutant proteins.

3.3. The Asn879Asp mutant had a lower ATPase activity with near wild type apparent Ca^{2+} affinity

The function of the PMCA mutant Asn879Asp was investigated measuring the ATPase activity. The liberation of Pi from ATP at 37 °C, was linear up to 70 min (Fig. 3A). In these conditions, the activity of the Asn879Asp enzyme was 40% of that of the wild type. The activity of the Asn879Asp was very sensitive to the presence of acidic lipids and no activity was detected in the presence of PC (not shown).

Fig. 3B shows the dependency of the ATPase activity with the concentration of Ca^{2+}. In the presence of EGTA and no added Ca^{2+} the enzymes should be devoid of Ca^{2+} since its concentration is extremely low. Under these conditions the Asn879Asp and wild type enzymes exhibited a small ATPase activity of the same magnitude. At increasing concentrations of Ca^{2+} the activity of the wild type enzyme increased reaching half maximal activity at 0.5 μM Ca^{2+}. The activity of the mutant enzyme also increased with the increase of Ca^{2+} indicating the persistence of a Ca^{2+} dependent activation. The mutant reached

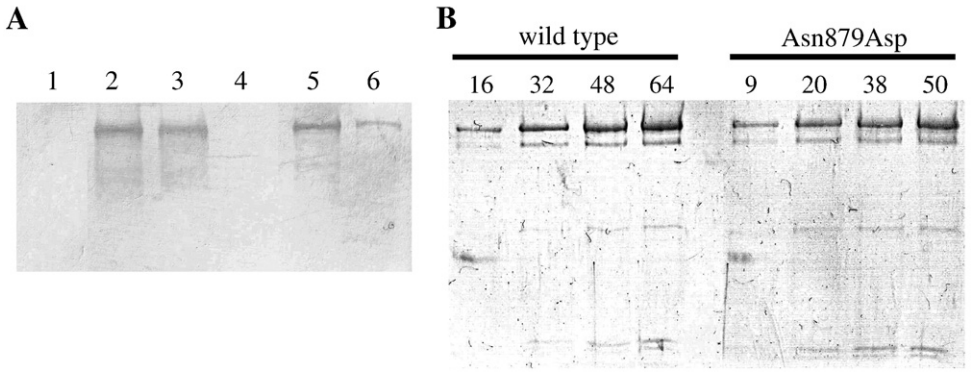

Fig. 1. The Asn879Asp mutant is expressed at a level similar to that of the wild type enzyme. (A) Immunoblot of total microsomal membranes from yeast cells expressing the wild type or the Asn879Asp proteins. Membranes from non-transformed yeasts, lanes 1 and 4, 4.8 and 3.2 μg of protein respectively; membranes from yeast transformed with DNA coding PMCA mutant Asn879Asp, lanes 2 and 5, 4.5 and 2.9 μg of protein, respectively; membranes from yeast transformed with DNA coding wild type PMCA lanes 3 and 6, 3.8 and 2.3 μg of protein, respectively. (B) SDS-PAGE stained with Coomassie Brilliant Blue of different amounts of purified proteins. The numbers on top of each lane indicates the ng of protein loaded.

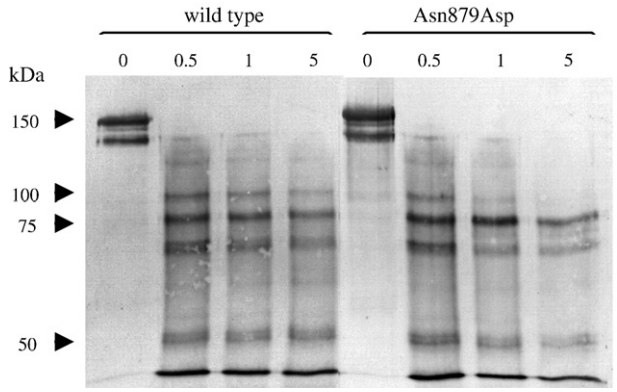

Fig. 2. The sensitivity to proteinase K degradation of the Asn879Asp protein is similar to that of the wild type enzyme. 100 ng of PMCA protein was digested as described in Materials and methods at 37 °C for the times indicated in the figure and then submitted to SDS-PAGE. The PMCA peptides were identified using the 5F10 antibody.

half maximal activity at 1.6 μM Ca^{2+}, and in the presence of saturating amounts of Ca^{2+} its maximal activity was about 30% of that of the wild type.

3.4. The Asn879Asp mutant formed less phosphorylated intermediate from ATP than the wild-type enzyme

The lower Ca^{2+}-ATPase activity of the Asn879Asp mutant may result from the impaired reaction of the enzyme with ATP and Ca^{2+}.

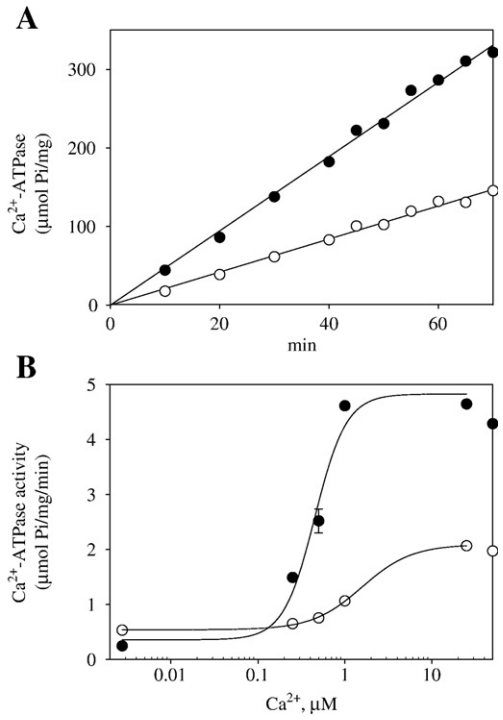

Fig. 3. The Asn879Asp mutant exhibits a reduced Ca^{2+}-ATPase, and Ca^{2+} concentration dependence similar to that of the wild type enzyme. (A) Time course of ATPase of wild type (filled circles) and Asn879Asp mutant (empty circles). The concentration of Ca^{2+} was 10 μM. (B) Ca^{2+} concentration dependence of Ca^{2+}-ATPase activity. The Ca^{2+}-ATPase activity was measured as indicated in Materials and methods. The symbols are the same as in panel A. The lines represent the best fit of the data given by the Hill equation plus a constant (v_0) with the following parameters: wild type Vmax = 4.7 ± 0.8 μmol/mg/min, $K_{1/2}$ = 0.4 ± 0.1 μM, n = 2.4 ± 1.2 v_0 = 0.4 ± 0.6 μmol/mg/min; Asn879Asp mutant Vmax = 1.5 ± 0.1 μmol/mg/min, $K_{1/2}$ = 1.5 ± 0.2 μM, n = 1.6 ± 0.2 v_0 = 0.5 ± 0.1 μmol/mg/min. The value of the activity at 50 μM Ca^{2+} was not included in the fitting.

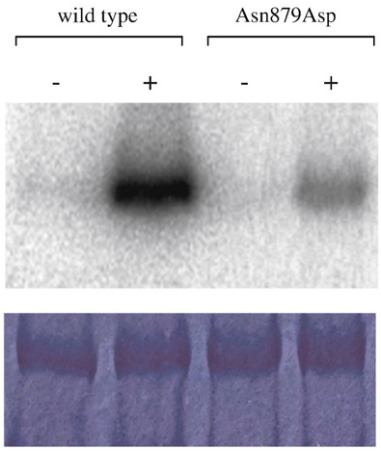

Fig. 4. The Asn879Asp mutant forms less phosphorylated intermediate from ATP than the wild type. 5 μg of purified Asn879Asp or wild type protein were phosphorylated as described in Materials and methods in the presence of 30 μM ATP and 5 mM EGTA (−) or 0.39 mM EGTA plus 0.48 mM $CaCl_2$ (+). In the lower panel the same gel stained with Coomassie Brilliant Blue is shown.

Fig. 4 shows that in the presence of Ca^{2+} and ATP a significant amount of phosphoenzyme was detected in the wild type enzyme. In contrast, the Asn879Asp mutant produced a lower amount of phosphoenzyme which according to the intensity of the bands was about 30% of that of the wild type. The mutant enzyme produced less phosphoenzyme than the wild type even when dephosphorylation was inhibited by adding La^{3+} to the reaction media (not shown). This result may indicate that in the mutant enzyme there is a lower amount of the E_1 conformer capable of reacting with ATP.

3.5. The Asn879Asp mutant exhibited lower apparent affinity for vanadate

The lower ATPase activity and apparent affinity for Ca^{2+} and the lower level of phosphoenzyme could be explained if the mutation caused the displacement of the enzyme toward the E_2 conformation. The affinity of the inhibition by the phosphate transition state analog vanadate has been usually taken as a measure of the abundance of the E_2 conformer of the enzyme during the ATPase reaction cycle. As shown in Fig. 5, the ATPase activity of the Asn879Asp mutant decreased as the concentration of vanadate in the reaction media increased. However, the concentration of vanadate needed for half maximal inhibition of the mutant's activity was about 25 μM compared with 1.8 μM for the wild type enzyme. Thus, this result

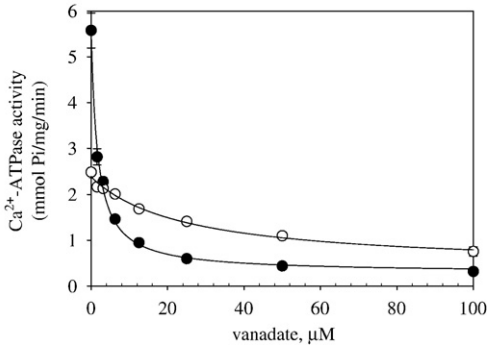

Fig. 5. The Asn879 is more resistant to inhibition by vanadate. The effect of vanadate on the Ca^{2+}-ATPase activity of the wild type and mutant PMCA was measured as indicated in Materials and methods. The symbols are as in Fig. 3. The lines are the best fit to the data given by the equation $v = V\text{max} / (1 + \text{Vanadate} / Ki) + v_0$, using the following parameters: wild type Vmax = 5.2 ± 0.1 μmol/mg/min, Ki = 1.6 ± 0.1 μM, v_0 = 0.3 ± 0.1 μmol /mg/min; Asn879Asp Vmax = 2.0 ± 0.1 μmol/mg/min, Ki = 25 ± 5 μM, v_0 = 0.4 ± 0.1 μmol /mg/min.

suggests that also the concentration E_2 conformer of the enzyme is lower in the mutant.

3.6. The mutation Asn879Asp nearly eliminated the ability of the enzyme to hydrolyze pNPP

It is known that the PMCA can hydrolyze pNPP and other non-nucleotide substrates. This activity is made possible by an E_2-like conformation of the PMCA which is attained in the absence of Ca^{2+}, and the binding of Ca^{2+} decreases the pNPPase activity [21]. In the absence of Ca^{2+} the wild-type enzyme hydrolyzed pNPP at a rate of about 1 μmol/mg protein/min. In the same condition the Asn879Asp mutant had a negligible pNPPase activity near 5% of that of the wild type (Fig. 6A). As shown in Fig. 6B increasing concentrations of Ca^{2+} did not affect the pNPPase activity of Asn879Asp while, as expected, decreased that from the wild type enzyme. At high concentrations of Ca^{2+} the activity of both enzymes became similar. It is known that ATP can rescue the pNPPase activity from the inhibition by Ca^{2+} [22]. The pNPPase activity of the mutant was very low even in the presence of saturating amounts of ATP (Fig. 6C).

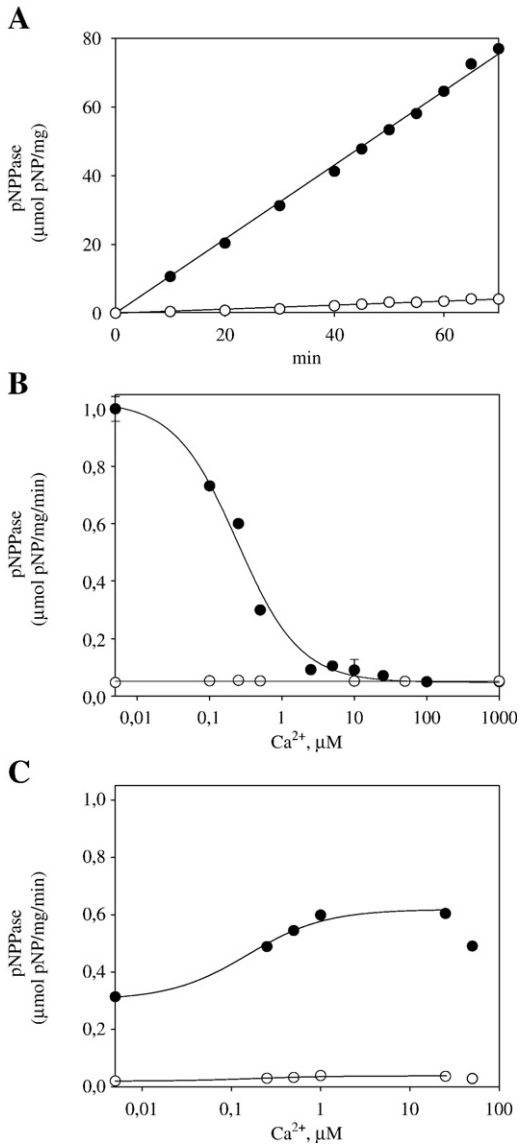

Fig. 6. The Asn879Asp exhibits a reduced phosphatase activity. (A) Time course of pNPPase of wild type and Asn879Asp mutant in the absence of Ca^{2+}. The activity was measured as indicated in Materials and methods in the presence of 0.5 mM EGTA without added $CaCl_2$. (B) Effect of Ca^{2+} on the pNPPase activity of the wild type and Asn879Asp mutant. The pNPPase was measured as indicated in Materials and methods at increasing concentrations of Ca^{2+} which were achieved by adding $CaCl_2$. The activity of the Asn879Asp mutant did not change significantly with Ca^{2+} and had constant value of 0.05 ± 0.01 μmol/mg/min. The data for the wild type was fitted by the equation $v = V_1.Km_1/(Km_1 + Ca^{2+}) + V_2$ with the following parameters: $V_1 = 1.0 \pm 0.1$ μmol/mg/min, $V_2 = 0.05 \pm 0.01$ μmol/mg/min, $Km_1 = 0.24 \pm 0.04$ μM. (C) Effect of Ca^{2+} on the ATP dependent-pNPPase activity the wild type and Asn879Asp mutant. The reaction media was identical to that of the results in panel B except for the presence of 3 mM ATP. The data were fitted by the equation $v = V_1.Ca^{2+}/(Km_1 + Ca^{2+}) + V_0$ with the following parameters: for the wild type $V_1 = 0.31 + 0.02$ μmol/mg/min, $V_0 = 0.30 + 0.02$ μmol/mg/min, $Km_1 = 0.15 + 0.04$ μM and for the mutant $V_1 = 0.018 + 0.004$ μmol/mg/min, $V_0 = 0.020 + 0.003$ μmol/mg/min, $Km_1 = 0.18 + 0.14$ μM. The value of the activity at 50 μM Ca^{2+} was not included in the fitting.

3.7. The pNPPase activity of the Asn879Asp increased with the increase of the concentration of H^+

It has been proposed that the transport of Ca^{2+} by the PMCA is accompanied by the counter transport of H^+. H^+ would bind and stabilize the E_2 form of the enzyme. Following this line of reasoning we tested the effect of pH on the ability of the wild type and mutant enzymes to hydrolyze pNPP. As shown in Fig. 7A, the wild type enzyme attained maximal pNPPase activity at a pH around 6.5 and it decreased at lower pH. In contrast the activity of the mutant enzyme increased as the pH decreased, and reached a maximal activity at a pH around 4.5. We tested whether the mutation caused a similar change in the optimal pH of the ATPase activity. Results in Fig. 7B show that the wild type enzyme reached the highest ATPase activity at a pH near 7.0. In contrast with the pNPPase activity the mutant exhibited maximal ATPase activity at a pH of 7.3, slightly higher than the wild type enzyme.

4. Discussion

Previous studies have shown that the Asn879 of PMCA and Asn796 of SERCA have a critical role in the function of these Ca^{2+} pumps as

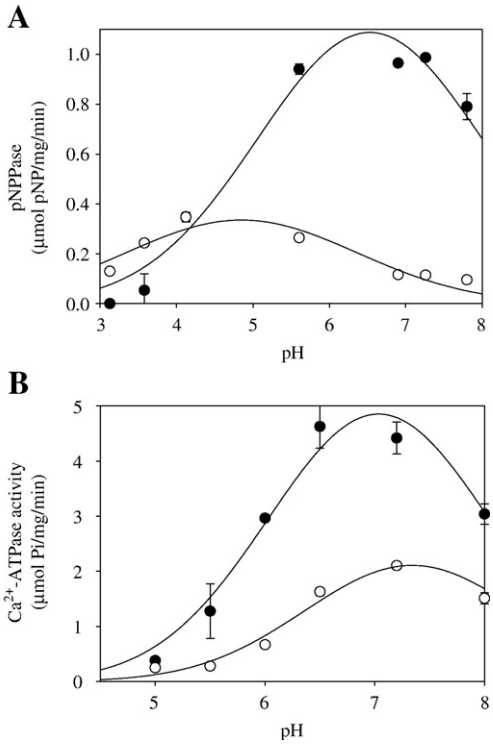

Fig. 7. Dependency with the pH of the wild type and Asn879Asp mutant. (A) The pNPPase activity was measured as indicated in Materials and methods in a media with 0.5 mM EGTA and without added $CaCl_2$ and ATP. (B) The ATPase activity was measured as indicated in Materials and methods in a media containing 10 μM Ca^{2+} and 3 mM ATP. The symbols are as in Fig. 3.

$$E_2H + P_i \rightleftharpoons E_2^- + H^+ \rightleftharpoons E_1^-$$

Scheme 1.

Ca^{2+} binding residues. Most studies however analyzed the consequences of the replacement of Asn by Ala eliminating the side chain oxygen of the lateral chain. Here, by changing Asn879 to Asp the Ca^{2+}-coordinating carbonyl oxygen was preserved and other functions of this residue were made more obvious.

The Asn879Asp PMCA mutant was successfully expressed in yeasts at a level similar to the wild type PMCA, and was obtained in purified form following the standard protocol. These results, and the fact that the mutant protein exhibited a wild type susceptibility to degradation by proteinase K, suggest that the mutation did not cause a global disruption of the protein structure. While the possibility that the structure of the PMCA protein was locally distorted as a consequence of the mutation cannot be discarded, we detected no signs of increased instability of the mutant protein under the conditions used for the activity measurements.

The substitution of Asn879 by Ala has been reported to eliminate the Ca^{2+}-dependent reactions of the PMCA [9,10] while in SERCA the change Asn796Ala eliminates Ca^{2+} binding at site II [5]. In contrast, we found that mutation Asn879Asp reduced the maximal ATP hydrolytic activity to 30–40% of that of the wild type enzyme and decreased the apparent affinity for Ca^{2+} about 3 fold. Thus, the substitution of Asn879 by Asp seems to have a milder effect on the affinity of the Ca^{2+} binding site than the substitution by Ala. Indeed, the lower activity of the mutant at saturating concentrations of Ca^{2+} should be attributed to the decrease of the rate of a reaction of the ATPase cycle other than the binding of Ca^{2+}. Consistent with this idea, we found that the pNPP hydrolysis, which is catalyzed by the PMCA in an E_2-like form, was severely impaired in the mutant either in the presence of Ca^{2+} + ATP or in the absence of Ca^{2+}. Thus, it would seem that the loss of the protonated amido group at position 879 of the PMCA affects the Ca^{2+}-independent reactions associated with the E_2 form of the enzyme. The fact that as the pH decreased, the pNPPase activity of the Asn879Asp mutant increased is also consistent with the proposed need for a protonated lateral chain at this position of the enzyme in the E_2 conformation.

The low sensitivity of the Ca^{2+}ATPase activity to inhibition by vanadate and the low pNPPase activity of the Asn879Asp mutant would indicate that amount of the E_2 conformation is reduced. These results could indicate that vanadate inhibition and the catalysis of pNPP hydrolysis may require the protonated form E_2. Because of the presence of a carboxylate, this form would be more difficult to attain in the mutant at physiological pH.

Previous studies of SERCA have shown that, the change Asn796Ala decreases the rate of dephosphorylation of the acylphosphate intermediate [8]. In contrast the lower activity of the PMCA Asn879Asp mutation cannot be explained by a reduction in the rate of dephosphorylation. Instead the parallel reduction of the Ca^{2+}-ATPase activity and the level of phosphoenzyme suggest a decreased amount of enzyme in the E_1 conformation which may result from the stabilization of the enzyme in the deprotonated E_2 form (represented as E_2^-, in Scheme 1). The accumulation of the enzyme in this form may explain the lower activity of the mutant either with ATP as a substrate or pNPP as a pseudosubstrate. If the pNPPase activity is attributed to the E_2H form of the enzyme, the decrease in the pNPPase activity may reflect that in the mutant the reaction $E_2H \rightarrow E_2^-$ is displaced toward E_2^-. However, this change is not expected to decrease the ATPase activity, and for this effect to take place is necessary to assume that in addition, the stabilization of E_2^- also decreases the rate of the transition between E_2^- and E_1^-. This would lower the amounts of EP and induce a change in the pH dependency of the pNPPase and ATPase activities of the mutant as experimentally observed.

Although the crystal structure of the PMCA is still not available, the functional relevance of Asn879Asp in the E_2 form of the enzyme is consistent with the position of Asn796 in the structure of SERCA. In the Ca^{2+} bound E_1 the amide of Asn796 is solely connected to a water molecule [6]. In contrast in E_2 (TG + BHQ) [23] the side chain amide of Asn796 is involved in interhelix hydrogen bonds with Ca^{2+} binding site I residue Glu771 in M5, and with the Ca^{2+} binding site II residue Glu309 in M4. Because the substitution of Asp for Asn introduces a negative charge it may destabilize the hydrogen bonds in which the amide group is involved. These hydrogen bonds may be preserved despite the change of the amide by carboxylate provided that protons are available. This effect can account for the increase of the phosphatase activity of the mutant at acidic pH.

It has been previously proposed that Asn796 participates in the countertransport of protons by SERCA [8,24]. In agreement with this proposal, our results show that in the PMCA the function of this residue cannot be ascribed solely to the binding of Ca^{2+}, and argue in favor of a role of Asn879 as one of the residues from a recently proposed "cation filter" group of residues of the P-ATPases that would alternatively coordinate both the transported and countertransported ions [25].

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