Deletions in the N-Terminal Segment of the Plasma Membrane Ca^{2+} Pump Impair the Expression of a Correctly Folded Functional Enzyme†

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ABSTRACT: Mutant cDNAs encoding h4 plasma membrane Ca^{2+} pumps with deletions in the N-terminal segment have been constructed and expressed in COS cells. As judged by immunoblotting, each construct was expressed at a high level similar to that of the wild-type enzyme. The removal of the first six amino acids had no effect on the Ca^{2+} transport activity, but deletions in the segment 15−75 reduced the activity to undetectable levels. The d(43−56)h4 mutant, lacking amino acids 43−56, was also efficiently expressed in stable form in CHO cells. The Ca^{2+} transport activity of d(43−56)h4 in this system was about 40% of that of the wild type. The d(43−56)h4 enzyme exhibited a similar affinity for Ca^{2+}, a slightly increased apparent affinity for ATP, and a slightly lower sensitivity to inhibition by vanadate than the wild-type enzyme. Analysis of the phosphoenzyme intermediate formed in the presence of lanthanum showed that the phosphorylation reaction was not affected, but the maximum amount of phosphoenzyme was reduced to the same extent as the Ca^{2+} transport activity. These results suggest that the expressed d(43−56)h4 was a mixture of fully active and inactive enzyme. The d(43−56)h4 enzyme was more easily degraded by proteases and had a higher sensitivity to heat inactivation than the wild type suggesting that the loss of function was due to the improper folding and instability of the mutant protein. On the basis of these findings, it appears that the N-terminal segment of the plasma membrane Ca^{2+} pump is neither essential for synthesis nor for catalytic activity but is critical for the expression of a correctly folded functional enzyme.

The plasma membrane Ca^{2+} pump (PMCA)[1] extrudes Ca^{2+} from the cytosol to the extracellular space against its concentration gradient at the expense of ATP hydrolysis. The pump is a single subunit P-type ATPase of molecular mass about 134 kDa with 10 membrane-spanning domains (M1−M10) and two major cytosolic loops between M2−M3 and M4−M5. These regions are highly conserved in all P-type pumps unlike the N and C terminal segments that are highly variable (*1*, *2*). It is well-known that the C-terminal 120 amino acid segment of hPMCA4b functions as an autoinhibitory domain and can be removed from the molecule, either by proteolysis or deletion mutagenesis, resulting in a fully active calmodulin-insensitive Ca^{2+} pump (*3*, *4*). In contrast, the function of the N-terminal segment of the PMCA is unknown.

We have previously shown that deletion of amino acids 18−75 of the hPMCA4b lead to the expression of an inactive pump indicating that unlike the C-terminal region, the N-terminal segment of the PMCA is important for a functional enzyme (*5*). Here we report on the functional consequences of smaller deletions involving amino acids from the segment 1−75 of the PMCA isoform h4 (Figure 1). We found that the activity of the enzyme was not affected by the removal of the first six residues, but it was substantially reduced by deletions involving the succeeding amino acids. The reduced activity can be accounted for by the expression of lower amounts of functional enzyme and a misfolded inactive pump. Thus, it seems that the N-terminal segment of the PMCA is not necessary for catalytic function

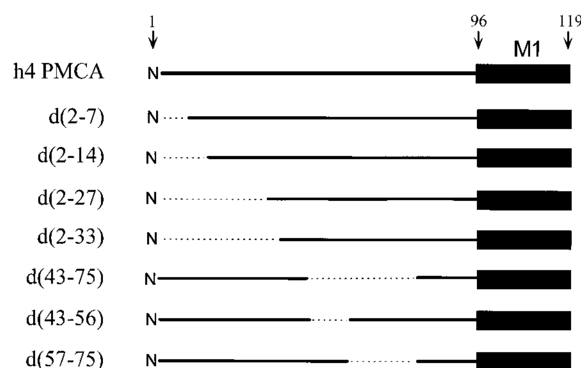

FIGURE 1: Scheme of the deletions in the N-terminal region of the plasma membrane Ca^{2+} pump. The numbers on the top refer to the amino acid position in the sequence of the wild-type Ca^{2+} pump human isoform 4 (h4 PMCA). The dotted lines indicate the deleted amino acid segment, and the black boxes correspond to the first transmembrane segment (M1).

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[1] Abbreviations: PMCA, plasma membrane Ca^{2+} ATPase; hPMCA4b, human plasma membrane Ca^{2+} ATPase isoform 4b; since this is the only PMCA isoform studied here it is also referred as “h4” enzyme. ct120, mutant of hPMCA4b lacking the C-terminal 120 amino acids; SERCA, sarcoplasmic/endoplasmic Ca^{2+} ATPase; SDS, sodium dodecyl sulfate.

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but is important to either generate or stabilize a properly folded protein.

EXPERIMENTAL PROCEDURES

Materials. Reagents were purchased from the following companies: enzymes used in DNA manipulations, New England Biolabs and Promega Corp.; oligonucleotide primers, DNAgency, Malvern, PA, USA; columns for DNA purification, Qiagen; ^{45}Ca, DuPont NEN; Immobilon transfer membranes and Nitrocellulose filters, Millipore; Immunochemicals, Vector Labs.; reagents for cell culture, thapsigargin and other chemicals, Sigma. The expression vectors pED and pMT2 were a generous gift of Dr. Randall J. Kaufman, Genetics Institute, Boston, MA. The vector pMM2 was derived from pMT2 as described previously (*6*).

Construction of Mutant cDNAs and Expression in COS-1 Cells. The construction of the cDNAs encoding h4(ct120) and d(18−75)h4(ct120) was described previously (*4*, *5*). The new constructs were obtained by polymerase chain reaction using the following oligonucleotide primers (nucleotide sequence 5′ → 3′): (2−7), aacccgtcgaccATGgtcttgcctgccaactcgat; (2−14) tgcaggtcgaccATGgccgagagccgtgaaggg; (2−27) tgcaggtcgaccATGgaactgaggaagctcat; (2−33) tgcaggtcgaccATGgagctgcgttcgagggatg; 308 tctccaggatgatgagcgtgacat; A1 ctgcaggtcgaccATGgcgaacccat; 3Q43R gacattaatacgcgtcagtgcatccctcgaacg; 5S56T gaatctctgcacgcgtctgaaaacctcccctgt; 3S56T ggttttcagacgcgtgcagagattctgtacacct;

Oligonucleotides (2−7), (2−14), (2−27), and (2−33) included the sequence recognized by the *Sal*I nuclease and the consensus initiation translational site (*7*), which is indicated in capital letters. They were used as the 5′-primer together with oligonucleotide 308 as the 3′-primer. The PCR products were digested with *Sal*I and *Dra*III (internal site present in the wild-type sequence) and the fragments ligated into the pMM2/h4(ct120) in place of the wild-type DNA.

A DNA coding for mutant d(43−75) was constructed using primers A1 and 3Q43R. Oligonucleotides A1 and 3Q43R contained a *Sal*I site and a *Mlu*I site, respectively. The product A1-3Q43R was digested with *Sal*I−*Mlu*I and the fragment, coding for residues 1−42, was inserted into the pMM2/d(18−75)h4(ct120). The cDNAs coding for mutants d(43−56) and d(57−75) were constructed using a similar strategy with primers 5S56T-308 and 3S56T-A1, respectively. The digested PCR products were inserted in pMM2/d(43−75)h4(ct120). The sequence of the *Sal*I−*Dra*III fragment from all the mutated DNAs was verified by dideoxy sequencing of double-stranded plasmids. The plasmid constructs used for transfections were purified on Qiagen columns according to the supplier's protocol.

The transfection of COS-1 cells (*8*) was carried out either by the DEAE-dextran-Chloroquine method (*9*) or using lipofectamine (Gibco) and harvested after 48 h. The microsomal fraction was isolated as described previously (*4*). Protein concentration was estimated by means of the Bio-Rad protein assay (Bio-Rad Laboratories, CA), with bovine serum albumin as a standard.

Stable Expression of Wild-Type h4 and d(43−56)h4 Mutant Ca^{2+} Pumps in CHO Cells. For stable expression of the Ca^{2+} pumps in CHO(dhfr-) cells, the pED vector was employed (*10*). The cDNA coding the h4 enzyme was cloned into the pED vector in two steps involving the insertion of the *Sal*I−*Eco*RI and *Eco*RI−*Eco*RI fragments obtained by digestion of the pMM2-h4 DNA. To express d(43−56)h4 in a stable form the pMM2/d(43−56)h4(ct120) DNA was digested with *Sal*I and *Bsp*EI, and the cDNA fragment was cloned into the corresponding position of pED-h4.

CHO (dhfr-) cells were obtained from the ATCC and maintained in Dulbecco's modified Eagle medium supplemented with 0.1 mM hypoxanthine, 0.01 mM thymidine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% of fetal calf serum. The cells were grown at 37 °C in humidified 5% CO_2/air on standard plastic culture dishes. Confluent dishes of 3.5 cm in diameter were transfected using lipofectamine with the empty vector pED or with the pED containing the cDNA coding for h4 or d(43−56)h4. Two days after transfection, the cells were split into three dishes of 15 cm in diameter and cultured in minimum essential medium Eagle alpha modification without ribonucleosides and deoxyribonucleosides, supplemented with antibiotics and 10% of dialyzed fetal calf serum. After 3 weeks, the colonies were isolated using cloning cylinders and five clones were analyzed for the integration of the transfected DNA by genomic PCR and for the expression of the pump protein by immunoblotting. Membranes were prepared from one of the positive clones, and the activity of the expressed enzyme was estimated by measuring the ATP-dependent thapsigargin-insensitive Ca^{2+} uptake into microsomal vesicles.

Detection of Expressed Ca^{2+} Pumps. SDS electrophoresis and immunoblotting were carried out as previously described (*11*). Proteins were electrophoresed on a 7.5% acrylamide gel according to Laemmli (*12*) and subsequently transferred to Millipore Immobilon 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 at 37 °C for 1 h with 5F10 or JA9 antibody (*13*) from ascitic fluid (dilution 1:1000). For staining, biotinylated anti-mouse immunoglobulin G and avidin-horseradish peroxidase conjugate were used.

Assay of Ca^{2+} Transport. Ca^{2+} uptake was assayed as described previously (*4*), in 500 μL of a reaction mixture containing 10 μg of microsomal protein, 100 mM KCl, 50 mM Tris-HCl (pH 7.3 at 37 °C), 5 mM NaN_3, 400 nM thapsigargin, 20 mM sodium phosphate, 95 μM EGTA, 8 mM $MgCl_2$, 6 mM ATP, and $CaCl_2$ to give different concentrations of free Ca^{2+} as indicated in each experiment. ^{45}Ca was present at a specific activity of about 2×10^5 cpm/nmol. The free Ca^{2+} concentration was calculated using the program of Fabiato and Fabiato (*14*). When the activity of the full-length Ca^{2+} pumps was measured, 240 nM calmodulin was added to the reaction medium. In the experiment shown in Figure 4, panel C, a lower concentration of ATP (375 μM) was used because high concentrations of ATP partially release the inhibition of the PMCA by vanadate (*15*, *16*).

The vesicles were preincubated at 37 °C for 5 min in the reaction mixture without ATP, and the reaction was initiated by the addition of ATP. The uptake was terminated after 5 min by filtering the samples through a 0.45-μm filter. The ^{45}Ca taken up by the vesicles was determined by counting in a liquid scintillation counter. Uptake activities were expressed per milligram of membrane protein.

The ATP dependence of the Ca^{2+} transport was measured in the same medium, but the total $MgCl_2$ was varied to ensure a constant level of free Mg^{2+}. The concentration of $MgCl_2$ in the absence of ATP was 4 mM, and ATP and $MgCl_2$ were added in equimolar amounts.

Formation of the Phosphorylated Intermediate. The reaction was carried out as described previously (*5*) in 250 μL of reaction buffer containing 15 μg of membrane protein, 100 mM KCl, 25 mM Tris-HCl (pH 7.0 at 4 °C), 400 nM thapsigargin, and 50 μM $CaCl_2$. When indicated, 50 μM of $LaCl_3$ was added to the reaction medium to stabilize the PMCA phosphoenzyme. The membranes were preincubated in the reaction buffer for 5 min, and the reaction was initiated by the addition of 1 μM (γ-^{32}P)ATP. The reaction was stopped at different times with 15 μL of 100% trichloroacetic acid. After 30 μg of bovine serum albumin was added, the denatured proteins were collected by centrifugation at 10000*g* for 10 min and washed twice with 1 mL of a cold solution containing 7% of trichloroacetic acid and once with 1 mL of distilled water. The proteins were dissolved in a gel loading buffer and separated by electrophoresis in a 7% SDS−acrylamide gel according to ref *17*. The gels were dried and exposed for 2−24 h at −70 °C. After the autoradiographs were produced, the slices of gel containing the PMCA phosphoprotein were cut and counted in a liquid scintillation counter.

Limited Tryptic Digestion of the Expressed Proteins. Ten micrograms of membrane protein were resuspended in a solution containing 100 mM KCl, 50 mM Tris-HCl (pH 7.3 at 37 °C), 5 mM NaN_3, 20 mM sodium phosphate, 95 μM EGTA, 8 mM $MgCl_2$, 6 mM ATP, and 0.15 mM $CaCl_2$ and 5 μg/mL of trypsin at a protein/trypsin ratio of 40:1. The mixture was incubated at 37 °C for different times, and the digestion was stopped by adding a 10-fold excess of aprotinin. The samples were incubated on ice for 10 min and then subjected to immunoblot analysis.

Thermal Inactivation. Ten micrograms of microsomal protein were incubated in Ca^{2+} uptake medium without ATP for 5 min at successively increasing temperatures following which the samples were immediately transferred to a water−ice bath. After the sample was incubated for 30 min on ice, ATP was added, and the Ca^{2+} transport was measured at 37 °C as described above.

RESULTS

Expression and Ca^{2+} Transport Activity of PMCA Mutants with Deletions in the N-Terminal Segment. Mutant PMCAs containing deletions in the N-terminal segment were transiently expressed in COS-1 cells using a C-terminally truncated h4(ct120) construct. Figure 2, panel A shows that in membranes from transfected cells antibody 5F10 recognized a band corresponding to the endogenous enzyme and a lower band corresponding to the expressed ct120 proteins. The intensity of the bands indicated that the mutant proteins were expressed at a level similar to that of the h4(ct120) enzyme.

The Ca^{2+} transport activity of the mutants is shown in Figure 2, panel B. The activity of mutant d(2−7)h4(ct120) was 95% of that of h4(ct120) indicating that deletion of the six residues following the initial methionine did not significantly affect the ability of the pump to transport Ca^{2+}. However, the removal of amino acids 2−14 resulted in a mutant enzyme with about 55% of the activity, and deletions further downstream in the segment 15−75 reduced the rate of Ca^{2+} uptake to a value close to that of control microsomes from cells transfected with the empty vector. Experiments using the full-length h4 enzyme instead of its ct120-truncated version indicated that the loss of activity was independent of the C-terminal regulatory domain (not shown).

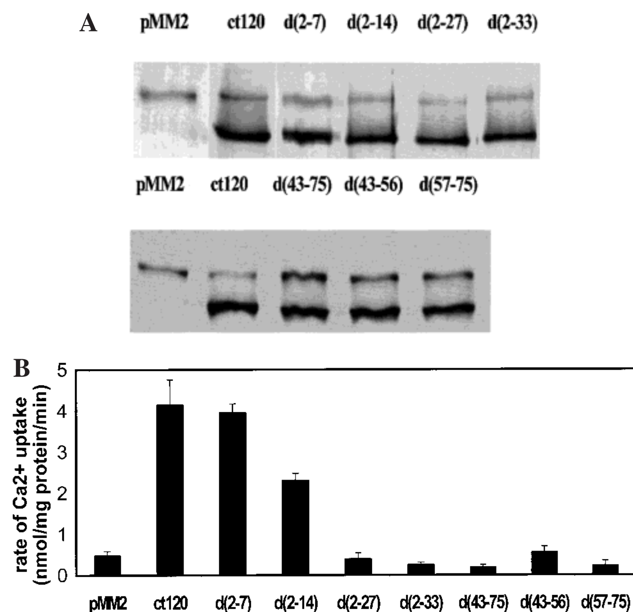

FIGURE 2: Expression level and Ca^{2+} transport activity of mutant Ca^{2+} pumps expressed in COS cells. (A) Immunoblot of microsomes from COS-1 cells transfected with the empty vector pMM2, and pMM2 containing the cDNAs encoding ct120 or the indicated N-terminally deleted ct120 mutants. Three micrograms of membrane protein were applied on each lane of a 7.5% SDS gel and subjected to immunoblot analysis using monoclonal antibody 5F10. (B) Ca^{2+} transport activity. The Ca^{2+} uptake in microsomal vesicles was measured for 5 min at 37 °C in a reaction mixture containing 1 μM Ca^{2+} and 6 mM ATP. The values shown in the figure are the average of two to seven determinations using different microsomal preparations. The error bars correspond to the standard deviation.

Ca^{2+} Transport Activity of the d(43−56)h4 Mutant Ca^{2+} Pump Expressed in Stable Form in CHO Cells. To obtain larger amounts of expressed PMCA, stable CHO cell lines expressing the h4 and d(43−56)h4 enzymes were isolated. In agreement with previous findings (*4*, *18*) immunoblot analysis showed that the h4 enzyme expressed in CHO cells had a migration similar to the PMCA from human erythrocytes but slightly faster than the endogenous PMCA (Figure 3, panel A). The intensity of the endogenous PMCA was significantly weaker in CHO cells expressing either the d(43−56)h4 or the wild-type enzyme than in CHO cells transfected with the empty vector pED. This probably reflects a downregulation of the endogenous enzyme, as it has been previously described (*19*, *20*). The measurements of the Ca^{2+} uptake showed that the d(43−56)h4 expressed in the CHO cell system retained a Ca^{2+} transport activity close to 37% of that of the wild type (Figure 3, panel B).

The Ca^{2+} transport activity of the d(43−56)h4 from transfected CHO cells was tested as a function of free Ca^{2+} concentration. As shown in Figure 4, panel A, despite the lower maximal velocity of d(43−56)h4, the Ca^{2+} dependency of the Ca^{2+} uptake was not significantly different between microsomes containing h4 and d(43−56)h4.

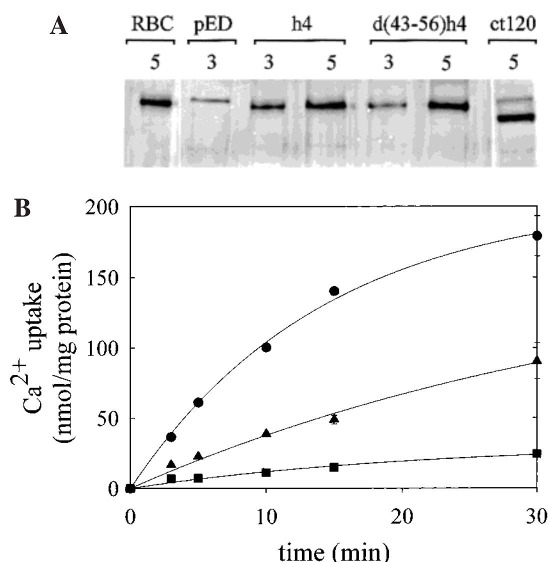

FIGURE 3: Stable expression of wild-type h4 and d(43−56)h4 mutant Ca^{2+} pumps in CHO cells. (A) Immunoblot using antibody 5F10 of microsomes isolated from CHO cells transfected with the empty vector pED and CHO cells containing the expressed h4 or d(43−56)h4 proteins. The number on top of the lane indicates the amount in microcrograms of microsomal protein loaded. RBC, membranes from human red blood cells; ct120, microsomes from CHO cells expressing the ct120-truncated mutant. (B) Time course of Ca^{2+} uptake by microsomes from CHO cells: control (pED) (squares), h4 (circles), or d(43−56)h4 (triangles). The Ca^{2+} uptake was measured at 37 °C in a reaction media containing 15 μM Ca^{2+} and 6 mM ATP.

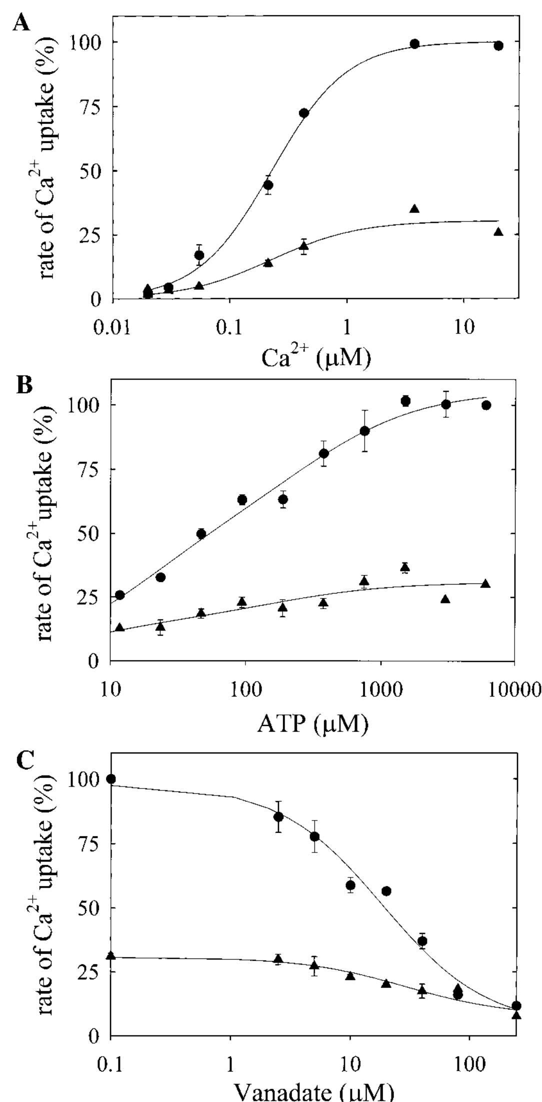

FIGURE 4: Kinetic properties of the Ca^{2+} transport by the d(43−56)h4 enzyme. (A) Ca^{2+} concentration dependence of Ca^{2+} uptake by h4 and d(43−56)h4. Ten micrograms of microsomal protein from CHO cells expressing h4 (circles) or d(43−56)h4 (triangles) were preincubated for 5 min at 37 °C in the reaction media, and the uptake was initiated by the addition of 6 mM ATP. The reaction was terminated after 5 min. The maximum rate of Ca^{2+} uptake of the wild-type enzyme was taken as 100%. The lines represent the best fit of the data given by the Hill equation. Data points are the average of two experiments. The V_{max} and $K_{1/2}$ values were h4, 100%, 0.23 μM; d(43−56)h4, 30%, 0.22 μM. (B) Ca^{2+} transport activity of the h4 and d(43−56)h4 pumps as a function of ATP concentration. The rate of Ca^{2+} uptake was measured after 5 min incubation at 37 °C as described under Experimental Procedures. The free Ca^{2+} was 15 μM. The symbols are the same as that for panel A. Data points are the average of two experiments. The best fit to the data was obtained by adjusting a double rectangular hyperbolic equation with the following parameters h4, V1 = 56%, V2 = 50%, Km1 = 17 μM, Km2 = 311 μM; d(43−56)h4, V1 = 16%, V2 = 15%, Km1 = 5 μM, Km2 = 179 μM. (C) Effect of vanadate on Ca^{2+} transport. Ca^{2+} uptake assays were carried out for 5 min at 37 °C in the presence of 0−250 μM vanadate as described under Experimental Procedures. The symbols are the same as that for panel A. The concentrations of ATP and free Ca^{2+} were 375 μM and 15 μM, respectively, and the rate of Ca^{2+} uptake at 0 μM vanadate of microsomes containing the h4 enzyme was taken as 100%. Data points for each curve are the average of two determinations. The lines represent the best fit to the data given by the following equation V = Vr + Vs/(1 + [vanadate]/Ki) with the following parameters h4, Vs = 94%, Vr = 4%, Ki = 19 μM; d(43−56)h4, Vs = 23%, Vr = 8%, Ki = 29 μM.

The activity of the mutant enzyme was also measured as a function of ATP concentration (Figure 4, panel B). The response to ATP of the d(43−56)h4 and h4 enzymes was similar, and both reached their maximum transport rate at about 1 mM ATP. However, at low concentrations of ATP the activity of the d(43−56)h4 enzyme became close to 50% of the corresponding wild-type activity suggesting that the affinity for ATP at the catalytic site was higher in the mutant than in the wild-type enzyme.

Vanadate is a potent inhibitor of all P-ATPases presumably by binding to the E2 form of these enzymes. As shown in Figure 4, panel C, concentrations of vanadate up to 5 μM reduced the activity of h4 to about 75% but did not significantly affect the activity of d(43−56)h4 indicating that the mutant had an apparent affinity for vanadate slightly lower than the wild type. This result suggests that in the d(43−56)h4 enzyme the proportion of E2 during the reaction cycle slightly decreased.

Formation of the Phosphorylated Intermediate. The ability of the d(43−56)h4 enzyme expressed in CHO cells to form the phosphorylated intermediate from ATP was investigated (Figure 5, panel A) both in the presence and the absence of lanthanum. Lanthanum is known to block the decomposition of the phosphoenzyme (*21*, *22*). In the absence of lanthanum, a band corresponding to the PMCA phosphoenzyme was only detected in microsomes containing the wild-type pump. As expected, the intensity of the wild type phosphoenzyme increased substantially in the presence of lanthanum. Under these conditions, a small amount of phosphoenzyme became visible in mutant d(43−56)h4.

Figure 5, panel B, shows the time course of phosphoenzyme formation by h4 and d(43−56)h4. Although the

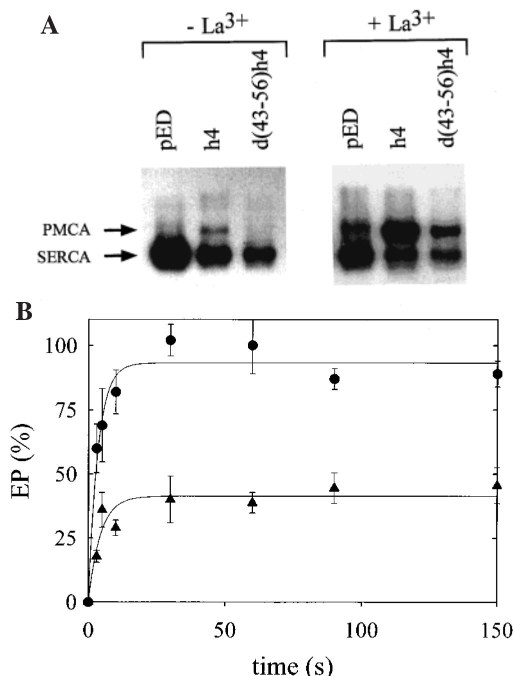

FIGURE 5: Phosphoenzyme formation by wild-type h4 and d(43−56)h4 mutant Ca^{2+} pumps. (A) The formation of the phosphoenzyme intermediate from ATP was studied on membranes obtained from CHO cells transfected with the empty vector pED, and from CHO cells expressing h4 or d(43−56)h4. Thirty micrograms of microsomal protein were phosphorylated at 4 °C for 1 min in the presence of 1 μM of (γ-^{32}P)ATP, 50 μM $CaCl_2$ with or without 50 μM $LaCl_3$ as described under Experimental Procedures. The phosphorylated proteins were separated by electrophoresis using a 7% acidic SDS−acrylamide gels, and the radioactivity was detected by autoradiography. (B) Time course of phosphorylation. Microsomal vesicles containing the expressed h4 (circles) and d(43−56)h4 (triangles) enzymes were preincubated for 5 min at 37 °C in the presence of 50 μM $CaCl_2$ and 50 μM $LaCl_3$. The reaction was initiated by the addition of 1 μM of (γ-^{32}P)ATP, and it was quenched at different times with 6% trichloroacetic acid. The phosphorylated proteins were separated by electrophoresis, exposed to X-ray films, and quantified by cutting gel slices and counting the radioactivity in a liquid scintillation counter. The maximum amount of radioactivity of the h4 enzyme was taken as 100%. The data points are average of three experiments.

amount of phosphoenzyme increased rapidly in both enzymes, the d(43−56)h4 mutant showed a maximal level of phosphoenzyme of about 40% of that of h4. This value is close to the relative Ca^{2+} transport activity of the mutant suggesting that the decrease of Ca^{2+} transport activity was accompanied by a similar reduction in the amount of phosphorylated intermediate.

Structural Integrity of the d(43−56)h4 Protein. CHO cell microsomes containing the d(43−56)h4 protein were exposed to trypsin, and the pattern of proteolytic fragments was compared with that produced by the wild-type h4 pump. The immunoblot of the proteolyzed samples with antibody 5F10, which reacts between amino acids 719−738, is shown in Figure 6, panel A. The treatment with trypsin caused a progressive disappearance of the intact wild-type enzyme and the production of peptides of 90−76 kDa as previously described in erythrocyte membranes (*17*). In contrast, most of the d(43−56)h4 protein was rapidly cleaved after 30 s of digestion.

The production of tryptic peptides from the N-terminal portion of the pump was followed using antibody JA9 (Figure 6, panel B). The epitope for JA9 has been previously located between amino acids 51−75, but it did also recognize the mutant d(43−56)h4. In agreement with previous studies (*23*, *24*), fragments of molecular mass of about 32−38 kDa accumulated as the digestion progressed. These and other smaller fragments were produced faster in the d(43−546)-h4 mutant than in the wild-type enzyme. Experiments (not shown) using the nonspecific protease Pronase also indicated that at short times of proteolysis the mutant enzyme was more susceptible to degradation than the wild-type protein.

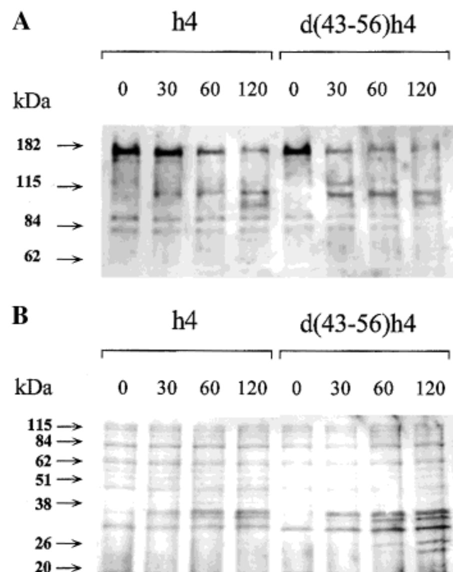

FIGURE 6: Tryptic digestion of membranes from CHO cells containing the h4 and d(43−56)h4 proteins. The treatment with trypsin was performed at 37 °C as described under Experimental Procedures. The number on top of each lane indicates the duration of proteolysis in seconds. (A) The proteins were separated in a 7.5% SDS−acrylamide gel, and the PMCA fragments were detected with antibody 5F10. (B) The proteins were separated in a 12.5% SDS−acrylamide gel, and the PMCA fragments were detected with antibody JA9.

The structural stability of the d(43−56)h4 protein was also assessed by comparing its heat inactivation profile with that of the h4 enzyme. As shown in Figure 7, the Ca^{2+} transport activity of the wild-type enzyme dropped to 50% of its initial value after incubation for 5 min at 49.5 °C while incubation at 46 °C over the same time period sufficed for a similar loss of activity of d(43−546)h4. This result suggests that the structure of the active d(43−56)h4 enzyme was less stable than the wild type.

DISCUSSION

We have performed several small deletions covering the N-terminal segment of the h4 plasma membrane Ca^{2+} pump. The mutant proteins were correctly expressed in the COS cell system, and none of the alterations significantly affected the level of pump protein present in microsomal membranes. The mutant d(2−7)h4(ct120) had a Ca^{2+} transport activity about equal to that of the h4(ct120) enzyme, indicating that the residues closer to the N-terminus of the protein were neither important for biosynthesis nor for function. Deletion of amino acids 2−14 decreased the activity to about 55%, and further deletions in the segment 15−75 reduced the activity below detectable levels.

The mutant d(43−56)h4, lacking only 14 amino acids, was also efficiently expressed in a stable form in CHO cells. The

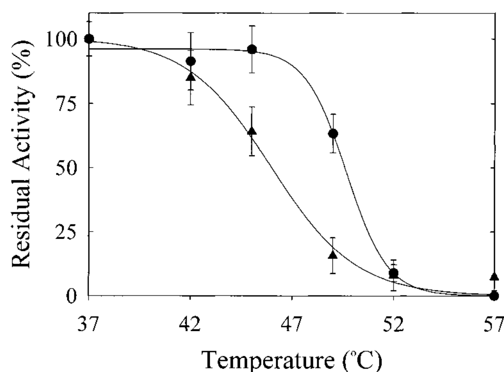

FIGURE 7: Thermal inactivation profiles for the Ca^{2+} transport activity of the h4 and d(43−56)h4 enzymes. CHO cell microsomes containing the h4 (circles) and d(43−56)h4 (triangles) enzymes were incubated for 5 min at different temperatures, and the residual Ca^{2+} transport activity was measured for 5 min at 37 °C as described under Experimental Procedures. The concentrations of ATP and free Ca^{2+} were 375 and 15 μM, respectively. The activity of each enzyme after incubation for 5 min at 37 °C was taken as 100%. The data points are average of three experiments with different microsomal preparations. The lines represent the best fit of the data given by the equation $Vr = Vm/(1 + e^{-(t - t50)/b})$ with the following values h4, Vm = 96%, b = −1, t50 = 49.7 °C; d(43−56)h4, Vm = 100%, b = −2, t50 = 46.0 °C.

Ca^{2+} transport activity of CHO cell microsomes containing the d(43−56)h4 mutant was clearly higher than that from control microsomes and about 30−40% of that of microsomes containing the wild-type pump. Apparently, the d(43−56)h4 produced in CHO cells had a higher relative activity than that produced in COS cells. This result suggests that the level of functional d(43−56)h4 enzyme was affected by the biosynthetic processing in the host cell.

Despite the lower maximal rate of transport, the d(43−56) enzyme expressed in CHO was functionally similar to wild type displaying a normal Ca^{2+} dependency and high apparent affinity for ATP. In addition the d(43−56) was slightly more resistant to vanadate than the wild-type pump. These results suggest that the loss of activity in the d(43−56) mutant was not caused by a reduction in the conversion of the enzyme from E2 to E1.

Although in the absence of lanthanum, it was not possible to detect the presence of phosphoenzyme in the d(43−56)h4 mutant, in the presence of lanthanum, a small amount of phosphoenzyme became visible. Under these conditions the maximal level of d(43−56)h4 phosphoenzyme was about 40% of that of h4. Because lanthanum blocks the decomposition of the phosphoenzyme, arresting the enzyme in a phosphorylated form, the amount of phosphoenzyme obtained in the presence of lanthanum is thought to be close to the total amount of active enzyme. Furthermore, the Ca^{2+} transport activity of d(43−56)h4 was proportionate with its maximal amount of phosphoenzyme, suggesting that some of the expressed d(43−56)h4 enzyme was fully functional while the rest was neither capable of transporting Ca^{2+} nor of phosphorylation. Further supporting this interpretation, we found that the d(43−56)h4 enzyme was phosphorylated at a normal rate. This is exactly what is expected if only the number of active enzyme units is reduced, since the rate constants for first-order exponential time courses are independent of enzyme concentration and so are unaffected by the presence of denatured enzyme. However, because lanthanum in addition to block dephosphorylation also slows the phosphorylation (*22*, *25*), differences in the rate of phosphoenzyme formation between the d(43−56)h4 and wild-type enzymes under normal conditions in the absence of lanthanum cannot be discarded.

Limited proteolysis experiments showed that the d(43−56)h4 protein was more sensitive to degradation by proteases than the wild-type enzyme. This result is consistent with the existence of at least a fraction of the expressed d(43−56)h4 protein in a structurally altered form. The increased rate of proteolysis of d(43−56)h4 may also result from the local exposure of peptide bonds that were partially protected by the N-terminal segment in the native structure.

The expression of nonfunctional d(43−56)h4 enzyme may indicate that the removal of the N-terminal segment impaired the initial folding of the protein, which is likely to be the rate-limiting step for the synthesis of an active PMCA when the level of translation is high. It is worth noticing that a similar situation is frequently found after overexpression of other mutant and wild-type proteins (*26*, *27*). Also in this case the limitations of the expression system for the proper processing of increasing amounts of newly synthesized polypeptides results in variable yields of functional products. Alternatively, it is possible that all the d(43−56)h4 protein, although initially similarly folded, was less stable than the wild type. Indeed, we found that the d(43−56)h4 activity was more sensitive than the wild type to heat inactivation suggesting that the d(43−56)h4 enzyme had a higher tendency to denaturation than the wild type. In all likelihood, the production of inactive d(43−56)h4 was the combined result of misfolding problems during biosynthesis and instability. Accordingly, although the N-terminal segment is not essential for the catalytic activity of the enzyme, it seems to be important to either generate or stabilize a correctly folded PMCA.

Previous studies have shown that deletions in the N-terminal region of the yeast plasma membrane H^+-ATPase (*28*) and SERCA (*29*, *30*) cause a rapid degradation of the protein and drastically reduce the expression levels. We found that this was not the case of the PMCA mutants with deletions in the N-terminal segment since their cellular expression was not hampered. This fact may indicate that the expression of PMCA is less tightly regulated by the quality control mechanisms that degrades misfolded proteins. On the other hand, it is tempting to speculate that regardless of the low conservation of amino acid sequence, the N-terminal segment may have a similar structural importance in different P-type pumps. Moreover, in agreement with our previous proposal for the N-terminal segment of the PMCA (*24*), the recent elucidation of the crystal structure of SERCA (*31*) has shown that the N-terminal segment is part of the so-called “domain A”, which also includes the segment between M2 and M3 highly conserved in all P-ATPases previously called the “transduction domain” or the “β-strand domain” (*32*).

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