Energy Transduction and Kinetic Regulation by the Peptide Segment Connecting Phosphorylation and Cation Binding Domains in Transport ATPases†

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ABSTRACT: The sarcoplasmic reticulum ATPase segment (Thr316-Leu356) connecting the extramembranous phosphorylation domain to the preceding transmembrane helix M4 (which is an integral component of the Ca^{2+} binding domain) retains a high degree of sequence homology with other cation transport ATPases. Single, non conservative mutations of *homologous* residues in this segment produces enzyme inhibition (Zhang et al., 1995). We have now produced single and multiple mutations of *non-homologous* residues in this segment of the Ca^{2+} ATPase to match the corresponding residues of the Na^+,K^+ ATPase. We find that the main characteristics of the ATPase mechanism (i.e., Ca^{2+} dependent phosphoenzyme formation and thapsigargin sensitivity) are retained even when the entire 41-amino acid (Thr316-Leu356) segment of the Ca^{2+} ATPase is rendered identical to the corresponding segment of the Na^+,K^+ ATPase by sequential mutations of the 14 non-homologous amino acids. However, the phosphoenzyme turnover (likely rate limited by the "$Ca_2{\cdot}E_1$-P → $Ca{\cdot}E_2$-P transition") is progressively reduced if four or more Ca^{2+} ATPase residues are mutated to the corresponding residues of the Na^+,K^+ ATPase. The time course of enzyme inactivation by EGTA (likely rate limited by the "E_1 to E_2 transition") is also prolonged. Our findings suggest that an analogous peptide segment provides a functional linkage for *energy transduction* between phosphorylation and cation binding domains in various cation transport ATPases. However, its *kinetic influence* on rate-limiting conformational transitions is dependent on matching specific structures in each ATPase.

The catalytic domain of the sarcoplasmic reticulum (SR) ATPase resides within the extramembranous region of the enzyme and contains an aspartyl (Asp351) residue undergoing phosphorylation as an intermediate step of the catalytic and transport cycle (Bastide et al., 1973; Degani & Boyer, 1973). On the other hand, the two calcium ions undergoing active transport are bound within a transmembrane domain formed by the four clustered helices M4, M5, M6, and M8 (Clarke et al., 1989). The SR ATPase segment (Thr316-Leu356) connecting the phosphorylation site to the preceding transmembrane helix (M4) retains a high degree of sequence homology with other cation transport ATPases (Inesi & Kirtley, 1992), and single non-conservative mutations of any of the *homologous* residues interferes with enzyme activity (Zhang et al., 1995). We have now produced single and multiple mutations of *non-homologous* residues in the Ca^{2+} ATPase to match the corresponding residues of the Na^+,K^+ ATPase. We then evaluated the functional properties of the resulting proteins to determine if and how chimeric mutations of increasing size would affect catalytic and transport function.

EXPERIMENTAL PROCEDURES

PCR Mutagenesis and Protein Expression. The chicken fast muscle SR Ca^{2+} ATPase (SERCA-1) cDNA (Karin et al., 1989) containing a c-myc tag and 11 unique restriction sites spaced at approximately 300-bp intervals (Zhang et al., 1993) was subcloned into pSELECT-1 vector (Promega, Madison, WI). This plasmid was used for amplification and as template DNA in polymerase chain reactions. The SERCA-1 gene sequence connecting the phosphorylation site with the M4 transmembrane region was amplified by PCR using oligonucleotide "flanking" primers 5′-GCTCATTAA-CATCGGCCACT-3′ and 5′-GCACAGATGGTGGCCAA-CTC-3′. The resulting 437-bp fragment contains *Bam*H1 and *Bsu*36I restriction sites to facilitate mutant cassette exchange with corresponding wild-type cDNA. Complementary mutagenic oligonucleotides of 27−36 bases in length were synthesized for each individual mutation. Specifically, these primers hybridized DNA sequences internal to the flanking primers and were used for PCR mutagenesis by the overlap extension method as described by Ho et al. (1989). Briefly, two overlapping fragments containing the mismatched base-(s) of the targeted sequence were amplified in separate PCR reactions. The PCR cocktails contained 1 μM each of flanking and mutagenic primers, 800 μM of dNTPs, 500 ng of SERCA-1 cDNA, 2.5 units of *Pfu* (*Pyrococcus furiosus*) DNA polymerase, and *Pfu* buffer in a final volume of 100 μL (Stratagene, Menasha,WI). The reaction products were analyzed on a 3% low melting agarose gel (FMC, Rockland, ME), and the correct molecular weight band was excised, melted at 72 °C for 5 min, and diluted with 500 μL of H_2O. In a subsequent PCR reaction, the diluted fragments were fused, and the entire cassette was amplified using both flanking primers. The mutant cassette was exchanged with corresponding wild-type cDNA in pSELECT-1 vector and sequenced by the dideoxy-chain-termination method using Sequenase (USB Corp.). Additive chimeric mutations were introduced by sequential PCR mutagenesis using mutant DNA as template. Finally, the mutated cDNA was subcloned

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into COS-1 expression vector pCDL-SRα296 (Takebe et al., 1988) for transfection and subsequent transient expression of protein under SV40 promoter as described by Sumbilla et al. (1993).

Microsomal Preparation and Immunodetection of Expressed Protein. The microsomal fraction of transfected COS-1 cells was prepared as described by Sumbilla et al. (1993). Immunodetection of expressed ATPase in the microsomal fraction was obtained by Western blotting and Elisa assays, using the mAb 9E10 antibody to the c-myc tag, and the CaF3-5C3 antibody to the SERCA-1 ATPase (Karin et al., 1989), as described by Sumbilla et al. (1993).

Northern Blot. Total cellular RNA was isolated and purified from COS-1 cells 3 days following transfection as described by Chomczynski and Sacchi, 1987. The RNA was separated by electrophoresis on a 1.2% agarose gel containing 15% formaldehyde and transferred by capillary diffusion to a Duralon-UV membrane (Stratagene, Menasha, WI). A radioactive 437-base pair probe specific for SERCA was generated by PCR using [α-^{32}P]dATP. The PCR reaction mixture contained 500 ng of wild-type Ca^{2+} ATPase cDNA, 20 μM each of *Bam*H1 and *Bsu*36I flanking primers (as described for mutagenesis), 100 μM each of dTTP, dCTP, dGTP, 50 μM dATP, 50 μCi of [α-^{32}P]ATP, and 1 unit of *Pfu* DNA polymerase (Stratagene, Menasha, WI) in a final volume of 100 μL of 1× *Pfu* buffer. The RNA was UV-linked to Duralon-UV membrane before pre-hybridization overnight at 37 °C in 50% formamide, 5× SSPE (0.9 M NaCL, 50 mM NaH_2PO_4, 5 mM EDTA, pH 8.0), 0.5% SDS, 5× Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin), and 100 μg of salmon sperm DNA/mL. Hybridization was performed under the same conditions in the presence of 5×10^5 cpm of [α-^{32}P]ATP-labeled probe per mL of hybridization solution containing 50% formamide, 5× SSPE, 0.5% SDS, 5× Denhardt's solution, and 10% dextran. The membrane was washed twice at room temperature in 2× SSPE, 0.2% SDS, and twice at 50 °C in 0.1% SSPE, 0.2% SDS before autoradiography.

Functional Studies. ATP dependent Ca^{2+} transport was measured by following the accumulation of radioactive calcium tracer in microsomal vesicles separated from the reaction mixture by filtration (0.45 μm Millipore filters). The reaction mixture contained 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, and variable concentrations of EGTA to yield the desired concentration of free Ca^{2+} [calculated according to Fabiato and Fabiato (1979)], 5−10 μg of microsomal protein/mL, 5 mM potassium oxalate, and 3 mM ATP. The reaction was started (30 °C) by the addition of oxalate and ATP and was terminated at sequential times by vacuum filtration. The loaded filters were washed with 2 mM $LaCl_3$ and 10 mM MOPS (pH 7.0) and were then processed for determination of radioactivity by scintillation counting. The observed rates of Ca^{2+} transport were corrected to reflect the level of expressed ATPase in each microsomal preparation, as revealed by immunoreactivity and with reference to microsomes obtained from COS-1 cells transfected with wild-type cDNA.

ATPase activity was assayed in a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM $CaCl_2$, 5 mM azide, 30 μg of microsomal protein/mL, 3 μM ionophore A23187, and 3 mM ATP. Ca^{2+} independent ATPase activity was assayed in the presence of 2 mM EGTA and no added Ca^{2+}. The reaction was started (37 °C) by the addition of ATP, and samples were taken at serial times for determination of P_i by the method of Lanzetta et al. (1979). The Ca^{2+} dependent activity was calculated by subtracting the Ca^{2+} independent ATPase (30%−35% of total ATPase in wild-type preparations) from the total ATPase and was corrected to account for the level of expressed protein in each microsomal preparation as revealed by immunoreactivity, and with reference to microsomes obtained from COS-1 cells transfected with wild-type cDNA.

Steady state levels of phosphorylated enzyme intermediate were obtained by adding 0.1 mL of 10 μM [γ-^{32}P]ATP to 0.4 mL of a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 30−100 μg of microsomal protein (the amount of microsomal protein was varied to approximate the amount of expressed ATPase contained by wild-type controls, as revealed by immunoreactivity). The components of the reaction mixture were preincubated in ice, and vortex mixing was carried out in the cold room. The reaction (carried out at 2 °C) was quenched after 10 s by the addition (vortex mixing) of 0.105 mL of 10.0 M perchloric acid. The suspension was transferred immediately onto an Eppendorf tube containing 100 μg of carrier protein (inactive microsomes or bovine serum albumin) and allowed to sit in ice for 5−10 min. After centrifugation at 5000 rpm for 10 min, the sedimented protein was washed twice with 0.125 M PCA and once with water. An aliquot of the solubilized sample was then subjected to gel electrophoresis (Weber & Osborn, 1969) at pH 6.3, and the radioactive phosphoenzyme was detected by autoradiography.

Thapsigargin inhibition of phosphoenzyme formation was performed as described above except for a preincubation (5 min, room temperature) of the reaction mix containing the protein with 500 nM thapsigargin before the addition of $CaCl_2$. Initialization of the reaction was with ATP as previously indicated.

The rates of phosphoenzyme decay were determined by first obtaining steady state levels of phosphoenzyme as explained above. Ten seconds following the addition of radioactive ATP, 0.5 mL of 1.0 mM non-radioactive ATP was added with rapid mixing, and samples were acid quenched with perchloric acid at serial times as described earlier. A zero-time base line was obtained by acid quenching before the chase. The entire procedure was carried out in ice and in the cold room. Washings, electrophoresis, and autoradiography were performed as described above.

The time course of enzyme inactivation by EGTA was determined by adding 2.0 mM EGTA to a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM MgCl2, 20 μM $CaCl_2$, and 30−100 μg of protein per mL. At serial times following addition of EGTA, 10 μM [γ-^{32}P]-ATP was added to start the phosphorylation reaction, which was then acid quenched 10 s after the addition of ATP. Control samples were obtained by allowing the phosphorylation reaction to occur in the absence of EGTA. The entire procedure was carried out in ice and in the cold room, and the reagents were added by rapid mixing.

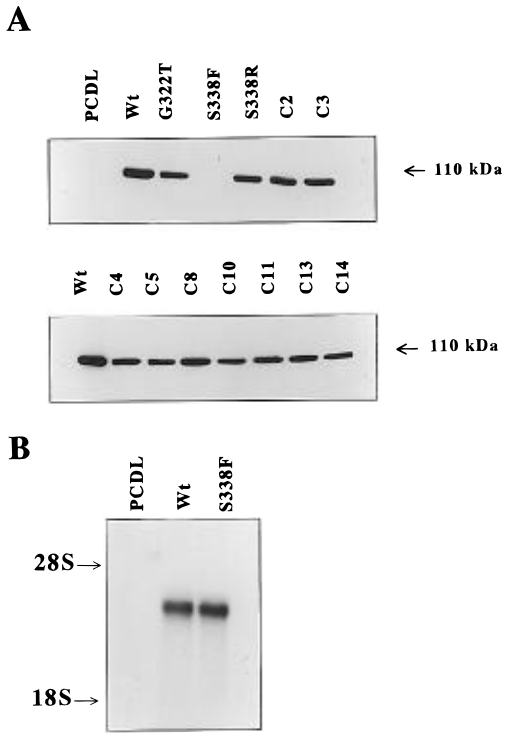

FIGURE 1: Western and Northern blots derived from COS-1 cells transfected with wild-type and mutant cDNAs. (A) Western blot. Microsomal vesicles were obtained from transfected COS-1 cells. Microsomal protein (15 μg) was separated by SDS gel electrophoresis and reacted with mAb CaF3-5C3. Control microsomes were obtained from cells transfected with plasmid (pCDL) containing no cDNA. (B) Northern blot. Total cellular RNA was isolated from COS-1 cells 3 days post-transfection. Purified RNA (10 μg) was separated overnight on a 1.2% agarose/15% formaldehyde gel and transferred by capillary diffusion to a Duralon-UV membrane. Ca^{2+} ATPase transcript was detected using a SERCA specific 437-bp probe (500 000 cpm/mL) generated by PCR.

RESULTS

Levels of Expression. Approximately 10% of the COS-1 cells transfected under our conditions overexpress and target the Ca^{2+} ATPase to the endoplasmic reticulum, as shown by in situ immunodetection of the c-myc tag at the carboxyl terminal (Zhang et al., 1995). In the experiments reported here, Western blot analysis of microsomal fractions obtained from the harvested cells revealed similar levels of expression for wild-type ATPase and ATPase mutants (Figure 1A). Minor variations of expression levels were generally related to the efficiency of transfection rather than the presence of mutations. At any rate, the expression levels were quantitated by densitometry of Western blots, and the resulting values were used to correct the functional parameters to be described below, with reference to the wild-type enzyme.

An exception to this general pattern was the Ser338 to Phe mutation which was not expressed at significant levels in the transfected COS-1 cells. As previously reported regarding similar cases (Zhang et al., 1995), we found that mRNA was produced at high levels (Figure 1B), suggesting that the lack of expression was not due to a transcriptional shortcoming but rather to impaired folding and assembly of the Ser338 to Phe mutant.

Ca^{2+} Uptake and ATP Hydrolysis. As originally reported by Maruyama and MacLennan (1988), microsomal vesicles obtained from transfected COS-1 cells sustain ATP dependent Ca^{2+} uptake. This is a most specific and useful functional signal which, in our present experiments (30 °C), occurred at rates varying between 20 and 30 nmol of Ca^{2+} per minute per mg of microsomal protein obtained from cells transfected with wild-type cDNA (Figure 2). Control microsomes obtained from cells transfected with plasmid and no cDNA insert sustained negligible Ca^{2+} uptake.

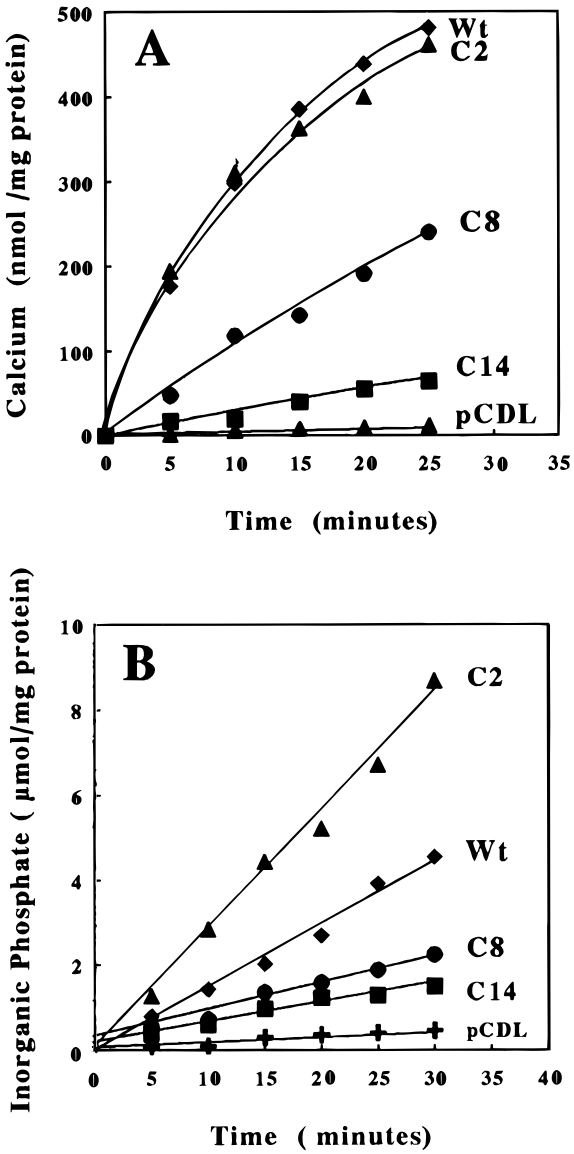

FIGURE 2: Examples of Ca^{2+} uptake and calcium dependent ATP hydrolysis by wild-type and mutant Ca^{2+} ATPases. Microsomal vesicles were obtained from COS-1 cells transfected with wild-type ATPase and ATPases containing two (C2), eight (C8), and fourteen (C14) chimeric mutations (refer to Table 1). Control microsomes were obtained from COS-1 cells transfected with plasmid (pCDL) containing no Ca^{2+} ATPase cDNA. (A) Calcium uptake was measured at 30 °C in a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 5 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM $^{45}CaCl_2$ (1.4 μM free Ca^{2+}), 5 mM potassium oxalate, 5 μg of microsomal protein/mL, and 3 mM ATP. Fractional samples were filtered through 0.45 μm Millipore filters, washed with 2 mM $LaCl_3$, 10 mM MOPS, pH 7.0, and processed for scintillation counting. (B) ATPase activity was measured at 37 °C in a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 3 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM $CaCl_2$, 5 mM sodium azide, 30 μg of microsomal protein/mL, 3 μM ionophore A23187, and 3 mM ATP. Ca^{2+} dependent ATP hydrolytic activity was determined by subtracting the Ca^{2+} independent ATPase from total ATPase activity as described in Experimental Procedures.

The Ca^{2+} ATPase segment (T316−L356) considered in this study includes 27 residues with homologous cor-

Table 1: Mutation scheme[a]

	316 320 325 330 335 340 345 350 356
Ca	T T C L A L G T R R M A K K N A I V R S L P S V E T L G C T S V I C S D K T G T L T
G322T	T
T323A	A
A331C	C
S338A	A
C344S	S
S338F	F
S338R	R
C2	A A
C3	A C A
C4	A C A S
C5	T A C A S
C8	T A K R C N A S
C10	T A K R C L K N A S
C11	T A K R C L K N E A S
C13	V T T A K R C L K N E A S
C14	V T T A K R C L K N E A S T
Na/K	T V C L T L T A K R M A R K N C L V K N L E A V E T L G S T S T I C S D K T G T L T

[a] The native sequences of the aligned segments of the Ca^{2+} ATPase and of the Na^+,K^+ ATPase are given on the top and on the bottom, respectively. Twenty-seven amino acids are homologous, and fourteen are not. The first five mutations are single replacements of non-homologous amino acids of the Ca^{2+} ATPase with the corresponding residue of the Na^+,K^+ ATPase. Mutations of Ser338 to Phe and Arg are single replacements with amino acids that are not the corresponding Na^+,K^+ ATPase residues. Mutations "C" are stepwise, multiple replacements of non-homologous Ca^+ ATPase amino acids with the corresponding Na^+,K^+ ATPase residues, to finally yield identical peptide segments.

Table 2: Functional Characterization of the Mutants[a]

chimeric mutation	Ca^{2+}-uptake rate	ATPase activity
pCDL (control)	{none}	{none}
wild-type	1.0	1.0
G322→T	0.65 ± 0.20	0.75 ± 0.31
T323→A**	1.56 ± 0.25	1.30 ± 0.20
A331→C**	1.76 ± 0.18	1.87 ± 0.29
S338→A**	0.86 ± 0.39	0.84 ± 0.09
C344→S**	0.76 ± 0.10	1.08 ± 0.02
S338→F	not expressed	
S338→R	0.59 ± 0.07	1.05 ± 0.08
C2	1.04 ± 0.10	1.36 ± 0.20
C3	0.75 ± 0.31	1.08 ± 0.13
C4	0.45 ± 0.09	0.62 ± 0.11
C5	0.38 ± 0.08	0.22 ± 0.03
C8	0.45 ± 0.11	0.44 ± 0.07
C10	0.07 ± 0.01	0.20 ± 0.07
C11	0.19 ± 0.04	0.21 ± 0.02
C13	0.09 ± 0.03	0.28 ± 0.07
C14	0.14 ± 0.10	0.34 ± 0.09

[a] Functional parameters are given as fractional values with reference to wild-type ATPase. These values reported for each mutant are averages of two to three measurements performed with two to three microsomal preparations obtained from COS-1 cells subjected to transfection in separate sessions. The functional values were corrected to reflect the level of ATPase expression in each preparation as determined by immunoreactivity. "**" indicates mutations previously reported by Zhang et al. (1995).

respondence in the Na^+,K^+ ATPase and 14 non-homologous residues (Table 1). We previously found (Zhang et al., 1995) that single non-conservative mutations of homologous residues produce strong inhibition of the Ca^{2+} ATPase. On the other hand, single mutations of the non-homologous residues to match the corresponding residues of the Na^+,K^+ ATPase (first five mutations in Table 1) produce little or no inhibition and in some cases enhancement of transport activity (Table 2).

We then produced an increasing number of additive mutations of non-homologous residues ("C" mutations in Table 1) in order to reach stepwise a total identity of the Ca^{2+} ATPase T316-L356 segment with the corresponding Na^+,K^+ ATPase segment. We found that the rates of transport and hydrolytic activity (Figure 2A,B) remained high even in the presence of three simultaneous mutations (Table 2). On the other hand, when a larger number of mutations were made to render stepwise the entire peptide segment identical to the corresponding segment of the Na^+,K^+ ATPase, a progressive inhibition of Ca^{2+} transport and ATPase activity was produced (Tables 1 and 2).

It is noteworthy that if only one of these residues (e.g., Ser338) was subjected to single non-conservative mutations to Phe or Arg (rather than being changed to the corresponding Na^+,K^+ ATPase residue), functional inhibition was produced (Tables 1 and 2).

Formation and Turnover of Phosphoenzyme Intermediate. We performed several experiments to uncover whether the ATPase inhibition produced by certain mutations involved the formation (step 2 in Figure 3A) or the turnover (steps 3 and 4 in Figure 3A) of the phosphoenzyme intermediate obtained by utilization of ATP in the presence of Ca^{2+}. We found that the phosphoenzyme levels obtained with all other mutants were similar to those obtained with wild-type enzyme (Figure 4), independent of whether the steady state ATPase activity was inhibited or not. This indicates that inhibition of intermediate formation is not a prominent feature of the effects of these mutations. We also found that formation of the phosphoenzyme remained totally Ca^{2+} dependent and thapsigargin sensitive (Figure 5), even when the entire peptide segment under study was rendered totally identical to the corresponding segment of the Na^+,K^+ ATPase.

We then conducted isotopic chase experiments to evaluate the effect of mutations on the decay of the phosphorylated enzyme intermediate (steps 3 and 4 in Figure 3A). This was accomplished by first obtaining steady state levels of radioactive phosphoenzyme in the presence of Ca^{2+} and

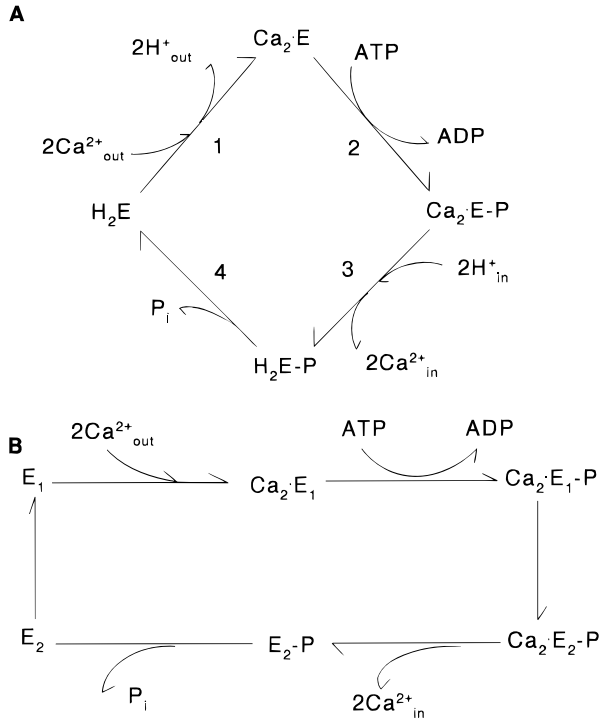

FIGURE 3: Reaction schemes for the Ca^{2+} ATPase. (A) Ca^{2+} binding in exchange for H^+, formation of phosphorylated enzyme intermediate by utilization of ATP, vectorial translocation of bound Ca^{2+}, and hydrolytic cleavage of P_i are the partial reactions that can be measured by chemical methods, and they are listed in the diagram for convenience of easy reference in the text. (B) This diagram (de Meis & Vianna, 1989) proposes two isomeric transitions ($E_1 \leftrightarrow E_2$ and $E_1P \leftrightarrow E_2P$) to account for the interconversion of enzyme states manifesting high affinity and cytosolic orientation or low affinity and lumenal orientation of Ca^{2+} binding, respectively.

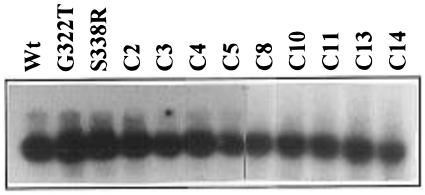

FIGURE 4: Autoradiographs demonstrating that similar steady state levels of phosphorylated enzyme are formed by wild-type and mutated ATPase even though some of the mutants exhibit a reduction of steady state velocity. Microsomal protein was incubated 10 s with mixing by Vortex at 3 °C in a reaction containing 20 mM MOPS, pH 7.0, 80 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM EGTA, 0.2 mM $CaCl_2$, and 2 μM [γ-^{32}P]ATP. The reaction was stopped by addition of 1 M perchloric acid, and the phosphorylated protein was washed twice with 0.125 M perchloric acid and once with water before solubilization, gel electrophoresis, and autoradiography.

[γ-^{32}P]ATP and then adding an excess of non-radioactive ATP. Following the isotopic chase, nearly all of the newly formed phosphoenzyme was non-radioactive, and the disappearance of radioactive phosphoenzyme was indicative of phosphoenzyme turnover. It was previously reported (Andersen et al., 1989; Clarke et al., 1993; Vilsen et al., 1989, 1991) that the turnover reaction is sensitive to certain ATPase mutations. We found that in all mutants exhibiting ATPase activity comparable to that of the wild-type enzyme, the phosphoenzyme turnover was also similar to that of the wild-type enzyme. On the other hand, the rate of phosphoenzyme decay was progressively reduced as the number of chimeric mutations was increased, consistent with the observed

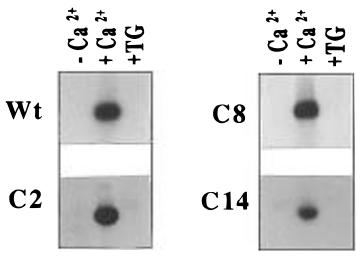

FIGURE 5: Autoradiographs showing Ca^{2+} dependency and thapsigargin (TG) sensitivity of phosphoenzyme formation. Microsomal vesicles were obtained from COS-1 cells expressing wild-type or chimeric mutant Ca^{2+} ATPase as indicated. Microsomal protein (30−100 μg) was preincubated at room temperature for 5 min in a reaction mixture containing 20 mM MOPS, pH 7.0, 80 mM KCl, 2.5 mM $MgCl_2$, and either 1 mM EGTA ($-Ca^{2+}$) or 0.2 mM EGTA ($+Ca^{2+}$) in the absence or presence of TG. The samples were transferred to ice, and 0.2 mM $CaCl_2$ was added (except for $-Ca^{2+}$). The samples were incubated at 3 °C with [γ-^{32}P]ATP for 10 s before quenching with 1 M perchloric acid. The phosphorylated protein was washed twice with 0.125 M perchloric acid and once with water before solubilization, gel electrophoresis, and autoradiography.

inhibition of steady state ATPase activity (Figure 6 and Table 2).

We considered that the observed reduction of phosphoenzyme turnover may be related to an effect of mutations on the rate-limiting phosphoenzyme conversion (Ca_2·E_1-P → Ca_2·E_2-P in Figure 3B) from a conformation sustaining Ca^{2+} binding of high affinity and cytosolic orientation to a conformation manifesting Ca^{2+} binding of low affinity and lumenal orientation. We then tested whether the corresponding transition in the absence of ATP ($E_1 \rightarrow E_2$) in Figure 3B) would also be affected by the mutations. To this aim we added EGTA to enzyme preincubated with Ca^{2+} (reverse of step 1 in Figure 3A) and *then* added ATP at serial time to monitor the time required for the enzyme to lose its ability to be phosphorylated by ATP (reaction 2 in Figure 3A). As shown in Figure 6A, we found that the time course of enzyme inactivation by Ca^{2+} dissociation was also delayed by the large chimeric mutations. We also found that this *global* effect on both E_1-P → E_2-P *and* $E_1 \rightarrow E_2$ transitions requires rather substantial structural changes as in the case of large chimeric mutations (e.g., C13 or C14) or of the single mutation Pro312 to Ala (Figure 6A,B). When we tested previously reported mutations (Chen et al., 1996) for this new parameter, we found (Figure 6B) that some single mutations interfere with phosphoenzyme turnover (E_1-P → E_2-P transition) without interfering with enzyme inactivation by EGTA ($E_1 \rightarrow E_2$ transition). On the other hand, single mutations which did not interfere with the E_1-P -- E_2-P transition, did not interfere with the $E_1 \rightarrow E_2$ transition either (Figure 6B).

DISCUSSION

Formation of a phosphorylated enzyme intermediate is a common mechanistic feature of cation transport ATPases. It was demonstrated by chemical analysis (Bastide et al., 1973; Degani and Boyer, 1973) that in the SR Ca^{2+} ATPase as well as in the plasmalemmal Na^+,K^+ ATPase, phosphorylation involves an aspartyl residue which is also found in corresponding positions of other cation ATPases. Analysis

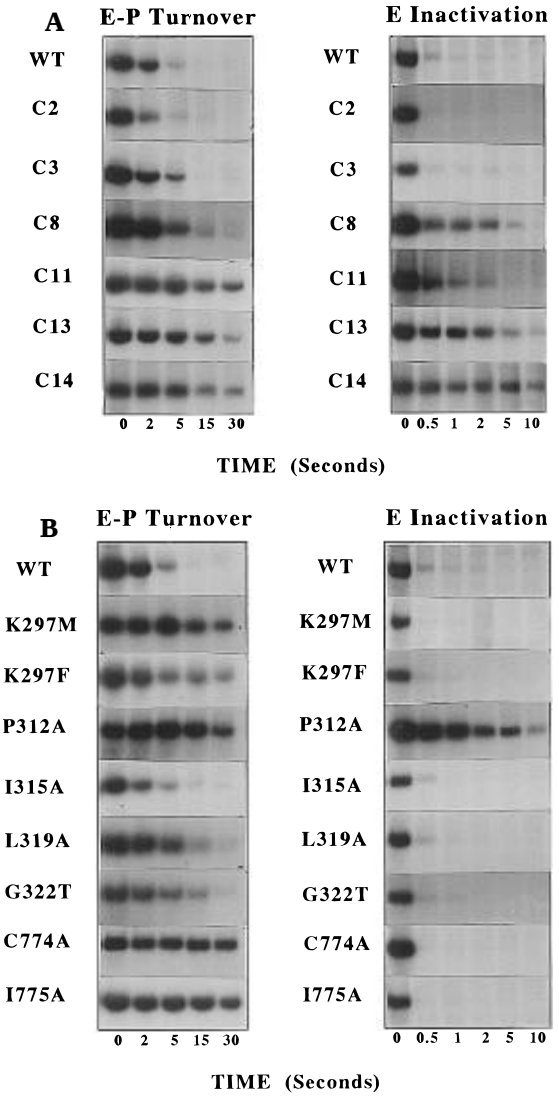

FIGURE 6: Autoradiographs of phosphoenzyme decay following a chase with non-radioactive ATP ("E_1-P to E_2-P transition") and enzyme inactivation following addition of EGTA ("E_1 to E_2 transition"). Phosphorylated enzyme was obtained by addition of [γ-^{32}P]ATP as described in the legend to Figure 5. *Decay of radioactive phosphoenzyme* was initiated by addition of 0.5 mM non-radioactive ATP 10 s after the initial mixing with [γ-^{32}P]ATP. The samples were incubated at 3 °C for serial times (seconds) before quenching with 1 M perchloric acid. The quenched protein was washed twice with 0.125 M perchloric acid and once with water before solubilization, gel electrophoresis, and autoradiography. The *time course of enzyme inactivation* was tested by adding 2 mM EGTA to enzyme preincubated with 20.0 μM Ca^{2+} and then adding [γ-^{32}P]ATP at serial times. The reaction was quenched with perchloric acid 10 s after the addition of ATP and processed for determination of phosphoenzyme as described above. The mutations listed in B were previously reported by Chen et al. (1996) and have been further characterized here for their time course of inactivation by EGTA.

of cDNA derived amino acid sequences (MacLennan et al., 1985; Shull et al., 1985) indicates that the phosphorylation (and catalytic) domain resides within the cytosolic (i.e., extramembranous) region of these enzymes. On the other hand, mutational (Clarke et al., 1989) and chemical (Sumbilla et al., 1991) evidence suggests that cation binding occurs within the membrane bound region of the Ca^{2+} ATPase, and most likely of the Na^+,K^+ ATPase as well (Glynn & Karlish, 1990; Andersen & Vilsen, 1995). The distance intervening between phosphorylation and Ca^{2+} binding domains is approximately 50 Å, as revealed by spectroscopic studies (Bigelow & Inesi, 1992). Therefore, vectorial translocation of bound Ca^{2+}, and likely that of other cations, is triggered by enzyme phosphorylation through long-range intramolecular linkages (Inesi et al., 1992).

Demonstration that cation binding and phosphorylation occur in two distinct and separate domains opened up the possibility of obtaining chimeric proteins by domain exchange. In early experiments most of the extramembranous region of the SR Ca^{2+} ATPase (containing the catalytic and phosphorylation domain) was replaced by the corresponding region of the Na^+,K^+ ATPase (Lemas et al., 1992; Luckie et al., 1992). It became apparent, however, that proteins derived from large chimeric exchanges do not sustain significant transport and hydrolytic activity, even though capable of forming high levels of phosphoenzyme intermediate by utilization of ATP in a Ca^{2+} dependent and thapsigargin sensitive manner (Norregaard et al., 1993; Sumbilla et al., 1993). These initial experiments indicated that utilization of ATP by the catalytic domain of the Na^+,K^+ ATPase can be rendered Ca^{2+} dependent by the large chimeric exchange, but the resulting phosphorylated intermediate undergoes negligible turnover. We then planned further experiments to find out whether smaller chimeric exchanges would also interfere with function. We considered for this purpose the peptide segment connecting the phosphorylation site to the preceding transmembrane helix M4, which is one of the four clustered helices involved in Ca^{2+} binding. This 41-amino acid segment, intervening between Thr316 and Leu356 of the SR Ca^{2+} ATPase, is highly homologous to the corresponding segments of other cation ATPases (Inesi & Kirtley, 1992). The Ca^{2+} ATPase 41-amino acid segment and the corresponding segment of the Na^+,K^+ ATPase can be aligned unambiguously due to the matching aspartyl residues undergoing phosphorylation in both enzymes. The alignment shows that, of 41 amino acids, 27 are homologous and 14 are not. Single non conservative mutations of *homologous* amino acids interfere with function and, in some cases, with assembly and recovery of expressed protein (Zhang et al., 1995; Figure 1 and Table 2). This suggests a prominent role of this segment in structure and function (Inesi, 1994).

Most of the experiments reported in this article were done to replace the 14 *non-homologous* amino acids of the Ca^{2+} ATPase with the corresponding amino acids of the Na^+,K^+ ATPase. We found that if a single amino acid was mutated, the activity was slightly decreased, or actually increased, depending on which amino acid was mutated. When we then increased sequentially the number of mutated amino acids, we found that the activity was not significantly changed by mutation of up to four amino acids. However, as the number of mutations was increased up to 14, thereby rendering the entire Ca^{2+} ATPase segment identical to that of the Na^+,K^+ ATPase, transport and hydrolytic activities were progressively reduced. It is of interest that the level of the phosphorylated enzyme intermediate was not reduced, but its turnover was inhibited (reactions 3 and 4 in Figure 3A, most likely rate limited by the Ca_2·E_1-P → Ca_2·E_2-P transition of Figure 3B). Furthermore, enzyme inactivation by EGTA was also inhibited (reverse of reaction 1 in Figure 3A, most likely rate limited by the E_1 → E_2 transition of Figure 3B). This indicates that chimeric exchange of a few amino acids at this critical location interferes with confor-

mational transitions which are involved in the basic mechanism of energy transduction. We can then derive the following conclusions from our experimental observations:

(a) Our mutational analysis adds specific structural qualification to the proposed involvement of protein conformational changes (de Meis & Vianna, 1979) and of common mechanistic features (Garrahan & Rega, 1988) in energy transduction by Ca^{2+} and Na^+,K^+ ATPases. It attributes a functional role to the peptide segment linking phosphorylation and Ca^{2+} binding domains.

(b) ATPase activation by Ca^{2+} binding, and formation of phosphorylated intermediate, can be carried out to yield normal levels of phosphoenzyme, following replacement of the Ca^{2+} ATPase segment linking phosphorylation and Ca^{2+} binding domains, with the corresponding segment of the Na^+,K^+ ATPase. Thapsigargin sensitivity is also retained, indicating that binding of this inhibitor must depend on other domains of the Ca^{2+} ATPase, such as transmembrane segment M3 (Norregaard et al., 1994).

(c) Vectorial displacement of bound Ca^{2+} by phosphorylation can also be obtained following the same replacement but at lower rates. Therefore a similar "tool" is used by the Ca^{2+} ATPase and the Na^+,K^+ ATPase to propagate the phosphorylation triggered perturbation to their cation binding domains. However, the "tool" (i.e., peptide conformation) is exquisitely specific for each enzyme, and has a very strong influence on the kinetics of the related enzyme transition. This specificity is evidently related to a requirement for matching complementarity by neighboring protein structures.

(d) The effects of substantial structural changes such as large chimerizations or proline mutation in the segment under study involve *both* E_1-P $\rightarrow$ E_2-P and $E_1 \rightarrow E_2$ transitions. This indicates that the segment linking phosphorylation and Ca^{2+} binding domains sustains a key role in triggering conformational changes which are instrumental in energy transduction. On the other hand, other more discrete point mutations interfere with the E_1-P $\rightarrow$ E_2-P transition but not with the $E_1 \rightarrow E_2$ transition. These observations and previous kinetic analysis (Fernandez-Belda et al., 1984; Petithory & Jencks, 1988; Myung & Jencks, 1991) indicate that the $E_1 \rightarrow E_2$ and E_1-P $\rightarrow$ E_2-P transitions include specific components which may be involved *globally* or *separately* by mutational perturbations.

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