Direct Demonstration of Ca\(^{2+}\) Binding Defects in Sarco-Endoplasmic Reticulum Ca\(^{2+}\) ATPase Mutants Overexpressed in COS-1 Cells Transfected with Adenovirus Vectors*

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Single mutations of specific amino acids within the membrane-bound region of the sarco-endoplasmic reticulum Ca\(^{2+}\) (SERCA)-1 ATPase interfere with Ca\(^{2+}\) inhibition of ATPase phosphorylation by P\(_i\) (1), suggesting that these residues may be involved in complexation of two Ca\(^{2+}\) that are known to bind to the enzyme. However, direct measurements of Ca\(^{2+}\) binding in the absence of ATP have been limited by the low quantities of available mutant protein. We have improved the transfection efficiency by means of recombinant adenovirus vectors, yielding sufficient expression of wild type and mutant SERCA-1 ATPase for measurements of Ca\(^{2+}\) binding to the microsomal fraction of the transfected cells. We find that in the presence of 20 \(\mu\)M Ca\(^{2+}\) and in the absence of ATP, the Glu\(^{771}\) \(\rightarrow\) Gln, Thr\(^{799}\) \(\rightarrow\) Ala, Asp\(^{800}\) \(\rightarrow\) Asn, and Glu\(^{908}\) \(\rightarrow\) Ala mutants exhibit negligible binding, indicating that the oxygen functions of Glu\(^{771}\), Thr\(^{799}\), Asp\(^{800}\), and Glu\(^{908}\) are involved in interactions whose single disruption causes major changes in the highly cooperative “duplex” binding. Total loss of Ca\(^{2+}\) binding is accompanied by loss of Ca\(^{2+}\) inhibition of the P\(_i\) reaction. We also find that, at pH 7.0, the Glu\(^{309}\) \(\rightarrow\) Gln and the Asn\(^{796}\) \(\rightarrow\) Ala mutants bind approximately half as much Ca\(^{2+}\) as the wild type ATPase and do not interfere with Ca\(^{2+}\) inhibition of the P\(_i\) reaction. At pH 6.2, the Glu\(^{309}\) \(\rightarrow\) Gln mutant does not bind any Ca\(^{2+}\), and its phosphorylation by P\(_i\) is not inhibited by Ca\(^{2+}\). On the contrary, the Asn\(^{796}\) \(\rightarrow\) Ala mutant retains the behavior displayed at pH 7.0. This suggests that in the Glu\(^{309}\) \(\rightarrow\) Gln mutant, ionization of acidic functions in other amino acids (e.g. Glu\(^{771}\) and Asp\(^{800}\)) occurs as the pH is shifted, thereby rendering Ca\(^{2+}\) binding possible. In the Asn\(^{796}\) \(\rightarrow\) Ala mutant, on the other hand, the Glu\(^{309}\) carboxylic function allows binding of inhibitory Ca\(^{2+}\) even at pH 6.2. In all cases mutational interference with the inhibition of the P\(_i\) reaction by Ca\(^{2+}\) can be overcome by raising the Ca\(^{2+}\) concentration to the mM range, consistent with a general effect of mutations on the affinity of the ATPase for Ca\(^{2+}\).

Activation of the sarco-endoplasmic reticulum Ca\(^{2+}\)

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(SERCA)-1 ATPase requires binding of Ca\(^{2+}\), which is then moved across the membrane upon utilization of ATP. Equilibrium binding isotherms, obtained with sarcoplasmic reticulum (SR) vesicles as an abundant source of enzyme, demonstrate that in the absence of ATP, two Ca\(^{2+}\)/ATPase bind cooperatively with \(K_s = 5 \times 10^{12} \text{M}^{-2}\) (2). As for the topology of Ca\(^{2+}\) binding within the ATPase molecule, involvement of six amino acid residues (Glu\(^{309}\), Glu\(^{771}\), Asn\(^{796}\), Thr\(^{799}\), Asp\(^{800}\), and Glu\(^{908}\)) within four clustered transmembrane helices (M4, M5, M6, and M8) was suggested by mutational analysis (1). The low yield of recombinant enzymes, however, has limited direct measurements of Ca\(^{2+}\) binding to the pertinent mutants (with the exception of the Glu\(^{309}\) \(\rightarrow\) Gln mutant; Skerjanc et al. (3)). In fact, involvement of the six amino acids in Ca\(^{2+}\) binding was suggested by their mutations interfering with the inhibitory effect of Ca\(^{2+}\) on enzyme phosphorylation by P\(_i\). The shortcoming of such experiments, however, was that they did not distinguish direct effects of mutations on Ca\(^{2+}\) binding from mutational effects on transmission of the Ca\(^{2+}\) binding signal within the ATPase protein.

Alternative studies were performed to test whether the pertinent mutants retain the ability to occlude Ca\(^{2+}\) following addition of Cr-ATP (4). In this case, however, there is some degree of uncertainty as to whether Cr-ATP may affect Ca\(^{2+}\) binding by stabilizing the enzyme in a state analogous to that of the phosphorylated intermediate (i.e. “E2^P”). Other studies have utilized the effect of Ca\(^{2+}\) on the susceptibility of wild type and mutated enzyme to proteolytic digestion (5).

We have now improved the efficiency of ATPase gene transfer into COS-1 cells by using recombinant adenovirus vectors. The resulting expression yields sufficient amounts of protein for direct measurements of Ca\(^{2+}\) binding in the absence of nucleotide substrate, using native microsomal vesicles as the source of enzyme. We describe here our recombinant adenovirus constructs, the characteristics of ATPase overexpression in COS-1 cells under control of the SV40 promoter, the direct measurements of Ca\(^{2+}\) binding by wild type and mutated enzyme, and related tests of Ca\(^{2+}\) inhibition of the enzyme reaction with P\(_i\).

MATERIALS AND METHODS

DNA Constructs and Vectors—The chicken fast muscle SR (SERCA-1) ATPase cDNA (6) was inserted into the pUC19 plasmid for amplification and then subcloned into the pSELECT-1 vector for site-directed mutagenesis by the Altered Sites In Vitro Mutagenesis System

1 The abbreviations used are: SERCA, sarco-endoplasmic reticulum Ca\(^{2+}\); SR, sarcoplasmic reticulum; Cr-ATP, chromium ATP; EGFP, enhanced green fluorescence protein; pfu, plaque-forming unit; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.
made available by Promogeta (Madison, WI) or by overlap expansion using polymerase chain reaction (7). Eleven unique restriction sites were introduced into the SERCA-1 cDNA, retaining the original coding sequence. These sites are spaced at approximately equal intervals and facilitate further mutagenesis by generating cassettes of approximately three bps that can be conveniently sequenced following mutagenesis and exchanged with the corresponding cassette in the wild type cDNA. Furthermore a c-myc tag was added to the 3’ end to monitor ATPase expression using anti-c-myc antibodies, independently of mutations in the enzyme sequence.

Site-directed mutagenesis were produced in the SERCA-1 cDNA by using the Altered Sites In Vitro Mutagenesis System (Promega) or by overlap expansion using the polymerase chain reaction (7). Enhanced green fluorescence protein (EGFP) cDNA (containing the Phc846 → Leu and Ser865 → Thr mutations of wild type green fluorescence protein) was obtained from CLONTECH (Palo Alto) in the form of the pEGFP-1 plasmid.

Wild type and mutated cDNA, as well as the EGFP cDNA, was subcloned into the shuttle plasmid pEElsp1A (Microbix Biosystems). In the final constructs, the cDNA was preceded by the SV40 promoter (cytomegalovirus promoter in the case of EGFP) and followed by the SV40 polyadenylation signal, both obtained from the mammalian expression plasmid pCDR-Sol2986 (8). The shuttle plasmid was either used directly for transfection of COS-1 cells by the DEAE-dextran method or by transfection with the replication defective adenovirus plasmid pJM17 (Microbix Biosystems) to obtain recombinant adenovirus vectors (9). The shuttle vector was constructed such that homologous recombination would result in an antisense direction of the SERCA cDNA with respect to the original adenovirus E1 gene and its promoter. The recombinant products were plaque-purified and cesium-banded, yielding concentrations on the order of 105–106 pfu/ml. Analogous procedures were used to obtain recombinant EGFP adenovirus vectors.

Cell Cultures and Transfections—Cultures of HEK293 and COS-1 cells were maintained as described by Graham and Prevec (9) and Sumbilla et al. (10), respectively. The growth medium for COS-1 cells was Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and containing Pen-Strep (100 units/ml) and Fungizone (1 µg/ml).

Transfections of COS-1 cells with wild type or mutated SERCA-1 cDNA or EGFP cDNA subcloned into the shuttle vector pEElsp1A as described above were conducted by the DEAE-dextran method as described by Sumbilla et al. (10).

Recombinant adenovirus vectors were used as follows: 6- or 15-cm plates of COS-1 cells (75–80% confluence) were aspirated to remove growth medium and then layered with 1 or 5 ml of infection medium (Dulbecco’s modified Eagle’s medium supplemented with 5% horse serum, 100 units Pen-Strep/ml, and 1 µg of Fungizone/ml) containing 1 × 105 pfu/ml corresponding to about 100 pfu/cell. 1 h after infection, 4 or 20 ml of growth medium was added, and 2 days later the cells were harvested for fluorescence microscopy with or without immunostaining and for Western blot analyses.

Immunostaining—Lawns of cultured cells were first washed with PBS and then fixed with 4% formaldehyde for 10 min. After repeated washings with PBS, blocking of nonspecific sites was obtained by a 10-min incubation with 1% bovine serum albumin and 0.5% lysine in PBS. This was followed by a 45-min incubation with the primary antibody at a concentration of 5–10 µg/ml of PBS containing 1% albumin, 0.5% lysine, and 0.25% saponin (permeabilization medium). After washing with PBS, the cells were incubated for 45 min with biotinylated anti-mouse secondary antibody (Vector Laboratory, Burlingame, CA) at a concentration of 5 µg/ml of permeabilization medium. The cells were then washed with PBS and incubated for 20 min with Fluorescein Streptavidin (Amersham Pharmacia Biotech) at a concentration of 5 µg/ml of permeabilization medium. The sample was then washed again with PBS, 70% EtOH, and 90% EtOH, allowed to dry, and processed for fluorescence microscopy using a Zeiss Axioskop equipped with a mercury vapor lamp and fluorescence accessories.

Microsome Preparation and Immunodetection of Expressed Protein—The procedure for microsome preparation was as described by Autry and colleagues (7,11), and the final product was stored in small aliquots at −70 °C. The microsomal protein was determined using bicinchoninic acid assay (Pierce). The expressed SERCA-1 ATPase was detected by Western blotting. For this purpose, microsomal protein was separated in 7.5% Laemmli (12) electrophoretic gels blotted onto nitrocellulose paper. This was then incubated with a monoclonal antibody (CaF3–5C3) to the chicken SERCA-1 (6) and in parallel with a monoclonal antibody (9E10) to the c-myc epitope (13). After incubation with secondary antibody (goat anti-mouse IgG- horseradish peroxidase conjugate), the bound antibodies were probed with an Enhanced Chemiluminescence-linked detection system (Amersham Pharmacia Biotech). Quantitation of immunoreactivity was obtained by densitometry and standardized with samples of the wild type ATPase used as controls for the functional studies.

Functional Studies—Studies of Ca2+ transport and phosphoenzyme formation with [32P]phosphate were carried out as described by Inesi et al. (14) and detected by autoradiography. ATPase hydrolytic activity was assessed by measuring P, production (15).

Ca2+ binding to the expressed ATPase in the absence of ATP was measured using the filtration method. The equilibrium mixture contained 20 mm MOPS, pH 6.8, 80 mm KCl, 3 mm MgCl2, 20 µM [32P]CaCl2 (including endogenous Ca2+), and 400 µm micromolar protein/ml. In the final experiments, the equilibrium mixture was determined by titrating with EGTA in the presence of 50 µM Arsenazo and recording differential light absorption changes (660 and 687 nm wavelengths) with a DW-2000 SLM-Aminco spectrophotometer.

Controls for nonspecific binding were conducted with microsomes preincubated with thapsigargin (6.75 µg/mg protein), and the binding observed with the inhibited microsomes was subtracted from the total binding obtained with noninhibited microsomes to yield “specific” Ca2+ binding.

The Ca2+ binding assay was started by adding either 2.7 µg of thapsigargin in 2 ml of 25% (w/v) sucrose to 493 µl of MeSO. The thapsigargin concentration was 400 µg of micromolar protein in 50 µl of medium. After a brief incubation at room temperature, the samples were kept on ice for 10 min, and then 1.0 ml of binding medium was added. Following a 10-min incubation on ice, 0.75 ml was placed on a Millipore filter (HAWP 0.65 µm, 25-mm diameter) under suction for approximately 30 s. The vacuum was then turned off, and the filter was blotted on a paper towel to remove excess moisture, folded, and inserted into a 7-ml scintillation vial. The filters were dissolved with 1 ml of N,N-dimethylforamide, scintillation fluid was added, and the radioactivity was measured by scintillation counting. The measured Ca2+ binding levels were finally adjusted to compensate for slight variations of SERCA-1 expression in various preparations, with reference to a wild type preparation as internal standard. The thapsigargin-sensitive (i.e. specific) binding accounted for approximately 25% of the total Ca2+ binding by microsomes obtained from cells expressing wild type SERCA. It is known that thapsigargin is a global SERCA inhibitor (16), and we established that at the concentration used thapsigargin inhibited the P0 reaction in the wild type enzyme and in all mutants studied.

RESULTS

Vector Efficiency and Transgenic Expression—A preliminary assessment of adenovirus vector efficiency in COS-1 cells was obtained by microscopic visualization of the number of COS-1 cells expressing EGFP. Fig. 1 (A and B) shows phase contrast and fluorescent images of COS-1 cells following the DEAE-dextran transfection procedure. A comparison of the two images shows that only a few cells express EGFP. On the other hand, in Fig. 1C, nearly all cells express EGFP following infection by recombinant EGFP adenovirus (40 pfu/cell). A quantitative assessment of the percentage of cells expressing EGFP as a function of adenovirus concentration is given in Fig. 2. An asymptotic level of 97–100% transfection efficiency was obtained at 100 pfu/seeded cell, although the amount of expressed protein continued to rise as the number of gene copies introduced per cell was further increased.

ATP-dependent Ca2+ Transport by Wild Type ATPase—ATP-dependent Ca2+ transport is a highly specific functional parameter of SERCA that can be conveniently measured by following the uptake of radioactive calcium tracer by microsomal vesicles. Microsomes obtained from control and S1 cells do not exhibit a significant rate of Ca2+ transport (Fig. 3) compared to the other lines we found under optimal conditions, the rate of Ca2+ transport by microsomes obtained from cells infected with the SERCA-1 adenovirus vector (100 pfu/cell) was approximately 10-fold higher than that of microsomes obtained from cells transfected by the DEAE-dextran method (Fig. 3). Conversely, their transport activity was approximately 10-fold lower than the rate of Ca2+ transport by native SR vesicles.
obtained from rabbit skeletal muscle (data not shown). Because SERCA-1 accounts for approximately 50% of the total protein in SR vesicles, it is apparent that transgenic SERCA-1 accounts for approximately 5.0% of the total microsomal protein obtained from COS-1 cells infected with recombinant adenovirus under our conditions. Similar conclusions were reached by comparative measurements of Ca\(^{2+}\)-dependent ATPase activity (not shown). They are also consistent with Western blots showing no SERCA in control samples (Fig. 3, inset, lane 1) and much greater amounts of recombinant SERCA in microsomes obtained from cells infected with adenovirus (Fig. 3, inset, lanes 4 and 5) as compared with microsomes obtained from cells transfected by the DEAE-dextran method (Fig. 3, inset, lanes 2 and 3).

ATP-independent Ca\(^{2+}\) Binding by Wild Type ATPase—Ca\(^{2+}\) binding in the absence of ATP may be considered as the initial step of a single catalytic and transport cycle. In fact it is well known that Ca\(^{2+}\) binding to the ATPase is required to activate the enzyme before ATP can be utilized. The bound calcium is then displaced vectorially and released against a concentration gradient upon enzyme phosphorylation by ATP. The cycle is...
Finally completed by hydrolytic cleavage of the phosphorylated enzyme intermediate.

We have previously used chromatography equilibrium columns to measure Ca\(^{2+}\) binding to SR ATPase under equilibrium conditions, in the absence of ATP. The resulting binding isotherms demonstrate that two Ca\(^{2+}\) bind cooperatively to each ATPase with \(K_a = 5 \times 10^{12} \text{ M}^{-2}\) at neutral pH (2). This method, however, is not suited for measurements of Ca\(^{2+}\) binding to COS-1 microsomes because of the low yield of recombinant enzyme. For this reason we used the filtration method, which has the advantage of being suited for small protein samples, even though it is less than optimal from an equilibrium theory viewpoint. Prior to its use, we determined the linear range for protein collection by Millipore filters using SR microsomal protein as a standard. It is shown in Fig. 4 that the linear range extended to 500 \(\mu\text{g}\) of microsomal protein/filter. We found that 20 \(\mu\text{g}\) Ca\(^{2+}\) provided an optimal ligand concentration for saturating the high affinity sites, yielding binding data consistent with those obtained by column chromatography (2). We also found that inhibition with thapsigargin provided a very useful tool to demonstrate the specificity of the high affinity Ca\(^{2+}\) binding to the SERCA enzyme. Therefore, the data reported here correspond to the difference between Ca\(^{2+}\) binding in the absence and in the presence of thapsigargin.

Using the filtration method under these conditions, we obtained Ca\(^{2+}\) binding levels of 7.0 nmol/mg of SR protein and 0.7 nmol/mg of microsomal protein derived from COS-1 cells infected with SERCA adenovirus vector. These values are consistent with the 10-fold difference observed in the Ca\(^{2+}\) transport and ATPase activities of the two preparations. No significant Ca\(^{2+}\) binding signal was detected with microsomes derived from COS-1 cells transfected by the DEAE-dextran method, because of the low SERCA content of these microsomes.

Expression and Characterization of Mutants—We observed in our Western blots only slight variations in the levels of SERCA-1 protein recovered in the microsomal fraction of COS-1 cells expressing the various SERCA mutants under the same conditions (Fig. 5). We obtained densitometric evaluations of the electrophoretic bands to relate the functional parameters measured for each mutant to a corresponding quantity of wild type SERCA-1. We found, however, that the Glu\(^{399}\) → Gln, Asp\(^{771}\) → Asn, Asn\(^{796}\) → Ala, Thr\(^{799}\) → Ala, Asp\(^{800}\) → Asn, and Glu\(^{908}\) → Ala mutants do not sustain significant rates of either ATP hydrolysis or coupled Ca\(^{2+}\) transport (see also Clarke et al. (1)). Furthermore, formation of phosphorylated intermediate through Ca\(^{2+}\)-dependent ATP utilization is totally inhibited in these mutants. On the other hand, phosphoenzyme formation by utilization of Pi still occurs at normal levels (see below).

Ca\(^{2+}\) Binding and Phosphorylation with Pi in ATPase Mutants—The main aim of our studies was to measure Ca\(^{2+}\) binding by the ATPase mutants, taking advantage of their overexpression in COS-1 cells infected with the adenovirus vectors. In fact, we were able to unambiguously demonstrate specific defects of Ca\(^{2+}\) binding in these mutants. It is shown in Table I that at neutral pH and in the presence of 20 \(\mu\text{M}\) Ca\(^{2+}\), some of the mutants (i.e. Glu\(^{771}\) → Gln, Thr\(^{799}\) → Ala, Asp\(^{800}\) → Asn, and Glu\(^{908}\) → Ala) displayed no significant binding, whereas others (i.e. Glu\(^{909}\) → Gln and Asn\(^{796}\) → Ala) bind approximately half as much Ca\(^{2+}\) as the wild type enzyme (Fig. 6). A 50% reduction of Ca\(^{2+}\) binding by the Glu\(^{909}\) → Gln mutation was also noted by Skerjanc et al. (3). Interestingly, we found that if the pH is reduced to 6.2, Ca\(^{2+}\) binding by the Glu\(^{909}\) → Gln mutant becomes negligible (0.02 ± 0.24 nmol/mg protein), whereas the Asn\(^{796}\) → Ala mutant binds approximately the same level of Ca\(^{2+}\) (0.44 ± 0.39 nmol/mg protein) as at neutral pH.

As mentioned above, putative Ca\(^{2+}\) binding defects of these mutants were originally suggested by the lack of inhibition of the Pi reaction by Ca\(^{2+}\) (1). With our present experiments we first confirmed that the Pi reaction is in fact inhibited by 20 \(\mu\text{M}\) Ca\(^{2+}\), at pH 7.0 or 6.2 when the wild type ATPase is used and is not inhibited when the Glu\(^{771}\) → Gln, Thr\(^{799}\) → Ala, Asp\(^{800}\) → Asn, and Glu\(^{908}\) → Ala mutants are used (Fig. 7). We also found that the Pi reaction is inhibited by 20 \(\mu\text{M}\) Ca\(^{2+}\) at pH 7.0 or 6.2 when the Asn\(^{796}\) → Ala mutant is used, similarly to the wild type ATPase. On the other hand, when the Glu\(^{909}\) → Gln is used, the Pi reaction is inhibited by 20 \(\mu\text{M}\) Ca\(^{2+}\) at pH 7.0 but not at pH 6.2 (Fig. 7; see also Ref. 17). With all mutants inhibition of the Pi reaction was obtained if the Ca\(^{2+}\) concentration was raised to the mM level (not shown, but see Ref. 18).

Identical results were obtained when the experiments were performed in the presence of the ionophore A23187, which increases the membrane permeability to Ca\(^{2+}\) and allows ATPase exposure to Ca\(^{2+}\) from both sides of the membrane.

**DISCUSSION**

Equilibrium and kinetic experiments have demonstrated that in the absence of ATP, SERCA binds Ca\(^{2+}\) with a stoichiometry of two Ca\(^{2+}\)/enzyme. Binding occurs with a sequential
and cooperative mechanism (2, 19, 20) whereby the enzyme is stabilized in the “E1” state. An important discovery for the understanding of the Ca\(^{2+}\) binding topology within the SERCA molecule was achieved when single mutations of specific residues in the transmembrane domain were found to interfere with Ca\(^{2+}\) inhibition of enzyme phosphorylation by P\(_i\) (1). Based on this interference, it was proposed that the six residues participate in Ca\(^{2+}\) complexation within the transmembrane domain (1). In fact it was shown by molecular modeling (21) that within the constraints imposed by the positions of these residues on four transmembrane helices, it is possible to arrange their oxygen functions to form a duplex calcium binding site with distribution and distances compatible with other duplex sites of known crystal structure, such as that of thermolysin (22).

Several attempts have been made to assign the oxygen functions of the six amino acids implicated in binding to either calcium ion of the duplex complex (21, 23, 24). Additional amino acids within a neighboring cytosolic segment have been also implicated (5). However, a shortcoming of this work has been the lack of direct Ca\(^{2+}\) binding measurements with the mutants, because of the low quantities of recombinant protein. The only mutant tested for binding was Glu\(^{909}\) → Gln expressed in insect cells infected with recombinant baculovirus (3). This measurement required solubilization of the membrane-bound enzyme, purification by affinity chromatography, and reconstitution in liposomes, leaving some uncertainty on the detergent effect on binding and the orientation of the reconstituted enzyme in the liposomal membrane. Alternatively, “Ca\(^{2+}\) occlusion” was measured in the presence of Cr-ATP (4), which stabilizes the enzyme in a state similar to that obtained by enzyme phosphorylation with ATP. An advantage of our present experiments is that Ca\(^{2+}\) binding was measured in the absence of ATP under equilibrium conditions yielding strictly the E1 state. Another advantage is the use of wild type and mutant proteins assembled in native microsomal membrane with no need for detergent solubilization.

Our measurements demonstrate that the Glu\(^{771}\) → Gln, Asp\(^{800}\) → Asn, Thr\(^{799}\) → Ala, and Glu\(^{908}\) → Ala mutations result in total loss of Ca\(^{2+}\) binding in the presence of 20 μM Ca\(^{2+}\) (Table I). The strong inhibition of Ca\(^{2+}\) binding in the first two mutants may be explained by considering that Glu\(^{771}\) and Asp\(^{800}\) could contribute electronegativity to both calcium ions, each donating its carboxyl oxygen to one calcium and its carbonyl oxygen to the other. Yet, it is remarkable that mutation of one of the acidic amino acids to its corresponding amide (thereby leaving the carboxyl oxygen in place) results in interference with binding of both calcium ions and with inhibition of the P\(_i\) reaction by Ca\(^{2+}\) (Table I).

The strong inhibition of Ca\(^{2+}\) binding by the Thr\(^{799}\) → Ala and Glu\(^{908}\) → Ala mutations is also remarkable, because Thr can contribute only one hydroxyl group to the coordination complex. Furthermore, Glu\(^{908}\) appears to contribute only a carbonyl group because the Glu\(^{908}\) → Gln mutation does not have functional consequences (18, 25).

It is then apparent that the oxygen functions of Glu\(^{771}\), Thr\(^{799}\), Asp\(^{800}\), and Glu\(^{908}\) provide important stabilization to the cooperative Ca\(^{2+}\) complex, either by direct interaction with Ca\(^{2+}\) or by participation in hydrogen bonding with water or peptide amide functions, so much so that mutational interference with a single one of these functions results in major disruption of the duplex binding site. These effects, however, can be overcome by increasing the Ca\(^{2+}\) concentration (18), indicating that mutational disruption affects the affinity of the enzyme for Ca\(^{2+}\).

It is noteworthy that our finding of strong binding inhibition in the Glu\(^{908}\) → Ala mutant in the absence of ATP (i.e. E1 state)
is in apparent contrast with the Ca\(^{2+}\) occlusion by the same mutant in the presence of Cr-ATP (4, 23). This may be explained by the high concentration of Ca\(^{2+}\) used in these experiments (23).

Although we observed strong inhibition of Ca\(^{2+}\) binding with the mutants mentioned above we found that in the presence of 20 \(\mu\)M Ca\(^{2+}\) and pH 7.0, the Glu\(^{309}\) \(\rightarrow\) Gln and Asn\(^{796}\) \(\rightarrow\) Ala mutations result in reduction of Ca\(^{2+}\) binding to approximately half the level observed with wild type enzyme (Fig. 6). At pH 6.2, on the other hand, the Glu\(^{309}\) \(\rightarrow\) Gln mutant exhibits no significant Ca\(^{2+}\) binding, whereas the Asn\(^{796}\) \(\rightarrow\) Ala mutant retains the same binding as at neutral pH. This suggests that ionization of acidic functions of other amino acids (e.g. Glu\(^{771}\) or Asp\(^{800}\)) occurs as the pH is shifted from 6.2 to 7.0, thereby facilitating Ca\(^{2+}\) binding in the Glu\(^{309}\) \(\rightarrow\) Gln mutant. On the other hand, in the Asn\(^{796}\) \(\rightarrow\) Ala mutant, the presence of the Glu\(^{309}\) acidic function allows binding of inhibitory Ca\(^{2+}\) even at pH 6.2.

The lack of Ca\(^{2+}\) inhibition of the P\(_{i}\) reaction in the mutants allowing no Ca\(^{2+}\) binding is well understandable. On the other hand, the strong inhibition of the P\(_{i}\) reaction in mutants permitting binding of half the normal Ca\(^{2+}\) level suggests that (a) these mutant molecules bind only one of two Ca\(^{2+}\) known to bind to the wild type enzyme and (b) the single bound Ca\(^{2+}\) is sufficient to inhibit the P\(_{i}\) reaction. Experimental demonstration of the inhibition of the P\(_{i}\) reaction by single Ca\(^{2+}\) binding is quite satisfactory, because it is observed in both Asn\(^{796}\) \(\rightarrow\) Ala and Glu\(^{309}\) \(\rightarrow\) Gln mutants at pH 7.0 and only in the former mutant at pH 6.2.

That a single Ca\(^{2+}\) may be sufficient to inhibit the P\(_{i}\) reaction was previously suggested based upon the effects of sequential (and negatively cooperative) binding of strontium to sarcoplasmic reticulum ATPase (26) and on different effects of various mutations on the inhibition of the P\(_{i}\) reaction by Ca\(^{2+}\) (23). Our direct measurements of Ca\(^{2+}\) binding at pH 7.0 and 6.2 in parallel with phosphorylation experiments confirm that in the Asn\(^{796}\) \(\rightarrow\) Ala and Glu\(^{309}\) \(\rightarrow\) Gln mutants, at suitable Ca\(^{2+}\) and H\(^{+}\) concentrations, a single Ca\(^{2+}\) is sufficient to inhibit the P\(_{i}\) reaction. This would be unlikely to occur in the wild type enzyme, because of the highly cooperative character of Ca\(^{2+}\) binding.

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