Probing the SERCA1a sarcoplasmic reticulum Ca\(^{2+}\)-ATPase phosphorylation-site mutant D351E with inorganic phosphate

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Abstract

The expression of sarcoplasmic reticulum SERCA1a Ca\(^{2+}\)-ATPase wild-type and D351E mutants was optimized in yeast under the control of a galactose promoter. Fully active wild-type enzyme was recovered in yeast microsomal membrane fractions in sufficient amounts to permit a rapid and practical assay of ATP hydrolysis and phosphoenzyme formation from ATP or Pi. Mutant and wild-type Ca\(^{2+}\)-ATPase were assayed for phosphorylation by Pi under conditions that are known to facilitate this reaction in the wild-type enzyme, including pH 6.0 or 7.0 at 25ºC in the presence of dimethylsulfoxide. Although glutamyl (E) and aspartyl (D) residue side chains differ by only one methylene group, no phosphoenzyme could be detected in the D351E mutant, even upon the addition of 40% dimethylsulfoxide and 1 mM 32Pi in the presence of 10 mM EGTA and 5 mM MgCl\(_2\). These results show that in the D351E mutant, increasing hydrophobicity of the site with inorganic solvent was not a sufficient factor for the required abstraction of water in the reaction of E351 with Pi to form a glutamylphosphate (P-E351) phosphoenzyme moiety. Mutation D351E may disrupt the proposed alignment of the reactive water molecule with the aspartylphosphate (P-D351) moiety in the phosphorylation site, which may be an essential alignment both in the forward reaction (hydrolysis of aspartylphosphate) and in the reverse reaction (abstraction of water upon formation of an aspartylphosphate intermediate).

Introduction

Sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA1a) from fast-twitch muscle is a membrane protein of 994 amino acid residues with a molecular mass of 110 kDa (1). The enzyme is a prototypical P-type ion-transport ATPase, whose topology includes 10 transmembrane segments (M1-M10) and three cytosolic domains (N, P, and A), connected to the membrane-embedded part by four helical stalks (1). The phosphorylation domain (P domain) contains D351, the residue which reacts with ATP during the catalytic cycle in the presence of calcium to form an acid-stable aspartylphosphate enzyme intermediate (Figure 1). According to the E1/E2 catalytic and transport mechanisms (2-4),
The enzyme is activated by binding of two 
Ca\textsuperscript{2+} ions (conversion of E2 to E1·2Ca, Figure 1, steps 1-2) leading to the phosphoryl transfer from MgATP to residue D351 to form an ADP-sensitive phosphoenzyme (E1–P·2Ca, Figure 1, steps 3-5). In the subsequent steps, 6 and 7, a reduction in affinity and a change in orientation of the Ca\textsuperscript{2+}-binding sites result in the release of ions into the sarcoplasmic reticulum (SR) lumen. After Ca\textsuperscript{2+} release, phosphoenzyme hydrolysis takes place and the enzyme returns to an unphosphorylated and Ca\textsuperscript{2+}-unbound form (E2, Figure 1, steps 8-9).

The enzyme cycle can be driven backwards, and the first step in this reverse cycle is formation of the aspartylphosphate intermediate (E2-P) from Pi in the absence of Ca\textsuperscript{2+} (Figure 1, steps 9 and 8) as originally shown by Masuda and de Meis (5). Affinity for Pi is low (\(K_m\) in the millimolar range) and varies with pH of the medium (5). Phosphorylation by Pi can be greatly favored in vitro by adding an organic solvent to the assay medium; affinity is increased by three orders of magnitude and the pH dependence is abolished (6). The implication is that phosphorylation of the enzyme by Pi is facilitated when hydrophobicity of the medium is increased by the addition of organic solvents (6). However, it has not been directly demonstrated if access of water to the catalytic site is the predominant factor that facilitates formation of phosphoenzyme from Pi in the presence of an organic solvent. This will depend on the difference of energy between the interaction of water with the solvent and of water and other required co-factors such as Mg ions with the chemical species of the amino acid side chains at the catalytic site (6).

Phosphorylation-site mutants of SR Ca\textsuperscript{2+}-ATPase have been successfully obtained by transient expression in mammalian cells (7, 8). In this system, mutants (D351E, N, S, T, H, and A) were expressed at levels similar to wild-type protein; however, they showed no activity when tested in the presence of ATP and calcium, confirming the requirement of phosphorylation of residue D351 for the activity of mammalian Ca\textsuperscript{2+}-ATPase (7). Mutations in this residue have been demonstrated to abolish phosphorylation by ATP, calcium occlusion and calcium transport (7, 9). Notably, this effect is observed even when D351 is replaced by E351 (7), a mutation that preserves the carboxyl reactive group of the side chain; the latter differs only by an additional \(\gamma\)-methylene group in the glutamyl residue when compared to aspartyl.

The phosphorylation site mutant D351E has not been tested so far for its reactivity towards Pi in the reverse cycle of the enzyme. In particular, D351E should be an interesting mutant that could be used to test the effect of the organic solvent and the requirement of coordination of Pi with specific side chain chemical species at the catalytic site. In the present report, we describe a simplified yeast expression and fractionation system which has yielded microsomal membrane fractions with a relative content of heterologous Ca\textsuperscript{2+}-ATPase that is amenable to di-

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**Figure 1.** Catalytic cycle of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase.
rect measurements of ATP hydrolysis and phosphoenzyme formation from ATP or Pi. Using this system, we report for the first time the assay of phosphorylation by Pi of mutant D351E in the reverse cycle of the enzyme, in parallel with wild-type and D351A controls.

Material and Methods

Strains and media

\textit{Saccharomyces cerevisiae} strain NY605 (MATa; ura3-12, leu2-3, 112, GAL2) (10) transformed according to Ref. 11 was used for the expression of rabbit SR Ca\textsuperscript{2+}-ATPase. Transformed NY605 cells were selected and propagated on agar plates with minimal medium containing 2\% (w/v) glucose supplemented with 30 mg leucine/L.

Plasmid constructions

The \textit{HindIII}-\textit{SacI} fragment containing the hexahistidine-tagged rabbit SERCA1a Ca\textsuperscript{2+}-ATPase cDNA (10) was transferred from plasmid pARF1 to the \textit{HindIII}-\textit{SacI}-digested pYES2 expression vector (Invitrogen) giving vector pYESCa. Point mutants D351E and D351A were obtained by site-directed mutagenesis with the overlap extension polymerase chain reaction (12). Fragments were subcloned into pBlueScript II (Stratagene, La Jolla, CA, USA) and the presence of a single desired mutation was verified by DNA sequencing. An \textit{SmaI} restriction fragment was excised and ligated back into the corresponding region in the full-length cDNA of the \textit{SmaI}-digested pYESCa plasmid. The pYES2 plasmid without insert was used for the negative control yeast transformants.

Preparation of sarcoplasmic reticulum membrane vesicles

SR vesicles used as positive control for Western blot and phosphorylation assays were purified from rabbit skeletal muscle by the method of Eletr and Inesi (13).

Isolation of yeast membranes

Transformed yeast cells were grown at 30\textdegree C in glucose minimal medium supplemented with leucine until OD\textsubscript{600} reached 1.4. Cells were then diluted to 0.5 OD\textsubscript{600} in galactose medium and were harvested after 16 h in culture. Preparation of cell lysates and low- and medium-speed centrifugation were essentially as described by Reis et al. (10). Following a low speed centrifugation (418 g for 10 min) for removal of unbroken cells and large debris, the lysate was spun at 24,000 g for 45 min. The resulting pellet containing the yeast microsomal membrane fraction was resuspended at A\textsubscript{660} = 30 in a medium containing 50 mM 3-[N-morpholino]propane sulfonic acid (MOPS), pH 7.5, 300 mM sucrose, 1 mM CaCl\textsubscript{2}, Protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 5 µg/mL chymostatin and 2 µg/mL each of leupeptin, pepstatin and aprotinin were included in the homogenization buffer. Protein concentrations were determined by the method of Lowry et al. (14) using bovine serum albumin as standard.

Integrity and quantitation of Ca\textsuperscript{2+}-ATPase in yeast membranes

Yeast microsomal membrane fractions (50 µg protein) of each test transformant were loaded onto 7.5\% SDS-PAGE (15). The yeast microsomal membrane fraction from negative control cells was loaded in parallel; 1 µg SR vesicle Ca\textsuperscript{2+}-ATPase was used as positive control. After electrophoresis, samples were electrophotographed to nitrocellulose membranes and incubated with monoclonal D12 antibody (kindly provided by Dr. Radovan Borojevic, Universidade Federal do Rio de Janeiro) against rabbit SR Ca\textsuperscript{2+}-ATPase (1:50), which is specific for rabbit fast-twitch Ca\textsuperscript{2+}-ATPase. For these qualitative assays, blots were developed with anti-mouse horseradish peroxidase-con-
jugated secondary antibody (GE Healthcare, Piscataway, NJ, USA) and ECL chemiluminescent reagents (GE Healthcare). For quantitative assays, dot-blot results were developed with anti-mouse IgG (1:5000; Sigma, St. Louis, MO, USA) and 1 µCi 125I-labeled protein G (GE Healthcare). The relative amounts of Ca2+-ATPase in yeast membrane fractions were determined using STORM 840 PhosphorImager (GE Healthcare) and the ImageMaster TotalLab software (GE Healthcare) using known amounts of SR vesicles Ca2+-ATPase as a calibration standard. The amount of yeast membrane fractions in the functional assays was adjusted according to the Ca2+-ATPase contents measured in each fraction.

ATPase assay

ATP hydrolysis was assayed at 30°C in a medium containing an ATP-regenerating enzyme system as described by Degand et al. (16), with some modifications. To a buffer containing 10 mM HEPES-KOH, pH 7.2, 10 mM PIPES-KOH, pH 7.2, 1 µg/mL A23187, 100 µM free Ca2+, 5 mM MgCl2, 100 mM KCl, we added 30 µg/mL of yeast membrane fraction. This mixture was pre-incubated for 5 min at 30°C in the presence of 3 mM azide, 50 mM KNO3, 50 ng/mL bafilomycin-A, 100 µM ammonium molybdate, and 0.1 µM N-aminocaproic acid. After the addition of 2 mM phosphoenolpyruvate and 10 µg/mL pyruvate kinase, the assay reaction was started by the addition of 3 mM ATP. Duplicate aliquots (100 µL) were taken just after initiating ATP hydrolysis (zero time point) and again after 20 min, and immediately added to 1 mL 20 mM H2SO4. Inorganic phosphate was measured by a modified procedure (17) of the malachite green reagent method (18). Hydrolytic activity was calculated as the difference between the rate obtained in the presence and in the absence of 8 µM thapsigargin, a specific inhibitor of Ca2+-ATPase, and was further corrected by subtracting the background activity found in the negative control yeast membranes.

Tryptic digestion

Tryptic digestion of 450 ng recombinant Ca2+-ATPase in yeast membrane fractions was carried out in 50 mM MOPS-Tris, pH 7.0, 100 mM KCl, 5 mM MgCl2 and 100 µM Ca2+ free at 37°C. The 24,000 g yeast membrane fraction was used and the amount of total yeast proteins in the assay was adjusted to contain 450 ng recombinant Ca2+-ATPase, as estimated for each wild-type or mutant preparation by quantitative dot-blot assays as described in the quantitation procedure above. Pre-incubation for 5 min was followed by trypsin addition (trypsin:total protein ratio 1:50), and the incubation was performed for 0, 0.5, 2, 5, 10, and 20 min. The reaction was terminated by the addition of soybean trypsin inhibitor. Reaction products were precipitated with 10% TCA followed by electrophoresis on 10.5% SDS-polyacrylamide gels (15). Fragments were detected by immunoblotting using the monoclonal D12 antibody specific for fast-twitch Ca2+-ATPase and a peroxidase-conjugated secondary antibody.

Phosphoenzyme formation

Formation of phosphoenzyme intermediates from [γ-32P]ATP was carried out at 0°C by incubating 500 ng Ca2+-ATPase from the yeast membrane fraction in 0.25 mL 20 mM MOPS-Tris, pH 7.0, 100 mM KCl, 5 mM MgCl2, 100 µM CaCl2, 5 mM azide, 50 mM KNO3, 50 ng/mL bafilomycin-A, and 100 µM ammonium molybdate. Phosphorylation reaction was started by the addition of 2 µM [γ-32P]ATP (25 Ci/mmol, GE Healthcare) and quenched after 10 s with 0.5 mL ice-cold quenching solution containing 9% (w/v) trichloroacetic acid and 27 mM NaH2PO4. The acid-precipitated enzyme was washed by centrifugation and resuspended.
in 50 µL 150 mM Tris-Cl, pH 6.8, 10 mM EDTA, 2% SDS, 16% glycerol (v/v), 0.04% bromophenol blue (v/v), and 0.84 M β-mercaptoethanol. Samples were separated by SDS-gel electrophoresis on 7% acrylamide gels at pH 6.0 (19). Gels were fixed and dried and the 32P-labeled radioactive band corresponding to the Ca2+-ATPase was quantitated using STORM 840 PhosphorImager (GE Healthcare) and compared with known amounts of dot-spotted [γ-32P]ATP. Background phosphorylation levels were determined in parallel experiments and subtracted from all data points.

Phosphorylation from [32P]Pi was assayed both at pH 6.0 and 7.0 at 25ºC. A 500-ng amount of Ca2+-ATPase of the yeast membrane fraction was incubated in a total volume of 0.25 mL 100 mM MES-Tris, pH 6.0, or 100 mM MOPS-Tris, pH 7.0, 5 mM MgCl2, 10-40% dimethylsulfoxide (DMSO), 50 ng/mL bafilomycin-A, 100 µM ammonium molybdate, 5 mM azide, 10 mM EGTA or 3 mM calcium. The phosphorylation reaction was started by the addition of 0.1-1 mM [32P]Pi (200 Ci/mol, Perkin Elmer, Waltham, MA, USA) and stopped after 15 s with 1 mL ice-cold quenching solution containing 9% (w/v) trichloroacetic acid and 27 mM NaH2PO4. Proteins were treated and quantitated as described above by comparison with known amounts of dot-spotted [32P]Pi.

Results

Optimization of the yeast expression system and isolation of Ca2+-ATPase from yeast microsomal membrane fractions

An expression system in the yeast S. cerevisiae under the control of galactose was developed for the production of recombinant protein. We have observed that the system which employed expression of rabbit Ca2+-ATPase under the control of a heat-shock promoter (10) resulted in the appearance at the yeast membrane fractions of a number of endogenous yeast proteins that were highly phosphorylated and interfered with measurements of heterologous Ca2+-ATPase phosphorylation (data not shown). We have raised the hypothesis that elimination of yeast heat-shock, with the reduction in the number of endogenous proteins phosphorylated, would permit the use of yeast microsomal membrane fractions for direct measurement of heterologous Ca2+-ATPase phosphorylation. Accordingly, we have constructed and tested pYESCa, an alternative expression vector using pYES2, which has a GAL-1 promoter under the control of galactose, as described in Methods. S. cerevisiae was transformed with pYESCa and grown under selective conditions, and subsequently tested for the expression of Ca2+-ATPase in liquid culture medium with galactose at different time intervals. Optimized growth and expression conditions were established and are described in Methods. A modified medium-speed centrifugation at 24,000 g had been shown to recover most of the heterologous Ca2+-ATPase expressed by heat-shock induction (10) and we confirmed that this medium-speed microsomal membrane fraction contained most of the Ca2+-ATPase under our expression conditions.

Figure 2A shows that D351E and D351A mutants were expressed in yeast cells at approximately the same level as the wild-type Ca2+-ATPase, as observed in Western blots of yeast microsomal membrane fractions developed with monoclonal D12 antibody. No Ca2+-ATPase band was detected in yeast negative control microsomal membranes, confirming that the microsomal membrane fractions have no cross-reactive contaminant proteins.

ATPase activity and phosphoenzyme formation from [γ32P]ATP

To establish the functionality of the Ca2+-ATPase produced in the heterologous yeast
membranes was insensitive to thapsigargin. Aspartyl residue 351 at the phosphorylation site of SERCA1a Ca\textsuperscript{2+}-ATPase was mutated to either a glutamyl or an alanine residue. Replacement of D351 with E or A resulted in complete abolition of ATP hydrolysis (data not shown), in agreement with previous literature reports (7, 9). In addition, Figure 2B shows that the wild-type heterologous enzyme was phosphorylated by ATP, while D351E and D351A mutants did not form a phosphoenzyme intermediate, again in agreement with previous literature results (7). It should be noted that negative control yeast microsomal membrane fractions had no detectable phosphorylation either from ATP or from Pi in the acid-gel assays (Figure 2B and C), again confirming that the yeast expression system under the control of galactose employed here provided a convenient and efficient preparation of membranes that can be isolated from yeast and used directly for phosphorylation experiments with no further purification, with no interference from endogenous yeast contaminants.

Assessment of the overall structural integrity of recombinant Ca\textsuperscript{2+}-ATPase

Since only the wild-type recombinant Ca\textsuperscript{2+}-ATPase exhibited a normal rate of ATP hydrolysis and formation of phosphoenzyme from ATP, and no activity was detected for either the D351E or D351A mutant (see above), we performed a limited trypsinolysis assay and followed the pattern of accumulation of tryptic digest bands as a function of time of digestion in order to investigate if the overall folding and membrane insertion of mutant Ca\textsuperscript{2+}-ATPase was similar to that of the wild-type recombinant enzyme. Figure 3 shows that the time course of digestion was similar for wild-type, D351E and D351A recombinant Ca\textsuperscript{2+}-ATPase, and an identical pattern of accumulation of four major tryptic bands (approximately 64, 40, 30, and 20 kDa) was obtained in each assay.
Phosphoenzyme formation from Pi

Phosphorylation by Pi was assayed at both pH 6.0 and 7.0 in the absence of calcium and in the absence and in the presence of different concentrations of the organic solvent DMSO (10-40%), under conditions that were shown by de Meis and collaborators (6) to favor formation of phosphoenzyme in the reverse direction of the catalytic cycle. Wild-type D351 Ca\(^{2+}\)-ATPase expressed in yeast was phosphorylated by Pi at levels similar to those obtained for the control SR Ca\(^{2+}\)-ATPase purified from rabbit muscle SR under all conditions tested (data not shown). As expected, inhibition of phosphorylation by Pi was observed in the presence of Ca\(^{2+}\). Figure 2C shows similar levels of phosphorylation of control SR ATPase and wild-type heterologous ATPase under optimal conditions in the presence of 40% DMSO and 1 mM \(^{32}\)Pi plus 10 mM EGTA and 5 mM MgCl\(_2\). Under these optimal conditions, phosphorylation by Pi of both SR ATPase and wild-type heterologous enzyme was inhibited but not totally abolished by the addition of calcium (see Figure 2C).

Conversely, no phosphoenzyme could be detected in the D351A mutant (Figure 2C) at pH 6.0 or 7.0, an expected result since the carboxyl functional group at the side chain of D351 had been replaced by a methyl group in alanine. Notably, the D351E mutant also had no detectable phosphoenzyme (Figure 2C) even though a carboxyl group side chain was present in the glutamyl residue. It should be noted that the Pi concentration that was used is 100-fold above the \(K_m\) for phosphorylation of wild-type Ca\(^{2+}\)-ATPase by Pi.

Discussion

The present study describes for the first time the assay of phosphorylation from Pi of D351E and D351A mutants in the reverse catalytic cycle of Ca\(^{2+}\)-ATPase. Phosphorylation was tested in the presence of an organic solvent that greatly favors such reverse reaction (6), and yet no phosphoenzyme was detected when native D351 was changed to E. The results can be rationalized in light of the recent structural information about Ca\(^{2+}\)-ATPase obtained at atomic resolution, as discussed below.

Heterologous expression of SR Ca\(^{2+}\)-ATPase was first established by Maruyama and MacLennan (7) using COS-1 mammalian kidney cells, and has been extensively used for the characterization of more than one-third of the residues of the Ca\(^{2+}\)-ATPase that have been replaced by site-directed mutagenesis. The exclusively transient expression of ATPase and the low transfection efficiency of the COS-1 cells are drawbacks of this system which have led to the development of alternative expression systems; the SR Ca\(^{2+}\)-ATPase has been expressed in baculovirus/Sf9 cells (20) and in yeast (10,16,21). Yeast stable transformation and relatively easy culture conditions make the latter system an efficient tool for mutant expression; however, the recovery of expressed Ca\(^{2+}\)-ATPase by yeast fractionation and membrane purification has been a limiting step that yields low amounts of recombinant Ca\(^{2+}\)-ATPase (10,16,21). An optimization of the yeast strain and of the plasmid vector (22) and an elaborate affinity purification of the Ca\(^{2+}\)-ATPase fused to a biotin acceptor domain (23) have provided enough protein for crystallization of the first recombinant Ca\(^{2+}\)-ATPase (23). In the yeast expression system previously developed by our group (10), recovery of recombinant Ca\(^{2+}\)-ATPase from the medium-speed 24,000-g yeast fraction by immunoprecipitation (20) was necessary before the phosphorylation measurements (10) because a number of endogenous yeast proteins were phosphorylated by \([\gamma-^{32}\text{P}]\text{ATP}\) as detected by acid gel electrophoresis, and interfered with detection of the 110-kDa recombinant Ca\(^{2+}\)-ATPase. Phosphorylation of these endogenous yeast proteins was much
reduced in the expression system optimized here, most likely due to the elimination of the heat-shock treatment that is no longer necessary because the heat-shock promoter that was previously used (10) has been replaced by a galactose-induced promoter as described in Methods. Thus, we have optimized a stably transformed yeast system that expresses SR Ca2+-ATPase under standard conditions in galactose medium, along with a simplified fractionation protocol that yields in a single medium-speed centrifugation step a microsomal yeast fraction enriched in recombinant Cu2+-ATPase that is adequate for enzymatic and phosphorylation assays of mutant Ca2+-ATPase without the need for immunoprecipitation or further purification.

Further evidence that the yeast expression system described here is adequate for obtaining recombinant Ca2+-ATPase is the finding that the overall structural integrity and a proper insertion of Cu2+-ATPase in the yeast membranes were maintained, as determined by limited tryptic proteolysis. The previously described T1 and T2 tryptic digestion cleavage sites of SR Cu2+-ATPase (24), which generate four characteristic tryptic fragments (A, B, Al, and A2), were maintained in all the recombinant Cu2+-ATPase expressed in yeast (Figure 3).

Transfer of the γ-phosphoryl group of ATP to the carboxyl moiety side chain of Cu2+-ATPase D351 residue is an obligatory step in the catalytic cycle. Mutation of D351 to glutamate, asparagine, serine, threonine, histidine or alanine, or the adjacent K352 to arginine, glutamine, or glutamate resulted in complete loss of Cu2+ transport and phosphorylation by ATP (7). Small changes in the position of the carboxyl group of D351 are deleterious to enzymatic activity, suggesting that a very rigid and precise alignment for D351 and K352 is necessary for utilization of ATP by the enzyme. Complete inhibition of ATP hydrolysis is produced by conservative mutation of D351 to E (7), whose difference is only for the presence of an additional γ-methylene group.

Because the D351 mutants reported in the literature were tested with ATP and Cu2+, there was the possibility that the lack of function of mutant D351E was due to a strict requirement of a stereochemical alignment of the adenosine moiety at the site for the effective transfer of γ-phosphate of ATP. If this was the case, phosphorylation by Pi of mutant D351E would be permitted, especially under extremely favorable conditions (e.g., 40% DMSO). Wild-type Cu2+-ATPase can be phosphorylated by Pi in the reverse direction of the catalytic cycle (Figure 1, step 9). This reaction yields equilibrium lev-
els of phosphoenzyme in the absence of Ca$^{2+}$ and in the presence of organic solvents such as DMSO at pH 6.0 phosphorylation of the enzyme by Pi is greatly facilitated, presumably by an increase in hydrophobicity which results in a reduced access of water to the catalytic site (5,6). The results of the present study show that hydrophobicity of the site is not sufficient for EP formation from Pi, and that the D351E mutation may disrupt the proposed essential coordination of Mg$^{2+}$ at neighboring K352 residues and other side chains at the phosphorylation site (see below), which would remain a critical factor even in the presence of a highly favorable hydrophobic medium.

It is known that P-type ATPases require Mg$^{2+}$ for activity as a non-transported cofactor (25) but this ion is not essential for nucleotide binding (26,27). Magnesium may have an important role in the mechanism of phosphorylation, probably by eliminating the electrostatic repulsion between the negatively charged γ-phosphate of nucleotide and the anionic carboxyl group of D351. This is confirmed in E1-AMPPCP crystals (PDB entries 1T5S and 1VFP) (28,29), where Mg$^{2+}$ is coordinated by the γ-phosphate, D351, T353, and D703. The E1-ADP:AlF$_4^-$ crystals contain two Mg$^{2+}$ ions (PDB entries 1TST and 1WPE) (28,30), one of them coordinated in the same manner as in E1-AMPPCP (with the exception that γ-phosphate is replaced by AlF$_4^-$), the other coordinated by the α- and β-phosphates and by AlF$_4^-$ . Notably, two Mg$^{2+}$ ions are also present in the E2-MgF$_4^{2-}$ crystal (1WPG) which simulates the E2-P state better (30). In this configuration of the enzyme, an atomic model of an aspartylphosphate could be taken from that of CheY (PDB accession 1QMP), a bacterial response regulator, which uses identical residues for the stabilization of phosphoryl group and Mg$^{2+}$ (30). The model of aspartylphosphate was superimposed with the main chain on D351; the oxygens of the phosphoryl group of the aspartylphosphate were superimposed on the fluorines of MgF$_4^{2-}$ without any change in the side-chain conformation of D351 (30). A water molecule was predicted to form a hydrogen bond with E183 in the TGES-conserved domain of Ca$^{2+}$-ATPase, at a distance of 2.8 Å from the carboxyl oxygen of D351 (see Figure 5b in Ref. 30), fixed by E183 and the carbonyl of T181. This would be the predicted reactive water molecule that would make an in-line attack on the aspartylphosphate to initiate phosphoenzyme hydrolysis (30) and is the same reactive water molecule that must be abstracted in the reverse reaction upon formation of an aspartylphosphate from Pi. In fact, E183 in the conserved TGES domain has been shown by mutagenesis to be important both in E2P formation from Pi and in E2P hydrolysis (31). Our mutagenesis study suggests that the carboxyl moiety of the D351E mutant is probably misplaced at the site in the sense that it will not permit this reactive water to appropriately coordinate with E183, so that no increase in hydrophobicity of the site by the inorganic solvent with a concomitant increase in reactivity of Pi could compensate for the lack of alignment of reactive residues. In fact, the well-documented effect of the inorganic solvent in increasing phosphorylation of wild-type Ca$^{2+}$-ATPase by Pi (2,6) probably is not a direct effect on water abstraction; rather, we propose that the organic solvent would facilitate the required movement of TGES to bring E183 into the catalytic site (32), thus catalyzing E2P formation from Pi (31).

Acknowledgments

We thank Dr. Radovan Borojevic, Universidade Federal do Rio de Janeiro, for producing a monoclonal D12 antibody against purified rabbit SR Ca$^{2+}$-ATPase.
References


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