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Calcium dependence of Pi phosphorylation of sarcoplasmic reticulum Ca^{2+}-ATPase at low water content: water dependence of the $E_2 \rightarrow E_1$ conversion

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Abstract

Enzymes entrapped in reverse micelles can be studied in low-water environments that have the potential of restricting conformational mobility in specific steps of the reaction cycle. Sarcoplasmic reticulum Ca^{2+}-ATPase was incorporated into a reverse-micelle system (TPT) composed of toluene, phospholipids, Triton X-100 and varying amounts of water (0.5–7%, v/v). Phosphorylation of the Ca^{2+}-ATPase by ATP required the presence of both water and Ca^{2+} in the micelles. No phosphoenzyme (EP) was detected in the presence of EGTA. Phosphorylation by Pi (inorganic phosphate) in the absence of Ca^{2+} was observed at water content below that necessary for phosphorylation by ATP. In contrast to what is observed in a totally aqueous medium, EP formed by Pi was partially resistant to dephosphorylation by Ca^{2+}. However, the addition of non-radioactive Pi to the EP already formed caused a rapid decrease in radiolabelled enzymes, as expected for the isotopic dilution, indicating the existence of an equilibrium ($E+Pi \leftrightarrow EP$). Phosphorylation by Pi also occurred in TPT containing millimolar Ca^{2+} concentrations in a range of water concentrations (2–5% v/v). The substrates *p*-nitrophenyl phosphate, acetyl phosphate, ATP and GTP increased the EP level under these conditions. These results suggest that: (1) the rate of conversion of the ATPase conformer E_2 into E_1 is greatly reduced at low water content, so that $E_2 \rightarrow E_1$ becomes the rate-limiting step of the catalytic cycle; and (2) in media of low water content, Pi can phosphorylate both E_1Ca and E_2. Thus, the effect of enzyme hydration is complex and involves changes in the phosphorylation reaction at the catalytic site, in the equilibrium between E_2 and E_1 conformers, and in their specificity for substrates.

Keywords: Ca^{2+}-ATPase; Water; Reverse micelle; Phosphorylation; Sarcoplasmic reticulum

1. Introduction

The Ca^{2+}-ATPase of sarcoplasmic reticulum (SR) (EC 3.6.1.38) converts chemical energy from ATP hydrolysis into a transmembrane Ca^{2+} concentration gradient. During ATP hydrolysis in aqueous media (Scheme 1), several phosphoenzyme species with different reactivities are formed [1,2]. They differ in their conformations, with the phosphate linked to

Abbreviations: Triton X-100, *p-t*-octylphenyl polyoxyethylene ether; Pi, inorganic phosphate; Wo, water:surfactant molar ratio; Tris, Tris(hydroxymethyl) aminomethane; MES, 2-[*N*-morpholino] ethanesulfonic acid; pNPP, *p*-nitrophenylphosphate; EGTA, ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SR, sarcoplasmic reticulum; EP, phosphoenzyme; AcP, acetylphosphate; FITC, fluorescein-5′-isothiocyanate; TNP-ATP, 2′,3′-*O*-(2,4,5-trinitrocyclohexadienylidene)adenosine 5′-triphosphate

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the enzyme as a chemically identical acylphosphate [3]. E_1-PCa is considered to be a 'high-energy' form because it can re-synthesize ATP when it binds ADP [4]. On the other hand, E_2-PCa and E_2-P are termed 'low-energy' forms, since they are not able to transfer the phosphate to ADP and since the E_2-P can also be formed from E_2 and inorganic phosphate (Pi) by reversal of steps 7 and 6 (Scheme 1) without any additional source of energy [5–10].

To account for the spontaneous phosphorylation by Pi, it has been proposed that the microenvironment of the catalytic site in E_2 would have less water than in E_1. This would cause desolvation of the Pi with the consequent increase in reactivity [7,11,12]. The catalytic site would alternate between hydrophilic (E_1) and hydrophobic (E_2) conformations, with more or less exposure to the aqueous medium, this change being essential for the energy-coupling process [12–19]. This hypothesis has received support from experiments on phosphorylation by Pi in organic solvents (dimethylsulfoxide, glycerol) that cause a pronounced increase in the apparent affinity for Pi by favoring the partition and providing a strong force for driving the Pi into the binding site [11–13,20]. Additional support was obtained from measurements of the fluorescence of enzyme-bound TNP-ATP, which increases dramatically when E_1-PCa is converted to E_2-P; in parallel, a blue shift of the maximal emission occurs, indicating an increase in hydrophobicity of the catalytic site during this transition [19]. On the other hand, by studying the effects of organic solvents in the different steps of the catalytic cycle, Suzuki and Kanazawa [21] showed that addition of dimethylsulfoxide, glycerol or dimethylformamide does not affect the rate of the conformational change involved in formation of the E_1ATPCa complex (step 2 in Scheme 1), but it greatly retards the subsequent phosphorylation reaction (step 3). With respect to phosphorylation of the Ca^{2+}-ATPase by Pi, it has also been proposed that organic solvents favor the reaction (step 6 in reverse), but not the binding of Pi to the enzyme [22,23].

In this study most of the attention was focused on the role of water on the phosphorylation reactions [7,11,13,14,17,19,21,23–25] and little on the interconversion between E_1 and E_2, which is associated with changes in the affinity for Ca^{2+} [24–30], and ATP [31]. Using fast kinetic techniques, Champeil et al. [22] and Mintz et al. [32] observed that the addition of organic solvents reduced the rate of intrinsic fluorescence changes induced by Ca^{2+}, attributed to the transition $E_2 \rightarrow E_1$.

The reverse-micelle system offers an alternative method for varying the amounts of water without enzyme denaturation, allowing the use of much lower water concentrations than those attained in studies where organic solvents were added to the medium. Previously, we showed that when the Ca^{2+}-ATPase of SR is incorporated into reverse micelles of toluene, phospholipids and Triton X-100 (TPT), it catalyzes Ca^{2+}-dependent but does not catalyze a Mg^{2+}-dependent hydrolysis of ATP, and this activity increases with the water content in the micelles [33]. We also found that at much lower water content phosphorylation by Pi but not by ATP occurs, leading to the proposal that the Ca^{2+}-ATPase can be trapped in an active E_2 conformation at this low water content. Here we describe the influence of water on the interconversion between E_1 and E_2 conformations, using phosphorylation by Pi, ^{32}P-^{30}P exchange and substrates that promote the $E_2 \rightarrow E_1$ conversion at different rates.

2. Materials and methods

2.1. Reagents

Toluene (spectroscopic grade), Triton X-100 (scintillation grade) and acetone were obtained from Merck; azolectin (phosphatidylcholine type IIS, from soybean), ATP, GTP, AcP, fluorescein isothiocyanate (FITC), α-tocopherol, sodium vanadate and *p*-nitrophenylphosphate (pNPP) were from Sigma Chemical Co; [^{32}P]Pi was purchased from IPEN (São Paulo, Brazil), and purified according to Kessler et al. [34]; [γ-^{32}P]ATP was prepared according to Walseth and Johnson [35].

2.2. Enzyme preparation

SR vesicles were prepared from rabbit hind leg muscle as described by Eletr and Inesi [36]. Ca^{2+}-ATPase was purified by method II of Meissner et al. [37] and stored in liquid nitrogen. Protein determinations were performed according to Lowry et al.

[38], using bovine serum albumin as a standard. The Ca^{2+}-ATPase activity of our purified preparations was 5–6 μmol Pi mg protein^{-1} min^{-1} under standard conditions (2 mM ATP, 50 μM $CaCl_2$, 5 mM $MgCl_2$, 100 mM KCl, 20 mM Tris-HCl, pH 7.5, 37°C). The maximal level of phosphorylation by ATP and Pi under standard aqueous conditions was 3.5 and 3.7 nmol EP/mg protein respectively.

2.3. Phospholipid purification

Crude soybean phospholipids (azolectin, 5 g) were resuspended in 100 ml 250 mM $MgCl_2$ and stirred on a vortex for 1 h at room temperature; phospholipids were extracted using 10 ml hexane. The organic phase was separated by centrifugation at 2000 rpm for 3 min in a clinical centrifuge, washed with 4 M NaCl (250 ml), and evaporated under a stream of nitrogen. This procedure removes most of the Ca^{2+} and Pi contaminants from the azolectin. The Pi content, measured as in [39], was reduced from 13 to 0.2–0.3 nmol/mg of lipids. Reduction in Ca^{2+} content was estimated from the concentration of EGTA needed to abolish the Ca^{2+}-ATPase activity in micelles made with different phospholipids. It was found that commercial azolectin contains Ca^{2+} contamination in the range of 25–35 nmol/mg of lipid; after purification these levels were reduced to 0.2–0.25 nmol/mg.

To remove neutral lipids the procedure described by Kagawa and Racker [40] was adopted. Briefly, to the residue treated as above was added a solution containing 50 ml acetone, 333 mg $MgCl_2 \cdot 6H_2O$ dissolved in 10 ml methanol and 10 μl α-tocopherol. After 30 min stirring at 4°C, phospholipids were filtered by suction through Whatman No. 42 filter paper on a Buchner funnel, then dissolved in 10 ml hexane and centrifuged as before. The supernatant was dried on a Buchi Re 111 Speed-vac and stored in the freezer.

2.4. Toluene/phospholipid/Triton X-100 (TPT) preparation

The mixture of organic solvent and surfactants was prepared by dissolving purified phospholipids (8.5 mg/ml) in a mixture of 15% (v/v) Triton X-100 in toluene. Water solutions were added using Hamilton syringes. Considering an average molecular weight of 700 Da for the phospholipids and 647 Da for the Triton X-100, water content of 1% (v/v) is equivalent to Wo = 2.2 mol of water per mol of surfactant. The NMR signal for water protons in the TPT system indicates that for water content up to 1% (v/v), the water inside the micelles is in a bound state [41]. Above this concentration, free water appears, and above 4.5% the proton properties resemble those of free water [41]. Water content can only be raised up to 7%, the limiting value for a stable emulsion in this system.

2.5. Incorporation of enzyme and substrates into TPT reverse micelles

Different amounts of purified Ca^{2+}-ATPase were resuspended in the buffer solutions described in the figure legends, and aliquots were injected into the TPT mixture in order to set the final desired water content. Subsequently, the tubes were vigorously stirred (15 s) in a vortex. Substrates were injected into TPT in the same way. The compounds used in the experiments have extremely low solubility in toluene, so the ionic composition inside the micelles is considered to be the same as in the original aqueous media [33,42,43]. The Mg^{2+} concentration was always maintained in excess over the substrate.

2.6. Phosphorylation by [^{32}P]Pi

Purified SR Ca^{2+}-ATPase (100 μg) resuspended in different buffer volumes containing 3 mM EGTA, 30 mM MES-Tris pH 6.0 and 10 mM $MgCl_2$ were incorporated into 0.75 ml TPT in order to obtain the desired water content. The reaction was started by adding 0.25 ml TPT containing 2 mM [^{32}P]Pi with the same buffer and water concentrations [33]. After 1 min of phosphorylation at 25°C, time necessary to attain the equilibrium (result not shown), the enzyme was denatured by the addition of 0.1 ml 50% (w/v) trichloroacetic acid in toluene. The turbid suspension was centrifuged at 3000 rpm for 3 min and the supernatant containing most of the phospholipids, toluene and Triton was discarded. The pellet was resuspended in 50 μl ethanol by vortexing, then vigorously stirred with 1 ml of a mixture containing 50 mM perchloric acid and 2 mM Pi in 70% (v/v) ethanol

and centrifuged as above. The pellet was resuspended in 1 ml 125 mM ice-cold perchloric acid in a sonicator bath, filtered through Millipore filters (HAWP 0.45 μm pore diameter) and washed six times with the same solution. Radioactivity on the filters was measured in a scintillation counter.

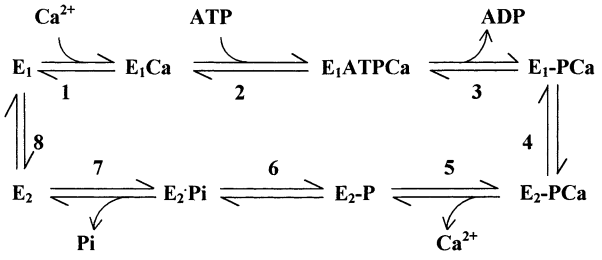
2.7. Dephosphorylation of the phosphoenzyme formed from [^{32}P]Pi

After 1 min of phosphorylation, ATPase dephosphorylation was promoted by the addition of Ca^{2+} (1 mM $CaCl_2$ in excess over EGTA) or by isotopic dilution with non-radioactive Pi (40 mM) previously incorporated into TPT containing the same volume fraction of water, 30 mM MES-Tris pH 6.0 and 10 mM $MgCl_2$. The reaction was stopped after 1 min at 25°C, using trichloroacetic acid in toluene as described above.

3. Results

3.1. Phosphorylation by ATP and Pi

Previously we have shown that phosphorylation of the Ca^{2+}-ATPase by ATP in the TPT system is dependent on water [33]. Here we show that phosphorylation is also dependent on Ca^{2+}. In contrast to the phosphorylation that is observed in a totally aqueous medium [1,28,44], phosphorylation of the enzyme by ATP in micelles prepared with minimal water is blocked, whether Ca^{2+} is present or not (Fig. 1). In the presence of Ca^{2+}, phosphorylation appears when



Scheme 1. Reaction cycle of the sarcoplasmic reticulum Ca^{2+}-ATPase, adapted from Carvalho et al. [47]. For simplicity, only one of the two Ca^{2+} ions is indicated, and this minimal model does not specify all the steps that have been described for Ca^{2+} binding and/or deocclusion (for review see [1,26–28,44]).

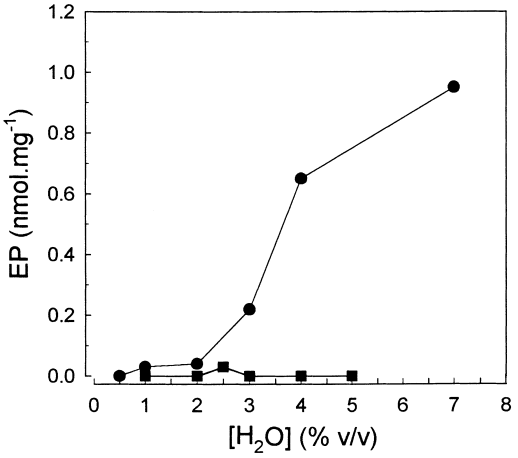


Fig. 1. Phosphorylation by [γ-^{32}P]ATP in TPT micelles in the presence and absence of Ca^{2+}. Purified sarcoplasmic reticulum Ca^{2+}-ATPase (100 μg) was resuspended in different volumes of buffer containing 5 mM $MgCl_2$, 80 mM KCl, 20 mM Tris-HCl pH 7.5 and either 0.5 mM $CaCl_2$ (●) or 3 mM EGTA (■), and incorporated into TPT in order to obtain the water concentrations shown on the abscissa (see Section 2). The reaction was initiated by the addition of [γ-^{32}P]ATP-Mg in TPT of the same water content (final ATP concentration 1 mM). The reaction was stopped after 5 s at 25°C. Phosphoenzyme was measured as described for Pi phosphorylation (see Section 2). The data are typical of four to six experiments with four different preparations.

the water content exceeds 2% (v/v), and increases steadily as the water content is raised to 7%. No phosphorylation is observed at any water content when Ca^{2+} is absent.

In the absence of Ca^{2+}, the Ca^{2+}-ATPase in TPT micelles is phosphorylated by Pi. Fig. 2 (filled circles) shows that this reaction is also dependent on the water content, but it reaches a maximum in 2% water, where ATP phosphorylation does not occur (cf. Fig. 1). Previous reports describing phosphorylation by Pi in the absence of Ca^{2+} in totally aqueous media have attributed it to the E_2 conformer [7–9]. In contrast to what is observed in a dimethylsulfoxide medium [13], the apparent K_m for this reaction in the micelles is 1.1 ± 0.2 mM for 2% v/v water content (not shown), in the same order of magnitude of those observed in an aqueous medium.

The contrast between the results of Figs. 1 and 2 (filled circles) could be interpreted as that either phosphorylation of E_2 by Pi requires less water than phosphorylation of E_1 by ATP, or alternatively that at low water content (less than 2%) the enzyme is trapped in the E_2 conformation and E_1 is not

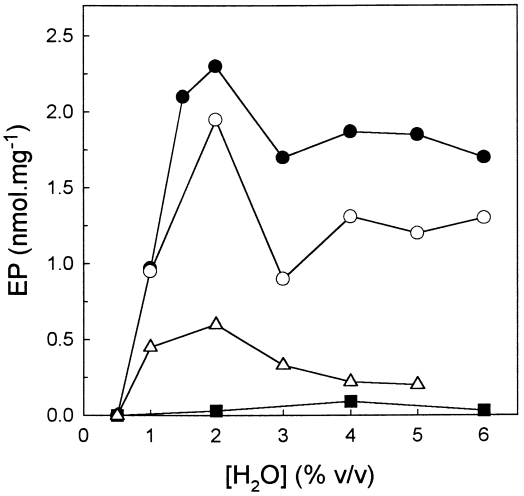


Fig. 2. Phosphorylation by [^{32}P]Pi (●), inhibition by vanadate (■) and dephosphorylation induced by Ca^{2+} (○) or non-radioactive Pi (△) in the presence of low water concentrations. ATPase (100 μg) was resuspended in TPT in different volumes of a buffer containing 30 mM MES-Tris pH 6.0, 3 mM EGTA, 10 mM $MgCl_2$ and either 0 (●) or 100 μM vanadate (■) using different volumes of buffer to obtain the various water concentrations. Reaction was started by adding [^{32}P]Pi in TPT of the same water content to a final concentration of 2 mM Pi, and allowed to proceed for 1 min at 25°C. In the two middle curves, dephosphorylation was induced after 1 min by adding 1 mM free Ca^{2+} (○) or 40 mM non-radioactive Pi (△), previously incorporated into TPT with the same concentrations of water, MES-Tris and $MgCl_2$. Dephosphorylation was interrupted after 1 min and EP was measured as described. The data are typical of four to six experiments with four different preparations.

available, irrespective of the presence or absence of Ca^{2+} in the reverse micelles.

3.2. Characteristics of EP formed from Pi in the micelles

In water, the addition of Ca^{2+} to the ATPase already phosphorylated by Pi induces the release of all Pi from the enzyme by shifting the reaction equilibria toward E_1Ca (steps 6, 7, 8 and 1 in Scheme 1) [5]. In the TPT micelles, on the other hand, when Ca^{2+} is added to the ATPase phosphorylated by Pi, only a partial dephosphorylation occurs. Fig. 2 (open circles) shows that the loss of EP after addition of Ca^{2+} ranges from almost none (in 1% water) to about 30% (in 3–6% water). The enzyme trapped in the micelles remains functional at low water concentrations, since addition of non-radioactive Pi to the EP already formed causes the labeling on the enzyme to decrease to the levels expected for the isotopic dilution (triangles in Fig. 2). Fig. 2 also shows that the enzyme is almost completely inhibited by the addition of vanadate, which has been shown in aqueous media to compete with phosphate, by binding to E_2 [45,46].

The incomplete dephosphorylation induced by Ca^{2+} in Fig. 3 may indicate that equilibrium was not attained under our conditions when Ca^{2+} is present. Fig. 3 shows that the decrease in EP attains the minimum value within the first minute of reaction, the residual EP being stable at least for 5 min. Addition of higher Ca^{2+} concentrations (up to 10 mM) did not modify the dephosphorylation curve (not shown). Figs. 2 and 3 indicate that EP attains an equilibrium with the medium and suggest that in a medium with low water mobility the transition between E_2 to E_1 may be strongly repressed.

Alternatively the results of Figs. 2 and 3 could be explained if, in contrast to what is observed in a totally aqueous medium, phosphorylation by Pi in the TPT micelles can take place even in the presence of Ca^{2+}. To test this hypothesis we preincubated the enzyme with Ca^{2+} in a totally aqueous medium to obtain E_1Ca and then transferred it into TPT micelles with variable water content. Indeed, in the

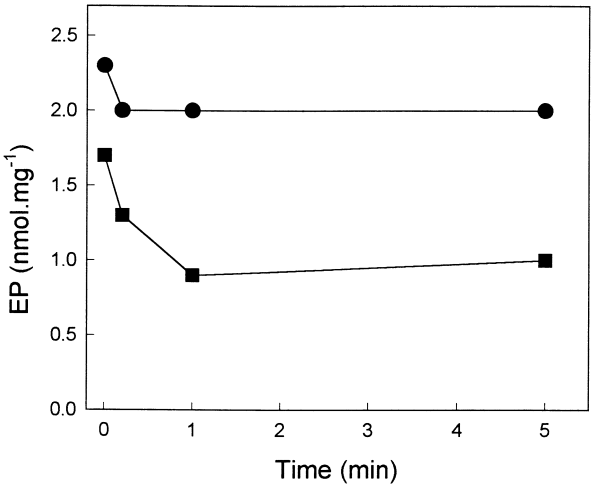


Fig. 3. Time course of dephosphorylation induced by Ca^{2+}. Phosphoenzyme was formed from [^{32}P]Pi in EGTA as described in Fig. 2, at water concentrations of 2% (●) or 3% (■). Dephosphorylation was induced by addition of 1 mM free Ca^{2+} in TPT, and the reaction was stopped at the indicated times. The data are typical of four to six experiments with four different preparations.

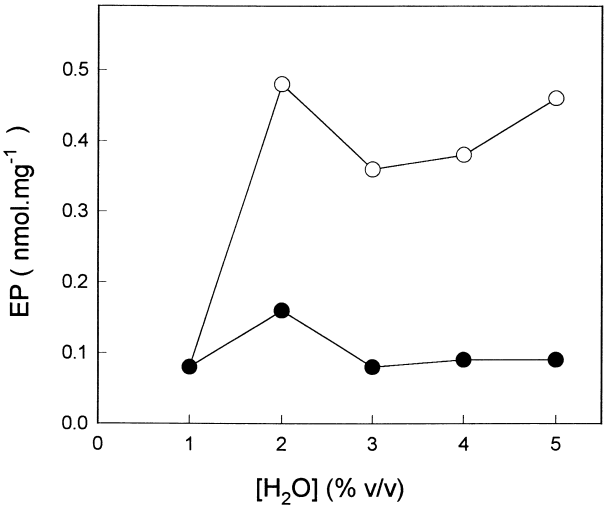


Fig. 4. Phosphorylation by [^{32}P]Pi in the presence of Ca^{2+} (○) and dephosphorylation induced by non-radioactive Pi (●). Enzyme was phosphorylated by Pi as in Fig. 2, but in the presence of 3 mM $CaCl_2$ instead of EGTA. After 1 min, EP level was measured in one aliquot and 40 mM non-radioactive Pi was added to another aliquot of each sample. Lower curve shows EP remaining 1 min after the cold Pi chase. Similar results were obtained in three other preparations.

presence of 3 mM Ca^{2+} and at 2 mM Pi, a small phosphorylation by Pi is observed (Fig. 4). This small phosphorylation attains maximal levels at less than 30 s and remains constant up to 5 min (not shown). The EP formed in the presence of Ca^{2+}, like that in the absence of Ca^{2+}, rapidly exchanges its bound phosphate with Pi added to the medium (Fig. 4, filled circles), and thus can be considered to be involved in an equilibrium reaction ($E_1Ca+Pi \leftrightarrow E_1Ca-P$). However, the low level of EP formed in the presence of Ca^{2+} ($\sim$0.5 nmol mg^{-1}) cannot entirely account for the residual EP (1–2 nmol mg^{-1}) that remains after Ca^{2+} is added to induce dephosphorylation of the EP that is formed without Ca^{2+} (cf. Fig. 4 with Figs. 2 and 3).

An additional clue to the source of the residual EP, i.e. after Ca^{2+} addition, in Figs. 2 and 3 is provided by the experiment in Fig. 5A, where a pronounced increase in the phosphoenzyme levels formed from [^{32}P]Pi in Ca^{2+}-containing medium is caused by the addition of 2 mM non-radioactive ATP. The increase at a water content of 1% is especially remarkable considering that at this water concentration, ATP phosphorylation is barely detectable (cf. Fig. 1). Similar data were obtained when 20 μM ATP was added (Fig. 5B). These results suggest that in the micelles, [^{32}P]Pi phosphorylates an enzyme conformation (E_2) that is produced during the hydrolysis of ATP even though hydrolysis in 1% water is extremely small. Other substrates of the Ca^{2+}-ATPase (GTP, pNPP and acetyl phosphate) also produce this effect (Fig. 5B). Taken together, one of the possible explanations for the data of Fig. 5

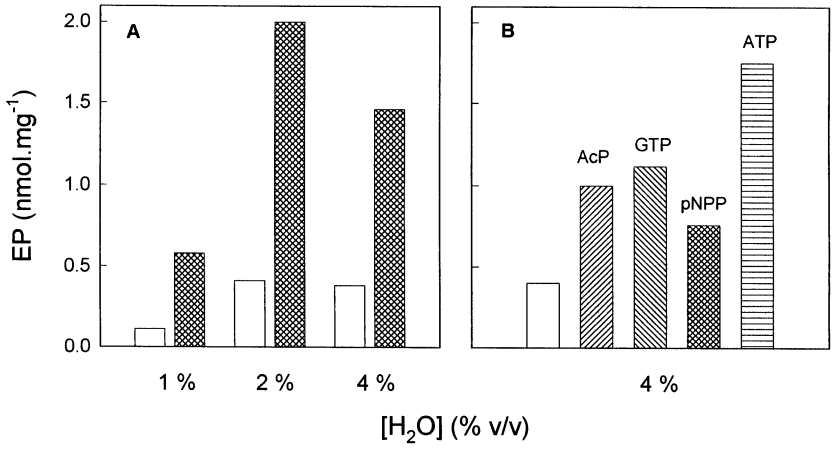


Fig. 5. EP formation from [^{32}P]Pi in the presence of Ca^{2+}: Stimulation by ATP (A) and other substrates (B). Phosphoenzyme was formed from [^{32}P]Pi in the presence of 0.5 mM $CaCl_2$ at different water concentrations, as in Fig. 4. Empty columns show EP levels attained after 1 min without any further additions. In (A), cross-hatched columns show EP level attained 30 s after adding 2 mM ATP-Mg in TPT to the enzyme preincubated with [^{32}P]Pi. In (B), cross-hatched columns show phosphorylation by [^{32}P]Pi 30 s after adding 2 mM AcP, 2 mM GTP, 3.5 mM pNPP or 20 μM ATP in TPT, as indicated. Data are typical of four experiments with two different preparations.

is that at low water content and in the presence of any hydrolyzable substrate, E_2 accumulates even in the presence of Ca^{2+}, and is phosphorylated by Pi (Scheme 1, steps 6 and 7 in reverse); and that this accumulation of E_2 is a result of the interconversion between E_2 and E_1 being impaired.

4. Discussion

Major differences between micellar and aqueous media are observed when the SR Ca^{2+}-ATPase is suspended in media of low water content: the Ca^{2+}-ATPase can be phosphorylated by Pi in the presence of Ca^{2+}; only part of the phosphoenzyme formed in EGTA is lost when Ca^{2+} is added; and the phosphoenzyme formed from Pi in the presence of Ca^{2+} increases when mM ATP is present. Taken together, these results indicate that dehydration of the protein affects more than one step of the catalytic cycle: in particular it severely impairs the conversion $E_2 \rightarrow E_1$ and modifies the specificity of the catalytic site at the E_1 conformation. The consequences of dehydration are thus more complex than previously proposed [33].

Regarding the substrate specificity of the catalytic site, it has been reported that in aqueous media in the presence of Ca^{2+}, E_1Ca is formed at the expense of E_2, and phosphorylation by Pi is nil. Here we show that at limited water content, the E_1Ca form can be phosphorylated by Pi, albeit with small efficiency (Fig. 4). The mechanisms that determine substrate specificity of the Ca^{2+}-ATPase are not clear. It is plausible that the reduction in water content promotes the loss of one or more molecules of water from the active site, and we speculate whether the displacement of water may be a general mechanism for determining the selection of different substrates by the E_1 conformer. Displacement of water might normally be accomplished by the P-O-P or C-O-P linkage, which is common to all hydrolyzable substrates of this enzyme. The phosphate moiety alone would be unsuccessful, since it lacks this structural feature, but if the catalytic site were to lose a water molecule as a result of the low water content of the surrounding medium, even Pi could be used by the E_1Ca conformer to form a phosphoenzyme.

Several pieces of evidence indicate that the low content of water severely impairs the interconversion $E_2 \rightarrow E_1$. Fig. 5A shows that ATP promotes a substantial increase in the level of E_2-P from Pi in 1% water. This result was unexpected, since at this water content a very low level of E_1-P is formed (Fig. 1) and ATP hydrolysis is almost undetectable, less than 1% of the rate in normal aqueous medium [33]. This probably means that the rate of conversion of E_2 into E_1 in 1% water is so low that some E_2 accumulates and is phosphorylated by Pi even though the forward reaction is almost nil. In the presence of ATP, the EP level formed from Pi (an index of E_2) increases from 1% to 2% water and then decreases in 4% water. This decrease may reflect acceleration of the conversion of E_2 into E_1, in accordance with the pronounced increase in the formation of E_1-P from ATP (Fig. 1) and in the velocity of ATP hydrolysis [33] as the water content is raised from 2% to 4%. Apparently, $E_2 \rightarrow E_1$ is affected to a greater extent than the other steps of the cycle. In this regard, it is noteworthy that the NMR signal from water protons in the TPT system at this very low water concentration indicates that bound water is the predominant species [41].

Additionally, the incomplete dephosphorylation by Ca^{2+} of the phosphoenzyme formed by Pi (Fig. 3) contrasts strikingly with the almost complete Pi exchange after the addition of non-radioactive Pi (Figs. 2 and 4). As previously mentioned, the direct phosphorylation of Ca^{2+}-ATPase by Pi in the presence of Ca^{2+} (Fig. 4) proceeds with a very low efficiency and does not account for the high levels observed after several minutes of Ca^{2+}-induced dephosphorylation (Fig. 2). Since the isotopic exchange data (Fig. 2) indicate that Pi can be released readily from E_2-P, one plausible mechanism is that rapid binding of Ca^{2+} to E_2-P in the micelles forms a stable E_2-PCa. This would be possible if E_2 converts slowly into E_1, so that there is a pseudo-equilibrium (E_2+Pi $\leftrightarrow$ E_2-P) during a short period of time after Ca^{2+} addition, thus allowing the conversion of most of the E_2-P into E_2-PCa while only a small fraction of E_2-P hydrolyzes to yield E_2 and then E_1Ca. Hydrolysis of E_2-P could account for the fast drop in Figs. 2 and 3. Such data, observed at all limiting water concentrations tested, underscore the importance of hydration in the $E_2 \leftrightarrow E_1$ interconversion.

It should also be noticed that in previous reports,

evidence has been presented to show that the interconversion of E_1 and E_2 in totally a aqueous medium is promoted by the action of ATP at a low-affinity regulatory site [6,47,48]. The stimulation of E_2-P formation from Pi is seen in water only when the regulatory site for ATP is not saturated, as for example when acetyl phosphate, GTP or a very low concentration of ATP ($\leq$10 μM) is used [47,49], promoting hydrolysis and the availability of E_2, but not $E_2 \rightarrow E_1$. Thus, in aqueous media, an increase in the ATP concentration accelerates $E_2 \rightarrow E_1$ and reduces E_2-P from Pi by depleting E_2. In the reverse micelles, however, ATP stimulates Pi phosphorylation even in the mM range (Fig. 5A), suggesting that acceleration of the $E_2 \rightarrow E_1$ conversion by ATP is also impaired in media of low water content. This result is in accordance with kinetic data that suggest lack of a regulatory ATP site that can accelerate ATP hydrolysis in TPT [33].

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