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Direct demonstration of an acid-labile phosphoenzyme in the cycle of the sarcoplasmic reticulum Ca^{2+}-dependent adenosinetriphosphatase

G.L. Alonso, D. Takara and D.A. González

Cátedra de Biofísica, Facultad de Odontología, Universidad de Buenos Aires, Buenos Aires (Argentina)

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The Ca^{2+}-dependent adenosinetriphosphatase (Ca^{2+}-ATPase) from the sarcoplasmic reticulum (SR) of rat skeletal muscles is phosphorylated by inorganic phosphate (P_i) in the absence of Ca^{2+}. The reaction can be described by the following simplified scheme:

$$E + P_i \overset{1}{\leftrightarrow} E \cdot P_i \overset{2}{\leftrightarrow} E-P + H_2O$$

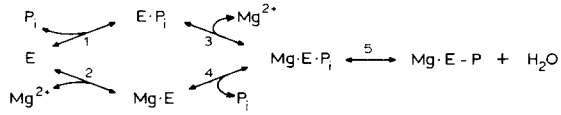
where E-P is a covalent, acid-stable and ADP-insensitive phosphoenzyme, and $E \cdot P_i$ is a noncovalent and acid-labile complex. The reaction is Mg^{2+}-dependent. Membrane fragments deposited on Millipore filters were successively perfused with two solutions, at constant flow. The effluent samples were analyzed. The perfused solutions were Ca^{2+} free and always contained 40% dimethylsulfoxide (DMSO), plus other reactants. Following the successive perfusion of solutions without and with [^{32}P]P_i, ^{32}P binding is only detected in the presence of Mg^{2+}, indicating the formation of the phosphoenzymes ($E \cdot P_i$ and E-P). Following perfusions of the phosphoenzymes with 5% trichloroacetic acid, ^{32}P release indicates the amount of the acid-labile moiety ($E \cdot P_i$). After phosphorylations, the filters were washed with acid and unlabeled P_i, and the remaining radioactivity was measured to evaluate the acid-stable phosphoenzyme (E-P). The acid-labile and acid-stable phosphoenzymes amounted, respectively, 0.72 ± 0.12, and 1.48 ± 0.10 nmol of P_i/mg of protein ($\pm$S.E., $n = 5$), after phosphorylations with 20 μM P_i. The results indicate: (1) The method allowed the evaluation of the acid-labile intermediate of the SR Ca^{2+}-ATPase cycle. $K_{eq} = k_2/k_{-2}$, in the above scheme, approaches 2.0. (2) The substrate of the phosphorylation reaction, in the presence of DMSO, is likely to be the $Mg \cdot P_i$ complex, since Mg^{2+} is necessary for step 1 in the above scheme.

Introduction

The phosphorylation of the SR Ca^{2+}-ATPase with P_i is the first step of the reaction leading to ATP synthesis from P_i and ADP [1]. This reaction can proceed in the absence of a transmembrane Ca^{2+} gradient [2]; it requires Mg^{2+}, and is inhibited by K^+ and high pH [2,3]. On the basis of kinetic arguments [4–7], it has been postulated that the formation of the covalent phosphoenzyme (E-P) is preceded by a noncovalent enzyme-substrate complex (Scheme 1):

$$E + P_i \overset{1}{\longleftrightarrow} E \cdot P_i \overset{2}{\longleftrightarrow} E-P + H_2O$$

To account for the effect of Mg^{2+}, it is generally accepted [8–11] that P_i and Mg^{2+} are bound to the enzyme in random sequence to form a ternary intermediate complex ($Mg \cdot E \cdot P_i$), prior to the formation of the covalent bound (Scheme 2):



E-P species are acid-stable; they can be measured after quenching the phosphorylation reaction with strong acids. The noncovalent complexes are acid-labile. Attempts for its direct experimental quantitation have been unsuccessful [12,13].

In this paper we demonstrate the release of P_i from phosphorylated SR membranes, by effect of strong acids. We used a method of continuous perfusion of the substrate containing media through SR membrane fragments previously deposited on Millipore filters. The method has been used in previous work from our laboratory [13,14]. We took advantage of the increased

Abbreviations: SR, sarcoplasmic reticulum; Ca^{2+}-ATPase, Ca^{2+}-dependent adenosinetriphosphatase; P_i, orthophosphate; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethyleneglycolbis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; DMSO, dimethylsulfoxide; Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

Correspondence: G.L. Alonso, Cátedra de Biofísica, Facultad de Odontología, Universidad de Buenos Aires, Buenos Aires, Argentina.

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affinity of the enzyme for P_i in the presence of DMSO [15], and of the increased sensitivity of the method at low ligand concentrations [14]. P_i released by acids reflects the amount of the $Mg \cdot E \cdot P_i$ species in equilibrium with $Mg \cdot E\text{-}P$.

Materials and Methods

Sarcoplasmic reticulum fragments from rat skeletal muscles were obtained as previously described [16]. The microsomes were stored at −70 °C in 250 mM sucrose and 5 mM histidine. The protein concentration was measured with the method of Lowry et al. [17] using bovine serum albumin as standard.

The Ca^{2+}-ATPase was phosphorylated using $[^{32}P]P_i$ as substrate. Phosphorylations and dephosphorylations were evaluated with the continuous perfusion method previously described [13,14]. Briefly, aliquots of the microsomal suspensions were deposited on Millipore filters (0.45 μm average pore and 13 mm diameter) and successively perfused with two different solutions, at constant flow. The perfusing media contained 40% (v/v) DMSO, 50 mM Mops-Tris (pH 6.0), $NaH_2{}^{32}PO_4$ and other reactants as indicated under Results, and, in some cases, ^3H_2O as a marker of the degree of mixing of the successively perfused solutions. The samples were collected at the output of the filter, after the media passed through the enzyme. The radioactivity of the effluent samples was determined by liquid scintillation counting. Changes in ^{32}P concentration indicate its binding to – or its release from – the SR membranes deposited on the filters.

$[^{32}P]$Orthophosphate was obtained from the Comisión Nacional de Energía Atómica de la República Argentina, and ^3H_2O from E.I. du Pont de Nemours & Co., NEN Res. Prod., Boston, MA. Other reagents were analytical grade.

Results

Fig. 1 shows the binding of P_i to the SR Ca^{2+}-ATPase, and its dependence on the presence of Mg^{2+} in the media. In three experiments (Fig. 1), two solutions were successively perfused through SR membrane fragments previously deposited on Millipore filters. In Fig. 1A the second solution contained $[^{32}P]P_i$ and ^3H_2O while the first one lacked radioisotopes. Both solutions contained Mg^{2+}. The lower amount of $[^{32}P]P_i$ related to ^3H_2O in the successively collected effluent samples indicates that a fraction of the perfused P_i is retained by the SR membranes.

In a similar experiment, without Mg^{2+} in any solution (Fig. 1B), the effluent concentrations of $[^{32}P]P_i$ and ^3H_2O are similar, indicating the absence of P_i binding when Mg^{2+} is not included in the media. Fig. 1B also discards unspecific P_i binding to the filter.

In Fig. 1C both solutions contained $[^{32}P]P_i$, while Mg^{2+} was only included in the second one. The downward deflection of the ^{32}P curve shows that the arrival of Mg^{2+} at the filter promotes P_i binding to the SR membranes.

Fig. 2 shows the successive perfusion of two solutions containing Mg^{2+} and $[^{32}P]P_i$. The second solutions were added with 5% trichloroacetic acid. The upward deflections of the the ^{32}P curves indicate P_i release promoted by the acid. The areas between the ^{32}P and

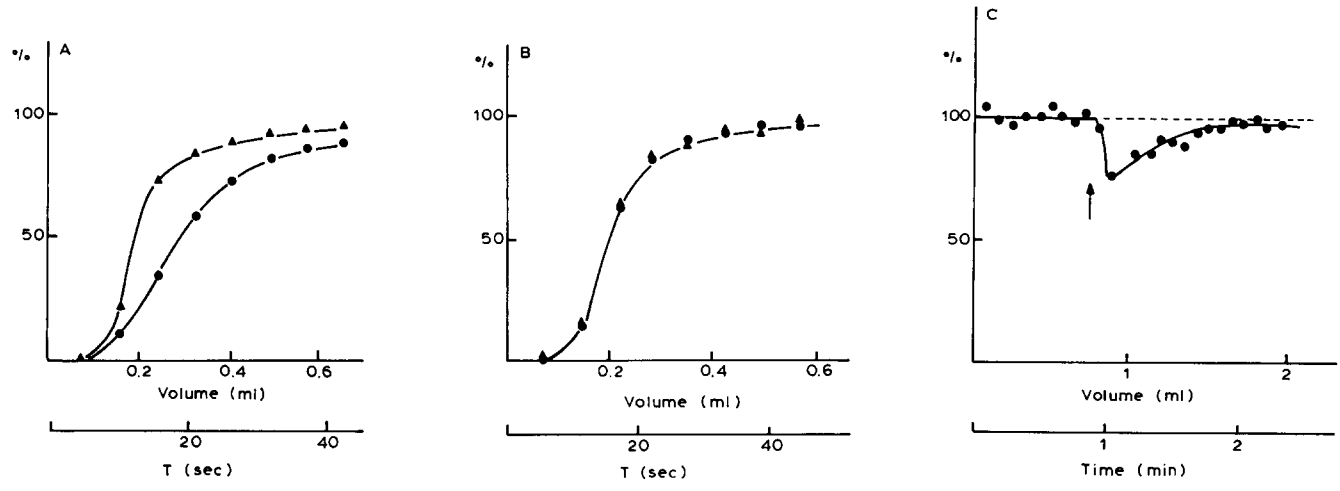

Fig. 1. Two solutions were successively perfused, at constant flow, through SR membrane fragments previously deposited on Millipore filters. All the solutions contained 40% DMSO, 50 mM Mops-Tris (pH 6.0) and 1 mM EGTA, plus the following additions: (A) Solution 1: 10 mM $MgCl_2$; solution 2: same as 1 plus 20 μM $NaH_2PO_4(^{32}P)$ and ^3H_2O. (B) Solution 1: 1 mM EDTA; solution 2: same as 1 plus 20 μM $NaH_2PO_4(^{32}P)$ and ^3H_2O. (C) Solution 1: 20 μM $NaH_2PO_4(^{32}P)$; solution 2 (arrow): same as 1 plus 10 mM $MgCl_2$. The radioactivity of the successively collected effluent samples was measured. The results are expressed as percentages of the perfused radioactivity: ●, ^{32}P; ▲, ^3H. In (B) ^{32}P and ^3H data are undistinguishable, in most cases. SR protein deposited on the filters amounted to 1.0 (A and B) or 1.5 (C) mg. Perfusions were at 0.91 (A), 0.76 (B), and 0.85 (C) ml/min, at 22 °C.

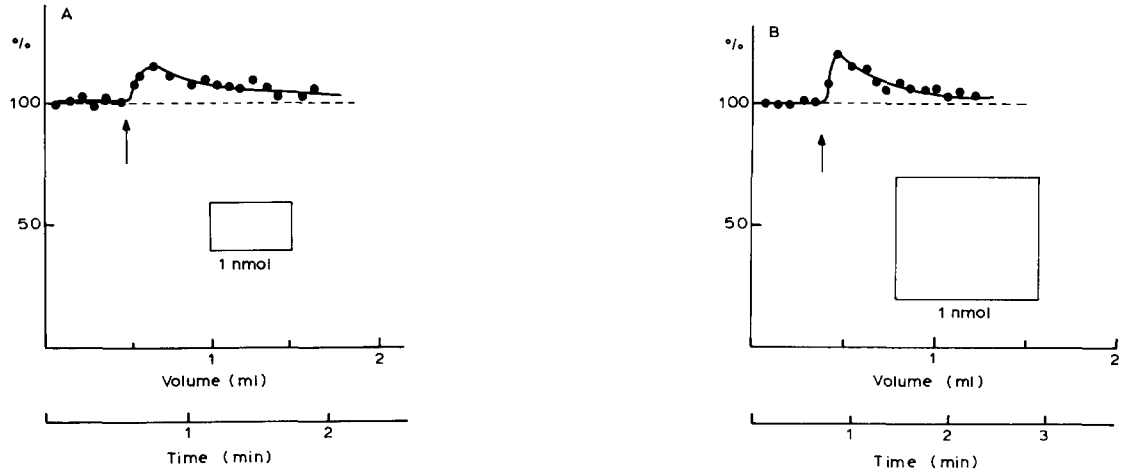

Fig. 2. (A) SR membranes (1.5 mg of protein) were deposited on a Millipore filter and successively perfused with two solutions at 0.82 ml/min and 22° C. Solution 1: 40% DMSO, 50 mM Mops-Tris (pH 6.0), 1 mM EGTA, 10 mM $MgCl_2$, and 20 μM $NaH_2PO_4(^{32}P)$; solution 2: same as 1 plus 5% trichloroacetic acid. (B) 1.0 mg of SR protein was perfused at 0.55 ml/min with 5 μM $NaH_2PO_4(^{32}P)$, being all the other reactants and conditions as in (A). The results are expressed as percentages of the radioactivity in the perfusing soltutions. The areas between the curves and the 100% horizontal lines express the amount of P_i released by the acid. It can be calculated by comparison with the standard areas indicated in the figures. P_i released averaged 0.72 ± 0.12 (S.E.) nmol/mg of protein, in five similar experiments with 20 μM P_i (A), and 0.32 nmol/mg of protein in the experiment B. After the experiments, the filters were exhaustively washed with 5% trichloroacetic acid and 15 mM unlabeled P_i, and the remaining radioactivity was measured. It amounted to 1.48 ± 0.10 (S.E., $n = 5$) nmol of P_i/mg of protein in (A) and 0.71 nmol of P_i/mg of protein in (B).

the 100% horizontal lines express the amounts of P_i released. In control experiments without Mg^{2+}, no changes in the effluent ^{32}P concentrations were observed as a consequence of the acid perfusions (data not shown). In other controls, after the acid perfusion of the phosphoenzymes, proteins were looked for in the effluent media; the negative results discarded an eventual effect of the acid on the retention of the protein by the filter.

After collecting the samples (Fig. 2), the filters were exhaustively washed with acid and unlabeled P_i. The radioactivity remaining in the filters was measured. It indicates the amount of acid-stable phosphoenzyme. In five similar experiments (Fig. 2A), P_i released by the acid averaged approximately one-half of the P_i remaining in the filters after the acid washings (Fig. 2, legend).

P_i released by acids is larger at the highest P_i perfusing concentration (Fig. 2A), but it is better visualized at the lowest concentration (Fig. 2B), because of the increase of the sensitivity of the method at lower concentrations of the ligand.

Discussion

A noncovalent and acid-labile phosphoenzyme ($E \cdot P_i$) is usually included as an intermediate step of the SR Ca^{2+}-ATPase cycle in most models of the reaction [6,18,19]. It is justified by some experimental results: (i) Initial velocities of E-P formation from P_i are dependent on the P_i concentration and demonstrate a saturation behaviour, indicating the occurrence of a phosphate-enzyme complex previous to the formation of the covalent bound [5,11]. (ii) The dephosphorylation velocity is larger when deduced from measurements of ^{18}O exchange between the phosphoenzyme and water, than when calculated from the decay of a ^{32}P-labeled phosphoenzyme [7]. The difference is attributed to the intermediate noncovalent complex, which formation from E-P (reversal of Scheme 1) not always leads to release of free P_i, since with a certain probability $E \cdot P_i$ returns to E-P.

Several attempts for the experimental determination of the $E \cdot P_i$ species, after phosphorylations with [^{32}P]ATP, have been reported. Froehlich and Taylor [4] interpreted that an initial burst of P_i production after the acid quenching of the reactions could be attributed to the formation of the acid-labile complex, which breakdown would contribute to free P_i measured. However, Verjovski-Almeida et al. [20], who also observed the P_i burst, pointed out that it disappeared upon changing the method for P_i determination. Chiesi and Inesi [12] concluded that the concentration of acid-labile phosphoenzyme is negligible in the presence of ATP and Ca^{2+}; the authors tried to detect it after quenching the reactions with Ca^{2+} chelators. We also failed to detect the acid-labile intermediate [13], using the same methodology as in the present experiments. Upon phosphorylations with [^{32}P]ATP, the enzyme-substrate complex ($E \cdot ATP$) coexists in equilibrium with the phosphoenzymes, and ^{32}P released by acids was attributed to $E \cdot ATP$ breakdown [13]. It hampered the detection of the $E \cdot P_i$ species, which, on the other hand, should be extremely low under the foregoing experimental conditions: the presence of Ca^{2+} and K^+, and high pH.

Now, we phosphorylated the Ca^{2+}-ATPase with

[^{32}P]P_i, in the absence of Ca^{2+}, thus avoiding the formation of the phosphate unreactive enzymatic species. The reversal of the ATPase cycle stops at the level of the ADP-insensitive phosphoenzyme species. Furthermore, it is well known that DMSO increases the P_i affinity of the enzyme [7,15]. It allowed us the use of low P_i concentrations. The continuous perfusion method increases its sensitivity at low ligand concentrations, because of the increase of the signal/noise ratio (Ref. 14, and Fig. 2).

The results from most authors [6,8,9,11] support Scheme 2 for the phosphorylation of the Ca-ATPase by P_i, in the absence of organic solvents. On the other hand, Champeil et al. [7] postulated that the substrate of the reaction, in the presence of DMSO, is the $Mg \cdot P_i$ complex (Scheme 3):

$$E + Mg \cdot P_i \xleftrightarrow{1} Mg \cdot E \cdot P_i \xleftrightarrow{2} Mg \cdot E\text{-}P + H_2O$$

In the absence of Mg^{2+}, we did not detect P_i binding indicating the formation of the $E \cdot P_i$ species (Scheme 2); obviously, P_i was not released by acids after the exposure of the enzyme to [^{32}P]P_i in the absence of Mg^{2+} (experiments not shown). Our results provide an evidence in favor of Scheme 3, since Mg^{2+} is necessary for the noncovalent binding of P_i to the enzyme, in the presence of DMSO.

However, our results do not allow us to discard the possibility that the lack of detection of the $E \cdot P_i$ species (Scheme 2) indicates that, in the presence of DMSO, the reaction runs preferentially through steps 2–4 rather than through steps 1–3. P_i would have a larger affinity for the $Mg \cdot E$ than for the E species, being $K_4 > K_1$ and $K_3 > K_2$. This entails a preferential order of binding; Mg^{2+} must bind first to the enzyme and only after Mg^{2+}, P_i would be able to bind. But this possibility is at variance with the observation of the same rate constant for phosphorylation, irrespective of the order of addition of Mg^{2+} and P_i [7].

The relative amounts of acid-labile and acid-stable phosphoenzymes (Fig. 2) indicate a value of approximately 2.0 for K_2 (k_2/k_{-2}) in Scheme 3. This value is somewhat higher than those calculated by Punzengruber et al. [6], and by Inesi et al. [21], and somewhat lower than that calculated by Lacapere et al. [11], from experiments in the absence of DMSO. Our K_2 value (Scheme 3) is very much lower than that calculated by Champeil et al. [7], who also used DMSO. We do not know the reasons for this difference. It could be accounted for by the use of 40% against 15% DMSO, or by the use of different experimental methodologies.

Even though we did not analyze the amount of P_i released by acids as a function of P_i in detail, from the two experiments shown in Fig. 2, and from the generally accepted maximal amount of phosphorylation sites on the enzyme [5,6,9], it can be estimated that $K_{0.5}$ for $Mg \cdot P_i$ (step 1, Scheme 3) lies in the low micromolar range. This greatly differs from values calculated from experiments in the absence of DMSO [6,9,11,19], and agrees with data reported for the apparent $K_{0.5,P_i}$ in phosphorylation reactions in the presence of DMSO [7,15]. Taking together our estimations for the equilibrium constants of the reactions in Scheme 3, and those calculated by other authors in the absence of DMSO [6,9,11,19,21], it must be concluded that the solvent affects primarily the first step of the reaction.

Acknowledgements

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