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K^+- and Mg^{2+}-dependent hydrolysis of acetyl phosphate catalyzed by the (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum

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The (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum catalyzes the hydrolysis of acetyl phosphate in the presence of Mg^{2+} and EGTA and is stimulated by Ca^{2+}. The Mg^{2+}-dependent hydrolysis of acetyl phosphate measured in the presence of 6 mM acetyl phosphate, 5 mM $MgCl_2$, and 2 mM EGTA is increased 2-fold by 20% dimethyl sulfoxide. This activity is further stimulated 1.6-fold by the addition of 30 mM KCl. In this condition addition of Ca^{2+} causes no further increase in the rate of hydrolysis and Ca^{2+} uptake is reduced to a low level. In leaky vesicles, hydrolysis continues to be back-inhibited by Ca^{2+} in the millimolar range. Unlike ATP, acetyl phosphate does not inhibit phosphorylation by P_i unless dimethyl sulfoxide is present. The presence of dimethyl sulfoxide also makes it possible to detect P_i inhibition of the Mg^{2+}-dependent acetyl phosphate hydrolysis. These results suggest that dimethyl sulfoxide stabilizes a P_i-reactive form of the enzyme in a conformation that exhibits comparable affinities for acetyl phosphate and P_i. In this conformation the enzyme is transformed from a Ca^{2+}- and Mg^{2+}-dependent ATPase into a (K^+ + Mg^{2+})-ATPase.

Introduction

The (Ca^{2+} + Mg^{2+})-ATPase of sarcoplasmic reticulum vesicles catalyzes ATP hydrolysis in a process that is coupled to Ca^{2+} transport across the membrane [1,2]. In the absence of Ca^{2+} it hydrolyzes, at a slow rate, several substrates such as nucleoside triphosphates, acetyl phosphate furylacryloylphosphate and *p*-nitrophenyl phosphate [3–10]. It has been proposed that this Mg^{2+}-dependent activity pertains to the (Ca^{2+} + Mg^{2+})-ATPase [11].

During the catalytic cyle the (Ca^{2+} + Mg^{2+})-ATPase can be phosphorylated by either nucleoside triphosphates or by P_i [11]. Both the kinetic of phosphorylation and the properties of the enzyme are modified when the water activity of the assay medium is modified by the addition of organic solvents [11–17].

In this study we show that dimethyl sulfoxide stimulates the Mg^{2+}-dependent hydrolysis of acetyl phosphate and abolishes Ca^{2+} transport. Furthermore, in the presence of organic solvent, the Mg^{2+}-dependent ATPase activity is stimulated by KCl and not by Ca^{2+}. These results suggest that the (Ca^{2+} + Mg^{2+})-ATPase is transformed into a (K^+ + Mg^{2+})-ATPase that does not transport Ca^{2+}.

Materials and Methods

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as described by Eletr and Inesi [18]. The vesicles were rendered leaky to Ca^{2+} according to the method described by Meissner et al. [19]. The purified (Ca^{2+} + Mg^{2+})-ATPase was prepared as described by MacLennan et al. [20,21].

The hydrolysis of acetyl phosphate in all experiments was carried out at 35 °C. The reaction was arrested by the addition of ice-cold HCl and P_i to a final concentration of 0.16 M and 1.2 mM, respectively. The concentration of acetyl phosphate was determined as described by Lipmann and Tuttle [22]. This method was not influenced by the presence of dimethyl sulfoxide in the medium.

Abbreviations: Mops, 4-morpholinepropanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; DMSO, dimethyl sulfoxide.

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Phosphorylation of the $(Ca^{2+} + Mg^{2+})$-ATPase by [^{32}P]phosphate was determined using Millipore filters [23]. The filters were washed 15 times with 5-ml samples of an ice-cold solution containing 0.1 M HCl and 2 mM P_i followed by 15 washes with 5 ml samples of deionized water. ^{32}P-labeled phospho protein was determined by liquid scintillation counting.

Protein concentrations were determined according to Lowry et al. [24] using bovine serum albumin as standard. Calcium uptake was measured with $^{45}CaCl_2$ as described previously [23].

[^{32}P]Phosphate was obtained from the Brazilian Institute of Atomic Energy and purified as previously described [23]. Acetyl phosphate was purchased from Sigma Chemical Co. and stored at −5 °C. Solution were freshly prepared shortly before use.

Results

Activation of Mg^{2+}-dependent acetyl phosphate hydrolysis by dimethyl sulfoxide and K^+

The Mg^{2+}-dependent hydrolysis of acetyl phosphate corresponds to 24% of the activity measured in the presence of Ca^{2+} and Mg^{2+} [10]. We now show that increasing concentrations of dimethyl sulfoxide stimulate the hydrolysis of acetyl phosphate measured in the presence of 5 mM $MgCl_2$, 2 mM EGTA and no added KCl (Fig. 1A). Maximal stimulation is obtained with 20% dimethyl sulfoxide. In the presence of dimethyl sulfoxide the Mg^{2+} dependent hydrolysis is further increased by the addition of KCl (Figs. 1A and 2). Acetyl phosphate hydrolysis measured in the presence of 20% dimethyl sulfoxide and 30 mM KCl is maximally stimulated and reaches a value that is 2.6 higher

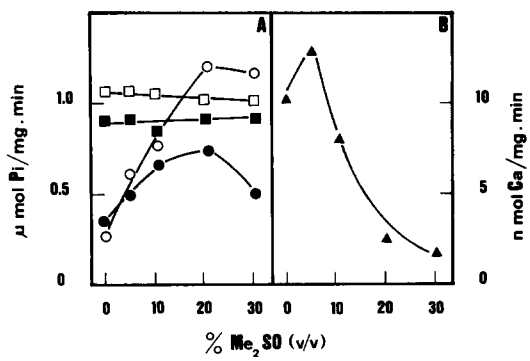


Fig. 1. Effect of dimethyl sulfoxide on the hydrolysisi of acetyl phosphate (A) and on Ca^{2+} uptake (B). In A, the assay medium for hydrolysis contained 50 mM Mops-Tris buffer (pH 7.0), 5 mM $MgCl_2$, 6 mM acetyl phosphate and either 2 mM EGTA (●, ○), or 0.1 mM $CaCl_2$ (□, ■). No added KCl (■, ●) or 120 mM KCl (□, ○) and the indicated concentrations of dimethyl sulfoxide. In B, calcium uptake was assayed in a medium containing 50 mM Mops-Tris buffer (pH 7.0), 5 mM $MgCl_2$, 6 mM acetyl phosphate, 0.1 mM $^{45}CaCl_2$. The reaction was started by the addition of sarcoplasmic reticulum vesicles to a final concentration of 0.15 mg protein/ml and arrested after 10 min at 35 °C.

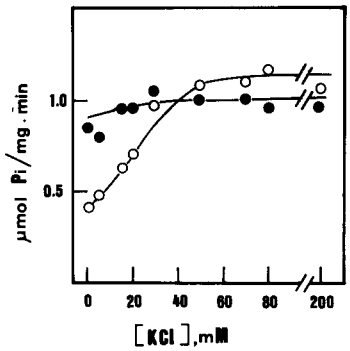


Fig. 2. K^+ dependence of acetyl phosphate hydrolysis in the presence of dimethyl sulfoxide. The reaction medium consisted of 50 mM Tris buffer (pH 7.0), 5 mM $MgCl_2$, 6 mM acetyl phosphate, 20% (v/v)dimethyl sulfoxide, either 2 mM EGTA (○) or 0.1 mM $CaCl_2$ (●) and the KCl concentrations shown on the abscissa. The reaction was started by the addition of sarcoplasmic reticulum vesicles to a final concentration of 0.15 mg protein/ml and arrested after 10 min at 35 °C.

than the activity measured without these additions (Fig. 2).

The hydrolysis of acetyl phosphate measured in presence of both Mg^{2+} and Ca^{2+} is not sensitive to dimethyl sulfoxide (Fig. 1A), however, the Ca^{2+} uptake is inhibited by dimethyl sulfoxide (Fig. 1B). In contrast to the Mg^{2+}-dependent activity the hydrolysis of acetyl phosphate measured in the presence of Ca^{2+} and Mg^{2+} (Figs. 1A and 2) and the Ca^{2+} uptake (data not shown) are only slightly modified by KCl regardless of whether or not dimethyl sulfoxide is added to the medium.

In order to test whether the Mg^{2+}-dependent activity pertains to the $(Ca^{2+} + Mg^{2+})$-ATPase (Table I), we measured the effect of dimethyl sulfoxide on the hydrolysis of acetyl phosphate in sarcoplasmic reticulum vesicles [18] and ATPase preparations purified as described by Meissner [19] and MacLennan [20,21]. After purification the Mg^{2+}-dependent activity falls from 0.51

TABLE I

The influence of dimethyl sulfoxide on the acetyl phosphase activity of different $(Ca^{2+} + Mg^{2+})$-ATPase preparations

The assay media contained 50 mM Mops-Tris buffer (pH 7.0), 5 mM $MgCl_2$, 120 mM KCl, 6 mM acetyl phosphate and either 2 mM EGTA (Mg^{2+}-dependent activity) or 1 mM EGTA and 1.03 mM $CaCl_2$ (Ca^{2+}- and Mg^{2+}-dependent activity). The reaction was started by the addition of the different $(Ca^{2+} + Mg^{2+})$-ATPase preparations (0.15 mg protein/ml) and arrested after 10 min. The values shown in the table are the averages + standard error of four experiments.

Enzyme	Rate (μmol/mg protein per min)			
	Mg^{2+} activity		$Ca^{2+} + Mg^{2+}$ activity	
	without DMSO	plus 20% DMSO	without DMSO	plus 20% DMSO
Vesicles	0.51 + 0.21	1.22 + 0.16	1.48 + 0.11	1.40 + 0.10
Meissner	0.38 + 0.02	1.10 + 0.17	1.53 + 0.22	1.30 + 0.25
Maclennan	0.06 + 0.12	1.19 + 0.30	1.25 + 0.30	1.20 + 0.30

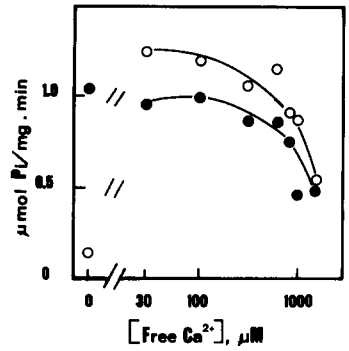


Fig. 3. Ca^{2+} dependence of acetyl phosphate hydrolysis. The assay medium consisted of 50 mM Mops-Tris buffer (pH 7.0), 5 mM $MgCl_2$, 120 mM KCl, 6 mM acetyl phosphate, 1 mM EGTA and either no addition (○), or 20% (v/v) dimethyl sulfoxide (●), and the concentrations of free Ca^{2+} shown on the abscissa. The reaction was started by the addition of purified ($Ca^{2+} + Mg^{2+}$)-ATPase to a final concentration of 0.15 mg protein/ml and arrested after 10 min at 35 °C.

$\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (in sarcoplasmic reticulum vesicles) to 0.06 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (in the MacLennan preparation). However, the rate of hydrolysis is stimulated by dimethyl sulfoxide in all three preparations (Table I). In each case, a comparison of the Mg^{2+}-dependent activity in the presence of dimethyl sulfoxide with the Ca^{2+}- and Mg^{2+}-dependent activity in the same preparation (Table I) reveals similar rates under the two conditions.

Ca^{2+} dependence of acetyl phosphate hydrolysis

The ($Ca^{2+} + Mg^{2+}$)-ATPase has two different affinities for Ca^{2+}. At concentrations from 0.1 to 2.0 µM, Ca^{2+} stimulates hydrolysis, whereas at concentrations higher than 500 µM the hydrolysis is inhibited by this ion [9–11]. We show that in presence of dimethyl sulfoxide and of 120 mM KCl, micromolar Ca^{2+} concentrations do not further activate acetyl phosphate hydrolysis (Fig. 3). However, the low-affinity binding site for Ca^{2+} is still operating, since hydrolysis is inhibited by this ion at concentrations higher than 500 µM (Fig. 3).

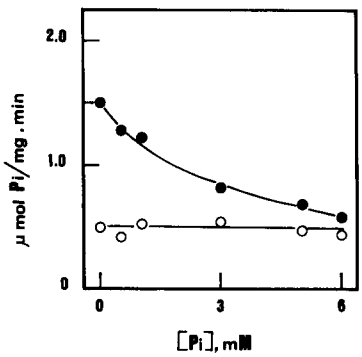


Fig. 4. Effect of P_i and dimethyl sulfoxide on the Mg^{2+}-dependent hydrolysis of acetyl phosphate. The assay medium composition was 50 mM Mops-Tris buffer (pH 7.0), 5 mM $MgCl_2$, 120 mM KCl, 6 mM acetyl phosphate, 2 mM EGTA either zero (○) or 20% (v/v) dimethyl sulfoxide (●) and the concentrations of P_i shown on the abscissa. The reaction was started by the addition of leaky vesicles (0.15 mg protein/ml) and arrested after 10 min.

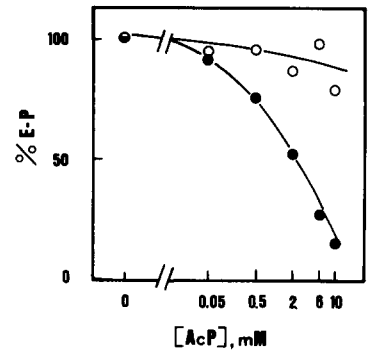


Fig. 5. Effect of acetyl phosphate on the equilibrium level of phosphoenzyme formed from P_i in the absence and in the presence of dimethyl sulfoxide. Phosphoenzyme from P_i was measured at 35 °C in an assay media containing 50 mM Mops-Tris buffer (pH 7.0), 5 mM $MgCl_2$, 2 mM either 2 mM [^{32}P]P_i (○) or 0.03 mM [^{32}P]P_i and 20% dimethyl sulfoxide (●), and the acetyl phosphate concentrations shown on the abscissa. The maximum phosphoenzyme level corresponds to 0.5 µmol EP/g protein in the absence of dimethyl sulfoxide and 0.3 µmol EP/g protein in the presence ofdimethyl sulfoxide. The reaction was started by the addition of leaky vesicles (0.3 mg protein/ml) and arrested after 15 s.

Effect of P_i

The Mg^{2+}-dependent hydrolysis of acetyl phosphate is not inhibited by 6 mM P_i in totally aqueous medium but it is inhibited by this P_i concentration when 20% dimethyl sulfoxide is included in the assay medium (Fig. 4).

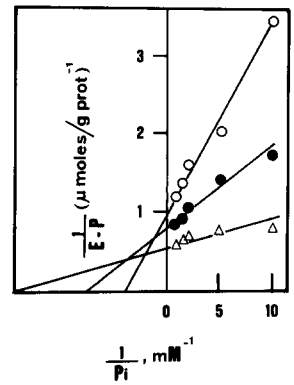


Fig. 6. Double-reciprocal plots of phosphoenzyme formation at various P_i and acetyl phosphate concentrations in the presence of dimethyl sulfoxide. Phosphoenzyme from P_i was measured at 35 °C in assay media containing 50 mM Mops-Tris buffer (pH 7.0), 5 mM $MgCl_2$, 2 mM EGTA, 20% dimethyl sulfoxide, and either no addition (△), 0.25 mM (●) or 2 mM (○) acetyl phosphate at the [^{32}P]P_i concentrations shown on the abscissa. The reaction was started by the addition of leaky vesicles (0.3 mg protein/ml) and arrested after 15 s.

Effect of acetyl phosphate on phosphoenzyme formation from P_i

Masuda and De Meis [25] demonstrated that in aqueous media substrates such as ATP and ADP, but not acetyl phosphate, competitively inhibit phosphoenzyme formation by P_i. When 20% dimethyl sulfoxide is included in the assay medium during steady-state acetyl phosphate hydrolysis, phosphoenzyme formation by P_i is inhibited by concentrations of acetyl phosphate higher than 0.05 mM (Fig. 5). In these experiments, phosphorylation by P_i was carried out in the absence of KCl because K^+ accelerates the dephosphorylation of the phosphoenzyme formed by P_i [26]. Since dimethyl sulfoxide increases the affinity for P_i, the concentrations of P_i used in purely aqueous medium were higher than in dimethyl sulfoxide. The aim was to use P_i concentration well below saturation to detect a possible competition between P_i and acetyl phosphate. Double-reciprocal plots of phosphoenzyme formation by P_i using different acetyl phosphate concentrations show that inhibition by acetyl phosphate is partially competitive (Fig. 6).

Discussion

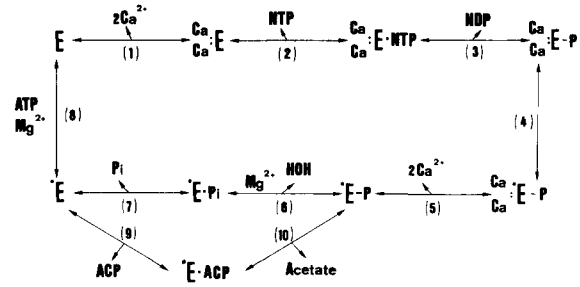
Mg^{2+}-dependent hydrolysis

The Mg^{2+}-dependent activity catalyzed by sarcoplasmic reticulum vesicles has been attributed by some authors to the $(Ca^{2+} + Mg^{2+})$-ATPase and by others to an enzyme that contaminates preparations of sarcoplasmic reticulum vesicles [9]. The latter explanation is consistent with loss of this activity when different methods are used to purify the $(Ca^{2+} + Mg^{2+})$-ATPase (Table I). The data presented show that in the presence of dimethyl sulfoxide the Mg^{2+}-dependent hydrolysis of acetyl phosphate can be detected even in a purified preparation (Table I), and that this activity is not enhanced by the addition of Ca^{2+}. These results suggest that this activity pertains to the $(Ca^{2+} + Mg^{2+})$-ATPase.

Reactions sequence

In the sequence proposed for the catalytic cycle (Reactions 1–8, Scheme I) [11], the ATPase is interconverted between the two forms E and *E, which bind Ca^{2+} with high and low affinity, respectively. The data reported here suggest that dimethyl sulfoxide stabilizes the *E conformation, since the $(Ca^{2+} + Mg^{2+})$-ATPase does not transport Ca^{2+} (Fig. 1B) and acetyl phosphate hydrolysis is no longer stimulated by low Ca^{2+} concentrations but it is still back-inhibited by high Ca^{2+} concentrations (Fig. 3).

In the absence of Ca^{2+}, dimethyl sulfoxide may facilitate partitioning of acetyl phosphate into the catalytic site in its hydrophobic form (Reaction 9, Scheme I), as has been proposed previously for P_i [13]. This



Scheme I. The catalytic cycle of the $(Ca^{2+} + Mg^{2+})$-ATPase. The sequence includes a transition between two distinct functional states of the enzyme. E and *E. The E form has a high affinity for Ca^{2+} ($K_s = 10^{-6}$ M) and the *E form has a low affinity for Ca^{2+} ($K_s = 10^{-3}$ M) [11]. In this cycle we include steps 9 and 10 to show how the enzyme might catalyze acetyl phosphate hydrolysis in the presence of dimethyl sulfoxide and no Ca^{2+}.

organic solvent stimulates 8.1-fold the Mg^{2+}-dependent activity of a purified $(Ca^{2+} + Mg^{2+})$-ATPase (Table I).

Partially competitive inhibition by acetyl phosphate of phosphoenzyme formation by P_i in the presence of organic solvent (Fig. 5 and 6) suggests that acetyl phosphate hydrolysis is being catalyzed by the *E form of the enzyme (reactions 6, 7, 9 and 10, Scheme I). Previous reports have demonstrated the existence of *E-P intermediates during Mg^{2+}-dependent hydrolysis of ATP [16,17] and furylacryloyl phosphate [8] in the presence of dimethyl sulfoxide.

Rate-limiting step

The effect of K^+ on the $(Ca^{2+} + Mg^{2+})$-ATPase is complex [5,6,26–28]. This ion promotes dephosphorylation of the *E-P form [26]. In the presence of dimethyl sulfoxide, the Mg^{2+}-dependent hydrolysis of acetyl phosphate is strongly activated by K^+ (Figs. 1 and 2). In contrast, in the presence of Ca^{2+} and Mg^{2+}, hydrolysis is only slightly activated by KCl, independently of the presence of dimethyl sulfoxide (Figs. 1A and 2). Activation of the Mg^{2+}-dependent hydrolysis of acetyl phosphate by K^+ is probably due to acceleration on the dephosphorylation of an *E-P intermediate formed during hydrolysis which would be stabilized by dimethyl sulfoxide and thus rate-limiting. In the presence of Ca^{2+} the effect of KCl is not evident (Figs. 1A and 2). This is probably related to the binding of Ca^{2+} to the enzyme form E and involvement of all the intermediary steps of the cycle in the hydrolysis of acetyl phosphate.

Acknowledgements

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