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Effects of a Ca^{2+} gradient and water activity on the phosphorylation of Ca^{2+}-ATPase by P_i

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The effects of dimethyl sulfoxide (20% v/v) on the phosphorylation of Ca^{2+}-ATPase of the sarcoplasmic reticulum by P_i vary depending on whether or not a Ca^{2+} gradient is formed across the vesicle membranes. In the absence of a Ca^{2+} gradient the solvent promotes a large increase in the affinity for P_i. This increase is no longer observed after the formation of a Ca^{2+} gradient. The enzyme affinity for Mg^{2+} is practically the same in the presence and absence of a gradient. Addition of dimethyl sulfoxide leads to an increase of the enzyme affinity for Mg^{2+} both in the presence and in the absence of a gradient.

Phosphorylation by P_i; Magnesium ion; Dimethyl sulfoxide; Sarcoplasmic reticulum; Ca^{2+} gradient

1. INTRODUCTION

The Ca^{2+}/Mg^{2+}-ATPase of the sarcoplasmic reticulum can operate both in the forward direction, hydrolysing ATP and pumping Ca^{2+} to the vesicles, and in the reverse direction, synthesizing ATP from ADP and P_i in a process that is coupled with the release of Ca^{2+} from the vesicles [1–5].

The synthesis of ATP is initiated by phosphorylation of the enzyme form *E by P_i (reactions 6 and 7 in Fig. 1). This reaction requires Mg^{2+}, it occurs both in the presence and absence of a transmembrane Ca^{2+} gradient, and in both conditions the binding of P_i and Mg^{2+} to the enzyme is random with heterotropic cooperativity [1–7]. A decrease of the apparent K_m for P_i is observed either when a Ca^{2+} gradient is formed across the vesicle membranes [8,9] or when empty vesicles are incubated in the presence of an organic solvent, such as dimethyl sulfoxide, which promotes a decrease in water activity of the medium [10–12].

In this report it is shown that effects of dimethyl sulfoxide on the apparent affinities of the ATPase for P_i and for Mg^{2+} vary greatly depending on whether or not a Ca^{2+} gradient is formed across the vesicle membranes.

2. MATERIALS AND METHODS

Sarcoplasmic reticulum vesicles were obtained from rabbit skeletal muscle as described by Eletr and Inesi [13]. The vesicles were actively loaded with calcium in a medium containing 50 mM MOPS-Tris (pH 7.0), 10 mM $MgCl_2$, 10 mM P_i, 0.3 mM $CaCl_2$, 2 mM ATP and 50–100 μg vesicle protein/ml. After 30 min incubation at 35°C, the loaded vesicles were sedimented by centrifugation at 40 000 × *g* for 30 min. The vesicles were kept on ice and, prior to the experiment, were resuspended in a small volume of ice-cold water.

Phosphorylation of the Ca^{2+}-ATPase by $^{32}P_i$ was assayed and corrected for non-specific binding as described previously [14].

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3. RESULTS

The apparent K_m values for P_i and Mg^{2+} were determined by measuring the equilibrium levels of phosphoenzyme. In the absence of a Ca^{2+} gradient the concentration of P_i required for half-maximal phosphorylation of the enzyme increases from 1.5 mM to a value higher than 10 mM when the pH of the medium is raised from 6.0 to 7.7 (Table I). The addition of 20% (v/v) dimethyl sulfoxide to the medium causes both a large decrease in the K_m for P_i and a loss of the pH dependence. These data confirm previous results [8–10], but in addition, we now show that the effect of dimethyl sulfoxide on the K_m for P_i is greatly decreased when a Ca^{2+} gradient is formed across the vesicle membranes. At pH 7.0 the K_m for P_i measured in the absence of a gradient decreased more than 35-fold when dimethyl sulfoxide was added to the medium (Fig. 2). In contrast, in the presence of Ca^{2+} gradient, the solvent has practically no effect on the K_m for P_i (Table I).

After the addition of organic solvent, values of the K_m for P_i measured in the absence of a Ca^{2+} gradient were about one order of magnitude smaller than those measured in the presence of a gradient (Table I). Thus the effect of the gradient on the enzyme affinity for P_i is to increase it in totally aqueous medium and decrease

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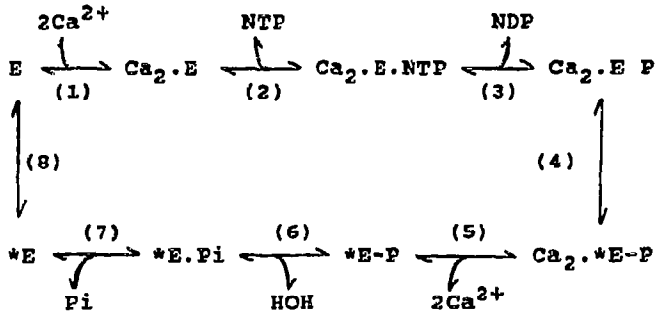


Fig. 1. Reaction sequence. The sequence includes two distinct functional states of the enzyme, E and *E. The Ca^{2+}-binding sites in the E form face the external surface of the vesicle and have a high affinity for Ca^{2+} ($K_s = 10^{-6}$ M at pH 7.0). In the *E form the Ca^{2+}-binding sites face the vesicle lumen and have a low affinity for Ca^{2+} ($K_s = 10^{-3}$ M). The enzyme form E is phosphorylated by ATP but not by P_i; conversely, the enzyme form *E is phosphorylated by P_i but not by ATP.

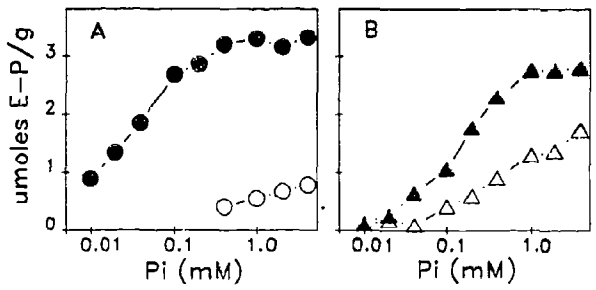


Fig. 2. P_i dependence: Effects of a Ca^{2+} gradient and dimethyl sulfoxide. The assay medium composition was 50 mM MOPS-Tris buffer (pH 7.0), 5 mM EGTA, 5 mM $MgCl_2$, 0.1 mg/ml vesicle protein and $^{32}P_i$ as indicated. The reaction was started by the addition of vesicles and arrested after 30 s at 35°C by the addition of 20% (w/v) TCA solution containing 2 mM P_i. (A) empty vesicles (no gradient) and (B) Ca^{2+}-loaded vesicles (gradient). (○, △) Totally aqueous medium; (●, ▲) 20% (v/v) dimethyl sulfoxide.

it in the presence of organic solvent (Fig. 2). This was observed at all pH values tested.

In aqueous medium, the formation of a Ca^{2+} gradient across the vesicle membrane has little effect on the Mg^{2+} dependence of the phosphorylation reaction (Table II). In contrast to the effect on the K_m for P_i, dimethyl sulfoxide promotes a decrease in the Mg^{2+} concentration needed for half-maximal phosphorylation, both in the absence and in the presence of a Ca^{2+} gradient (Fig. 3). In fact, at pH 6.0 the effect of the organic solvent on the Mg^{2+} affinity was more pronounced in the presence of the gradient than in empty vesicles. At pH 7.0, the effect of solvent was practically the same in empty vesicles and in Ca^{2+}-loaded vesicles (Table II). These data show that after formation of the gradient, the solvent selectively increases the affinity

Table I

P_i dependence at different pH values

pH	K_m for P_i (mM)			
	Empty vesicles		Ca^{2+}-loaded vesicles	
	H_2O	20% dimethyl sulfoxide	H_2O	20% dimethyl sulfoxide
6.0	1.50 + 0.11 (7)	0.04	0.86 + 0.07 (7)	0.49 + 0.11 (3)
7.0	3.20 + 0.25 (4)	0.09	0.42 + 0.17 (4)	0.62 + 0.13 (3)
7.7	> 10.00	0.03	1.23 + 0.19 (3)	0.29 + 0.14 (3)

The assay media and experimental conditions were as in Fig. 2. The values in the table are either the average of two experiments or the average ± SE of the number of experiments shown in parentheses.

Table II

Mg^{2+} dependence at different pH values

pH	K_m for Mg^{2+} (mM)			
	Empty vesicles		Ca^{2+}-loaded vesicles	
	H_2O	20% dimethyl sulfoxide	H_2O	20% dimethyl sulfoxide
6.0	0.77	0.36 + 0.20 (3)	1.16	0.05
7.0	1.30	0.10 + 0.06 (7)	0.57 + 0.05 (7)	0.03 + 0.03 (3)
7.7	n.d.	0.10 + 0.04 (7)	0.34 + 0.14 (3)	0.04 + 0.05 (3)

The assay media and experimental conditions were as in Fig. 3. The values in the table are either the average of two experiments or the average ± SE of the number of experiments shown in parentheses. At pH 7.7 in totally aqueous medium, the Mg^{2+} dependence could not be determined (n.d.) due to the very low enzyme affinity for P_i (see Table I).

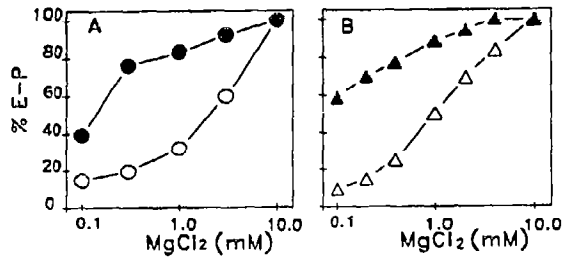
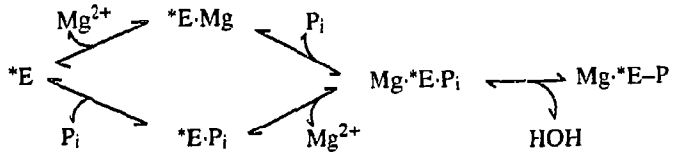


Fig. 3. Mg^{2+} dependence: Effects of a Ca^{2+} gradient and dimethyl sulfoxide. The assay medium composition was 50 mM MOPS-Tris buffer (pH 7.0), 5 mM EGTA, 1.0 mM (A) or 0.2 mM (B) $^{32}P_i$, 0.1 mg/ml vesicle protein and $MgCl_2$ as indicated. The reaction was started by the addition of vesicles and arrested after 30 s at 35°C by the addition of 20% (w/v) TCA solution containing 2 mM P_i. (A) empty vesicles (no gradient) and (B) Ca^{2+}-loaded vesicles (gradient). (○, △) Totally aqueous medium; (●, ▲) 20% (v/v) dimethyl sulfoxide. In (A) 100% phosphoenzyme (E-P) was (○) 1.5 and (●) 2.6 μmol/g. In (B) 100% was (△) 1.3 and (▲) 2.7 μmol/g.

for Mg^{2+} while having little or no effect on the enzyme affinity for P_i.

4. DISCUSSION

Free P_i and not the complex $Mg \cdot P_i$ is the true substrate for the phosphorylation reaction [6,7]. Mg^{2+} and P_i bind in a random sequence to the enzyme and the binding of one ionic species facilitates the binding of the other:



The data presented in this report suggest that the gradient and organic solvent would selectively increase the binding of either P_i or Mg^{2+} to the enzyme. Thus, the sequence of binding would be ordered rather than random. With the gradient, the organic solvent increases the affinity of *E for Mg^{2+} more than it does for P_i. As a result, Mg^{2+} would bind first to the enzyme and the organic solvent would have little effect on the apparent affinity for P_i because this was already enhanced by the binding of Mg^{2+}.

Conversely, in the absence of a gradient, the organic solvent increases the enzyme affinity for P_i more than that for Mg^{2+}. The enzyme would first bind P_i and the effect of the solvent on the apparent affinity for Mg^{2+} would become less pronounced.

At present we do not know why dimethyl sulfoxide decreases the K_m for P_i and Mg^{2+} to different extents depending on whether or not a gradient is formed across the membrane. Perhaps this is related to the equilibrium between the enzyme forms *E and *E:2Ca which is established when a gradient is formed across the vesicle membranes [15,16].

The effects of pH and organic solvents on the phosphorylation reaction measured in the absence of a gradient have been discussed in previous reports [10,12].

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