MECHANISM OF CALCIUM-INDEPENDENT PHOSPHORYLATION OF SARCOPLASMIC RETICULUM ATPase BY ORTHOPHOSPHATE

Evidence of magnesium–phosphoprotein formation

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1. Introduction

Magnesium affects several reaction steps of the calcium transport cycle in sarcoplasmic reticulum membranes [1,2]. Recent studies on the effect of magnesium on sarcoplasmic reticulum function [3] indicate a dual role of magnesium on the phosphorylation of sarcoplasmic reticulum transport ATPase from ATP, as evaluated from analysis of the exchange rate of γ-phosphate between ATP and ADP [4] in the presence of saturating calcium concentrations:

(i) That magnesium activates the enzyme directly;
(ii) That it represents part of the substrate MgATP for the phosphorylation reaction [3].

From studies on the role of magnesium in calcium-independent and calcium-dependent phosphorylation of sarcoplasmic reticulum transport ATPase from orthophosphate [5–19], the formation of a magnesium–phosphoprotein in calcium-independent phosphorylation was suggested [18]. Based on the good fit of a reaction scheme to the data the following features were proposed:

(i) The phosphoprotein ($Mg \cdot E{-}P_i$), in which the phosphate is covalently bound to the enzyme, is in equilibrium with the Michaelis complex ($Mg \cdot E \cdot P_i$), the concentration of which is determined by the concentration of free orthophosphate and free magnesium;
(ii) The binding of orthophosphate and magnesium appears to be interdependent, both ligands apparently bind randomly [18].

However a good fit of experimental data to an assumed reaction sequence does not provide conclusive evidence for a reaction mechanism unless other mechanisms, which might produce the same experimental results, are not conclusively ruled out. For example, no attempts have been made in investigations on the phosphorylation of sarcoplasmic reticulum ATPase from orthophosphate [5–19] to distinguish whether free orthophosphate or magnesium–phosphate is the true substrate for phosphorylation.

Here, all possible reaction mechanisms for calcium-independent phosphoprotein formation were considered and distinguished theoretically. Elimination of certain mechanisms was achieved by comparing the data of phosphoprotein formation assayed at different concentrations of free orthosphosphate and free magnesium with the theoretical pattern obtained from model equations for various assumed reaction sequences.

It is demonstrated that there is only one possible reaction mechanism in calcium-independent phosphorylation of sarcoplasmic reticulum ATPase:

The enzyme binds free magnesium and free orthophosphate at random – as was assumed in [18].

2. Materials and methods

Preparation of sarcoplasmic reticulum from rabbit skeletal muscle was performed according to [20] and

in [18]. Sarcoplasmic reticulum protein was measured by the Biuret method and the Folin method standardized against crystalline bovine serum albumin [21]. Phosphorylation of sarcoplasmic reticulum from ortho[^{32}P]phosphate was performed as in [18,19]; the media are given in the appropriate legends. Calculation of the concentrations of total ortho[^{32}P]-phosphate, total magnesium and total calcium in order to obtain the desired free concentrations of ortho-[^{32}P]phosphate, magnesium and calcium for the phosphoprotein assays was performed as detailed in [18].

Reagents: Ortho[^{32}P]phosphate was purchased from Radiochemical Centre (Amersham); EGTA (ethyleneglycol-bis-(2-aminoethyl ether) *N,N,N',N'*-tetraacetic acid) from Fluka AG. (Buchs); all other reagents (analytical grade) were bought from E. Merck (Darmstadt). The ionophore, a 5-bromoderivative of Ro X-537A (Ro 20-006, see [22]), was a generous gift from Hoffmann La Roche, Vienna.

3. Results and discussion

Figure 1 shows an experiment on the phosphorylation of sarcoplasmic reticulum ATPase performed with ionophore X-537A plus EGTA-treated sarcoplasmic reticulum vesicles with increasing concentrations of free orthophosphate in the presence of different concentrations of free magnesium. The reciprocal of the phosphoprotein formed (E–P) is plotted versus the reciprocal concentration of free orthophosphate in the assay medium.

The characteristic findings in fig.1 are:

(i) The slopes of the regression lines increase inversely with the concentration of free magnesium;

(ii) The lines have a common intersection on the left of the ordinate (mode e in fig.3). These results exclude the possibility that free magnesium does not bind directly to the enzyme (see Appendix).

Figure 2 shows a plot of the reciprocal of phosphoprotein formed (the experimental data are the same as in fig.1) versus the reciprocal concentration of magnesium–phosphate in the assay medium.

The characteristics of fig.2 are:

(i) The slopes of the lines increase with increasing concentrations of free magnesium in the medium;

(ii) The intercepts of the lines on the ordinate decrease with increasing concentrations of free magnesium (mode f in fig.3).

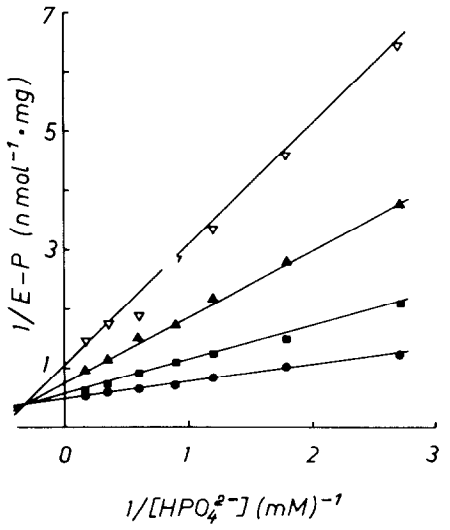


Fig.1. Dependence of calcium-independent phosphorylation of sarcoplasmic reticulum ATPase from ortho[^{32}P]phosphate on free HPO_4^{2-} and free magnesium concentrations. Sarcoplasmic reticulum vesicles (20 mg/ml) were preloaded with 20 mM $CaCl_2$ in the presence of 100 mM KCl, 5 mM histidine buffer (pH 7.0) on ice for ~15 h followed by ionophore X537A plus EGTA treatment for 10 min at 20°C prior to phosphorylation. Phosphorylation was performed at pH 7.0 and 20°C in a medium containing 40 mM histidine, 15 mM EGTA, 0.03 mM ionophore X537A, 10 nM free calcium, different concentrations of free magnesium, various amounts of tetraethylammonium chloride in order to keep the ionic strength constant (at 0.2) and 0.4 mg protein/ml. Phosphorylation was started by addition of ortho[^{32}P]phosphate, after the protein was kept in the above medium without P_i for 10 min, and stopped 10 s later by addition of a solution containing 0.5 M perchloric acid and 100 mM phosphoric acid. Since the ionophore X537A was dissolved in dimethylsulfoxid the medium for phosphorylation contained in addition 0.3% dimethylsulfoxid. Concentrations of free magnesium: (▽)1 mM; (▲) 2 mM; (■) 5 mM; (●) 20 mM. The regression lines in the figure are derived by non-linear least squares approximation according to eq. (4) in the Appendix, as detailed in [18].

These features of phosphoprotein formation exclude unequivocally that magnesium–phosphate ($MgHPO_4$) is the true substrate for the calcium-independent phosphorylation of the sarcoplasmic reticulum ATPase and, in addition, that the binding of free magnesium and free orthophosphate to the enzyme is in sequential order (see Appendix).

From the above precluding procedure, the mechanism for calcium-independent phosphorylation of

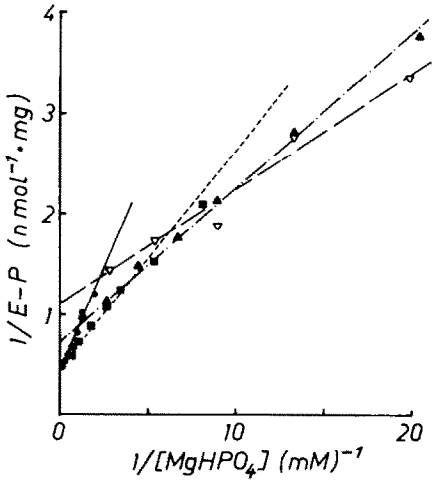


Fig.2. Double reciprocal plot of phosphoprotein versus $MgHPO_4$ at different concentrations of free magnesium. The phosphoprotein values and the symbols used are the same as in fig.1. The concentrations of $MgHPO_4$ have been calculated as detailed in [18]. The individual regression lines were calculated according to [23].

sarcoplasmic reticulum ATPase can only be formulated as follows:

1. Mg^{2+} bind directly to the enzyme;
2. Free orthophosphate is the true substrate for phosphoprotein formation.

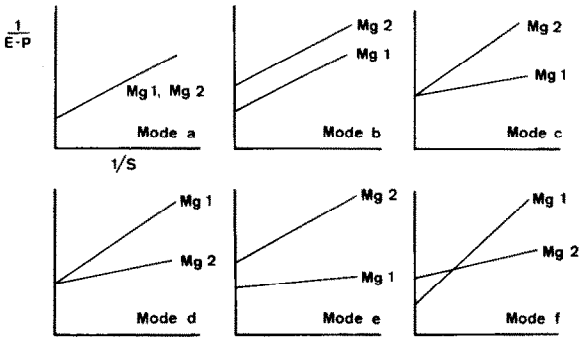
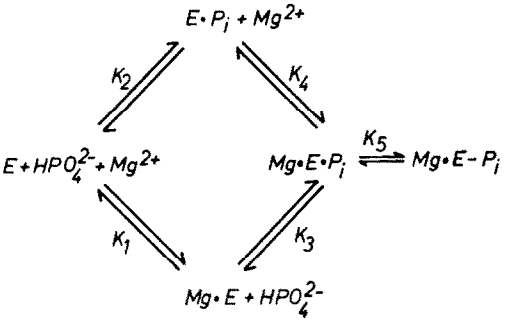


Fig.3. Theoretical pattern of calcium-independent phosphorylation of sarcoplasmic reticulum ATPase by orthophosphate. The characteristics of phosphoprotein formation in the presence of two different concentrations of free Mg^{2+} (Mg 1 > Mg 2) are presented schematically in double reciprocal plots. Ordinate: Reciprocal of the phosphoprotein formed (1/E–P). Abscissa: Reciprocal of phosphate concentration ($1/P_i$ = A-Plot; $1/MgP_i$ = B-plot; for explanation see text).

Scheme 1



Reaction sequence in calcium-independent phosphorylation of sarcoplasmic reticulum ATPase from orthophosphate. (see Appendix 2.3. and [18])

3. The ternary complex ($Mg \cdot E \cdot P_i$) is formed by random binding of free magnesium and free orthophosphate to the enzyme.
4. The binding of both ligands is interdependent, i.e., the binding of one reactant influences the binding of the other and vice versa. This is inferred from the common intersection of the lines in fig.1 above the substrate axis giving different values for the calculated dissociation constants of the binary complexes and the ternary complexes (see Appendix 2.3 and [18]).
5. The phosphoprotein formed in calcium-independent phosphorylation is a magnesium–phosphoprotein.

This only possible reaction scheme, which describes calcium-independent phosphoprotein formation, is shown in scheme 1, which is the one assumed in [18].

4. Conclusion

The system in Appendix 2.3, i.e., the reaction sequence with Mg^{2+} and P_i as the randomly binding ligands, remains the only possible mechanism, which does not contradict the experimental results given in fig.1 and fig.2. The binding of Mg^{2+} and of P_i to the enzyme are interdependent [18] (see also the text). All other possibilities are excluded.

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Appendix

In order to permit formulation of a reaction mechanism of calcium-independent phosphorylation of sarcoplasmic reticulum ATPase from inorganic phosphate, the following questions have to be answered:

(1) Is the activating effect of magnesium on phosphoprotein formation from orthophosphate due to a direct binding of free magnesium to the enzyme or due to the binding of magnesium–phosphate ($MgHPO_4$)?
(2) Is free orthophosphate (P_i) or the magnesium–phosphate complex the true substrate for phosphoprotein formation?
(3) If Mg^{2+} bind directly to the enzyme, in which order does the binding of Mg^{2+} and P_i (or $MgHPO_4$) to the ATPase occur?
(4) Does the binding of the ligands Mg^{2+} and P_i (or $MgHPO_4$) to the enzyme occur independently on one another?

Since there is evidence that phosphoprotein formation from P_i does not occur in the absence of free magnesium, a clear distinction has to be made between the 7 theoretically possible reaction sequences listed below. A model equation was derived for each reaction scheme from which predictions on the pattern of intercepts and/or slopes of the double reciprocal plots of phosphoprotein versus free orthophosphate (A-plot) or phosphoprotein versus magnesium–phosphate (B-plot) can be made (fig.3). From a comparison of the experimentally obtained data of phosphoprotein formation as given in fig.1 (A-plot; mode e in fig.3) and fig.2 (B-plot; mode f) with the predicted pattern of phosphoprotein formation obtained from the model equations for each assumed reaction scheme, certain reaction mechanisms can be eliminated.

Symbols: E_o, total enzyme; E, free enzyme; K, equilibrium constants for the corresponding reaction steps; Mg, Mg^{2+}; P_i, ionized orthophosphate; MgP_i, $MgHPO_4$; K_o, dissociation constant of the $MgHPO_4$ complex.

1. Mg^{2+} is not a direct activator, MgP_i is the substrate

$$E + MgP_i \underset{}{\overset{K_1}{\rightleftharpoons}} E \cdot P_i \cdot Mg \overset{K_5}{\rightleftharpoons} E{-}P$$

$$K_5 \frac{E_o}{E{-}P} = \frac{1}{MgP_i} K_1 + K_5 + 1$$

$$= \frac{1}{P_i} \frac{K_1 K_o}{Mg} + K_5 + 1 \qquad (1)$$

Pattern in fig.3: A-plot ($1/E{-}P$ versus $1/P_i$) shows

mode c; B plot ($1/E{-}P$ versus $1/MgP_i$) shows mode a. This mechanism can be eliminated.

2. Mg^{2+} is a direct activator and P_i is the substrate

2.1. *Sequential order reaction a*

$$E + Mg \underset{}{\overset{K_1}{\rightleftharpoons}} Mg \cdot E$$

$$Mg \cdot E + P_i \overset{K_2}{\rightleftharpoons} Mg \cdot E \cdot P_i \overset{K_5}{\rightleftharpoons} E{-}P$$

$$K_5 \frac{E_o}{E{-}P} = \frac{1}{P_i} K_2 \left(\frac{K_1}{Mg} + 1\right) + K_5 + 1$$

$$= \frac{1}{MgP_i} \frac{K_2}{K_o} (K_1 + Mg) + K_5 + 1 \qquad (2)$$

Pattern in fig.3: A-plot shows mode c; B-plot shows mode d. Eliminated.

2.2. *Sequential order reaction b*

$$E + P_i \overset{K_1}{\rightleftharpoons} E \cdot P_i + Mg \overset{K_2}{\rightleftharpoons} Mg \cdot E \cdot P_i \overset{K_5}{\rightleftharpoons} E{-}P$$

$$K_5 \frac{E_o}{E{-}P} = \frac{1}{P_i} \frac{K_1 K_2}{Mg} + \frac{K_2}{Mg} + K_5 + 1$$

$$= \frac{1}{MgP_i} \frac{K_1 K_2}{K_o} + \frac{K_2}{Mg} + K_5 + 1 \qquad (3)$$

Pattern in fig.3: A-plot shows mode e (i.e., identical to fig.1); B-plot shows mode b. Eliminated.

2.3. *Random order reaction*

The reaction scheme is shown in scheme I. $E{-}P = Mg \cdot E{-}P$

$$K_5 \frac{E_o}{E{-}P} = \frac{1}{P_i} \left(\frac{K_2 K_4}{Mg} + K_3\right) + \frac{K_4}{Mg} + K_5 + 1$$

$$= \frac{1}{MgP_i} \frac{1}{K_o} (K_2 K_4 + MgK_3) + \frac{K_4}{Mg} + K_5 + 1 \qquad (4a)$$

or

$$K_5 \frac{E_o}{E{-}P} = \frac{1}{P_i} K_3 \left(\frac{K_1}{Mg} + 1\right) + \frac{K_4}{Mg} + K_5 + 1$$

$$= \frac{1}{MgP_i} \frac{K_3}{K_o} (Mg + K_1) + \frac{K_4}{Mg} + K_5 + 1 \qquad (4b)$$

Equations (4a) and (4b) are equivalent since: $K_1 \cdot K_3 = K_2 \cdot K_4$

Pattern in fig.3: A-plot shows mode e (identical to fig.1); B-plot shows mode f (identical to fig.2). This reaction mechanism cannot be eliminated.

3. Mg^{2+} is the activator and MgP_i is the substrate

3.1. *Sequential order reaction a*

$$E + Mg \overset{K_1}{\rightleftharpoons} Mg \cdot E;$$

$$Mg \cdot E + MgP_i \overset{K_2}{\rightleftharpoons} Mg \cdot E \cdot P_i \cdot Mg \overset{K_5}{\rightleftharpoons} E{-}P$$

$$K_5 \frac{E_o}{E{-}P} = \frac{K_2}{MgP_i} \left(\frac{K_1}{Mg} + 1\right) + K_5 + 1$$

$$= \frac{1}{P_i} \frac{K_o K_2}{Mg} \left(\frac{K_1}{Mg} + 1\right) + K_5 + 1 \qquad (5)$$

Pattern in fig.3: A-plot and B-plot show mode c. Eliminated.

3.2. *Sequential order reaction b*

$$E + MgP_i \overset{K_1}{\rightleftharpoons} E \cdot P_i \cdot Mg$$

$$E \cdot P_i \cdot Mg + Mg \overset{K_2}{\rightleftharpoons} Mg \cdot E \cdot P_i \cdot Mg \overset{K_5}{\rightleftharpoons} E{-}P$$

$$K_5 \frac{E_o}{E{-}P} = \frac{1}{MgP_i} \cdot \frac{K_1 K_2}{Mg} + \frac{K_2}{Mg} + K_5 + 1$$

$$= \frac{1}{P_i} \frac{K_1 K_2 K_o}{(Mg)^2} + \frac{K_2}{Mg} + K_5 + 1 \qquad (6)$$

Pattern in fig.3: A-plot shows mode e, but not identical to fig.1 (no common intersection); B-plot shows mode e. Eliminated.

3.3. Random order reaction

$$E + Mg \underset{}{\overset{K_1}{\rightleftharpoons}} Mg \cdot E$$

$$Mg \cdot E + MgP_i \overset{K_3}{\rightleftharpoons} Mg \cdot E \cdot P_i \cdot Mg$$

$$E + MgP_i \overset{K_2}{\rightleftharpoons} E \cdot P_i \cdot Mg$$

$$E \cdot P_i \cdot Mg + Mg \overset{K_4}{\rightleftharpoons} Mg \cdot E \cdot P_i \cdot Mg$$

$$Mg \cdot E \cdot P_i \cdot Mg \overset{K_5}{\rightleftharpoons} E\text{–}P$$

$$EP = Mg \cdot E\text{–}P \cdot Mg$$

$$K_5 \frac{E_o}{E\text{–}P} = \frac{1}{MgP_i}\left(\frac{K_2K_4}{Mg} + K_3\right) + \frac{K_4}{Mg} + K_5 + 1$$

$$= \frac{1}{P_i}\frac{K_o}{Mg}\left(\frac{K_2K_4}{Mg} + K_3\right) + \frac{K_4}{Mg} + K_5 + 1 \qquad (7)$$

Pattern in fig.3: A-plot shows mode e, but not identical to fig.1. B-plot shows also mode e. Eliminated.