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THE ROLE OF MAGNESIUM IN MUSCLE PHOSPHORYLASE KINASE ACTIVITY

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SUMMARY

Phosphorylase kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.38), the enzyme which regulates glycogen breakdown by converting α-glucan phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) into its active form, requires ATP and divalent metal ions for its activity.

We have shown that:

1. $MgATP^{2-}$ chelate is the substrate for the reaction with an apparent K_m of 0.07 mM $\pm$ 0.02 (S.D.).

2. Free Mg^{2+} in mM concentration is also required for activity. The apparent K_a for free Mg^{2+} is 0.6 mM $\pm$ 0.2 (S.D.).

3. Free ATP^{4-} is not inhibitory.

4. Mn and Ca at mM concentrations are inhibitors of the enzyme even though μM concentrations of Ca are required for activity[1,2]. With both metals the inhibition is not competitive with respect to $MgATP^{2-}$ and Mg^{2+}. Mn as well as Al, Ba and Co, in the absence of Mg, will allow the phosphorylase kinase reaction to proceed but only at approx. 5% of the rate observed in the presence of Mg.

INTRODUCTION

Phosphorylase kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.38), the enzyme which activates α-glucan phosphorylase (α-1,4-glucan:orthophosphate glycosyltransferase, EC 2.4.1.1) by phosphorylation of specific serine hydroxyl groups in the phosphorylase protein molecule, requires both ATP and magnesium salts in mM concentrations[3] and calcium salts in μM concentrations[1,2]. The kinase is inhibited when the total ATP concentration $[ATP_t]$ exceeds the total Mg concentration $[Mg_t]$.

Kinetic experiments indicate that two magnesium atoms are involved in the reaction. This is concluded from the fact that the Hill coefficient for magnesium is 1.99 and from the fact that plots of the kinase velocity against $[Mg_t]$ are sigmoidal, but plots of the velocity against $[Mg_t]^2$ are hyperbolic[4].

These results can be explained by assuming that $MgATP^{2-}$ is the substrate of the reaction, for which a K_m of 0.07 mM ± 0.02 (S.D.) can be calculated, and that either an additional Mg^{2+} is also required or that ATP^{4-} is an inhibitor and that a high $[Mg_t]$ is required in order to keep the ATP^{4-} concentration low. Activation of the kinase by free Mg^{2+} has previously been postulated[4,5] but inhibition by ATP^{4-} could never be excluded.

It is now concluded that Mg^{2+} is required on the basis of experiments described below, where a second chelator, sodium citrate, was used in order to keep ATP^{4-} constant at predetermined values while Mg^{2+} was varied. A second approach, to use other metals besides magnesium to bind ATP, proved unsuccessful since all metals tested proved inhibitory to phosphorylase kinase at the mM concentrations required for these experiments. The results described in this paper have been previously presented in brief form[6].

METHODS

Enzyme isolation and assays

Rabbit skeletal muscle phosphorylase kinase ("40 000 rev./min pellet") was isolated as described by Krebs *et al.*[3] except that EDTA was omitted. The phosphorylase kinase used in the experiments described below was largely in the activated form, *i.e.* with a high ratio of activities at pH 6.8 to 8.2. All assays were performed at pH 8.2, measuring both the activated and non-activated forms. Phosphorylase *b* (3 times crystallized) was isolated as described by Fischer and Krebs[7].

Phosphorylase kinase was assayed by incubating with phosphorylase *b* in a reaction mixture of 120 μl containing 33 mM Tris–HCl (pH 8.2), 70–100 units/ml phosphorylase *b* and ATP, magnesium acetate and other additions as indicated. The digest was incubated for 10 min at 30 °C and the kinase reaction was stopped by dilution and chelation of Mg^{2+} by addition of 2 ml of 40 mM glycerophosphate and 30 mM cysteine in 5 mM EDTA (pH 7.0). Phosphorylase *a* was then determined by mixing 50 μl of the digest with 50 μl of 150 mM glucose 1-phosphate, 20 mg/ml glycogen (pH 6.5). After 10 min of incubation, the reaction was stopped by addition of 1.0 ml of 0.036 M H_2SO_4; phosphate formed from glucose 1-phosphate was determined by reading the absorbance at 700 nm, 2 min after addition of 1.0 ml of ammonium molybdate reagent (10 g ammonium molybdate per l 1 M H_2SO_4) containing 4 g/100 ml $FeSO_4$. Phosphorylase kinase activity is expressed as the amount of enzyme required to convert 1 unit of phosphorylase *b* into phosphorylase *a* per min.

Calculation of the concentration of ionic species

For the calculation of the ionic species, the association constants given in Table I were used. We attempted to select values determined under conditions resembling ours (33 mM Tris–HCl, pH 8.2). Ionic species in mixtures containing one metal ion and one chelator were calculated from the quadratic equation which can be derived from the expression for the association constant. An example of the equations that may be obtained in the case of Mg and ATP is shown in Table II. In a similar way, a cubic equation can be derived when two chelators and one metal are used (compare ref. 13). The cubic equation obtained in the case of ATP, citrate and

TABLE I

ASSOCIATION CONSTANTS OF DIVALENT METAL IONS AND CHELATORS SELECTED FOR USE IN Tris–HCl, pH 8.2

Metal ion	*Chelator*	*Association constant (mM^{-1})*	*Reference*
Mg^{2+}	ATP^{4-}	24.5	8, 9
Ca^{2+}	ATP^{4-}	8.5	8
Mn^{2+}	ATP^{4-}	56.2	10, 11
Mg^{2+}	$Citrate^{3-}$	1.59	12

TABLE II

CALCULATION OF IONIC SPECIES WHEN ONLY ONE CHELATOR AND ONE METAL ARE PRESENT

$$24.5 = \frac{[MgATP^{2-}]}{[Mg^{2+}]\,[ATP^{4-}]}\ mM^{-1}$$

If $Mg_t > ATP_t$

$$24.5 = \frac{ATP_t - ATP^{4-}}{(Mg_t - ATP_t + ATP^{4-})\,(ATP^{4-})}$$

$$24.5\,[ATP^{4-}]^2 + (24.5\,Mg_t - 24.5\,ATP_t + 1)\,[ATP^{4-}] - ATP_t = 0$$

If $ATP_t > Mg_t$

$$24.5 = \frac{Mg_t - Mg^{2+}}{(Mg^{2+})\,(ATP_t - Mg_t + Mg^{2+})}$$

$$24.5\,[Mg^{2+}]^2 + (24.5\,ATP_t - 24.5\,Mg_t + 1)\,[Mg^{2+}] - Mg_t = 0$$

TABLE III

CALCULATION OF THE CONCENTRATION OF IONIC SPECIES PRESENT IN REACTION MIXTURES CONTAINING ATP, CITRATE, AND Mg WHEN $ATP_t + citrate_t > Mg_t$

$$K_{ATP} = \frac{[MgATP^{2-}]}{[Mg^{2+}]\,[ATP^{4-}]} = 24.5\ mM^{-1}$$

$$K_{Cit} = \frac{[Mg\,Cit^{-}]}{[Cit^{3-}]\,[Mg^{2+}]} = 1.59\ mM^{-1}$$

$$Mg_t = Mg^{2+} + MgCit^{-} + MgATP^{2-} = Mg^{2+}\{1 + K_{Cit}(Cit^{3-}) + K_{ATP}(ATP^{4-})\}$$
$$ATP_t = ATP^{4-} + MgATP^{2-} = ATP^{4-}\{1 + K_{ATP}(Mg^{2+})\}$$
$$Cit_t = Cit^{3-} + MgCit^{-} = Cit^{3-}\{1 + K_{Cit}(Mg^{2+})\}$$
$$0 = \{(24.5)(1.59)\}(Mg^{2+})^3 + \{24.5 + 1.59 + (24.5)(1.59)(Cit_t + ATP_t - Mg_t)\}(Mg^{2+})^2 + \{1.59\,Cit_t + 24.5\,ATP_t + 1 - (24.5 + 1.59)(Mg_t)\}(Mg^{2+}) - Mg_t$$

Mg when $ATP_t + citrate_t > Mg_t$ is given in Table III. The roots for the cubic equation can be obtained with the help of the IBM 360/65 computer.

Computer analysis of kinetic data

Kinetic data were analysed with the IBM 360/65 computer, using the program Hyperb written by Hanson *et al.*[14], based on the method of Bliss and James[15]. This program fits a hyperbola to the experimental points by the maximum likelihood method and provides values for K_m and the maximum velocity (V) together with standard deviations or confidence limits for these values. For the purpose of clarity, we have re-drawn the data as Lineweaver–Burke plots, using the computer generated values for V and K_m, indicating the standard deviations on the x- and the y-axes.

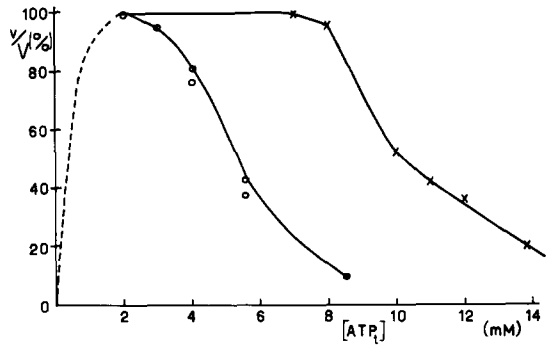

Fig. 1. Rabbit muscle phosphorylase kinase activity as a function of total ATP concentration at two different magnesium acetate concentrations. The reaction mixture (120 μl) contained 33 mM Tris–HCl (pH 8.2), approx. 70–100 units/ml phosphorylase *b*, 2 mM (○) or 8 mM (×) magnesium acetate and ATP. The mixture was incubated at 30 °C for 10 min. The kinase reaction was stopped and the formed phosphorylase *a* was determined as indicated under Methods.

RESULTS

In a number of experiments where only ATP and Mg were used, we determined that the K_m for $MgATP^{2-}$ of rabbit muscle phosphorylase kinase was 0.07 mM ± 0.02 (S.D.). At saturating levels of $MgATP^{2-}$, but under conditions where the total ATP concentration exceeded the total Mg concentration, the phosphorylase kinase was inhibited (Fig. 1). This inhibition might be due to the fact that the enzyme's free Mg^{2+} requirement is not being met or that the excess free ATP is an inhibitor, either through direct interaction with the enzyme or through chelation of the minute

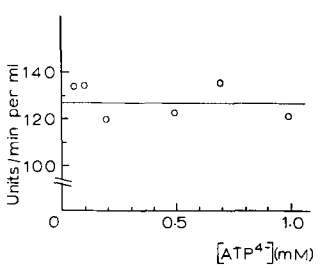

Fig. 2. Rabbit muscle phosphorylase kinase activity as a function of ATP^{4-} concentration, where the Mg^{2+} concentration is kept constant at 1.0 mM. Reaction conditions were as in Fig. 1 except that in order to obtain 1.0 mM Mg^{2+} and the desired ATP^{4-} concentrations, the following total ATP, magnesium acetate and sodium citrate concentrations were used for the points from left to right:

[ATP$_t$] (mM)	*[Sodium citrate] (mM)*	*[Magnesium acetate] (mM)*
2.55	0	4.06
2.55	1.0	4.06
5.10	1.0	6.51
12.75	1.0	13.86
17.85	1.0	18.76
25.50	1.0	26.11

Free ion concentrations were calculated as indicated in Methods.

amounts of Ca^{2+} required for activity[2,3]. However, when we provided the enzyme with saturating amounts of $MgATP^{2-}$ and a constant Mg^{2+} concentration, while varying the free ATP concentration as shown in Fig. 2, no inhibition by free ATP could be detected. This experiment also showed that our reagents contained enough Ca^{2+} to satisfy the kinase's requirement for that metal.

It thus appeared that the phosphorylase kinase requires free Mg^{2+}. This was proven by an experiment (Fig. 3) in which the $MgATP^{2-}$ concentration was saturating, the free ATP^{4-} concentration was kept constant, and the free Mg^{2+} concentration

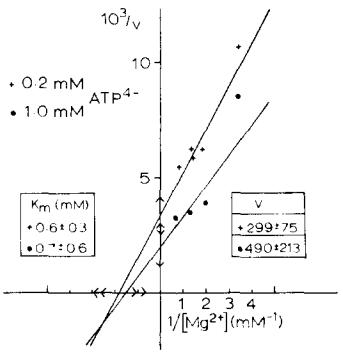

Fig. 3. Reciprocal plot of rabbit muscle phosphorylase kinase activity with respect to Mg^{2+} concentrations at two different concentrations of ATP^{4-}. Arrowheads on the x-axis indicate standard deviation of the $1/K_m$ values. Reaction conditions were as in Fig. 1 except that in order to obtain the desired ATP^{4-} concentrations the following total ATP, magnesium acetate, and sodium citrate concentrations were used:

[ATP^{4-}] (mM)	*[ATP_t] (mM)*	*[Sodium citrate] (mM)*	*[Magnesium acetate] (mM)*
0.2	0.69	2.0	0.87
0.2	1.67	2.0	2.40
0.2	2.65	2.0	3.84
0.2	3.63	2.0	5.18
0.2	5.59	2.0	7.76
0.2	6.57	2.0	9.02
1.0	3.45	2.0	2.82
1.0	8.35	2.0	8.30
1.0	13.25	2.0	13.64
1.0	18.15	2.0	18.90
1.0	27.95	2.0	29.32
1.0	32.85	2.0	34.50

Free ion concentrations were calculated as described in Methods.

was varied. It can be seen from Fig. 3 that at the two ATP^{4-} concentrations, identical K_a values (0.6 mM $\pm$ 0.2 (S.D.)) for Mg^{2+} were obtained, ($0.90 < P < 0.95$). The two V values were not significantly different ($0.5 < P < 0.6$).

Although these experiments clearly prove the point that Mg^{2+} is required for phosphorylase kinase activity, we tried to prove the same point in an independent way by trying to find a metal ion that would not interfere with the phosphorylase kinase activity. This would give us the opportunity to use two metal ions and one chelator, ATP, and to devise conditions where we varied [ATP^{4-}] while keeping

TABLE IV

EFFECTS OF VARIOUS CATIONS ON MUSCLE PHOSPHORYLASE KINASE ACTIVITY

A. In the absence of magnesium.

Reaction mixture	*Activity (units/min per ml)*	*% Activity*
ATP 3 mM + Al 10 mM	34	5
+ Ba 10 mM*	32	5
+ Co 10 mM	38	6
+ Cu 10 mM*	0	0
+ Sr 10 mM	0	0
+ Zn 10 mM*	0	0
+ Mn 12 mM	—	6
+ Mg 10 mM (control)	615	100

B. In the presence of magnesium.

Reaction mixture	*Activity (units/min per ml)*	*% Inhibition*
ATP 3 mM + Mg^{2+} 10 mM	615	0
+ Al 2 mM	358	42
+ Ba 2 mM*	497	19
+ Co 2 mM	124	80
+ Cu 2 mM*	0	100
+ Sr^{2} 2 mM	336	45
+ Zn 2 mM*	0	100
+ Ca 2 mM	—	32
+ Mn 2 mM	—	76

* A precipitate was observed in these reaction mixtures.

[Mg^{2+}] constant and *vice versa*. At the same time, we tested whether other metal ions could replace Mg either as the ATP chelate or as the free ion.

Table IV shows that all metals tested in the presence of Mg were inhibitors of the phosphorylase kinase reaction. At the same time, we confirmed the observation of Krebs *et al.*[3] that Mn can replace Mg. However, maximal velocities in the presence of all metals tested, including manganese, were considerably lower than with Mg. Fig. 4 shows that both Mn and Ca are inhibitors. The inhibition is not competitive with respect to $MgATP^{2-}$, the K_m values in the absence and the presence of these metals do not differ significantly, whereas there is no overlap between the widely different V values which are significantly lower in the presence of either Mn or Ca. Fig. 5 shows that the inhibition by Ca^{2+} is also not competitive with respect to free Mg^{2+}. Although the data suggest that the inhibition might be non-competitive this can not be stated with certainty since only one inhibitor concentration was tested.

Mn, Ba and Co in the absence of Mg will allow some (approx. 5% of V) phosphorylase kinase activity, but it is unlikely that any of these metals play a significant role *in vivo*. No metals were found which were neither inhibitors nor activators in the phosphorylase kinase reaction, and, thus, it was not possible to confirm our results of the experiments with citrate in an independent way.

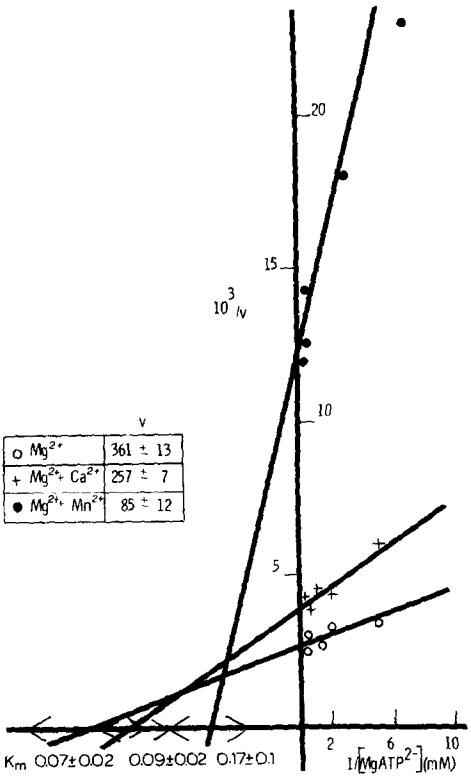

Fig. 4. Reciprocal plot of rabbit muscle phosphorylase kinase activity with respect to the substrate, $MgATP^{2-}$ concentration, in the presence of Mg alone, Mg + Ca, and Mg + Mn. Arrowheads on the x- and y-axes indicate standard deviation of the $1/K_m$ and $1/V$ values, respectively. Reaction conditions were as in Fig. 1 except that the following concentrations of total ATP, total $CaCl_2$, total $MnSO_4$ and total magnesium acetate were used:

$[ATP_t]$ (○, +, ●) (mM)	$[Ca_t]$ (+) (mM)	$[Mn_t]$ (○) (mM)	$[Mg_t]$ (○, +, ●) (mM)
0.2	2.0	2.0	10
0.5	2.0	2.0	10
1.0	2.0	2.0	10
2.0	2.0	2.0	10
3.0	2.0	2.0	10

Free ion concentrations were calculated as described in Methods.

DISCUSSION

We consider the following mechanism to be established for the phosphorylase kinase reaction: $MgATP^{2-}$ is the substrate; Mg^{2+} in mM concentration is required in addition to the ATP chelate; Ca is required in μM concentrations[1,2]; and ATP^{4-} is not an inhibitor at physiological concentrations. It can be calculated from the ex-

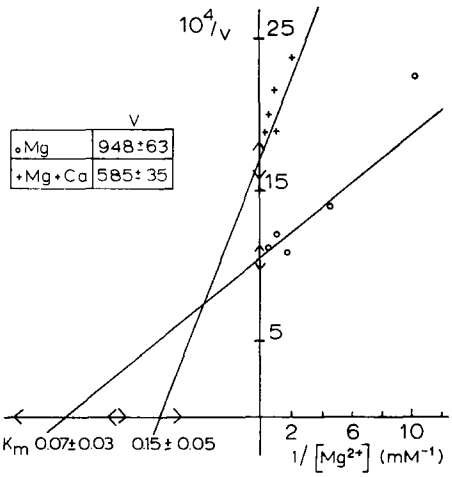

Fig. 5. Reciprocal plot of rabbit muscle phosphorylase kinase activity with respect to the activator, Mg^{2+}, in the presence and absence of Ca. Arrowheads on the x- and y-axes indicate standard deviation of the $1/K_m$ and $1/V$ values, respectively. Reaction conditions were as in Fig. 1 except that the following concentrations of total ATP, total magnesium acetate and total $CaCl_2$ were used:

$[ATP_t]$ (○, +) (mM)	$[Mg_t]$ (○, +) (mM)	$[Ca_t]$ (+) (mM)
8.0	10.0	1.0
8.5	10.0	1.0
9.0	10.0	1.0
9.5	10.0	1.0
10.0	10.0	1.0
11.0	10.0	1.0
14.0	10.0	1.0

Free ion concentrations were calculated as indicated in Methods.

periment shown in Fig. 2, that ATP^{4-} could only be a competitive inhibitor but with a K_i in excess of 40 mM.

From the present experiments, it is not possible to conclude that the free Mg^{2+} concentration *in vivo* plays a role in the control of the phosphorylase kinase reaction; however, we consider this to be a real possibility, since the concentration of free Mg^{2+} required *in vitro* is higher (K_a 0.6 ± 0.2 mM) than would be expected *in vivo*. Therefore, small changes in the intracellular concentration of free Mg^{2+} could significantly change the phosphorylase kinase activity.

The method employed to distinguish between a Mg^{2+} requirement and ATP^{4-} inhibition for phosphorylase kinase may also be employed for the study of other enzymes which are inhibited by $[ATP_t]$ in excess of $[Mg_t]$; among these enzymes are phosphofructokinase, pyruvate carboxylase and the glycogen synthetase-inactivating protein kinase[16].

Phosphorylase kinases from other sources such as liver, adipose tissue and leucocytes show behaviour similar to that from skeletal muscle in that they are also

inhibited by excess ATP and show similar K_m values for $MgATP^{2-}$ and free Mg^{2+} (Huijing, F., unpublished observations). In addition, the residual phosphorylase kinase activity in the leucocytes of patients with glycogen-storage disease caused by a low phosphorylase kinase activity[17–19] requires both $MgATP^{2-}$ and Mg^{2+} in concentrations that are not different from those required in control leucocytes.

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