The Mg^{2+} requirements of nonactivated and activated rat liver phosphorylase kinase

Inhibition of the activated form by free Mg^{2+}

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Received 13 January 1984

Incubation of rat liver phosphorylase kinase in the presence of MgATP results in a time-dependent increase in activity, i.e., activation. Determination of the magnitude of activation depends, in large part, on the relative concentrations of Mg^{2+} and ATP used in the phosphorylase kinase activity assay, such that as the Mg^{2+} to ATP ratio increases less activation is detectable. Prior to activation, maximal activity of nonactivated phosphorylase kinase requires a 2–3-fold molar excess of Mg^{2+} (i.e., free Mg^{2+}) over ATP. MgATP-dependent activation of the enzyme results in an alteration in the free Mg^{2+} requirement such that the activity of the activated enzyme is sharply inhibited by the free cation. Inhibition by free Mg^{2+} of the activated enzyme is rapidly reversed by removal of free Mg^{2+} but is not affected by addition of Ca^{2+}. Both nonactivated and activated forms of the enzyme appear to be inhibited by free ATP^{4-}. The results show that the use of high concentrations of free Mg^{2+} in the phosphorylase kinase activity assay can blunt or completely obscure changes in enzyme activity following activation of the enzyme.

Liver *Glycogenolysis* *Phosphorylase kinase* *Activation* *Divalent cation* *Free Mg^{2+}*

1. INTRODUCTION

The phosphorylation and activation of phosphorylase kinase by cyclic AMP-dependent protein kinase is considered an integral component of cyclic AMP-mediated hormonal stimulation of liver glycogenolysis. The view that activation by phosphorylation of liver phosphorylase kinase occurs in vivo is based on a variety of in vitro studies using partially purified [1–3] and purified fractions [4] of the enzyme and by analogy to the established regulatory features of skeletal muscle phosphorylase kinase (review [5]). These studies have also noted that, while similar in some respects, the liver and muscle enzymes are not identical. Whereas muscle phosphorylase kinase exhibits a complete dependence on Ca^{2+} for activity, the liver enzyme appears to have an incomplete or partial requirement for the divalent cation [4,6–10]. In addition, the nonactivated forms of the enzymes from the two tissues display clearly different pH optima – the liver enzyme having an activity optimum at a pH at which the muscle enzyme is nearly inactive [1,4,11]. The above evidence suggests that (even though similar to the muscle enzyme) an evaluation of liver phosphorylase kinase should not depend solely on the established methodology used for the study of skeletal muscle phosphorylase kinase.

The recent availability of highly purified rat liver phosphorylase kinase [4] has allowed a closer examination of the regulatory features of this enzyme, especially those aspects involving the effects

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00145793/84/$3.00

of phosphorylation on activity. It will be shown that activation of the enzyme alters the Mg^{2+} requirement such that the activated enzyme is sharply inhibited by free Mg^{2+} (i.e., Mg^{2+} in excess of ATP). This characteristic of the activated form of the liver enzyme offers a plausible reason for the apparent lack of activation in vitro as reported by some [10,12] as well as further substantiating the idea [13] that liver and skeletal muscle phosphorylase kinase have distinct and different properties.

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP was made as in [14]. Rabbit skeletal muscle phosphorylase *b* was prepared as in [15]. All other chemicals were from the sources previously reported [4].

2.2. *Preparation of rat liver phosphorylase kinase*

For all preparations used in these studies the initial fractionation steps through the high-speed (120000 × *g*) centrifugation and the purification of the enzyme initially associated with the high-speed pellet ('glycogen pellet') were as described in [4]. Treatment of the high-speed supernatant fluid with polyethylene glycol (PEG) 8000 (formerly 'PEG 6000') was as in [2]. The frozen (−70°C) PEG pellet from 30 rats was resuspended in 50 ml of 0°C buffer [20 mM triethanolamine–HCl (pH 7.5), 20% (v/v) glycerol, 1 mM dithiothreitol, 0.02% NaN_3] containing 1 mg each of the protease inhibitors leupeptin, antipain, pepstatin and chymostatin. The suspension was clarified by centrifugation at 120000 × *g* for 90 min and the supernatant fluid containing the majority of phosphorylase kinase activity was applied to a 5 × 85 cm Sepharose 6B column. The single peak of activity ($M_r = 1.3 \times 10^6$) was then applied directly to a 30 ml column of heparin–Sepharose Cl-6B [4], the column washed with 10 bed volumes of buffer containing 0.04 M NaCl and the enzyme eluted with buffer containing 0.3 M NaCl. The eluted material was diluted with 2.5 vols of buffer and applied to a 20-ml column of DEAE–cellulose (Whatman DE-52), and the column washed with 10 bed volumes of buffer containing 0.1 M NaCl. The enzyme was eluted from the column with a 100 ml linear NaCl gradient (0.1–0.35 M). The single peak of enzyme activity was precipitated by the addition of cold neutral saturated ammonium sulfate to a final concentration of 40%. The hazy solution was centrifuged at 40000 × *g* for 20 min and the supernatant fluid discarded. The precipitate was resuspended in 5 ml buffer containing 0.25 M NaCl and applied to a 2.5 × 85 cm Sepharose 6B column equilibrated in buffer containing 0.25 M NaCl. The enzyme eluting as a single symmetrical peak ($M_r = 1.3 \times 10^6$) was diluted with 3 vols of buffer and applied to a 1 ml column of DEAE–cellulose. The enzyme was eluted with 2 ml of buffer containing 0.5 M NaCl, dialyzed overnight against 250 vols of buffer and stored at −70°C. At this stage, the enzyme had been purified some 12000–15000-fold and comprised 30–50% of total protein, as determined by electrophoresis in the presence of 0.1% SDS.

3. RESULTS AND DISCUSSION

As is the case for skeletal muscle phosphorylase kinase, the nonactivated and activated forms of liver phosphorylase kinase display distinctly different catalytic properties, e.g., pH optima [1,4], time course of reaction [2,4], heparin stimulation [2]. An additional difference between the two forms of the liver enzyme is the Mg^{2+} requirement of each (fig.1). Prior to activation (lower curve) free Mg^{2+} (i.e., Mg^{2+} in excess of ATP) is required for maximal activity with a 2–3-fold molar excess of Mg^{2+} over ATP (1 mM in this case) being optimal. However as the enzyme is progressively activated* (middle and upper curves) the Mg^{2+} requirement is altered such that free Mg^{2+} becomes inhibitory. Thus after a 1 min period of activation there is no detectable increase in activity when the assay contains 10 mM Mg^{2+} although activation is clearly evident at Mg^{2+} concentrations equal to or less than the ATP concentration. After a prolonged period of activation (6 min, upper curve)

* For the experiments described herein, activation was accomplished in the absence of the cyclic AMP-dependent protein kinase and by analogy to the skeletal muscle enzyme [5] could be termed "autoactivation". Activation of liver phosphorylase kinase in the presence of the catalytic subunit of the cyclic AMP-dependent protein kinase does not qualitatively alter the characteristics of the activated enzyme, although the resulting activity is greater [2,4].

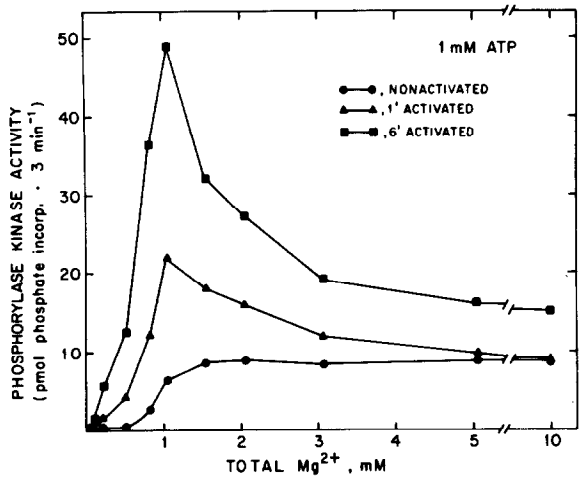

Fig.1. The Mg^{2+}-requirements of the nonactivated and the activated forms of rat liver phosphorylase kinase. Rat liver phosphorylase kinase (purified from the glycogen pellet) was activated for the indicated times by incubation at 30°C in the presence of 3 mM $Mg(C_2H_3O_2)_2$, 50 μM ATP and then diluted with 9 vols of cold 20 mM triethanolamine–HCl (pH 7.5), 10 mM NaF. For nonactivated ('0'' activated) phosphorylase kinase, the enzyme was diluted in cold TEA–NaF buffer and then the same volume of the $Mg(C_2H_3O_2)_2$–ATP solution used for the activation incubation was added. Kinase activity was then determined in a 3 min incubation (30°C) in the presence of 1 mM [γ-^{32}P]ATP, 2 mg/ml skeletal muscle phosphorylase *b*, 20 mM triethanolamine–HCl (pH 7.5) and the indicated amounts of $Mg(C_2H_3O_2)_2$. In addition the activity assay contained in a final volume of 50 μl, 10 μl of the diluted activated or nonactivated enzyme. The reaction was terminated by transferring 40 μl of the incubation mixture onto Whatman 31ET filter papers and further processed as in [2].

an increase in activity is detectable at the highest Mg^{2+} but this is still much less than the increases seen at the lower Mg^{2+} concentrations.

Although activation results in increased enzyme activity at all Mg^{2+} concentrations, the degree of activation is much greater when free Mg^{2+} is very low (Mg^{2+} = ATP) or almost nonexistent (Mg^{2+} < ATP) (table 1). This table lists the activity of the enzyme following a 6 min activation relative to that of the nonactivated enzyme at the different total Mg^{2+} concentrations used in the activity determination. It is obvious that very large increases in activity (30-fold) can be seen when Mg^{2+} is less than ATP, although the absolute activity is less than when Mg^{2+} and ATP are equimolar.

Table 1

The expression of activation of rat liver phosphorylase kinase as a function of the Mg/ATP in the activity assay

Total $Mg(C_2H_3O_2)_2$ (with 1 mM ATP) (mM)	Relative activity[a] (activated/ nonactivated)
0.51	31
0.81	14
1.06	7.7
1.56	3.7
2.06	3.0
3.06	2.4
5.06	1.8
10.06	1.7

[a] The activities of the nonactivated and 6 min activated enzymes are taken from fig.1

Fig.2 shows that in the presence of 0.5 mM, 1.0 mM or 1.5 mM ATP, essentially the same maximum activity of the activated enzyme is observed when Mg^{2+} and ATP are equimolar. With the nonactivated enzyme, maximum activity is observed with approx. 2 mM free Mg^{2+}. These data suggest that both forms of the enzyme have free Mg^{2+} binding sites, although exerting opposite effects on activity when Mg^{2+} is bound. Additionally the data can be interpreted as evidence that free ATP^{4-} is inhibitory to the nonactivated enzyme, as has been suggested to be the case for the skeletal muscle enzyme [16]. That free ATP^{4-} inhibits the activated form of the liver enzyme is evident in fig.2 when the ATP concentration is increased from 0.5 mM to 1.5 mM while maintaining total Mg^{2+} at 0.5 mM.

The apparent inhibitory effect of free Mg^{2+} on the activated enzyme alternatively could be explained by the activation of a contaminating phosphoprotein phosphatase which requires free Mg^{2+} for activity. This phosphatase could dephosphorylate and inactivate the kinase as free Mg^{2+} was increased. However, if this were the case, the inhibition of the activated kinase by free Mg^{2+} should not be rapidly reversed by removal of free Mg^{2+} during the kinase reaction. The experiment shown in fig.3 was designed to determine if the inhibition by free Mg^{2+} was rapidly reversible. For this experiment the activated enzyme was initially incubated in the absence (upper curve) or

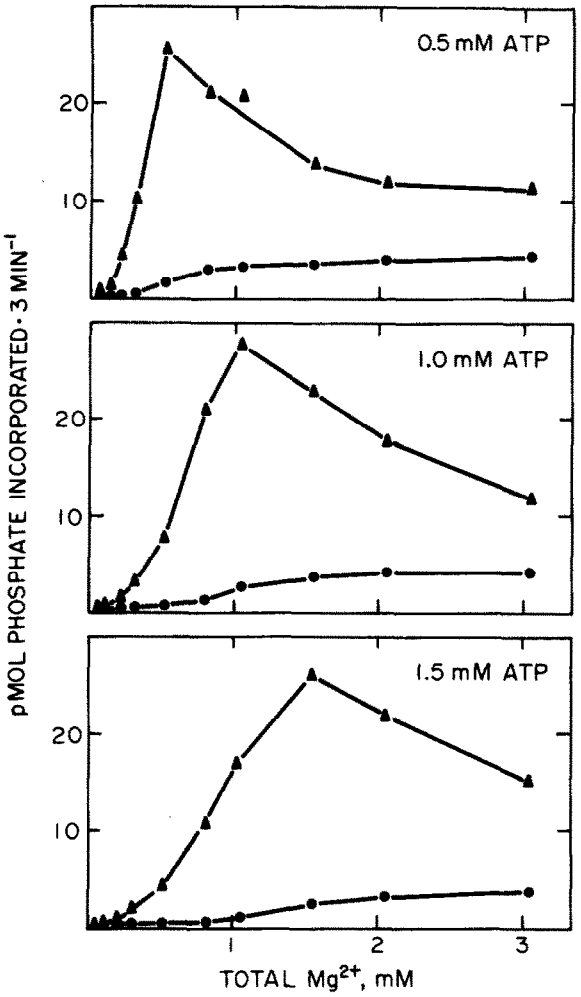

Fig.2. The Mg^{2+}-requirement of activated liver phosphorylase at different ATP concentrations. Rat liver phosphorylase kinase (partially purified from the high-speed supernatant fluid fraction) was activated (6 min) and enzyme activity determined as in fig.1 in the presence of 0.5 mM, 1.0 mM, or 1.5 mM ATP and 12.5 mM NaF.

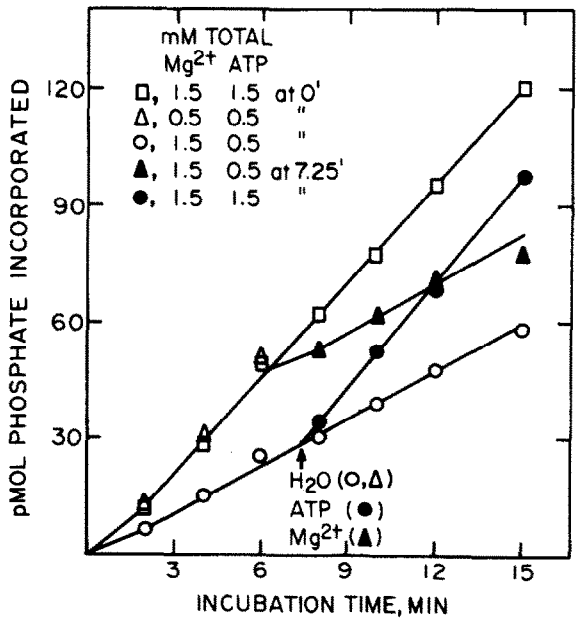

Fig.3. Time courses of the Mg^{2+}-inhibition and the reversal of Mg^{2+}-inhibition of activated liver phosphorylase kinase. Rat liver phosphorylase kinase (partially purified from the high-speed supernatant fluid fraction) was activated (6 min) as in fig.1. The diluted, activated enzyme (60 μl) was added to the incubation mixture containing (final volume of 300 μl) initially 20 mM triethanolamine–HCl (pH 7.5), 2 mg/ml skeletal muscle phosphorylase *b*, 12.5 mM NaF and (□—□) 1.5 mM $Mg(C_2H_3O_2)_2$–1.5 mM ATP, or (Δ—Δ) 0.5 mM $Mg(C_2H_3O_2)_2$–0.5 mM ATP, or (○—○) 1.5 mM $Mg(C_2H_3O_2)_2$–0.5 mM ATP. At the indicated times, 30-μl aliquots were transferred onto filter paper and processed as described in fig.1. At 7.5 min (arrow), H_2O (○—○, Δ—Δ), $Mg(C_2H_3O_2)_2$ (▲—▲), or ATP (●—●) were added to give final total concentrations of 1.5 mM $Mg(C_2H_3O_2)_2$ (Δ—Δ) or 1.5 mM ATP (○—○). Reaction volume was increased 10% by these additions.

presence (lower curve) of free Mg^{2+} establishing a constant reaction rate for both. During the course of the reaction, addition of either Mg^{2+} (upper curve) or ATP (lower curve) immediately altered the rates of both reactions such that removal of the free Mg^{2+} by complexation with the added ATP (●—●) increased the reaction rate to that of the incubation initiated in the absence of free Mg^{2+} (upper curve). Conversely, addition of excess Mg^{2+} (▲—▲) reduced the reaction rate to that of the incubation initiated in the presence of free Mg^{2+} (lower curve). The rapid reversal of the Mg^{2+} inhibition and the near-constant reaction rates seen under these conditions strongly argue that there is no significant contaminating phosphatase activity and additionally suggest that there is no Mg^{2+}-stimulated protease, which could also account for the inhibition.

A third possible mechanism by which free Mg^{2+} could inhibit the activated enzyme is by displace-

ment of Ca^{2+} from a Ca^{2+}-binding site which has been altered by activation. Fig.4 shows that the Ca^{2+}-requirement of the activated enzyme is not changed by free Mg^{2+}, suggesting that at least the affinity for Ca^{2+} remains the same even though the enzyme remains inhibited. Furthermore, Ca^{2+} does not reverse the Mg^{2+} inhibition, which argues against Mg^{2+}-displacement of Ca^{2+} from its binding site.

The in vitro activation of liver phosphorylase kinase under conditions where its subunits are phosphorylated is consistent with its proposed role in the mediation of the cyclic AMP-dependent pathway of hormonal stimulation of hepatic glycogenolysis [17]. The evidence suggests that the nonactivated form of the liver enzyme is similar to the skeletal muscle enzyme in that both require free Mg^{2+} for maximum activity. For the skeletal muscle enzyme this has been interpreted as evidence for a binding site for free Mg^{2+} on the enzyme [16,18] although it could be explained on the basis of free ATP^{4-} inhibition [17]. Either or both interpretations could be correct for the nonactivated liver enzyme.

The reason(s) for the marked inhibition of the activated enzyme by excess Mg^{2+} are unexplained but could result from the exposure of an inhibitory Mg^{2+}-binding site by phosphorylation of either or both of the α- and β-subunits of the enzyme. A practical consequence of the Mg^{2+} inhibition is that the greater activity of the activated enzyme may be blunted or completely obscured in in vitro measurements utilizing high Mg^{2+}. This could explain the inability of some workers to demonstrate the activation of rat liver phosphorylase kinase by the cyclic AMP-dependent protein kinase [10,12].

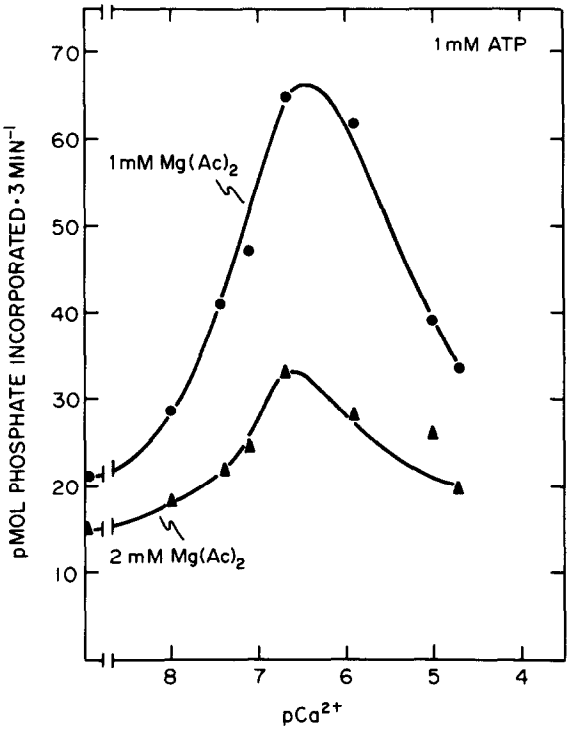

Fig.4. The influence of Mg^{2+}-inhibition on the Ca^{2+}-requirement of activated liver phosphorylase kinase. Rat liver phosphorylase kinase (purified from the glycogen pellet) was activated (6 min) and activity was determined as in fig.1 in the presence of 1 mM (○—○) or 2 mM (△—△) $Mg(C_2H_3O_2)_2$, 1 mM ATP, and 0.1 mM EGTA. $CaCl_2$ was added to give the calculated pCa^{2+} [19].

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