# ACTIVATION OF MUSCLE PHOSPHORYLASE b KINASE BY $Mg^{++}$ [1]

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Phosphorylase b kinase (E.C. 2.7.1.38) catalyzes the conversion of phosphorylase b to phosphorylase a (E.C. 2.4.1.1) (Krebs et al., 1958). In muscle the kinase exists in two forms: one which is inactive in the physiological pH range ("non activated form"), and the other which is active in this range ("activated form") (Krebs et al., 1959 and 1964). The activation of the kinase is known to be produced by: I) an ATP-$Mg^{++}$ dependent phosphorylation, stimulated by cyclic 3',5'-AMP; II) a $Ca^{++}$-requiring reaction, and III) the action of trypsin (Krebs et al., 1959 and 1964; Meyer et al., 1964; DeLange et al., 1968).

This paper reports that muscle phosphorylase b kinase can also be activated by incubation with $Mg^{++}$.

Phosphorylase b kinase was prepared from rabbit skeletal muscle by the method of Krebs et al. (1964) with some modifications. Acid precipitation was carried out at pH 5.2. The precipitate was resuspended in 4 mM EDTA (20 ml/100 g tissue) and the pH was adjusted to 7.0. The suspension ("acid precipitate") was centrifuged for 120 minutes at 105,000 x g. The precipitate ("unwashed precipitate") was purified by resuspension in 0.05 M NaCl

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(10 ml/100 g tissue) and centrifugation at 105,000 x g for 90 minutes. The sediment obtained after three cycles of resuspension and centrifugation ("washed precipitate") was suspended in cold water and incubated as described below.

Unless otherwise indicated, the activation reaction mixture contained: 25 mM N-ethylmorpholine-HCl, pH 7.0, 5 mM $MgCl_2$, and enzyme (0.02 to 0.04 ml). The total volume was 0.1 ml. The incubations were performed at 30° and were stopped by the addition of 2.9 ml of an ice-cold solution containing 2.5 mM glycerophosphate, pH 6.8, 0.5 mM EDTA and 10 mM mercaptoethanol. Phosphorylase b kinase activity was assayed at pH 6.8 and pH 8.2 according to Krebs et al. (1964). A unit of phosphorylase b kinase was defined as that amount of enzyme producing 1 nmole of phosphorylase a per minute per milliliter of assay mixture.

Figure 1 shows the time course of the effect of $Mg^{++}$ on the activity of the kinase. There was an evident activation when the kinase assay was performed at pH 6.8. When the assay was carried out at pH 8.2, the activity did not vary. As it can also be seen, cyclic adenylate did not modify the effect of $Mg^{++}$.

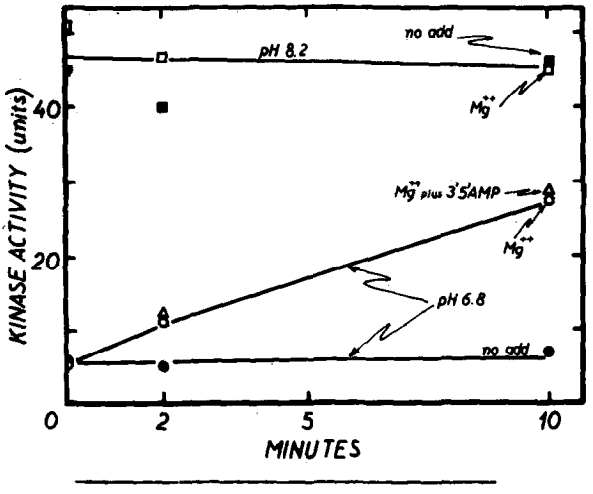


Figure 1. Time course of phosphorylase b kinase activation by incubation with $Mg^{++}$. The "acid precipitate" was dialyzed against water for 4 hours. Aliquots of 0.02 ml of this preparation, were incubated as described above. The concentration of cyclic 3',5'-AMP was $2 \times 10^{-6}$ M, when added. Activities were expressed as kinase units in the activation incubation mixture.

The activation of phosphorylase b kinase by $Mg^{++}$ led to an increase in the maximum velocity at pH 6.8, when the enzyme was assayed at high concentrations of phosphorylase b. No appreciable change in the apparent $K_m$ for phosphorylase b was observed.

The existence of a $Mg^{++}$-dependent activation of the kinase, raises some

questions on the requirement of ATP for the activation of the enzyme in the absence of cyclic adenylate. As can be seen in figures 2 and 3-A, at equimolar concentrations of ATP and $Mg^{++}$, the kinase activation was negligible. Under these conditions the presence of cyclic 3',5'-AMP was an important requirement for the conversion of the enzyme. On the contrary, at $Mg^{++}$ concentrations greater than those of ATP, the rate of the reaction was similar to that observed in the presence of $Mg^{++}$ alone (figure 2). Cyclic adenylate also increased the activation observed with ATP and a $Mg^{++}$ concentration higher than that of ATP (figure 2). Furthermore, the time course obtained in these conditions (cyclic 3',5'-AMP, ATP and excess of $Mg^{++}$) seems to be the summation of that observed in the presence of $Mg^{++}$ alone, plus the one corresponding to the incubation with cyclic 3',5'-AMP and equimolar concentrations of ATP and $Mg^{++}$.

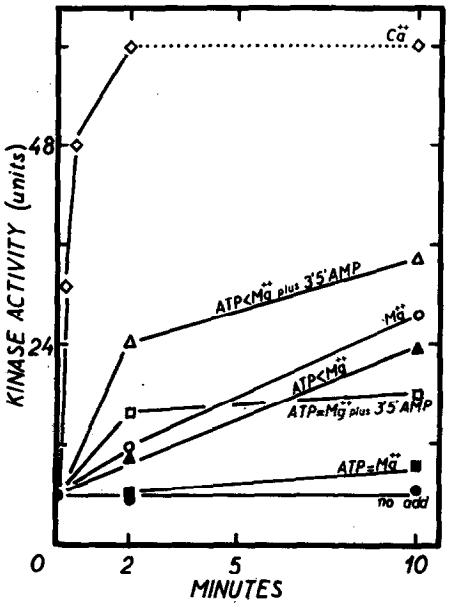


Figure 2. Time course of the activation of phosphorylase b kinase by different additions. Concentrations of the added substances were: ATP=$Mg^{++}$, 5 mM ATP and 5 mM $MgCl_2$; ATP=$Mg^{++}$ plus 3',5'-AMP, same as ATP=$Mg^{++}$ but containing $2 \times 10^{-6}$ M cyclic 3',5'-AMP; ATP<$Mg^{++}$, 2.5 mM ATP plus 5 mM $MgCl_2$; ATP<$Mg^{++}$ plus 3',5'-AMP, as ATP<$Mg^{++}$ but containing $2 \times 10^{-6}$ M cyclic 3',5'-AMP; $Mg^{++}$, 5 mM $MgCl_2$; $Ca^{++}$, 5 mM $CaCl_2$. Kinase activities were assayed at pH 6.8. Other conditions were as in figure 1.

The effect of varying $[Mg^{++}]$ on the kinase activation was determined in the absence and presence of equimolar concentrations of ATP and $Mg^{++}$ (figure 3-B). The stimulatory effect of ATP-$Mg^{++}$ over that of $Mg^{++}$ was only evident at low concentrations of the cation.

The kinase activation by $Mg^{++}$ does not appear to require the presence of ATP since dialysis of the enzyme or addition of hexokinase plus glucose to the $Mg^{++}$ containing mixture did not modify the activation. Moreover, the

concentration of ATP in the enzyme preparation was found to be less than $10^{-5}$ M. On the other hand, the addition of ATP to the $Mg^{++}$ containing mixture, elicited an additional stimulation of the kinase conversion at concentrations of the nucleotide above $10^{-4}$ M (figure 3-A).

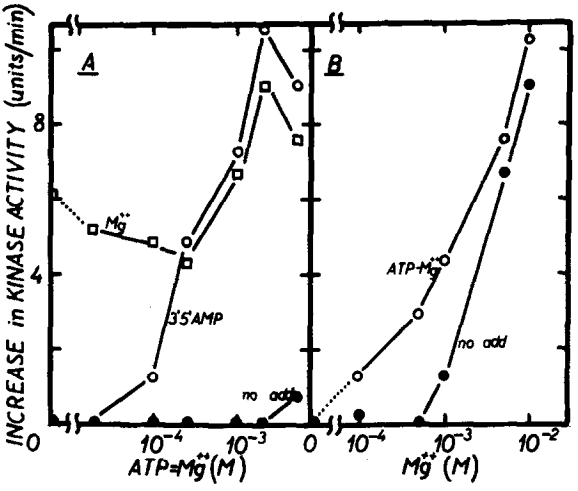


Figure 3. A. Phosphorylase b kinase activation varying [ATP] and [$Mg^{++}$] at equimolar concentrations, in the presence and absence of $2x10^{-6}$ M cyclic 3',5'-AMP or 5 mM $MgCl_2$.
B. Phosphorylase b kinase activation varying the concentration of $Mg^{++}$, in the presence or absence of ATP and $MgCl_2$ at equimolar concentrations (5 mM). The incubation time was 2 minutes. Initial phosphorylase b kinase activity (pH 6.8) in the activation reaction mixture was 16.5 units. Activations were expressed as increases in kinase activities (pH 6.8) per minute, in the activation mixture. Other conditions were as in figure 1.

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The kinase preparations that showed a $Mg^{++}$ effect were also activated by $Ca^{++}$. Using a dialyzed "acid precipitate" as enzyme source, and the cations at the same concentration (5 mM), $Ca^{++}$ was about ten-fold more effective than $Mg^{++}$ (figure 2). Some evidence indicates that the kinase activation by $Mg^{++}$ requires a factor(s) different from that involved in the $Ca^{++}$-activation. When the "acid precipitate" was purified by successive centrifugations at 105,000 x g, the preparation lost the ability to be activated by $Ca^{++}$ to a greater extent than by $Mg^{++}$. However, the activating effect of these cations on the "washed precipitate" could be restored by the addition

of a resuspended "unwashed precipitate". However, when the latter fraction was chromatographed on TEAE-cellulose, a fraction was excluded from the column that restored the $Mg^{++}$ effect. This fraction was almost free of phosphorylase b kinase activity and of the protein factor required for the $Ca^{++}$-activation.

A sample of the $Mg^{++}$-activated kinase was purified by centrifugation at 105,000 x g, and the enzyme thus obtained was incubated in the presence of mercaptoethanol, As can be seen in figure 4, the kinase lost activity when assayed either at pH 6.8 or 8.2. However, the activities at both pH's were partially restored when the inactivated kinase was incubated in the presence of ATP, $Mg^{++}$ and cyclic 3',5'-AMP. These results suggest the possibility that the $Mg^{++}$-activated kinase can be reverted to a form inactive both at pH 6.8 and 8.2 ("inactive kinase").

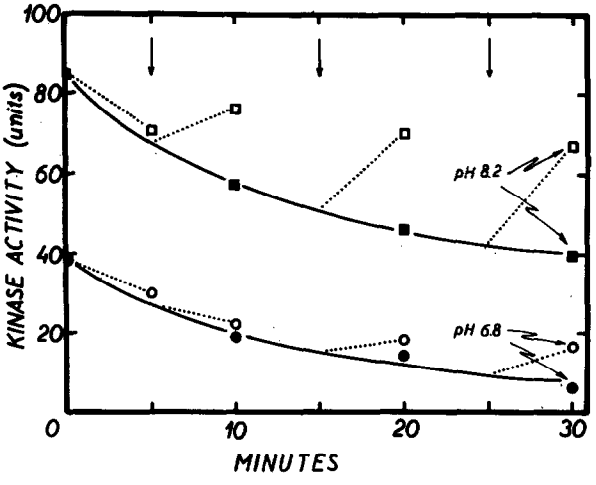


Figure 4. Reversion of the magnesium-activated phosphorylase b kinase. The "unwashed precipitate" obtained from 20 ml of "acid precipitate" was resuspended in 5 ml and dialyzed for 3 hours against water. The dialyzed preparation (ratio of activity at pH 6.8 to that at pH 8.2, 0.16) was incubated at 30° for 10 minutes with the following additions: N-ethylmorpholine buffer, pH 7.0, 150 μmoles; mercaptoethanol, 120 μmoles; and $MgCl_2$, 60 μmoles. The total volume was 6 ml. Reaction was stopped by the addition of an ice-cold solution containing 50 mM glycerophosphate buffer, pH 6.8, 25 mM mercaptoethanol, 65 mM NaF, and 12.5 mM EDTA. This preparation was centrifuged for 90 minutes at 105,000 x g and the pellet thus obtained was treated by the procedure of resuspension-centrifugation (two cycles) as was described above. The pellet obtained in the final step of purification was resuspended in one milliliter of water, and aliquots of 0.04 ml

of this resuspended fraction were incubated at 30° in the presence of 20 mM mercaptoethanol, and 25 mM N-ethylmorpholine-HCl, pH 7.0. The total volume was 0.1 ml. Reaction was stopped at the indicated times as described above. Five minutes before the reaction was stopped (vertical arrows), 0.02 ml of a solution containing 25 mM ATP, 25 mM $MgCl_2$, and $2x10^{-5}$ M cyclic 3',5'-AMP were added to some incubations (open symbols) but not to others (closed symbols).

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The results reported in this paper provide evidence for an additional regulatory mechanism of the levels of "activated" phosphorylase b kinase. It is possible that this mechanism has physiological significance, since the kinase becomes activated at $Mg^{++}$ concentrations above 1 mM (figure 3-B). $Mg^{++}$ concentrations in skeletal muscle were found to be approximately 10 mM (Walser, 1967). It is possible that under anaerobic conditions and during muscular contraction, an appreciable fraction of the total $Mg^{++}$ may be transformed to the free ionic form by a decrease in the ATP concentration. Under these conditions, the $Mg^{++}$ requirement for kinase activation would be fulfilled.

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