LIVER PHOSPHORYLASE PHOSPHATASE: REGULATION OF ACTIVITY BY ATP AND Mg IONS

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Received 16 September 1974

1. Introduction

The activity of phosphorylase phosphatase (phosphorylase phosphohydrolase, EC 3.1.3.17) from various sources [1–5] can be increased by an incubation with ATP in the presence of Mg ions. We [3] have presented arguments that led to the conclusion that this increase in activity was of a stable character; indeed, it persisted after a several 100-fold dilution, after a Sephadex G-25 filtration and after an ammonium–sulphate precipitation followed by dialysis.

The liver enzyme has been separated [6] by gel filtration into three forms: two forms (mol. wt = 215 000 and 77 000) are active in the absence of ATP–Mg and one form (mol. wt. = 138,000) needs to be activated by a preincubation with these factors. After sucrose density gradient centrifugation, the mol. wt. values were 155 000 and 59 000 for the spontaneously active forms and 59 000 for a form, active only after incubation with ATP–Mg. Furthermore the active forms with high molecular weight were inhibited by ATP–Mg [6]. In line with our previous conclusion [3], the enzyme (previously activated by ATP–Mg) was recovered as active in a sucrose density gradient; however, no more active enzyme than originally present was recovered after a G-200 filtration [6].

In this paper we report that the effects of ATP–Mg (activation of ATP–Mg dependent form and inhibition of the spontaneously active form) are of a reversible nature. Furthermore the K_A and the K_i of the two forms for ATP and Mg were determined.

2. Materials and methods

The active liver phosphorylase was prepared as indicated previously [3]. Phosphorylase phosphatase was isolated from dog liver perfused with 0.15 M NaCl and measured by a method previously described [5]. Phosphatase activity is expressed as U of phosphorylase *a* inactivated per min and per mg of protein. Proteins were determined by a modification of the method of Kabat and Mayer described by Sutherland et al. [7].

Preparative separation of the different forms of phosphorylase phosphatase was done as follows: 18 ml of the enzyme fraction mixed with 1.8 mg of Blue Dextran and 3.6 mg of DNP-L-alanine were eluted through a column (8 × 135 cm) of Sephadex G-200 with 10 mM Tris pH 7.4 at 4°C. The flow rate was maintained at 75 ml/hr with a peristaltic pump. (The ultraviolet absorption of the column eluate was monitored at 280 nm) After the phosphatase assay, the peak fractions with minimal crosscontamination were pooled, concentrated by ultrafiltration (Amicon Model 202; Filter UM-10) and kept frozen in small aliquots, with negligible loss of activity for several weeks.

Blue Dextran, Sephadex G-25 fine and Sephadex G-200 were purchased from Pharmacia, DNP-L-alanine from Serva, γ-labeled ^{32}P-ATP from New England Nuclear.

3. Results and discussion

We show in table 1 that a maximal ATP–Mg activation of the phosphatase is reversibly canceled by a filtration through a long column of Sephadex G-25; a more than hundredfold decrease in the ATP content (measured as ^{32}P-labeled ATP) was so achieved, and this was further enhanced by a 300-fold dilution in the assay.

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Table 1
Reversibility of the ATP–Mg activation

Sample	Control	ATP–Mg activated
Before G-25 filtration	0.22	0.59
After G-25 filtration	0.13	0.22
After ATP–Mg reactivation	0.53	0.64

A phosphorylase phosphatase preparation [5] was incubated for 20 min at 30°C in 10 mM Tris pH 7.4 either without or with 1 mM ATP and 4 mM $MgCl_2$. Both samples were then filtered at 4°C through a Sephadex G-25 (fine) column (1 × 55 cm) equilibrated with 10 mM Tris pH 7.4 and 10 μM EDTA. Both filtrates were then reincubated for 20 min at 30°C with 1 mM ATP and 4 mM $MgCl_2$, and diluted 300 fold with cold 10 mM Tris pH 7.4.

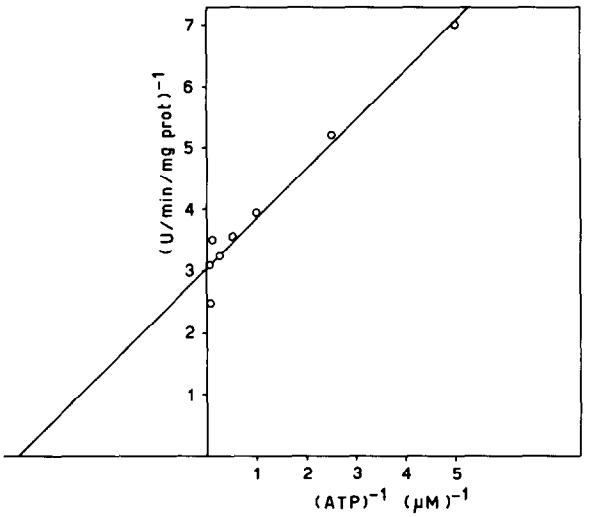

Fig. 1. Activation of phosphorylase phosphatase by ATP–Mg. The enzyme form, susceptible to the ATP–Mg activation, was incubated for 15 min at 30°C in 10 mM Tris pH 7.4 and 0.1 mM $MgCl_2$, in the presence of varying concentrations of ATP (from 0.2 μM to 40 μM); phosphatase activity was measured after the addition of a small volume (1/20 of the final volume) containing phosphorylase *a* and caffeine.

This observation probably explains the erasion of the ATP–Mg effect brought about by a Sephadex G-200 filtration [6]. All the other methods used before to decrease the ATP concentration were not efficient enough to lower it below the level where activation (of diluted enzyme preparations) still occurs. The persistence of the ATP–Mg effect in sucrose density gradient centrifugation can also be accounted for on the same grounds: the ATP-concentration, as estimated from the radioactivity of included ^{32}P-labeled ATP, was still 0.1 mM in those fractions where the phosphatase was recovered, high enough to maintain its effect on the phosphorylase phosphatase.

Large scale G-200 filtration was used next to provide us with essentially uncontaminated preparations of spontaneously active form (mol. wt. = 215 000) and ATP–Mg activated form (mol. wt. = 138 000) of phosphorylase phosphatase, in order to study some of their properties.

The form which is activated by ATP–Mg, has a K_A for ATP–Mg of about 0.3 μM (fig. 1); with ATP alone no activation was observed. The low concentration of Mg^{++} (0.1 mM) used in the experiment shown in fig. 1 has no effect on the enzyme, and much higher concentrations are required for activation (fig. 2). We calculated for Mg^{++} a K_A of 55 mM; this value is underestimated however, since Mg^{++} are inhibitory in the phosphatase assay, as indicated by the departure from linearity at the higher Mg^{++} concentrations.

The spontaneously active form is inhibited by free ATP with a K_i of 7 μM (fig. 3) and by Mg^{++} with a K_i

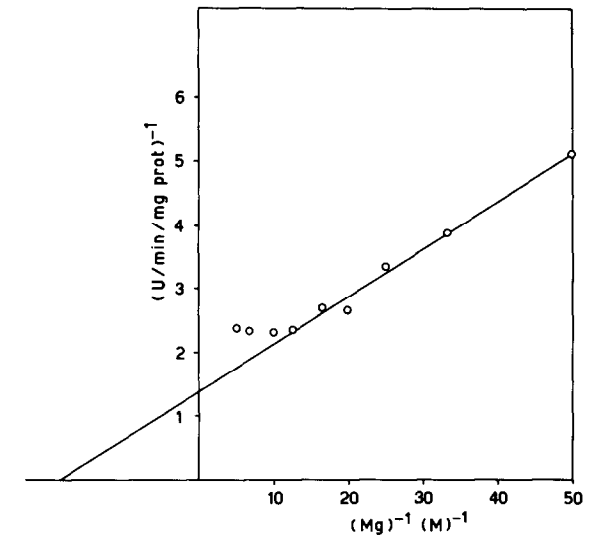

Fig. 2. Activation of phosphorylase phosphatase by Mg ions. The enzyme form, susceptible to the ATP–Mg activation, was incubated for 20 min at 30°C in 10 mM Tris pH 7.4 in the presence of $MgCl_2$ (from 20 mM to 200 mM); phosphatase activity was measured after a 100-fold dilution.

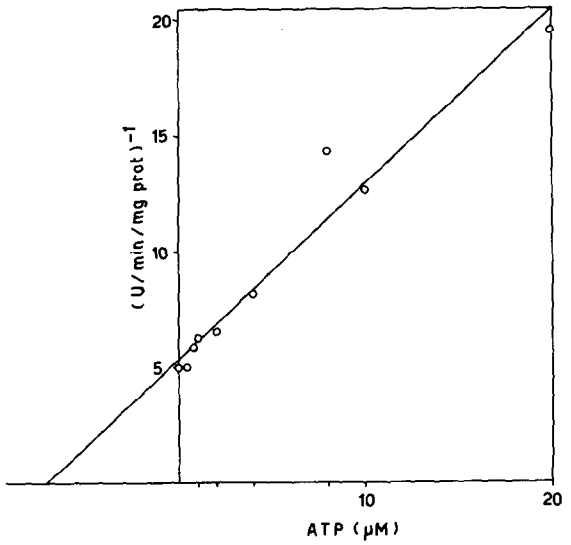

Fig. 3. Inhibition of the spontaneously active form of phosphorylase phosphatase by ATP. The enzyme was preincubated for 20 min at 30°C in 10 mM Tris pH 7.4 in the presence of ATP (from 0 μM to 20 μM); phosphatase activity was measured as indicated in fig. 1.

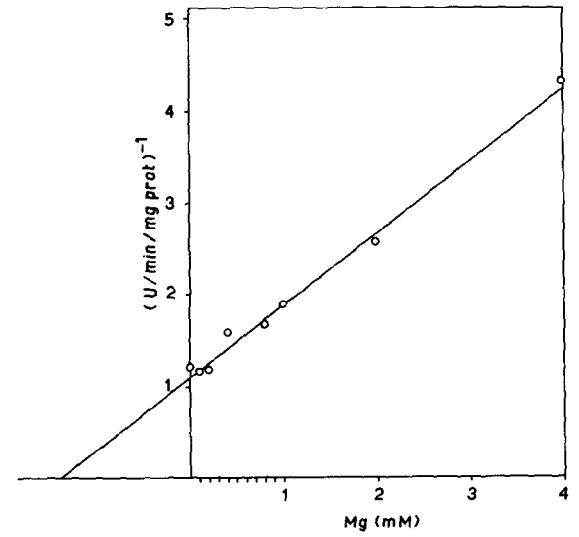

Fig. 4. Inhibition of the spontaneously active form of phosphorylase phosphatase by Mg ions. The enzyme was incubated for 10 min at 30°C in 10 mM Tris pH 7.4 in the presence of $MgCl_2$ (from 0 mM to 4 mM); phosphatase activity was measured as indicated in fig. 1.

of 1.4 mM (fig. 4). In a previous publication [6] it was stated that this form was inhibited by ATP–Mg. This inhibition is reversible. Since free ATP is so strongly inhibitory (fig. 3), we propose that the inhibition recorded with ATP–Mg is due to the uncomplexed nucleotide. Indeed, the K_i for the free ATP as calculated from the data of Nanninga [8], is about 10 μM for Mg^{++} concentrations from 50 to 400 μM.

4. Conclusions

We can draw two conclusions from these experiments: a) since the activation of the phosphatase by ATP-Mg is readily reversible (table I) its nature is probably not a covalent modification; its time and temperature (Q_{10} = 1.4) dependency [3–5] suggests a conformational change rather than a simple association, limited only by the diffusion velocity. b) Depending on the enzyme form, the effect of ATP, in the presence of Mg ions, is either stimulatory or inhibitory. We are tempted to believe that the in vitro spontaneously active form will be inhibited in the liver, due to the presence of ATP and Mg ions; on the contrary, the form which in vitro has to be activated by a preincubation with ATP–Mg, is expected to be fully active in vivo.

Acknowledgements

The authors are grateful to Mrs M. Buckinx-Veeckmans and Mrs R. Cabergs-Verbiest for expert technical assistance. This work was supported by the 'Fonds voor Wetenschappelijk Geneeskundig Onderzoek'.

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