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INACTIVATION AND REACTIVATION OF LIVER PHOSPHORYLASE *b* KINASE

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Summary

When crude rat liver preparations were incubated at 30°C, a gradual loss of phosphorylase kinase (ATP:phosphorylase *b* phosphotransferase, EC 2.7.1.38) activity was observed. This inactivation was Mg^{2+} dependent and was partially inhibited by sodium fluoride. Addition of Mg^{2+} ATP to the liver preparations, at any time throughout the incubation, caused a reactivation of the phosphorylase kinase and this was accelerated by micromolar concentrations of cyclic AMP. The reactivation process could be completely abolished by the addition of a heat stable protein kinase inhibitor, implicating cyclic AMP dependent protein kinase in the activation reaction. Both the low and the high activity forms of the enzyme required micromolar quantities of Ca^{2+} for full activity (K_A = 0.6 μM). The two forms exhibit quite different pH dependencies and at the physiological pH of liver (pH 7.4) their activities differed by a factor of 5—10. Conversion of the lower activity form into the higher seems to affect only the V. K_m for muscle phosphorylase *b* (EC 2.4.1.1) was about 1 mg/ml for both enzyme forms.

Introduction

The sequence of events from hormonal impulse, via cyclic AMP, to the activation of glycogen phosphorylase (EC 2.4.1.1) and subsequent enhanced glycogenolysis, has been very well documented for the muscle system [1,2]. Our knowledge of these individual reactions in liver is still fragmentary, and it has been mainly by analogy with the muscle system that the cyclic AMP promoted phosphorylase activation in this tissue has been explained [3,4]. Our understanding of liver phosphorylase kinase [5—7] in particular lags behind the

Abbreviations: EGTA, ethylene glycol-2-(2-aminoethyl)-tetraacetic acid.

knowledge we already have of the muscle enzyme [2,8]. Its dependence on Ca^{2+} [6,7] and its activation following glucagon administration [6,9] mediated via cyclic AMP dependent protein kinase (EC 2.7.1.37) [9] have been established recently, but the exact molecular mechanisms involved in these processes still remain to be clarified. In this report we show that liver phosphorylase kinase can be inactivated in a Mg^{2+}-dependent reaction, presumably catalyzed by a phosphatase, and subsequently reactivated by the cyclic AMP dependent protein kinase. Some properties of the inactivated and reactivated liver phosphorylase kinase are discussed.

Materials and Methods

1. Materials

ATP and cyclic AMP were obtained from Sigma Chemical Co (St. Louis, Mo.) and Sephadex G-25 from Pharmacia (Uppsala, Sweden). Crystalline rabbit muscle phosphorylase *b*, isolated according to Fisher et al. ref. 10, was a gift of Dr. G. Defreyn. Protein kinase inhibitor was isolated up to the trichloroacetic acid precipitation step, as described by Walsh et al. [11]. After dialysis against 5 mM potassium phosphate pH 7.0, the preparation was clarified by centrifugation at 34 000 $\times g$ for 20 min. All chemicals used were of reagent grade.

2. Methods

Preparation of liver fractions. For these experiments, we used fed male Wistar rats, weighing about 150 g each. The animals were deeply anesthetized with ether, and bled from the jugular veins, after which the liver was quickly removed and cooled on ice. About 10 g liver was homogenized in two volumes of buffer containing 250 mM sucrose, 10 mM phosphate and 0.5 mM dithothreitol, pH 7.4, using a Potter-Elvehjem, glass-Teflon homogenizer. The homogenate was centrifuged for 15 min at 10 000 $\times g$ and the supernatant decanted through glass wool. In experiments with crude extracts (S_1) this was diluted two fold in the same buffer, bringing the protein concentration to 25 mg per ml. For the experiments with high speed supernatants, the 10 000 $\times g$ supernatant fraction was centrifuged for 1 h at 40 000 rev./min in a Beckman L2-65 B ultracentrifuge using a Ti 60 rotor and the clear supernatant again decanted through glass wool in order to remove traces of lipid material. This fraction contained over 90% of the phosphorylase kinase activity measured in the crude extracts. This high speed supernatant solution (A_{40}) (containing 30 mg of protein per ml) was then used either as such, or was first filtered through a small Sephadex G-25 (coarse) column: 1 ml of sample was applied to a 1 by 15 cm Sephadex column equilibrated with the same homogenizing buffer, and 1 ml of eluate (containing about 10 mg of protein) was collected after a volume equal to the void volume of the column had been eluted. This fraction is referred to as the high speed filtrate: $A_{40}F$. Crude extracts desalted over a Sephadex G-25 column are referred to as S_1F.

Enzyme assays. The amount of protein kinase inhibitor required to completely abolish the protein kinase activity (assayed as described before [9] with $5 \cdot 10^{-6}$ M cyclic AMP and using histone f_{2b} as substrate) was determined in each enzyme fraction and was then used in those experiments where it was

decided to block protein kinase activity.

For the assay of phosphorylase kinase, 20 μl of the rat liver preparation were used as enzyme source, in a total of 200 μl reaction mixture containing: 1 mM ATP, 10 mM magnesium acetate, 0.2 mg phosphorylase *b* (7 units, measured in the presence of 1 mM AMP), 50 mM NaF, 10 mM phosphate buffer and enough protein kinase inhibitor to completely block the protein kinase activity. The reaction was started with the addition of the enzyme preparation, unless mentioned otherwise. The phosphorylase kinase reaction was stopped after 0, 2, 4, 6, 10 and 15 min of incubation by diluting a 20 μl aliquot of the reaction mixture into 400 μl of cold 10 mM β-glycerolphosphate buffer pH 6.8, containing 45 mM β-mercaptoethanol and 20 mM NaF. 100 μl of these diluted samples were then used for the determination of phosphorylase *a* activity at 30° C for 1 h [12]. Linear reaction rates for phosphorylase kinase were obtained over a period of at least 10 min of incubation. 1 munit of phosphorylase kinase catalyzed the formation of 1 munit of phosphorylase *a* per min under these conditions.

Inactivation and reactivation of rat liver phosphorylase kinase. Crude extracts or high speed supernatant fractions of rat liver were preincubated at 30° C for various lengths of time, and assayed for phosphorylase kinase activity. The experimental set up for the reactivation process was as follows: after 60 min preincubation of the crude extract at 30° C, 20 μl of it were added to 120 μl of a reaction mixture containing 50 mM NaF, 1 mM ATP, 10 mM magnesium acetate and 10 mM phosphate buffer pH 7.4, allowing catalysis by endogeneous protein kinase. This activation was then stopped at different time intervals by the addition of 50 μl of protein kinase inhibitor solution and the phosphorylase kinase assay was started by the addition of 10 μl of a 20 mg per ml phosphorylase *b* solution.

Results

Inactivation and reactivation of phosphorylase kinase in crude extracts of rat liver.

When crude rat liver extracts were preincubated at 30° C, and assayed for phosphorylase kinase at various times, a gradual loss of the enzyme activity was observed (Fig. 1). This inactivation could be slowed down considerably (about 50%) but could not be completely prevented by the addition of 100 mM NaF to the preincubation medium. After 60 min of preincubation, the phosphorylase kinase activity had reached a constant level of about 25 munits/mg protein. This low activity form of the enzyme is referred to as "inactivated phosphorylase kinase".

Reactivation of this enzyme could be achieved by addition of Mg^{2+} ATP to the incubation medium, and this process was accelerated by cyclic AMP and prevented by the heat stable protein kinase inhibitor. Results are also shown in Fig. 1. When the activation was done in the presence of cyclic AMP ($5 \cdot 10^{-6}$ M), a much faster reactivation of the phosphorylase kinase was observed, and the presence of protein kinase inhibitor during this activation time completely prevented this (Fig. 1). Cyclic AMP only increased the rate of the reactivation of the phosphorylase kinase but did not influence the final activity level. This

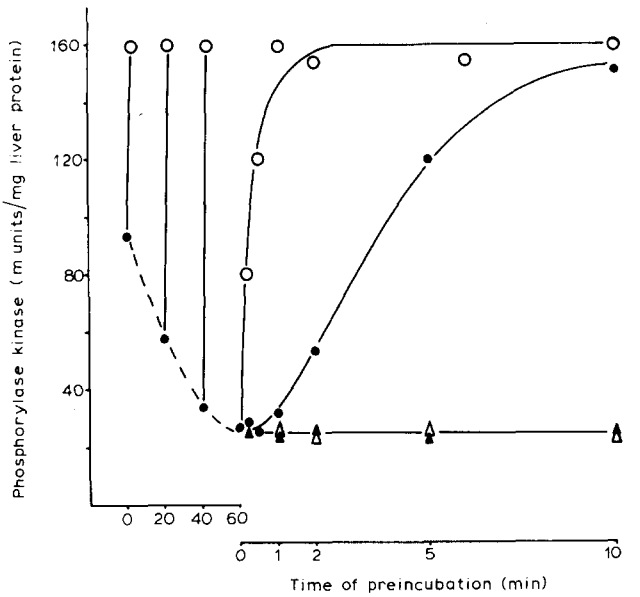

Fig. 1. Inactivation and reactivation of phosphorylase kinase. Crude extracts of rat liver were assayed for phosphorylase kinase activity after various times of preincubation at 30°C, as outlined in Methods. After 0, 20, 40 and 60 min of preincubation (- - - - -), reactivation of the enzyme was achieved by addition of MgATP, in the presence (○) or absence (●) of $5 \cdot 10^{-6}$ M cyclic AMP for various times, and again assayed for phosphorylase kinase activity as outlined in Methods. Incubation with MgATP in the presence of the protein kinase inhibitor in the presence (△) or absence (▲) of $5 \cdot 10^{-6}$ M cyclic AMP.

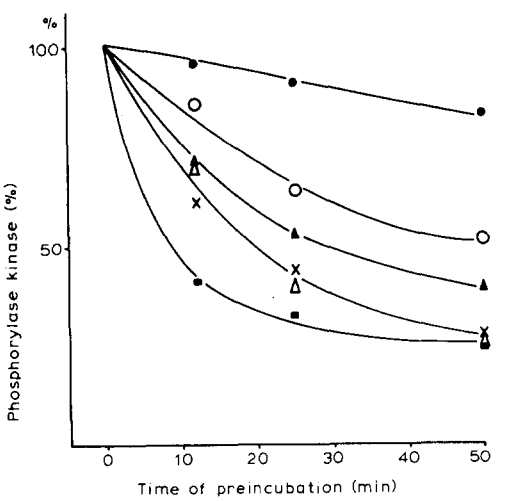

Fig. 2. Mg^{2+} dependency of the inactivation of phosphorylase kinase. Inactivation of the phosphorylase kinase was followed in crude extracts (S_1) (X), and crude extracts that were first filtered over a Sephadex G-25 column (S_1F) in the absence (●) or presence of respectively: 0.5 mM (○), 2.0 mM (▲), 5.0 mM (△), and 10.0 mM (■) magnesium acetate. Assays for phosphorylase kinase were done as outlined in Methods. The phosphorylase kinase activity at zero time was taken as 100% (corresponding to 210 munits per mg protein for the S_1F fractions, and 95 munits per mg protein for the S_1 fraction).

high activity form is referred to as "activated phosphorylase kinase". Neither preincubated crude extracts, nor partially inactivated enzyme preparations were activated to the same activity level (Fig. 1), showing that the inactivation of the enzyme can be reversed at any time of the incubation.

Mg^{2+} dependency of the inactivation of phosphorylase kinase

The inactivation of phosphorylase kinase did not require additional metal ions when measured in crude extracts. However, when crude extract fractions were filtered through a Sephadex G-25 column (S_1F), very little inactivation of phosphorylase kinase occurred (Fig. 2). Addition of 10 mM magnesium acetate to these S_1F fractions completely restored, and even increased the rate of inactivation beyond that observed with the crude extracts (S_1) (Fig. 2). A dose response for Mg^{2+} in the inactivation reaction is also shown in Fig. 2.

pH Profiles of inactivated and activated phosphorylase kinase

The pH dependency of the two forms of liver phosphorylase kinase was determined using a high speed supernatant fraction as enzyme source. The

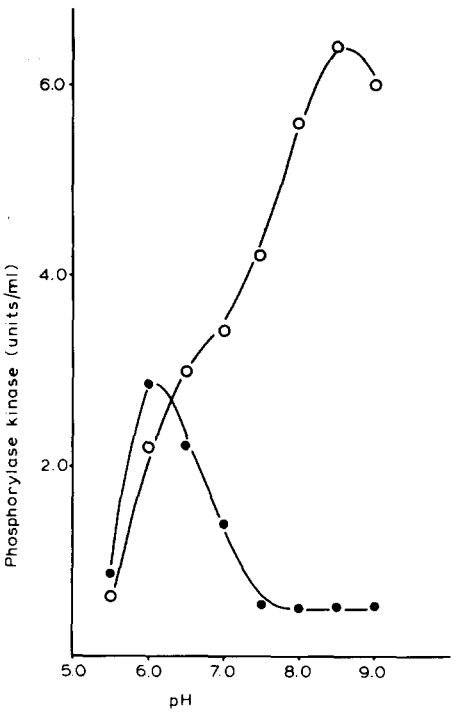

Fig. 3. pH-profiles of inactivated and activated phosphorylase kinase. High speed supernatant fractions, desalted over a Sephadex G-25 column ($A_{40}F$) were prepared as outlined in Results and diluted 3-fold into different pH buffers going from pH 5.5 to pH 9.0, containing each 50 mM Tris, 50 mM phosphate and 50 mM 2-(*N*-morpholino)-ethanesulfonic acid previously adjusted to the desired pH. 40 µl of these samples were then incubated with 160 µl of a reagent mixture containing: 0.2 mg phosphorylase *b*, protein kinase inhibitor, 1 mM ATP, 10 mM magnesium acetate, 100 mM NaF and 40 µl of the pH adjusted buffers. Phosphorylase kinase activities were followed in each case over a period of 10 min. Inactivated phosphorylase kinase (●) and reactivated phosphorylase kinase (○). The ordinate shows units of phosphorylase *a* formed per ml.

inactive phosphorylase kinase was prepared by incubating the A_{40} at 30° C for 50 min in the presence of 10 mM magnesium acetate. Part of this fraction was then further incubated at 30° C in the presence of 100 mM NaF, 1 mM ATP and $5 \cdot 10^{-6}$ M cyclic AMP for 5 min in order to get completely activated phosphorylase kinase. Both fractions were then passed through a Sephadex G-25 column, and the phosphorylase kinase activity determined at different pH values. The pH profiles for the two forms of phosphorylase kinase are shown in Fig. 3. The inactivated enzyme had a pronounced pH optimum at pH 6.0 whereas the activated form had its maximal activity at pH 8.5.

Ca^{2+} dependency of activated and inactivated phosphorylase kinase

Both enzymatic forms of phosphorylase kinase were about 70% inhibited when 0.5 mM EGTA was included in the reaction mixture (pH 7.4) and high speed supernatants were used as enzyme source. Readdition of 0.3 mM $CaCl_2$ in the presence of 0.5 mM EGTA, completely restored the enzyme activity for both forms. According to Portzehl et al. [13], this corresponds to a free calcium concentration of 4 μM. Half maximal stimulation of both enzyme forms occurred in the presence of 0.6 μM Ca^{2+}.

K_m for phosphorylase b

High speed supernatant fractions were used in the determination of the K_m of the two enzyme forms. These preparations were essentially free of phosphorylase activity; i.e. no detectable amounts of phosphorylase *a* were formed in the course of a usual phosphorylase kinase assay when no exogeneous phosphorylase *b* was included in the reaction mixture. At the physiological pH of liver (7.4) the K_m values for the substrate phosphorylase *b* were found to be 1.0 mg per ml for both the activated and inactivated phosphorylase kinase.

Discussion

When crude extracts or high speed supernatants from rat liver were incubated at 30° C, a gradual loss of phosphorylase kinase activity was observed (Fig. 1). Eventually, a constant level (25 munits/mg liver protein) of phosphorylase kinase activity was reached. This inactivation could be reversed by incubating the enzyme fraction with MgATP, in the presence or absence of cyclic AMP, and the heat stable protein kinase inhibitor completely prevented the reactivation. This activation-inactivation pattern completely parallels the phosphorylation-dephosphorylation scheme catalyzed by the cyclic AMP dependent protein kinase and the phosphorylase kinase phosphatase, respectively, as is known for the muscle system. Cyclic AMP accelerated the protein kinase promoted activation of phosphorylase kinase, but eventually the same level of activity was reached in the presence or absence of the cyclic nucleotide (160 munits/mg liver protein). The inactivation process, presumably catalyzed by a phosphatase, could not be completely blocked by the addition of NaF. When the crude liver extract or the high speed supernatant fraction was desalted over a Sephadex G-25, very little phosphatase activity was left (Fig. 2). The inactivation reaction could be completely restored, or even accelerated by addition of 5—10 mM magnesium acetate (respectively) to the preincubation mixture. Presum-

ably fluoride ions inhibit the inactivation reaction by removing essential Mg^{2+} from the phosphorylase kinase phosphatase.

Both the inactivated and activated phosphorylase kinase require micromolar quantities of Ca^{2+} for full activity, when assayed in the crude extracts or high speed supernatant fractions. Half maximal stimulation of both enzyme forms was seen with 0.6 μM calcium. These results confirm the already known calcium dependency of liver phosphorylase kinase [6,7] and are in agreement with recent data of van de Werve et al. [14] who have studied the calcium dependency of this enzyme before and after glucagon treatment.

When no Ca^{2+} was added exogeneously, 0.5 mM EGTA caused a 70% inhibition of the enzymatic activity of both forms. In contrast with the muscle enzyme, no complete inhibition could be obtained, not even by higher concentrations of EGTA as has also been reported by others [6,7,14]. While in skeletal muscle, the Ca^{2+} requirement of the phosphorylase kinase constitutes the link between muscle contraction and stimulation of glycogenolysis, in liver, its dependency on Ca^{2+} makes the phosphorylase kinase the target enzyme in the regulation of liver glycogen breakdown by non-cyclic AMP mediated hormonal stimuli [14,15].

The pH profile of the activated phosphorylase kinase is very similar to that of the rabbit skeletal muscle enzyme [16]. However, the inactivated enzyme exhibits a quite different pH dependency. Its optimal pH is around pH 6.0, whereas the muscle enzyme shows very little activity below pH 8.0 [16]. In agreement with these results is the observation that a homogeneous preparation of the catalytic subunit of rabbit liver phosphorylase kinase also has a pronounced pH optimum at 6.0 (Vandenheede, J.R., unpublished results). At the physiological pH of liver (7.4) the activated enzyme form is 5—10 times more active than the inactivated enzyme form.

At pH 7.4, the K_m for the substrate phosphorylase *b*, was found to be 1.0 mg per ml for the inactivated as well as for the fully activated phosphorylase kinase. This is in sharp contrast with the skeletal muscle enzyme where activation causes a 10-fold decrease in the K_m for phosphorylase *b* [17]. This would then indicate that the difference in activity between the two forms of liver phosphorylase kinase, does not seem to be due to an altered affinity of the enzyme for its substrate phosphorylase *b*.

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