A DEINHIBITOR PROTEIN NEUTRALIZING THE EFFECT OF THE PROTEIN INHIBITORS ON DOG LIVER PHOSPHORYLASE PHOSPHATASE

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Received 30 April 1977

1. Introduction

The level of active phosphorylase is the result of a balance between the activities of phosphorylase kinase and of phosphorylase phosphatase. In recent years, attention has been increasingly focused on the enzymes responsible for the dephosphorylation of phosphoproteins. Among them phosphorylase phosphatase and the protein inhibitors [1–5] of this enzyme have been studied in some detail. Several types of phosphorylase phosphatase have been extracted from dog liver [6]. Two of these are present in the cytosol: one type of enzyme (mol. wt. 77 000–215 000) is spontaneously active in vitro, but is presumed to be inhibited in vivo owing to the presence of small amounts of free ATP; the other is an ATP–Mg-dependent phosphorylase phosphatase (mol. wt 138 000), expected to be fully active in vivo. A third and still different phosphorylase phosphatase (mol. wt 51 000) is associated with particulate glycogen; it is active as such and presumably also active in vivo.

Protein kinase-dependent (inhibitor-1) and -independent (inhibitor-2) proteins extracted from dog liver [3,4] inhibit the ATP–Mg-dependent phosphorylase phosphatase from the cytosol as well as the spontaneously active enzyme associated with particulate glycogen [7]. The ATP-inhibited phosphatase extracted from the cytosol is not affected by either protein inhibitor.

Now we have isolated from the glycogen pellet a protein factor which cancels the effect of these protein inhibitors on phosphorylase phosphatase.

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2. Materials and methods

The glycogen pellet and the phosphorylase phosphatase (EC 3.1.3.17) from the cytosol were prepared according to Goris et al. [6]. The glycogen pellet was dissolved in a volume of 10 mM Tris–HCl, pH 7.4, one-fifth of the weight of the liver.

The preparation of the phosphorylase phosphatase inhibitors-1 and -2 has been described [4]. Inhibitor-1 was activated by incubation with a preparation of the catalytic unit [8] of protein kinase (EC 2.7.1.37) in the presence of 10 mM Tris–HCl, pH 7.4, 0.5 mM ATP and 2.5 mM Mg^{2+}, for 15 min at 30°C; after boiling for 5 min denatured protein was eliminated by centrifugation, and the activated inhibitor-1 was isolated by filtration through Sephadex G-25.

The heat-stable protein deinhibitor was extracted from the glycogen pellet by incubation in the presence of 1 M NaCl (added as solid salt) for 15 min at 30°C. The supernatant (260 000 × *g* for 2 h), after addition of 2 mg/ml β-lactoglobulin A [9], was dialyzed against 10 mM Tris–HCl, pH 7.4. After boiling for 5 min the protein deinhibitor was free of any phosphorylase phosphatase and kinase (EC 2.7.1.38) activity.

The preparation of liver phosphorylase (EC 2.4.1.1) and the assay of phosphorylase phosphatase have been described [10]. Phosphorylase phosphatase inhibitor activity was measured on 15–20 mU phosphorylase phosphatase. Deinhibitor activity was determined in

boiled preparations (5 min at 100°C) in the same assay system in the presence of a titrated amount of inhibitor that assured near-complete inhibition of phosphorylase phosphatase.

Treatment of the deinhibitor with 0.7 mg/ml trypsin (Worthington; lyophilysed trypsin 200 U/mg) or 1 mg/ml pronase P (Serva) in 10 mM Tris–HCl, pH 7.4, during 15 min at 30°C, was stopped by boiling for 5 min. When trypsin was used on phosphatase preparations, which are thermolabile, the proteolytic reaction was arrested by the addition of a four-fold excess (w/w) of trypsin inhibitor (type I-S from Sigma).

3. Results and discussion

Phosphorylase phosphatase isolated from the glycogen complex by DEAE-cellulose chromatography could be inhibited completely by inhibitor-1 as well as by inhibitor-2 [10]. When we examined the effect of the protein inhibitors on the enzyme present in the crude glycogen fraction the phosphorylase phosphatase activity was not or only slightly affected although no detectable amount of inhibitor was present in the glycogen pellet. The phosphorylase phosphatase isolated from the glycogen complex was inhibited by added particulate glycogen (0.5–6% of a preparation according to Drochmans [11]) and this inhibition was also observed in addition to the effect of the protein inhibitors-1 and -2. Therefore other factor(s) present in the glycogen pellet were assumed to be responsible for the reduced inhibition of the

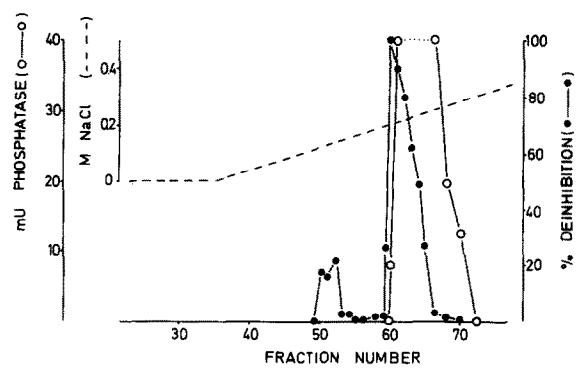

Fig.1. Elution profile of phosphorylase phosphatase and the phosphorylase phosphatase deinhibitor of the glycogen pellet from a column of DEAE-cellulose. 10 ml of the dissolved glycogen pellet was diluted with 2 vol. 20 mM Tris, pH 7.8, and applied to a DEAE-cellulose (Whatman DE-52) column (0.9 × 15 cm) equilibrated with 10 mM Tris–HCl, pH 7.8. The eluate obtained by application of a NaCl gradient in the same buffer, was passed immediately through a continuous flow dialyzing device (DOW Hollow Fiber, model b/HFD) for dialysis against 10 mM Tris–HCl, pH 7.4. Fractions (3 ml) were collected and assayed for phosphorylase phosphatase and deinhibitor activity. Deinhibitor activity was measured on the phosphorylase phosphatase extracted from the cytosol in the presence of 0.5 mM ATP, 2.5 mM Mg^{2+} and inhibitor-2.

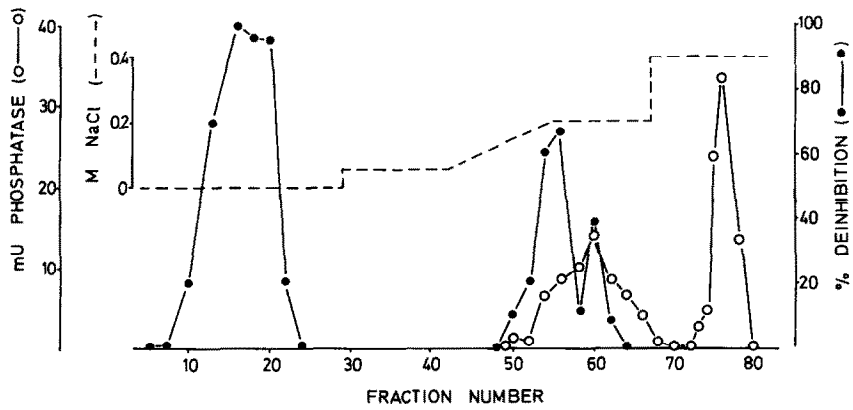

Fig.2. Separation by DEAE-cellulose chromatography of phosphorylase phosphatase and the phosphorylase phosphatase deinhibitor of the glycogen pellet after mercaptoethanol treatment. 10 ml of the dissolved glycogen pellet was incubated during 15 min at 30°C with 0.2 M mercaptoethanol. The preparation was stored at −18°C for at least 1 h, thawed and diluted with 20 ml solution containing 20 mM MES-buffer, pH 6.5, and 5 mM mercaptoethanol. DEAE-Cellulose chromatography and assays were performed as detailed in fig.1, except for the elution with MES-mercaptoethanol buffer and the NaCl gradient.

phosphatase by the protein inhibitors. The effect of this factor(s) (a 'deinhibitor') persisted after boiling of the glycogen fraction for up to 30 min.

Phosphorylase phosphatase and deinhibitor could not be readily separated by chromatography of the glycogen pellet on DEAE-cellulose (fig.1). This figure shows that the phosphorylase phosphatase extracted from the glycogen pellet by the previously described method [10] still contains heat-stable deinhibitor, well separated from glycogen which is not retained by the DEAE-cellulose. Separation of the phosphorylase phosphatase and the deinhibitor was obtained when the glycogen fraction was treated with mercaptoethanol prior to DEAE-cellulose chromatography (fig.2). Under these experimental conditions most of the deinhibitor was not retained by the DEAE-cellulose; a small amount eluted with the first peak of phosphorylase phosphatase. The fractions eluting at 0.4 M NaCl contained phosphorylase phosphatase, but no measurable amount of deinhibitor. These fractions were pooled and used for further experiments.

The deinhibitor-free phosphorylase phosphatase was sensitive to the activated inhibitor-1 as well as to inhibitor-2 (fig.3). The inhibition was, as might be expected, more pronounced than the inhibition observed with the glycogen phosphorylase phosphatase previously used [7] since the latter fraction contains deinhibitor.

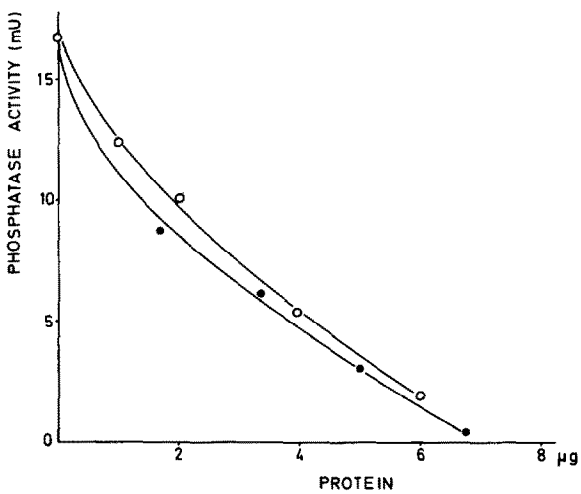

Fig.3. Effect of inhibitor-1 and -2 on the deinhibitor-free phosphorylase phosphatase prepared from the liver glycogen pellet. Phosphorylase phosphatase activity was measured in the presence of increasing concentrations of activated inhibitor-1 (●——●) or inhibitor-2 (○——○).

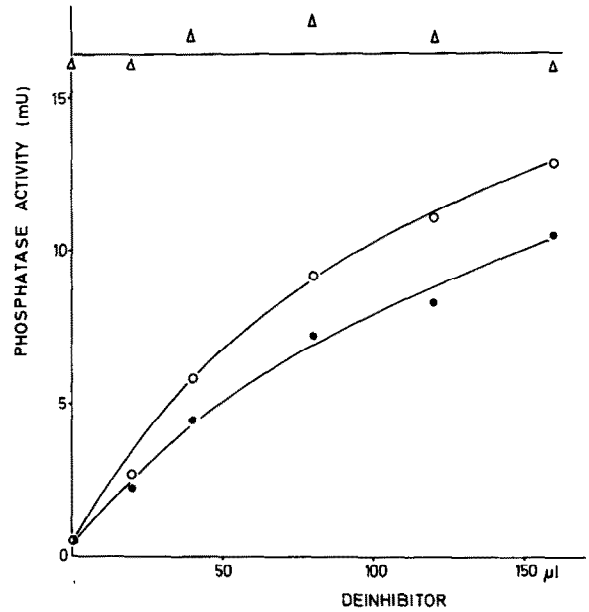

Fig.4. Deinhibition of deinhibitor-free phosphorylase phosphatase prepared from the liver glycogen pellet. Phosphorylase phosphatase activity was measured in the presence of 7 µg activated inhibitor-1 (●——●), 10 µg inhibitor-2 (○——○) or in their absence (△——△), and increasing concentrations of deinhibitor.

As shown in fig.4, the effect of both activated inhibitor-1 and inhibitor-2 was neutralized by the deinhibitor. The deinhibition of phosphorylase phosphatase was concentration dependent, but the effect of inhibitor-2 was suppressed more easily than the effect of the activated inhibitor-1. Similar results were obtained with the ATP–Mg-dependent phosphatase from the cytosol. Indeed this feature was made use of in the assays of deinhibitor activity in fig.1 and 2.

The deinhibitor is thermostable, resisting at boiling during 30 min without loss of activity, and could be recovered quantitatively after alcohol precipitation (5 vol. 95% ethanol at 25°C). However since the deinhibitor is non-dialyzable, and completely destroyed by pronase (not shown) or trypsin (fig.5), it was concluded that the deinhibitor is a protein.

Activity of the phosphorylase phosphatase present in the glycogen pellet, and isolated by DEAE-cellulose

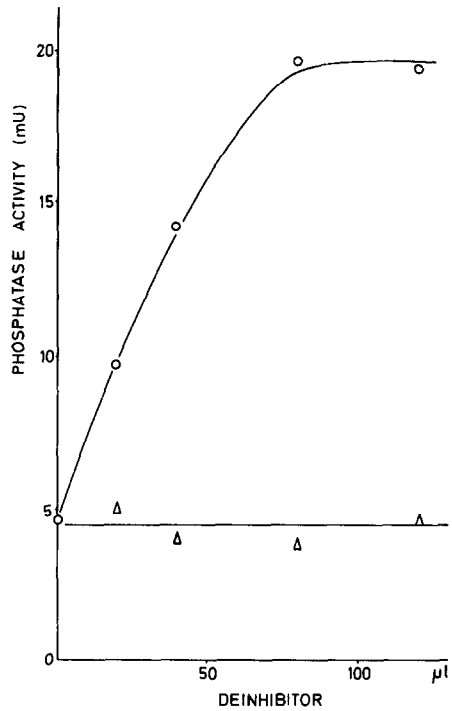

Fig.5. Effect of trypsin on the deinhibitor. The activity of deinhibitor-free phosphorylase phosphatase (20 mU) was measured in the presence of inhibitor-2 (8 µg) and increasing concentrations of untreated (o——o) or trypsin-treated deinhibitor (Δ——Δ).

chromatography (fig.1) was not affected by trypsin treatment, but the sensitivity of the phosphatase to both the activated inhibitor-1 and inhibitor-2 was increased. This observation could be accounted for by the destruction of the contaminating protein deinhibitor.

We have considered the possibility that the deinhibitor activity would be due to a modified phosphorylase phosphatase protein, devoid of phosphatase activity but capable of competitive binding of the inhibitors. However, our observations that the deinhibitor, unlike the phosphatase, is trypsin-labile, and that the phosphatase could be separated from the deinhibitor (fig.2), argue against this hypothesis. Furthermore heat-denaturation of the trypsin-treated phosphatase and of the deinhibitor-free phosphatase (fig.2) did not generate deinhibitor activity.

Since glycogen synthesis requires the existence of phosphorylase in the inactive b form, phosphorylase phosphatase should be active under these conditions; glycogenolysis, on the other hand would evidently be favored by a low activity of phosphorylase phosphatase. Hepatic glycogenolysis can be promoted via cyclic AMP and protein kinase-mediated activation of inhibitor-1 of phosphorylase phosphatase; the reverse reaction is catalyzed by a protein phosphatase that is now under investigation. No mechanism is known for the regulation of inhibitor-2 activity. The association with liver glycogen of a deinhibitor, neutralizing both inhibitor-1 and inhibitor-2, could constitute the mechanism by which phosphorylase phosphatase is kept in the active state during accumulation of glycogen. We speculate that during cyclic AMP-initiated glycogenolysis, inhibitor-1 is phosphorylated and overcomes the protective effect of the deinhibitor. Such a mechanism could account for an inhibition of phosphorylase phosphatase during cyclic AMP-stimulated glycogenolysis.

Acknowledgements

The authors are grateful to R. Verbiest and R. Bollen for expert technical assistance. This work was supported by the 'Fonds voor Wetenschappelijk Geneeskundig Onderzoek'.

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