A PROTEIN INHIBITOR OF RABBIT LIVER PHOSPHORYLASE PHOSPHATASE

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SUMMARY. Extracts of rabbit liver contain a heat-stable, non-dialysable inhibitor of phosphorylase phosphatase. The inhibitory activity is destroyed by trypsin treatment or by ethanol precipitation. The kinetics of the inhibition are non-competitive with respect to phosphorylase *a*. The inhibitory activity is polydisperse on gel permeation chromatography. The mechanism of the inhibition is due to a direct interaction of the protein inhibitor with the enzyme.

During the development of a procedure for the isolation of rabbit liver phosphorylase phosphatase, a consistent activation of enzyme activity was noted during an ethanol precipitation step (1,2). In a study of rat tissue extracts we have shown that the activation by the ethanol treatment is accompanied by the conversion of the enzyme activity from multiple molecular weight forms into a single form of lower molecular weight, *ca.* 32,000 (3). We have also proposed that phosphorylase phosphatase exists in an inactive form consisting of a complex of the low molecular weight enzyme (the catalytic subunit) with an inhibitor protein (3). In this report we present evidence for the existence of a proteinaceous inhibitor of phosphorylase phosphatase and a preliminary characterization of the form present in rabbit liver.

METHODS

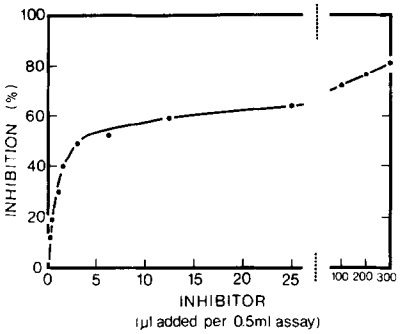
Preparation of phosphorylase phosphatase inhibitor. All procedures were carried out at 4°C unless otherwise indicated. Freshly excised rabbit livers were homogenized in a commercial blender (low speed, 20 seconds) with three volumes of IED buffer (50 mM imidazole chloride, 5 mM EDTA, 0.5 mM dithiothreitol, pH 7.45). After centrifugation (10,000g, 20 minutes) the crude extract was divided into 10 ml portions in test-tubes. These were heated in a boiling water bath until the temperature of the contents reached 90°C, and then for a further 5 minutes. After cooling, the suspensions were pooled with an additional 1.5 volumes of IED buffer. The suspension was homogenized in a blender (15 seconds)

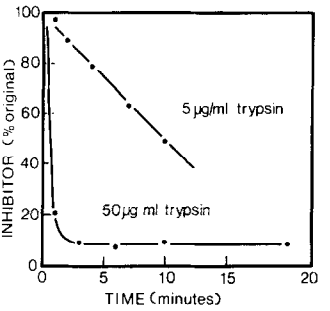
and again centrifuged (10,000g, 20 minutes). The supernatant was brought to 75% saturation with ammonium sulfate. The ammonium sulfate precipitate was collected by centrifugation, resuspended and dialysed exhaustively against IED buffer. The preparation was then centrifuged at 100,000g (90 minutes) to remove glycogen. An approximately 10-fold purification of the inhibitor was obtained by this procedure, assuming complete recovery of the inhibitor. The experiments described in this report were carried out with a preparation which contained 57 mg protein/ml. *Phosphorylase phosphatase assay.* This was determined by the conversion of phosphorylase *a* into phosphorylase *b* (2). The assay system contained 0.2 mg/ml rabbit muscle phosphorylase *a*, 50 mM imidazole chloride, 0.5 mM EDTA, 0.5 mM dithiothreitol and 5 mM theophylline, pH 7.2. One unit of enzyme activity was that which converted 0.2 mg of phosphorylase *a* per minute at 30°C. *Inhibitor activity.* The inhibitor activity was determined as the percentage inhibition of phosphorylase phosphatase activity in the assay system described above to which 0.025 units of phosphorylase phosphatase was added (per 0.5 ml assay volume). *Phosphorylase phosphatase.* For most of the experiments a partially purified preparation of rabbit liver phosphorylase phosphatase was used; this had been carried to the DEAE-Sephadex stage (1,2) and had a specific

TABLE I. THE INHIBITION OF PHOSPHORYLASE PHOSPHATASE BY A HEAT-STABLE, NON-DIALYSABLE INHIBITOR

ENZYME SOURCE	INHIBITOR SOURCE	INHIBITION (%)
A. Rabbit liver	Rabbit liver	61
Rabbit liver	Rat liver	57
Rabbit liver	Rat heart	56
Rabbit liver	Rat muscle	57
B. Rabbit liver (ethanol-activated)	Rabbit liver	70
Rabbit liver (crude extract)	Rabbit liver	7
Rat liver (ethanol-activated)	Rabbit liver	77
Rat liver (crude extract)	Rabbit liver	10

For the rabbit liver inhibitor, 5 µl of inhibitor preparation (METHODS) were added per 0.5 ml assay. For the inhibitor of the rat tissues, 100 µl of inhibitor solution were used; this was 3:1 (v/w) homogenate which was heated to 100°C for 5 minutes, and extensively dialysed after centrifugation. In part A, partially purified rabbit liver enzyme (185 units/mg) was used. In part B, the crude extracts and the ethanol-activated enzymes were prepared as described previously (3).





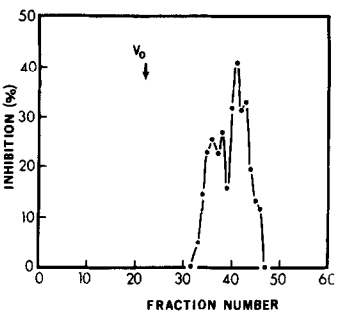


Figure 1. Concentration Dependence of the Inhibition of Rabbit Liver Phosphorylase Phosphatase by the Heat-Stable Protein Inhibitor.

Figure 2. The Effect of Trypsin on the Inhibitor of Phosphorylase Phosphatase. The inhibitor preparation (51 mg/ml protein) was incubated with either 5 or 50 µg/ml of bovine pancreas trypsin (TPCK-treated; Worthington Biochemical Corp.) at 30°C. Trypsinization was stopped by the addition of a four-fold excess by weight of soybean trypsin inhibitor (Worthington Biochemical Corp., Type SI). The inhibitory activity was then determined as described in the text.

Figure 3. Behavior of the Phosphorylase Phosphatase Inhibitor Preparation on Biogel 0.5m Chromatography. The rabbit liver inhibitor preparation (1 ml) was chromatographed on a Biogel 0.5m column (exclusion limit, M.W. 500,000; 1.6 x 60 cm) equilibrated with 0.1M NaCl in IED buffer. Fractions of 2 ml each were collected and assayed for inhibitory activity. V_0 refers to the void volume.

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activity of 185 units/mg protein. In some experiments homogeneous phosphorylase phosphatase (2) having a specific activity of 2,100 units/mg protein was used.

RESULTS AND DISCUSSION

Demonstration of a heat-stable, macromolecular inhibitor of phosphorylase phosphatase in mammalian tissues.

Phosphorylase phosphatase in tissue extracts may be activated by treatment with a high concentration of ethanol at room temperature (3). Using the activated form of phosphorylase phosphatase, the presence of a heat-stable, non-dialysable inhibitory factor is readily demonstrated in several mammalian tissues (Table I). Activated rabbit liver phosphorylase phosphatase is inhibited by a heat-treated and dialysed extract of rabbit liver, as well as by similarly treated extracts of rat liver, heart and skeletal muscle (Table I). The rabbit liver inhibitor also inhibits ethanol-activated phosphorylase phosphatase from rat liver

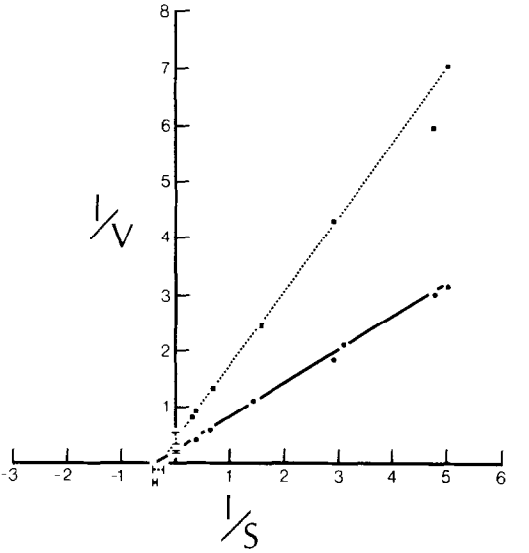


Figure 4. Kinetics of the Inhibition of Phosphorylase Phosphatase by the Protein Inhibitor.
Values of S on the double reciprocal plot are μM rabbit muscle phosphorylase *a* (dimer); values of V are moles phosphorylase *a* converted per mole of phosphorylase phosphatase per second. (■ ····· ■): with 2.5 μl of inhibitor preparation per 0.5 ml assay. (● —— ●): no inhibitor added. Lines represent the best fit obtained by the method of Bliss and James (8) using the computer program of Hanson *et al.* (9) adapted for the Univac 1106 system by Dr. J.F. Woessner of the University of Miami. Values obtained for K_m were: minus inhibitor, 2.7 μM (2.4 - 3.0); plus inhibitor, 3.1 μM (2.3 - 4.4). Values obtained for V_{max} were: minus inhibitor, 4.5 (4.2 - 4.8); plus inhibitor, 2.4 (1.9 - 2.8). Values in parentheses indicate the 95% confidence limits for the data.

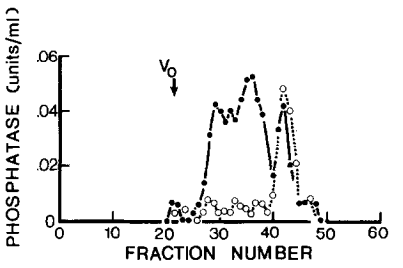
(Table I). On the contrary, if non-activated phosphorylase phosphatase is used, as in untreated liver extracts, little inhibition is observed (Table I).

Properties of the Phosphorylase Phosphatase Inhibitor from Rabbit Liver.

The concentration dependence of the inhibition of rabbit liver phosphorylase phosphatase by a rabbit liver inhibitor preparation is shown in Fig. 1. The maximal inhibition observed in this experiment was about 80%.

The inhibitory activity was not affected by incubation with RNase, DNase, or α-amylase, but was almost completely destroyed by trypsin treatment (Fig. 2), or by the ethanol treatment described previously (3). The inhibitory activity was also acid-stable, since it could be recovered without loss after trichloroacetic acid (15%) precipitation.

The behavior of the inhibitor preparation on a Biogel 0.5m agarose column is



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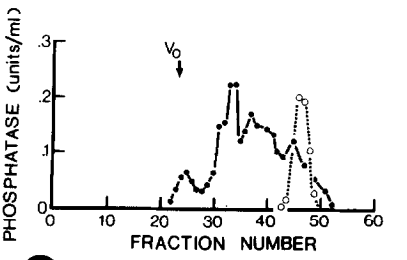

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Figure 5. Behavior of a Mixture of the Rabbit Liver Inhibitor and Purified Rabbit Liver Phosphorylase Phosphatase on Biogel 0.5m Chromatography. Purified phosphorylase phosphatase (4 units) was mixed with 1.5 ml of inhibitor solution. Column conditions were as for Fig. 3. The fractions were assayed for phosphorylase phosphatase activity before (o·····o) and after treatment with trypsin (●——●). Conditions for trypsin treatment were 22 µg/ml of trypsin for 6 minutes at 30°C followed by addition of a four-fold excess by weight of soybean trypsin inhibitor.

Figure 6. Behavior of Rabbit Liver Phosphorylase Phosphatase Activity on Biogel 0.5m Chromatography.
(o·····o): the elution profile of homogeneous rabbit liver phosphorylase phosphatase (M.W. 34,000, ref. 2).
(●⁓●): the elution profile of phosphorylase phosphatase activity in a crude extract of rabbit liver. Column conditions as in Fig. 3.

shown in Fig. 3. The inhibitory activity was eluted over a wide span, indicating the presence of multiple molecular weight forms. These extended over an approximate range of molecular weight from 50,000 - 250,000.

These results establish the existence of a protein inhibitor of phosphorylase phosphatase in rabbit liver. Since the inhibitory activity is destroyed by the same ethanol treatment which activates phosphorylase phosphatase, the suggestion that the latter effect may be due to the selective removal of a protein inhibitor (3) appears to be confirmed. The possible functional or physiological significance of this inhibitor is unknown, although it may be noted that a protein inhibitor (M.W. less than 50,000) of glycogen synthase phosphatase has been shown to be present in mouse Ehrlich ascites carcinoma cells which do not synthesize glycogen (4). It is of interest that both the glycogen synthase phosphatase inhibitor (4) and a protein inhibitor of cAMP-dependent protein kinase from rabbit muscle (5) have in common with our inhibitor a stability to heat and acid treatment.

Mechanism of the Inhibition of Phosphorylase Phosphatase.

The kinetics of the inhibition of phosphorylase phosphatase were examined (Fig. 4). The inhibition was non-competitive with respect to rabbit muscle phosphorylase *a* (Fig. 4), so that the inhibition was not due to the presence of a competitive phosphoprotein substrate. An indication that the inhibitor affects the enzyme and not the phosphorylase substrate comes from preliminary observations (6) that the inhibitor also inhibits the glycogen synthase phosphatase activity (7) of homogeneous rabbit liver phosphorylase phosphatase.

Physical evidence that the inhibitor interacts with phosphorylase phosphatase was obtained in experiments in which a mixture of homogeneous rabbit liver phosphorylase phosphatase (M.W. 34,000) and the rabbit liver inhibitor was chromatographed on a Biogel 0.5m agarose column (Fig. 5). A peak of phosphorylase phosphatase activity was found (Fig. 5) at the volume where the enzyme elutes when chromatographed on its own (Fig. 6). However, when the column fractions were treated with trypsin to destroy the inhibitor, the enzyme itself being stable to this treatment (6), most of the phosphorylase phosphatase activity was now revealed in regions of much higher molecular weight (Fig. 5). These results show that the inhibitor is capable of interacting with phosphorylase phosphatase to form rather stable complexes of higher molecular weight, and of much lower activity. As the inhibitor is itself polydisperse (Fig. 3), this would account for the generation of multiple molecular weight forms of the inactive enzyme. The elution profile (Fig. 5) of the reconstituted inactive, or less active forms, does in fact bear a striking resemblance to that of phosphorylase phosphatase activity in a crude rabbit liver extract (Fig. 6).

The demonstration of a protein inhibitor of phosphorylase phosphatase and the preliminary examination of its properties presented here, lends support to our hypothesis that phosphorylase phosphatase may exist in inactive forms which consist of stable enzyme-inhibitor complexes (3). Rigorous proof that phosphorylase phosphatase exists in an inactive form in which a catalytic subunit is specifically complexed with a unique inhibitor protein subunit awaits the

isolation and characterization of the holoenzyme. However, all the properties of the heat-stable inhibitor protein are consistent with the possibility that it may represent a specific inhibitor moiety.

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