MULTIPLE FORMS OF PROTEIN PHOSPHATASE INHIBITORS IN MAMMALIAN TISSUES

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Summary: The heat-stable inhibitors of phosphorylase phosphatase from rat skeletal muscle, rat liver, beef heart, and beef adrenal cortex were studied. Two major types of inhibitors can be separated from all these tissues by chromatography on DEAE-cellulose. The two types of inhibitors from rat muscle are the same as those reported for rabbit muscle. The type-1 inhibitor, but not the type-2, is regulated by phosphorylation and dephosphorylation reactions. There is also a phosphorylatable inhibitor in beef heart and adrenal cortex, but such an inhibitor could not be demonstrated in liver.

INTRODUCTION

Regulation of glycogen metabolism in mammalian tissues has been studied in great detail at the level of phosphorylase, phosphorylase kinase, and glycogen synthase (1-6). These enzymes exist in phospho- and dephospho- forms, and their activities are controlled by direct phosphorylation and dephosphorylation reactions (1-6). Phosphorylase phosphatase, another glycogen metabolizing enzyme, may also exist as active and inactive forms (7-9), but the reactions involved in the conversion have been difficult to study since the enzyme appears to have multiple molecular forms (9-11), copurifies with other phosphoprotein phosphatase activities (12,13), and undergoes activation during purification (14).

In crude preparations, the enzyme is inactivated by ATP and activated by ATP-Mg (7,8,15). The enzyme from muscle and adrenal cortex (7,8) has been shown to be inactivated by cyclic AMP in the presence of ATP-Mg, but such an effect has not been observed for the liver enzyme (15). In rabbit muscle we found that this inhibitory effect of cyclic AMP is associated with the phosphorylation of a heat-stable protein by the cyclic AMP-dependent protein kinase (16). When phosphorylated, this heat-stable protein is a potent inhibitor of phosphorylase phosphatase. We have also described another heat-stable inhibitor of phosphorylase

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phosphatase from rabbit muscle (17,18), which does not require phosphorylation for inhibitor activity. This latter type of inhibitor from rabbit muscle was also observed by Cohen et al. (19) and was shown to be the specific inhibitor for the phosphatase that dephosphorylates phosphorylase, synthase, and β-phosphorylase kinase.

We are interested in the physiological function(s) of these inhibitors. Studies with an isolated hind limb perfusion system showed that the activities of phosphorylase phosphatase and its inhibitor are inversely affected by the administration of epinephrine; results of the studies will be described elsewhere (20). The present study demonstrated the appearance of multiple forms of phosphatase inhibitors in various mammalian tissues. In adrenal cortex and muscle, where there is an effect of cyclic AMP on phosphatase activity, there is a phosphorylatable inhibitor. The nonresponsiveness of liver phosphatase to cyclic AMP appears to be due to the absence of such an inhibitor in liver.

MATERIALS AND METHODS [γ-^{32}P]ATP was obtained from New England Nuclear Corporation. ATP and cyclic AMP were purchased from P-L Biochemicals. DEAE-cellulose (DE-52) was obtained from Whatman. Acrylamide and sodium dodecylsulfate were from Bio-Rad Laboratory. Phosphorylase a and b were products of Sigma Chemical Company.

Rabbit muscle phosphorylase phosphatase used in the inhibitor assay was prepared as previously described (16). ^{32}Phosphorylase phosphatase activity was measured by the release of ^{32}Pi from [^{32}P]phosphorylase a (16). [^{32}P]phosphorylase a was prepared by enzymatic phosphorylation of phosphorylase b according to the method of Torres and Chelala (21). A unit of phosphatase is defined as that amount of enzyme which releases one μmole of [^{32}P]phosphate per min at 30°. Inhibitor activity was determined as the percentage inhibition of rabbit muscle phosphorylase phosphatase in an assay which contained 5 μU of phosphatase.

Rabbit muscle cyclic AMP-dependent protein kinase for the phosphorylation of inhibitor was prepared by the method of Wastila et al. (22) and was further purified by hydroxyapatite column chromatography (23). Rabbit muscle Mn^{++}-stimulated phosphoprotein phosphatase was prepared by the method of Kato and Bishop (10). Standard conditions for the phosphorylation of the inhibitor were incubation of the inhibitor for 10 min with protein kinase, 120 mU/ml; cyclic AMP, 0.05 mM; ATP, 0.1 mM; and magnesium acetate, 5 mM. Conditions for dephosphorylation were phosphatase, 3 mU/ml; $MnCl_2$, 5 mM; and incubation for 10 min.

Inhibitors from various tissues were isolated and separated on DEAE-cellulose columns as described for rabbit muscle inhibitors (18). Different inhibitor fractions were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis according to the procedure of Shapiro et al. (24).

RESULTS AND DISCUSSION DEAE-cellulose column chromatography of rat skeletal muscle phosphatase inhibitors is shown in Fig. 1. Prior to protein kinase

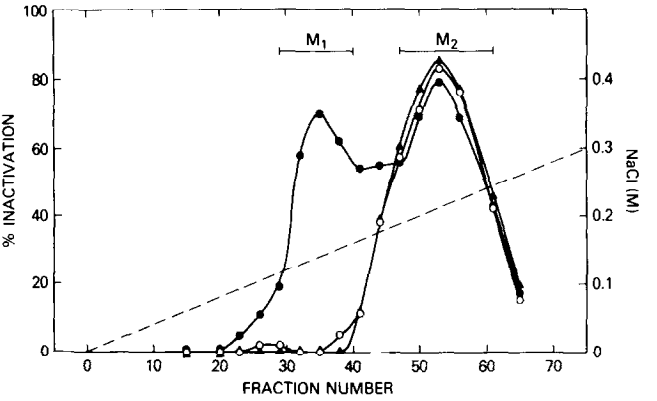


Fig. 1 . DEAE-cellulose column chromatography of rat muscle phosphatase inhibitors. Extract was made from 80 g of fresh hind limb muscle of rats. Column for separation was 2.5 x 15 cm. Linear gradient of 400 ml of 0-0.4 M NaCl was used for elution. Fractions of 4 ml were collected. Inhibitor activity without treatment, (○); after protein kinase treatment, (●); after phosphatase treatment, (▲).

treatment, a single activity peak was located at 0.21 M NaCl. After treatment with protein kinase, a second inhibitor peak was located at 0.14 M NaCl. The elution sequence and response to phosphorylation of these two peaks are similar to those of rabbit muscle (18) and were designated as M_1 and M_2. With the treatment of Mn^{++}-stimulated rabbit muscle phosphatase, activity under M_2 was not affected.

Separation of rat liver inhibitors on DEAE-cellulose column is shown in Fig. 2. Unlike that of muscle, two phosphatase inhibitor peaks were located at 0.14 M and 0.22 M NaCl and were designated as L_1 and L_2 respectively. Following treatment with protein kinase the activity of L_1 was greatly reduced, and the latter portion of the peak was inhibited to a greater extent than the portion eluting at a lower salt concentration. There was no change in L_2 activity. A different pattern emerged following treatment of the fractions with phosphatase; L_1 activity was not altered, but L_2 activity was increased.

Fig. 3 shows the DEAE-cellulose chromatography of inhibitors from beef adrenal cortex. A major peak of activity designated A_2 was located at 0.22 M

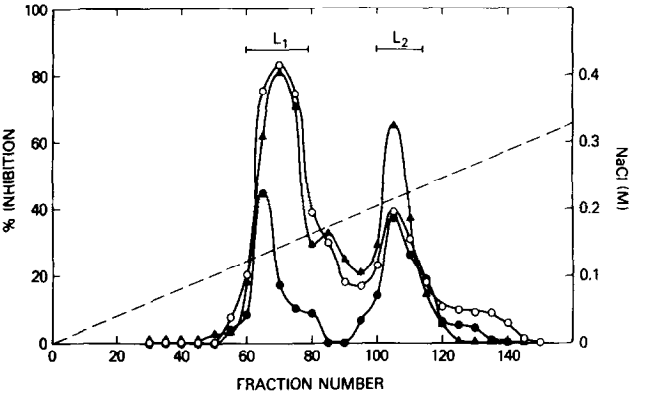
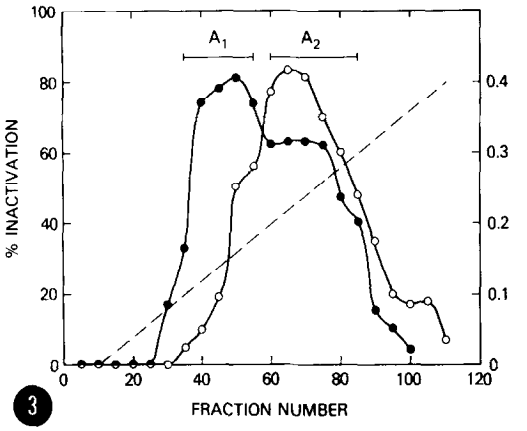


Fig. 2 DEAE-cellulose column chromatography of rat liver phosphatase inhibitors. 60 g of fresh rat liver was used. Column size was 2.5 x 18 cm. Gradient was 1000 ml of 0-0.4 M NaCl. Fractions of 5 ml were collected. Inhibitor activity without treatment, (○); with kinase treatment, (●); with phosphatase treatment, (▲).

NaCl and had a leading shoulder of activity at 0.16 M NaCl. Following treatment with protein kinase a major activity peak (A_1) appeared at a salt concentration of 0.14 M and A_2 activity was slightly reduced.

Beef heart inhibitors were separated on a DEAE-cellulose column (Fig. 4). The majority of activity (H_2) was located between salt concentrations of 0.2 M to 0.24 M NaCl. Two minor peaks of activity were located at 0.10 M and 0.17 M NaCl. After the treatment with protein kinase, a distinct peak of activity (H_1) was located at 0.15 M NaCl, and H_2 activity was reduced and appeared to contain more than one molecular species.

The two major types of inhibitor fractions from each column (indicated by bars) were pooled and concentrated by Amicon ultrafiltration using a UM-2 membrane. Effect of protein kinase on inhibitor activity of the concentrated fractions were reexamined (Table I). After treatment with protein kinase, reactions were maximally diluted to reduce the reactant concentration of ATP-Mg and cyclic AMP in the final mixtures. M_1, A_1, and H_1 were activated by protein kinase; L_1, A_2, and H_2 were inactivated by this treatment; and L_2 and M_2 remained unchanged. In



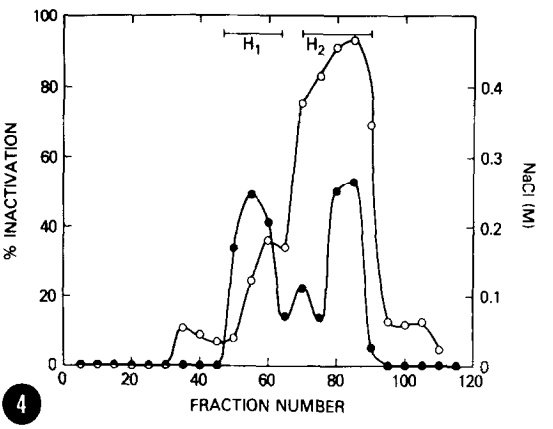


Fig. 3 DEAE-cellulose column chromatography of beef adrenal cortex phosphatase inhibitors. 220 g of fresh tissue was used where medulla portions had been removed. Column was 4 x 20 cm. Gradient was 1200 ml of 0-0.4 M NaCl. Fractions were 10 ml. Inhibitor activity before (0) and after (●) protein kinase treatment.

Fig. 4 DEAE-celluloase column chromatography of phosphatase inhibitors from beef heart. 300 g of tissue was used. Column size was 5 x 15 cm. Gradient was 1400 ml of 0-0.4 M NaCl and fractions were 10 ml. Inhibitor activity before (0) and after (●) protein kinase treatment.

all the cases, when protein kinase was omitted, no such changes could be observed (data not shown).

Concentrated inhibitor fractions were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis. As shown in Fig. 5, each inhibitor fraction gave one major activity peak with some minor contamination of the other peak. The origin of the minor activity peak between the two major inhibitor fractions in liver and the peak in the high-molecular-weight region in the heart preparation are presently unknown. Except for L_1 and A_2, all the inhibitors appear to have a molecular weight of 30,000 $\pm$ 5,000, which is close to that of inhibitor-1 and inhibitor-2, 26,000 and 33,000 respectively, of rabbit muscle.

The separation profile of rat muscle heat-stable phosphatase inhibitors on DEAE-cellulose is identical to that of rabbit muscle (18). There are two types of inhibitor: type M_1 is activated by phosphorylation with the cyclic AMP-

Table 1

Effect of protein kinase on phosphatase inhibitors. Conditions for protein kinase treatment were as described in the Methods. After the treatment, liver and muscle fractions were diluted 50-fold and adrenal and heart fractions were diluted 100-fold into the phosphatase assay in mixture.

	% Inactivation	
Fraction	Before treatment	After treatment
L_1	59	11
L_2	49	51
M_1	3	83
M_2	64	65
A_1	29	80
A_2	40	30
H_1	46	70
H_2	74	20

dependent protein kinase and is inactivated by dephosphorylation; type M_2 is not affected either by phosphorylation or dephosphorylation. Though the molecular weight of M_1 and M_2 are not exactly the same as those of rabbit muscle, these two inhibitors appear qualitatively similar with respect to the relationship between their state of phosphorylation and their activity. It is the type-1 inhibitor that makes muscle phosphatase sensitive to inactivation by cyclic AMP (8). The mechanism involves the conversion of an inactive (dephospho-) inhibitor to an active (phospho-) inhibitor by a phosphorylation catalyzed by the cyclic AMP-dependent protein kinase. The phosphorylation site has recently been determined (25).

There are multiple forms of phosphatase inhibitors in rabbit liver (26); this

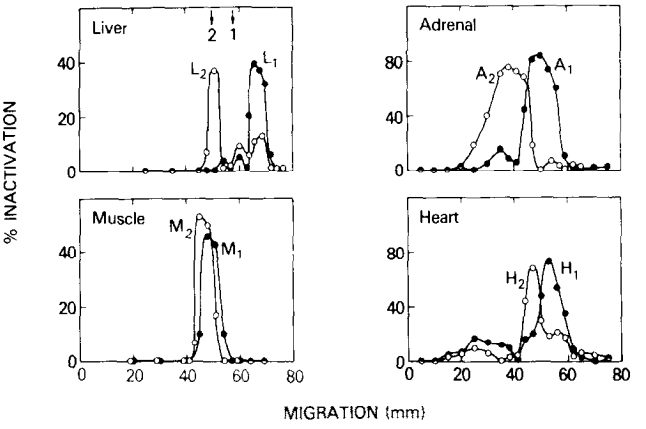


Fig. 5 Sodium dodecylsulfate-polyacrylamide gel electrophoresis of phosphatase inhibitors. M_1, A_1, and H_1 were treated with protein kinase before electrophoresis. Gels were sliced into 1 mm fractions, eluted with buffer, and assayed for inhibitor activity. Type 1, (●); type 2, (○). Arrows indicate migrations of the two types of inhibitors from rabbit muscle.

also appears to be true in rat liver (Fig. 2). Protein kinase did not affect L_2 activity, but it inactivates L_1. Liver phosphatase in crude preparations has been reported to be activated by ATP-Mg, and this activation could be related to the changes in inhibitor activity. The inactivation of L_1 was kinase-dependent, and the reaction was maximally diluted before final testing so that the final concentration of ATP-Mg in the assay was minimal and was not enough to affect phosphatase activity. There could be more than one species of protein in L_1 since the reduction in activity was mostly at the higher salt region (Fig. 2), but L_1 appeared as a single activity peak on gel electrophoresis.

Both A_1 from adrenal and H_1 from heart appeared to be phosphorylatable inhibitors. The shoulder activity at 0.16 M NaCl in Fig. 3 and that at 0.17 M NaCl in Fig. 4 should be the phospho-form of the corresponding inhibitor. Upon treatment with protein kinase the dephospho-form of the inhibitor, which appeared at a lower salt concentration than the phospho-form, became fully activated. A_2 and H_2 were like L_1 in that their activities were inactivated by protein kinase and in that the inactivation was kinase-specific. The possibility that the inacti

vation was due to a contaminant protein in the kinase preparation, however, cannot be ruled out.

While the different types of inhibitors need to be further characterized, the appearance of these inhibitors in various mammalian tissues calls attention to the possibility that they may have physiological roles in the regulation of the phosphatase. Preliminary studies using an isolated rat hind limb perfusion system showed that the phosphatase and inhibitor activities were inversely affected by the administration of epinephrine, which caused a rise in tissue cyclic AMP level and in the active form of glycogen phosphorylase (20).

It is presently unknown how liver and muscle phosphatase are regulated in glycogen metabolism. Our present data may explain the difference between the two enzymes in their sensitivities toward cyclic AMP. In muscle (8) adrenal (7), and perhaps heart, the inhibitory effect of cyclic AMP on phosphatase may be due to the presence of a phosphorylatable inhibitor. The lack in liver of an inhibitor which becomes active through phosphorylation may render liver phosphatase insensitive to cyclic AMP or may account for the activation of phosphatase by ATP-Mg (15).

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