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Reversible Inhibition of Skeletal Muscle Phosphoprotein Phosphatase by ATP, Phosphate and Fluoride *

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SUMMARY

A phosphoprotein phosphatase preparation which showed activity towards glycogen synthase, phosphorylase, phosphorylase kinase, and phosphohistones was reversibly inhibited (70-90%) by preincubation with free ATP (apparent K_i about 0.3 mM). Other nucleotides (ADP<AMP-P(NH)P<GTP<ATP) and EDTA were only partially effective in inhibiting phosphatase activity. Inhibition was also achieved by preincubation with 50 mM KF or by 16 hr. dialysis against 5 mM potassium phosphate buffer. The inhibition by all these ligands was reversed by a second preincubation with $MnCl_2$ (apparent K_a 3μM) prior to assay. Other divalent metals ($Co^{++} > Zn^{++} > Mg^{++}$) were partially effective in reversing the inhibition. It is concluded that ATP by virtue of its special structure and metal binding capacity possibly removes a catalytically important metal ion from the enzyme.

INTRODUCTION

Divalent cations Mg^{++} and Mn^{++} have been generally shown to stimulate phosphohistone and glycogen synthase phosphatase activities (1,2) and inhibit phosphorylase phosphatase activity (2,3). Previous reports indicate that phosphorylase phosphatase preparations from dog liver (4) and bovine adrenal cortex (5) occur in active and inactive forms. The active form can be converted into an inactive form by incubation with free ATP, and the inactive form can be activated by preincubattion with either $MgSO_4$ or Mg-ATP. Torres and Chelala (6) have observed that phosphorylase phosphatase in pigeon breast muscle extracts can be inactivated by including ATP, ADP, AMP, GTP, UTP, CTP, or pyrophosphate in the incubation medium and reactivated by including ATP-Mg^{++} alone in the assay. Recently, ATP has also been shown to inactivate a partially purified rabbit skeletal muscle phosphoprotein phosphatase, which is then reactivated by $MnCl_2$ (7). In this report we have studied the effects of free ATP, complexed ATP, phosphate buffer, fluoride ions and metal ion on phosphatase activity. To eliminate the effects

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of these agents on protein substrates, the enzyme was preincubated with these agents a the latter removed by dilution or dialysis.

MATERIALS AND METHODS

Preparation of enzymes and 32 P-substrates: Rabbit skeletal muscle glycogen synthase phosphorylase b, phosphorylase kinase and phosphoprotein phosphatase were prepared as described by Soderling et al. (8), Fischer and Krebs (9), Brostrom et al. (10), and Khatra and Soderling (11), respectively. Catalytic subunit of cAMP-dependent protein kinase was prepared from rabbit skeletal muscle by the method of Reimann and Corbin (12). Phosphorylase, histones, glycogen synthase and phosphorylase kinase were phosphorylated as described by Krebs et al. (13), Meisler and Langan (14), Soderling et al. (8), and Hayakawa et al. (15), respectively. [γ-^{32}P] ATP was prepared according to Glynn and Chappel (16).

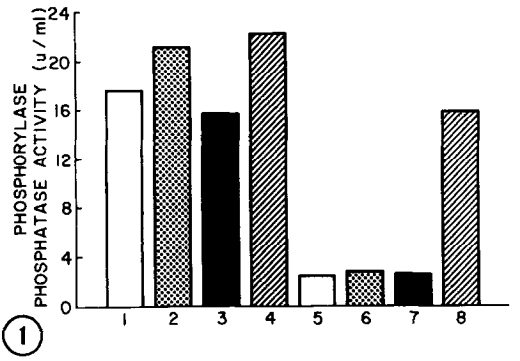
Phosphoprotein Phosphatase assays: Phosphoprotein phosphatase activity was measured by the release of ^{32}P from ^{32}P-phosphoproteins. A typical assay mixture for phosphorylase phosphatase contained in a total volume of 50 μl, 2.5 mM dithiothreitol, 0.025% BSA 20 mM glucose, 1 mM caffein, 50 mM triethanolamine buffer (pH 7.5), 25 μg of ^{32}P-phosphorylase, and 5-10 μl of phosphoprotein phosphatase. For glycogen synthase and phosphorylase kinase phosphatase activities, the assay mixtures were the same except no glucose or caffein were used. Histone phosphatase assay mixture was also similar except that no caffein or glucose were used and 0.1 M NaCl as well as 10 μM (in terms of alkali-labile ^{32}P) phosphohistone were present. The amounts of phosphatase added and the times of incubation were such that no more than 20% of added ^{32}P was released from phosphohistones and no more than 40% ^{32}P was released in the case of the other substrates. Fifty microliters of 30 mM silicotungstic acid in 40 mM H_2SO_4 were then added to stop the phosphohistone phosphatase reaction. In the case of other substrates 50 μl of 25% TCA was used. After centrifugation 50 μl of supernatant were counted for ^{32}P. One unit of phosphatase activity is defined as the amount of enzyme which liberates one nmole of phosphate per minute at 30° under the assay conditions.

Preincubation procedure: One or two preincubations of the phosphatase were usually performed. In the first preincubation 20-70 μl of phosphoprotein phosphatase was incubated with or without different concentrations of the given effector for 5-10 minutes at 30° in a total volume of 100 μl. The preincubation was stopped by adding 0.9 to 4.9 ml of cold Buffer A (50 mM Tris-Cl buffer containing 20% sucrose and 15 mM mercaptoethanol, pH 7.5). In the second preincubation, 90 μl aliquots of the phosphatase obtained from the first preincubation were then incubated at 30° in a total volume of 100 μl with or without given concentrations of divalent metal ions for 5-10 minutes. This reaction was stopped by diluting 5-10 fold in buffer A but now containing EDTA at a concentration which after dilution would give at least a two-fold excess over the metal ion concentrations. Total dilutions from the first preincubation into the phosphatase assays were between 1250-2500 for the phosphatase and 250-500 for the effectors.

Materials: Purified AMP-P(NH)P was kindly supplied by Dr. Roger A. Johnson from this department. Most of the metals were obtained from Fischer Scientific Company. Other chemicals were from Sigma Chemical Company.

RESULTS

Effect of phosphate and fluoride: A 16 hour dialysis of the phosphatase preparation against 5 mM potassium buffer (pH 7.0) containing 1 mM EDTA and 15 mM mercaptoethanol led to 80% loss in its activity (see Fig. 1). Preincubation of the phosphate-treated enzyme with 10 mM Mn^{++} followed by dilution in EDTA-containing buffer (see Methods)



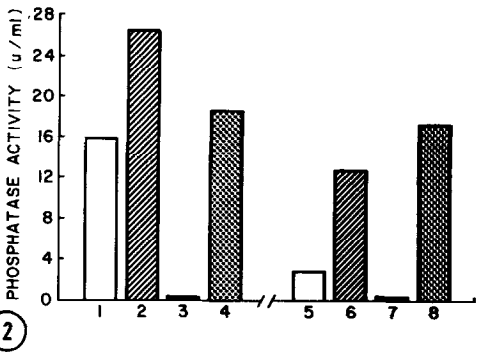


Figure 1. Effect of potassium phosphate buffer on phosphorylase phosphatase activity. One ml aliquots of phosphatase were dialyzed against 1 liter of pH 7.0 buffer containing 1 mM EDTA, 15 mM mercaptoethanol, and 5 mM Tris-Cl (bars 1-4) or 5 mM potassium phosphate (bars 5-8) for 16 hr at 4°. Aliquots (25 μl) from these were then preincubated with either no metal (open bars) or 10 mM concentrations of $CaCl_2$ (dotted bars); $MgCl_2$ (solid bars) or $MnCl_2$ (crossed bars) at 30° for 10 min in a total volume of 100 μl. The preincubation was stopped by adding 0.9 ml of buffer A containing 2 mM EDTA, and phosphorylase phosphatase activity was measured.

Figure 2. Effect of fluoride on phosphorylase phosphatase activity. The phosphatase was first preincubated with or without 50 mM potassium fluoride for 5 min and diluted 50-fold in buffer A followed by a second preincubation with or without 10 mM $MnCl_2$ for 5 minutes and a dilution of 5-fold in Buffer A containing 4 mM EDTA. Phosphatase activities were measured both with phosphorylase a (bars 1-4) and phosphohistones as subs trate (bars 5-8). Open bars represent phosphatase treated with 0 fluoride and then 0 $MnCl_2$; crossed bars represent phosphatase treated with 0 fluoride and then 10 mM $MnCl_2$; solid bars represent enzyme treated with 50 mM KF and then 0 $MnCl_2$; and dotted bars represent phosphatase treated with 50 mM KF and 10 mM $MnCl_2$.

resulted in almost complete recovery of the enzyme activity. Mg^{++} and Ca^{++} were not effective in restoring the activity. Preincubation of phosphatase with 50 mM potassium fluoride followed by dilution produced about 90% inhibition of the phosphatase activity toward phosphatase a and phosphohistones (Fig. 2). A second preincubation of this fluoride-inhibited enzyme with Mn^{++} resulted in 90% recovery of the enzyme activity. The carry over concentration of fluoride (40 μM) from the first incubation into the phosphatase assay was not inhibitory to the control enzyme (data not shown).

Effect of ATP and other nucleotides: Figure 3 shows the effect on phosphatase activity of preincubation with 1 mM ATP. The phosphatase activity was assayed with glycogen synthase D, phosphorylase a, phosphorylase kinase and phosphohistones. The ATP treated enzyme lost about 60% of its activity towards phosphorylase kinase and about 80% of its activity towards other substrates. The concentration of ATP require to inhibit one half

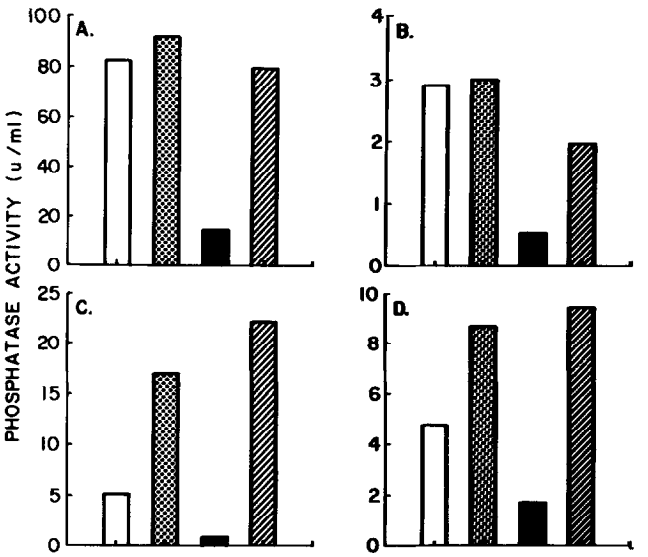


Figure 3. Effect of ATP on phosphoprotein phosphatase activity assayed with different substrates. Phosphoprotein phosphatase was preincubated with and without 1 mM ATP for 10 min at 30° and diluted 10-fold in buffer A. Aliquots from this were then incubated with and without 0.5 mM $MnCl_2$ for 10 min and again diluted 10-fold in buffer A. Aliquots of these were then assayed for phosphoprotein phosphatase activity with different substrates. Open bars represents those treated with no ATP and no $MnCl_2$; dotted bars represents those treated with no ATP and then $MnCl_2$; closed bars represents those incubated with ATP and then no $MnCl_2$; and crossed bars represent those treated with ATP and the $MnCl_2$. ^{32}P-substrates were (A) phosphorylase a, (B) glycogen synthase D, (C) phosphohistones and (D) phosphorylase kinase.

the phosphorylase phosphatase activity was about 0.3 mM. In each case a second preincubation with $MnCl_2$ resulted in reversal of inhibition. The effect of some other nucleotides on histone phosphatase activity is shown in Table 1. ATP gave the maximum inhibition followed by GTP and AMP-P(NH)P. Again in each case $MnCl_2$ reversed the inhibition. The phosphoprotein phosphatase showed no significant hydrolysis of ATP (not shown). This suggests that this inhibition by ATP is not due to its conversion into AMP or IMP. No inhibition by ATP was observed when either Ca^{++} or Mg^{++} (a large excess over ATP concentrations) was included in the preincubation mixture (data not shown). When [γ-^{32}P] ATP was incubated with the enzyme, no incorporation of ^{32}P into protein was observed. Furthermore, sucrose gradient centrifugation of the control and ATP-treated enzyme showed no shift in the sedimentation coefficient. On storage at -20°, the ATP-treated enzyme rapidly lost activity.

TABLE 1

Effect of Nucleotides on Phosphohistone Phosphatase Activity

Nucleotide	Phosphohistone Phosphatase Activity (U/ml)	
	Preincubated without Mn^{++}	Preincubated with Mn^{++}
None	6.81	25.0
AMP	8.91	26.4
IMP	8.76	23.2
ADP	5.21	18.0
ATP	1.55	25.0
GTP	3.37	18.2
AMP-P(NH)P	4.02	23.0

The phosphatase was preincubated with 1 mM concentration of given nucleotides for 10 minutes and diluted 50-fold in Tris buffer. Aliquots from each were then incubated with and without 10 mM Mn^{++} for 5 minutes and diluted 5-fold in Tris buffer containing 10 mM EDTA. These were then assayed for phosphohistone phosphatase activity.

Table 2

Effect of Metals on Control and ATP-treated Phosphoprotein Phosphatase

Metal Salt	Phosphorylase Phosphatase Activity		Histone Phosphatase Activity	
	Treated with no ATP	Treated with ATP	Treated with no ATP	Treated with ATP
None	100%	100%	100%	100%
$CaCl_2$	95	109	104	109
$CoCl_2$	35	667	44	587
$MgCl_2$	88	231	99	311
$MnCl_2$	89	808	102	626
$NiSO_4$	56	142	91	177
$Zn(CH_3COO)_2$	76	352	70	103

Phosphoprotein phosphatase was preincubated with and without 1 mM ATP for 10 minutes at 30° and chromatographed on G-25 equilibrated with 50 mM Tris, 30 mM mercaptoethanol. Aliquots from each were then incubated with 5 mM concentration of different metals for 10 minutes at 30°, diluted 4-10 fold in Tris buffer containing 2-5 mM EDTA(4- fold excess over metal ion concentration) and assayed for phosphatase activities. The given value represents the percent of activity relative to the enzyme preincubated with no metal.

Effect of Metals: Both the control and ATP-treated enzyme forms were strongly inhibited by Cu^{++}; Fe^{++}; Hg^{++} and Pb^{++} whereas Ba^{++}; Ca^{++} and Sr^{++} had no effect (data not shown). However, $Mn^{++} > Co^{++} > Zn^{++} > Mg^{++}$ activated the ATP-treated enzyme **(Table 2)**. The control enzyme was inhibited by Co^{++} and activated to variable extents by Mn^{++}. With phosphohistone as the substrate similar results were obtained, except that Zn^{++} did not reactivate the ATP-treated enzyme. By adding increasing

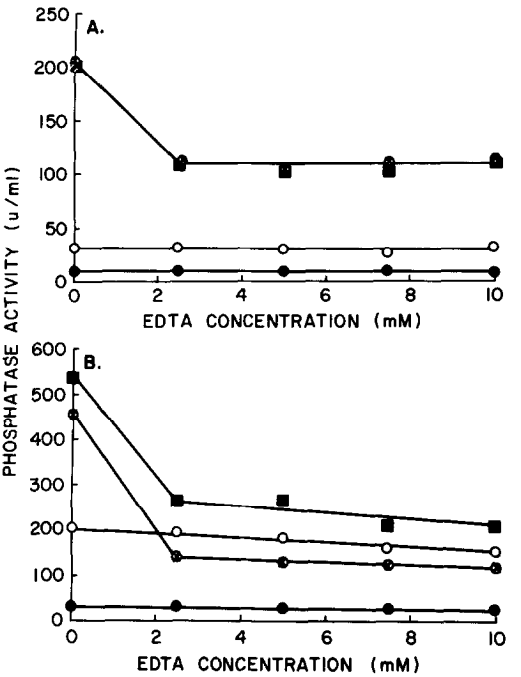


Figure 4. Effect of EDTA on control, Mn^{++} treated and ATP-treated phosphoprotein phosphatase activities. Phosphoprotein phosphatase was preincubated with or without 1 mM ATP for 15 minutes and diluted 10-fold in buffer A. Aliquots from these were then incubated with or without 1 mM $MnCl_2$ for 10 minutes and diluted another 10-fold in buffer A. Aliquots from each were then assayed for phosphohistone phosphatase (A) and phosphorylase phosphatase (B) activity in the presence of given concentrations of EDTA. (0——0); (■——■); (●——●), and (◐——◐) represent the enzyme with no ATP then no Mn^{++}; no ATP then 1 mM Mn^{++}; 1 mM ATP then no Mn^{++}; and 1 mM ATP then 1 mM Mn^{++}, respectively.

concentrations (4-20 μM) of Mn^{++} to the assay mixture an apparent K_a of 3 μM was found for Mn^{++} using phosphorylase *a* as the substrate (data not shown). The Mn^{++} reactivated enzyme exhibited a sedimentation ccoefficient similar to the control enzyme (data not sh

Effect of EDTA and other metal ion chelators: Inclusion of increasing concentrations of EDTA (see Fig. 4) in the assay mixture had no effect on the ATP-treated or control phosphatase activities which had not been preincubated with Mn^{++}. Preincubation of the control enzyme with Mn^{++} lead to 2-fold and about 7-fold increases in its activities towards phosphorylase *a* and phosphohistones, respectively. EDTA only inhibited this activated enzyme about two-fold in each case. On the other hand, incubation of ATP-treated phosphatase with Mn^{++} resulted in 13- to 16-fold increases in its activities towards

phosphorylase a and phosphohistones. However, inclusion of increasing concentrations of EDTA in the assay of this Mn^{++} reactivated enzyme resulted in only about 50% inhibition in the case of both substrates. Furthermore, incubation of the control phosphatase for 20 minutes with 10 mM o-phenanthroline, 5 mM 8-hydroxyquinoline, 10 mM ethylene-glycol-bis (β-aminoethylether)-N,N[1] tetraacetic acid) or 10 mM cyclohexanediamine tetra-acetic acid did not significant affect its activity.

DISCUSSION

The results presented in this manuscript show that a 400-fold purified phosphoprotein phosphatase isolated from the glycogen protein complex of rabbit skeletal muscle (12) is reversibly inhibited by phosphate, fluoride and ATP. The mechanism of this reversible inhibition of phosphoproprotein phosphatase activity by ATP is not clear. Although phosphorylation of phosphatase protein by protein kinase in the presence of low ATP-Mg^{++} (50 μM) has been reported (17), this does not seem to be the case in the present study. Firstly, free ATP was needed for the inhibition to occur and no protein kinases are known which utilize free ATP and secondly, some inhibition by AMP-P(NH)P, ADP and GTP was seen (Table 2). Two other possibilities seem likely. Free ATP may simply be binding to the phosphatase protein and changing its conformation, or it may, by virtue of its metal binding capacity, be simply removing a metal ion from the enzyme molecule. Similar reversible inhibition of phosphoprotein phosphatase by fluoride, phosphate, and to a lesser extent by other nucleotides would tend to indicate that ATP, phosphate or fluoride may be removing some metal from the enzyme. The proposed ability of the ATP to remove a metal from the enzyme more effectively than metal chelators such as EDTA may reside in its structure. Due to its special structural features, ATP may bind to or enter into the catalytic site of the enzyme and remove a metal ion which is essential for phosphatase activity. Although Mn^{++} stimulated the inhibited enzyme maximally, no attempt was made to determine whether the phosphatase originally contained bound Mn^{++} ion. The published stability constant for ATP-Mn^{++} is 13μM (18) whereas the apparent K_a of the phosphatase for Mn^{++} is about 3 μM. This suggests a higher affinity of the enzyme for Mn^{++} compared for that of ATP. However, a higher molar ratio of ATP to phosphatase protein would favor the binding of Mn^{++} to ATP.

The lack of inhibition by other metal chelators was surprising. The structure of EDTA may restrict its access to the metal on the enzyme or there may be two metal binding sites on the enzyme, only one of which is accessible to EDTA. Alternatively, the partial inhibition by EDTA may be a substrate directed effect.

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