Vol. 95, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1980 Pages 1192-1199

DETHIOPHOSPHORYLATION OF THIOPHOSPHORYLASE a BY A MULTIFUNCTIONAL PHOSPHOPROTEIN PHOSPHATASE OF Mr=35,000*

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Received June 23,1980

SUMMARY: It is generally believed that proteins thiophosphorylated by adenosine 5'-O-(3-thiotriphosphate) (ATPγS) are resistant to phosphoprotein phosphatases. We have found that a general phosphoprotein phosphatase of Mr=35,000 purified from bovine heart, rabbit muscle or liver, can efficiently catalyze the dethiophosphorylation of [^{35}S]thiophosphorylated phosphorylase ([^{35}S]thiophosphorylase a). The rate of the dethiophosphorylation reaction in the presence of 1mM Mn^{2+} was about one-third as that of dephosphorylation of [^{32}P]phosphorylase a. The dethiophosphorylation reaction was almost completely inhibited by EDTA and was stimulated several fold by Mn^{2+}. On the other hand, the dephosphorylation of [^{32}P]phosphorylase a was not significantly affected by these reagents. The present findings indicate that thiophosphorylated proteins in general may not be resistant to phosphoprotein phosphatases.

INTRODUCTION

Considerable biochemical evidence has accumulated indicating that the covalent modification of enzymes and regulatory proteins through a cycle of phosphorylation and dephosphorylation reactions is an important control mechanism involved in the regulation of a variety of biological processes (1). It has been reported that protein kinases can utilize ATPγS instead of the normal substrate, ATP, to thiophosphorylate their receptor protein(s) (2-5), and that the resultant thiophosphorylated proteins are generally resistant to phosphoprotein phosphatases (2-4). These data imply that one could control the state of phosphorylation of a protein in a biological system without interference from phosphatase action. Owing to the

* This work was supported by a Grant-in-aid from the New York Heart Association and by the National Institutes of Health Grant HL-22962.

0006-291X/80/151192-08$01.00/0

potential usefulness of protein thiophosphorylation for the studies of hormones, cyclic nucleotides, and Ca^{2+} actions in various biological processes, we have examined the activity of a multifunctional phosphatase of Mr=35,000 (6-12) towards [^{35}S] thiophosphorylase a. The results indicate that thiophosphorylase a can be efficiently dethiophosphorylated by the Mr=35,000 phosphatase and the rate of this reaction is inhibited by EDTA, and enhanced by Mn^{2+}.

MATERIALS AND METHODS

Preparation of Substrates

Adenosine 5'-0-(3-[^{35}S]triphosphate) ([γ-^{35}S]ATPγS), and [γ-^{32}P]ATP were purchased from New England Nuclear. Non-labelled ATPγS was from Boehringer. Rabbit skeletal muscle phosphorylase kinase was from Sigma. Crystalline phosphorylase b was prepared from rabbit skeletal muscle according to Brostrom et al (13). Phosphorylase b was converted to either [^{35}S]phosphorylase a or [^{32}P]phosphorylase a, using [γ-^{35}S]ATPγS or [γ-^{32}P]ATP as the phosphorylating agent, respectively, according to a modified method of Torres and Chelala (14). The [^{35}S]thiophosphate or [^{32}P]orthophosphate content was about 10 nmol/mg protein. The specific radioactiviy of [^{35}S]thiophosphorylase a or [^{32}P]phosphorylase a used in the assay varied in the range of 50-180 cpm per pmol.

Preparation of Phosphoprotein Phosphatase

The Mr=35,000 phosphoprotein phosphatase was purified from bovine heart, rabbit skeletal muscle or liver by a modified procedure described previously (10). The purified enzyme had a specific activity of about 1,000-2,000 unit/mg (phosphorylase a as a substrate) and was stored at -20°C in a buffer containing 20 mM Tris·HCl, pH 7.4, and 50% glycerol. The enzyme was diluted with a buffer containing 20 mM Tris·HCl, pH 7.4 and 0.2% bovine serum albumin prior to assays.

Enzyme Assay

Phosphatase activity was measured by the release of [^{35}S] thiophosphate or [^{32}P]orthophosphate at 30°C from [^{35}S]thiophosphorylase a or [^{32}P]phosphorylase a, respectively. The standard assay mixture contained 50 mM Tris·HCl, pH 7.4, 2.5 mM theophylline, 2 mM $MnCl_2$, 5 µM substrate and enzyme in a total volume of 25 µl. The reaction was initiated by the addition of enzyme and terminated with 25 µl of 8% (w/v) trichloroacetic acid. The radioactivity released was separated from the remaining substrate by the paper chromatographic method described previously (10). Blank values, i.e. radioactivity obtained in the absence of enzyme, were subtracted from all assays. Conversion of thiophosphorylase a to phosphorylase b by phosphatase was

also determined by measuring the disappearance of thiophosphorylase a activity in the absence of AMP by a modified method as described by Hurd et al (15). Protein concentration was determined by the method of Lowry et al (16). Bovine serum albumin was used as a standard.

RESULTS

As shown in Table I, the Mr=35,000 phosphoprotein phosphatase from bovine heart was found to efficiently catalyze the dethiophosphorylation of [^{35}S]thiophosphorylase a. The reaction was almost completely inhibited by 2mM EDTA, but was stimulated by about two to five-fold by 2mM Mn^{2+}. Co^{2+}, Ca^{2+}, and Mg^{2+}, were ineffective, while Zn^{2+} was inhibitory. By contrast, the dephosphorylation of [^{32}P]phosphorylase a was not significantly affected by 2mM EDTA or divalent cations, with the exception that Zn^{2+} was inhibitory. Thus, in the presence of 2mM Mn^{2+}, the

Table I.

Addition (2mM)	Activity (pmol Released/10 Min) [^{32}P]Phosphorylase a	[^{35}S]thiophosphorylase a
None	25.1	3.76
EDTA	24.0	0.64
$CaCl_2$	19.1	3.8
$MgCl_2$	24.7	4.1
$MnCl_2$	31.8	10.2
$CoCl_2$	29.7	1.8
$ZnCl_2$	1.6	0.59

Effects of various divalent cations on phosphoprotein phosphatase activity towards the thio or normal phosphorylase a.

The enzymic activity was measured in a volume of 50µl containing, 50mM Tris·HCl, pH 7.4, 1mM dithiothreitol 2.5mM theophylline, 0.092µg of bovine heart phosphatase, 5µM [^{32}P]phosphorylase a or [^{35}S]thiophosphorylase a and 2mM of EDTA or various divalent cations as indicated. After incubating at 30°C for 10 min, the reaction was terminated by the addition of 20µl of 8% trichloroacetic acid and the radioactivities released were determined as described in the text.

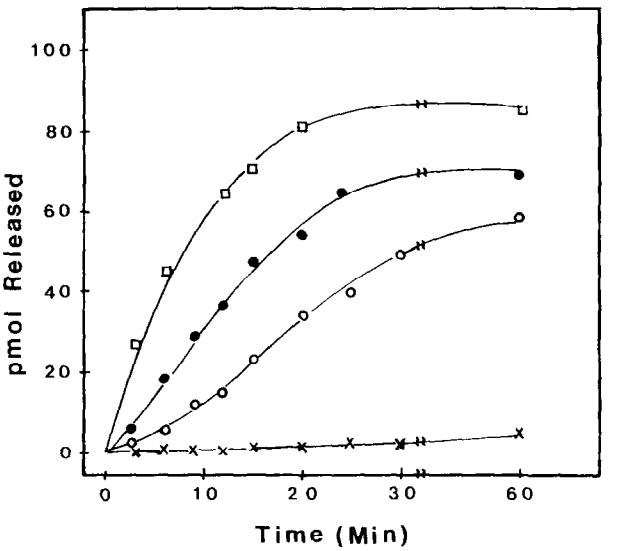

Figure 1. Time course of phosphoprotein phosphatase activity towards the thio and normal phosphorylase a.

The enzymic activity was measured at 30°C in a total volume of 0.3ml containing 50mM Tris·HCl, pH 7.4, 1mM dithiothreitol 2.5mM theophylline, 0.092µg of bovine heart phosphatase, and 5µM [^{32}P]phosphorylase a in the presence of 1mM $MnCl_2$ (□—□); or 5µM [^{35}S]thiophosphorylase a in the absence (○—○) and in the presence of 1mM $MnCl_2$ (●—●) or 2mM EDTA (✕—✕). At the indicated time intervals, aliquotes of 25µl were removed from the reaction mixtures and mixed with 25µl of 8% trichloroacetic acid. The radioactivities released were determined as described in the text.

amount of radioactivity released from [^{35}S]thiophorylase a in 10 min incubation time was about 10-30% as that from [^{32}P]phosphorylase a.

In contrast to the dephosphorylation of phosphorylase a, the dethiophosphorylation reaction showed an obvious initial lag period (Fig. 1). In the presence of 1 mM Mn^{2+}, this initial lag period became less pronounced. Although the rate of dethiophosphorylation was increased about two to three-fold in the presence of Mn^{2+}, the extent of dethiophosphorylation of thiophosphorylase a was only slightly higher than that in the absence of this divalent cation, as the incubation time was prolonged. After 60 min incubation, the radioactivity released from [^{35}S]

phosphorylase a was more than two-thirds as that from [^{32}P] phosphorylase a. As shown in Fig. 1, the enzymic activity towards [^{35}S]thiophosphorylase a was almost completely inhibited by 2 mM EDTA. When the reaction was carried out for 10 min in the presence of 1mM $MnCl_2$, the rate of dethiophosphorylation is linear with the increase of phosphatase concentration (data not shown). The Mr=35,000 phosphoprotein phosphatase purified from rabbit skeletal muscle and liver showed properties similar to the enzyme from bovine heart. Conversion of thiophosphorylase a to phosphorylase b catalyzed by the rabbit liver phosphatase has been carried out. The results indicated that Mn^{2+} greatly enhanced the rate of the conversion reaction.

Fig. 2 shows the effects of Mn^{2+} concentration on the bovine heart phosphatase toward [^{35}S]thiophosphorylase a. Mn^{2+} stimulated the reaction in a concentration dependent manner, and reached a plateau at about 1mM. The concentration for half maximum stimulation was about 0.1mM.

DISCUSSION

Gratecos and Fischer (2) have studied the action of phosphorylase phosphatase towards thiophosphorylase a. Under the conditions they used, thiophosphorylase a was resistant to the phosphatase and, therefore, behaved as a competitive inhibitor with respect to normal phosphorylase a. We also found that thiophosphorylase a was resistant to the phosphatase, but only when EDTA was present. Without added EDTA, thiophosphorylase a was efficiently hydrolyzed by the phosphatase and the hydrolytic reaction was enhanced in the presence of added Mn^{2+}. The Mr=35,000 phosphatase used in the present studies has been described as being active towards a variety of phosphoproteins including phosphorylase a, glycogen synthase D, phosphorylase

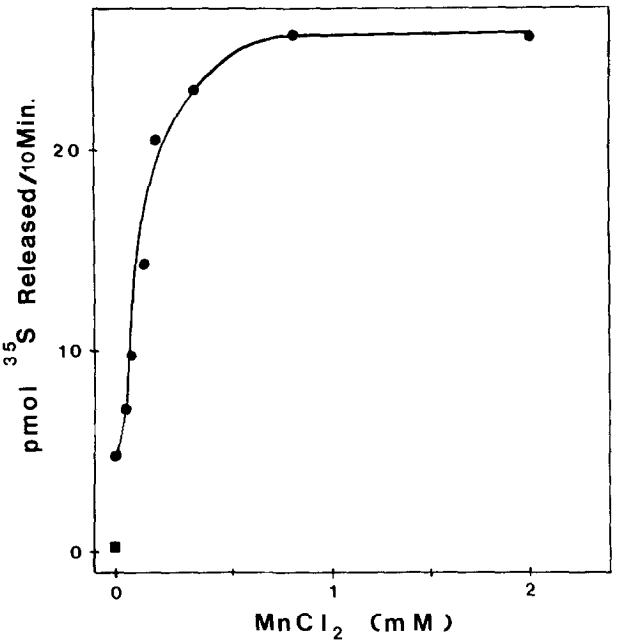

Figure 2. Effects of $MnCl_2$ concentration on phosphoprotein phosphatase activity towards [^{35}S]thiophosphorylase a.

The enzymic activity was measured in a total volume of 25µl containing 50mM Tris·HCl, pH 7.4, 2.5mM theophylline, 5µM[^{35}S]thiophosphorylase a, and 0.18µg bovine heart phosphatase in the presence of 2mM EDTA, (■), or various concentrations of $MnCl_2$ (●—●) as indicated. The reaction was carried out at 30°C for 10 min. [^{35}S]thiophosphate released was determined as described in the text.

kinase, pyruvate kinase, hormone sensitive lipase, phosphorylated regulatory subunit of type II cyclic-AMP-dependent protein kinase, troponin, histone, and casein (6-12). Furthermore, this Mr=35,000 phosphatase has been found to be widely distributed in animal tissues (17). It appears that a number of investigators have interpreted their data based on the assumption that the thiophosphorylated proteins in general are resistant to phosphoprotein phosphatase activity. We wish to report that it cannot be taken for granted that dethiophosphorylation of thiophosphorylated proteins does not occur under certain experimental conditions.

Previously, we have reported that the Mr=35,000 phosphatase may be a metalloenzyme which contains a tightly bound divalent cation(s) probably Mn^{2+}, in the active site (11,18). The tightly bound divalent cation(s) could not readily be chelated by EDTA, and thus addition of EDTA into the assay mixture did not significantly affect the dephosphorylation reaction. The present results indicate that the dethiophosphorylation reaction, in contrast to the dephosphorylation reaction, was stimulated by Mn^{2+} and drastically inhibited by EDTA. These data suggest that the Mr=35,000 phosphatase may contain an additional loosely bound divalent cation(s), probably Mn^{2+}, which is required for the hydrolysis of the thio- but not the normal phosphorylase a. The data also suggest that divalent cations, such as Mn^{2+}, may play a role in regulating the substrate specificity of the Mr=35,000 phosphatase. Further studies, however, are required for clarifying these points.

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