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STUDIES ON INACTIVATION AND REACTIVATION OF HOMOGENEOUS RABBIT LIVER PHOSPHOPROTEIN PHOSPHATASES BY INORGANIC PYROPHOSPHATE AND DIVALENT CATIONS

RAMJI L. KHANDELWAL and SALIM A.S. KAMANI

Department of Oral Biology, Faculty of Dentistry, The University of Manitoba, Winnipeg, Manitoba R3E 0W3 (Canada)

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Summary

Preincubation of two homogeneous rabbit liver phosphoprotein phosphatases (phosphoprotein phosphohydrolases, EC 3.1.3.16) (Khandelwal, R.L., Vandenheede, J.R. and Krebs, E.G. (1976) J. Biol. Chem. 251, 4850—4858) with ATP, ADP and PP_i caused a time- and concentration-dependent inactivation of the enzyme activity. A 50% inactivation of phosphoprotein phosphatase I required relatively low concentration of inactivating metabolite and less preincubation time as compared to the inactivation of phosphoprotein phosphatase II. AMP, adenosine, adenine, P_i, EDTA, EGTA, 1,10-phenanthroline and diethyl dithiocarbamate were without effect on both enzymes. Pretreatment of both enzymes by metal-chelating agents followed by PP_i did not augment the effect observed with PP_i alone. Both inactivated enzymes could be reactivated by cobalt or manganese in the presence of dithiothreitol. Although the extent of reactivation by these two metal ions was almost similar, cobalt required a ten times lower concentration than manganese for this process. No difference in inactivation or reactivation of both enzymes was observed with different substrates, phosphorylase *a*, histone or casein, employed in the assay. P_i and PP_i added during the assay inhibited activities of both phosphatases with phosphorylase *a* and casein substrates. With histone as substrate, PP_i slightly inhibited enzyme activities at lower concentrations (0.01—0.25 mM) but activated at higher concentrations. P_i activated both enzymes with this substrate; maximal activation being observed at a concentration of 5 mM.

Abbreviations: Mes, 2-[*N*-morpholino]ethanesulfonic acid; SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

Introduction

Phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16), which catalyzes the dephosphorylation of phosphoproteins, has recently been shown to be involved in the regulation of certain physiological processes [1—6]. A detailed investigation on protein dephosphorylation and its subsequent role in cellular regulation, however, is hampered by an apparent existence of multiple molecular forms of phosphoprotein phosphatase in mammalian tissue extracts [7—10]. In the last few years, several investigators have been able to convert these less active, multiple-molecular forms into an active low molecular weight form (M_r = 30 000—35 000) in all tissues examined [7—9, 11—13]. The exact relationship between higher and lower molecular weight forms of the enzyme is not yet established, but it has been suggested that higher molecular weight form is a native enzyme whereas lower molecular weight form represents a catalytic subunit [8,14—16]. The higher molecular weight form of the enzyme, therefore, would consist of a catalytic subunit with one or more regulatory subunits [15—20].

The catalytic subunit of phosphoprotein phosphatase has been purified to homogeneity from rabbit liver [9,13], rabbit skeletal muscle [10] and heart muscle from rabbit, dog and ox [11,12,21,22] and its properties have been studied in detail. The enzyme activity is generally independent of divalent cations and exhibits a broad specificity for the dephosphorylation of phosphorylated substrates. The substrates thus far examined are phosphorylase *a*, glycogen synthase D, phosphorylase kinase, R-subunit of cyclic AMP-dependent protein kinase II, hormone-sensitive lipase, inhibitor subunit of troponin, histone and casein.

The purified catalytic subunit has been found to undergo interconversion between active and inactive forms [14,23,24]. It can be inactivated by ATP, ADP or PP_i, but not by AMP, adenosine, adenine and P_i. The inactivated enzyme can be reactivated by Mn^{2+} or Co^{2+}, but not by any other divalent cation.

We have previously reported the purification of two homogeneous low molecular weight forms (I and II) of phosphoprotein phosphatase from rabbit liver [9]. The enzyme was recovered in two distinct peaks after chromatography on Sepharose-4B-histone. The molecular weights calculated by sodium dodecyl sulfate gel electrophoresis were 30 500 and 34 000 for phosphoprotein phosphatase I and II, respectively. Both of these phosphatases were able to dephosphorylate all the phosphorylated substrates employed in the study. In a subsequent report, we showed that preincubation of homogeneous phosphoprotein phosphatase II with PP_i caused complete inactivation of the enzyme; this inactivation was completely reversed by Co^{2+} [14]. The present paper reports the differences in the rate of inactivation of purified homogeneous rabbit liver phosphoprotein phosphatase I and II with ATP, PP_i and other related compounds, and reactivation by Co^{2+} and Mn^{2+}. With the use of three different substrates, it has also been shown that the effects of these compounds are on the enzyme and not on protein substrates.

Materials and Methods

Materials. Crystalline rabbit muscle phosphorylase *b* was isolated as described by Fischer and Krebs [25]. Skeletal muscle phosphorylase kinase was isolated by the method of Hayakawa et. al [26] and was a gift from Drs. J.H. Wang and T.J. Singh (Faculty of Medicine, University of Manitoba). The catalytic subunit of cyclic AMP-dependent protein kinase was a generous gift from Drs. E.G. Krebs and T.S. Huang (University of Washington, Seattle). Homogeneous rabbit liver phosphoprotein phosphatases (I and II) were purified as described previously [9]. Vitamin-free casein was obtained from Nutritional Biochemicals and partially dephosphorylated by heating at 100°C for 10 min at pH 9.5 before use [9]. [γ-^{32}P]ATP and Aquasol-2 (liquid scintillation solvent) were obtained from New England Nuclear. Histone II-A was obtained from Sigma Chemical Co. All other chemicals were of reagent grade.

Preparations of ^{32}P-labelled substrates. The preparation of all ^{32}P-labelled substrates has been previously described in detail [9]. Briefly, rabbit skeletal muscle [^{32}P]phosphorylase *a* was prepared from phosphorylase *b* using [γ-^{32}P]ATP, Mg^{2+} and phosphorylase kinase, as described by Krebs et. al [27]. ^{32}P-labelled histone II-A was prepared essentially according to Meisler and Langan [28]. The 20% trichloroacetic acid-insoluble pellet was washed once with water and then dissolved in a small volume of 25 mM 2-[*N*-morpholino]-ethanesulfonic acid (Mes) buffer (pH 7.0). The dissolved suspension was first dialyzed against the same buffer and then dialyzed against 10 mM Tris-HCl, pH 7.0. The ^{32}P-labelled histone contained 12—15 nmol bound ^{32}P per mg histone. ^{32}P-labelled casein was prepared using purified catalytic subunit of protein kinase, employing essentially the same procedure as used for making phosphohistone. Phosphorylated casein was precipitated by 5% trichloroacetic acid, washed with water and suspended in 25 mM Mes (pH 7.0). The dissolved casein was dialyzed overnight against the same buffer with one change of the buffer. The ^{32}P-labelled casein contained 0.35—0.39 mol bound ^{32}P per mol casein (M_r = 24 000). Phosphorylated histone and casein were stored at —20°C whereas phosphorylase *a* was stored at 4°C.

Enzyme assays. Phosphoprotein phosphatase activity was determined by the release of $^{32}P_i$ from ^{32}P-labelled substrates at 30°C [9]. Reaction mixtures for the dephosphorylation of [^{32}P]phosphorylase *a* contained 50 mM Tris-HCl (pH 7.4), 1 mM caffeine, 0.5 mg/ml phosphorylase *a*, 0.5 mM dithiothreitol, and phosphoprotein phosphatase preparation in a total volume of 50 μl. Reaction mixtures for the dephosphorylation of ^{32}P-labelled histone contained 50 mM Tris-HCl (pH 7.0), 0.5 mg/ml ^{32}P-histone, 0.5 mM dithiothreitol, 0.1 M KCl and phosphoprotein phosphatase, in a total volume of 50 μl. With ^{32}P-labelled casein as substrate, reaction mixtures were the same as for the dephosphorylation of ^{32}P-labelled histone except that 0.1 M KCl and ^{32}P-labelled histone were replaced by ^{32}P-labelled casein. In all cases, the reactions were started by the addition of phosphoprotein phosphatase to the reaction mixture and terminated by the addition of 0.2 ml 10% trichloroacetic acid for all substrates except ^{32}P-labelled histone. In this instance, the reactions were stopped with 0.2 ml 5% trichloroacetic acid containing 0.25% sodium tungstate. After storing at 4°C for 20 min, all tubes were centrifuged in an International clinical

centrifuge for 10 min. Radioactivity was estimated in aliquots of the clear supernatants using 5 ml Aquasol-2 as solvent. In all cases, the amount of phosphoprotein phosphatase added was such that less than 10% of the substrate was dephosphorylated in a 10-min incubation period. Reaction rates were linear with time and were proportional to the amount of enzyme under the above conditions. Blank values, i.e., the counts obtained in the absence of enzyme, were subtracted from all the assays. One unit of phosphoprotein phosphatase was defined as that amount of enzyme which released 1 nmol P_i from the phosphorylated substrate per min. Specific activity was defined as the number of units per mg protein.

Analytical methods. Protein was determined by the method of Lowry et al. [29] with bovine serum albumin as the standard.

All the results reported in this paper have been repeated with at least two preparations of the enzyme and phosphoprotein substrates.

Results

Effect of ATP and its degradation products on inactivation of rabbit liver phosphoprotein phosphatases

It has been shown previously that the preincubation of phosphoprotein phosphatase II with PP_i results in a time-dependent inactivation of the enzyme [14]. The effect of PP_i, ATP and a number of other related compounds was further examined using both purified liver phosphoprotein phosphatases in this

TABLE 1

EFFECT OF PREINCUBATION WITH METAL-CHELATING AGENTS, ATP AND ITS DEGRADATION PRODUCTS ON THE ACTIVITIES OF PURIFIED RABBIT LIVER PHOSPHOPROTEIN PHOSPHATASE

Purified rabbit liver phosphoprotein phosphatases I (11.7 μg) and II (1.6 μg) were preincubated with 0.5 mM metal-chelating agent or 0.25 mM ATP and its degradation products in a buffer system of 100 mM Tris-HCl, (pH 7.5), containing 5% sucrose/0.5 mM dithiothreitol/1 mg/ml bovine serum albumin, in a total volume of 0.1 ml. After preincubation of 15 min at 30°C 10-μl aliquots from each tube were withdrawn and diluted with 490 μl 10 mM Tris-HCl, pH 7.0. Phosphatase activity was determined in diluted samples with [^{32}P]phosphorylase *a* as the substrate. *P* values relative to the control values were obtained by Student's *t*-test.

Additions	Phosphoprotein phosphatase I	Phosphoprotein phosphatase II
None	100	100
+ATP	63.2 *	71.8 *
+ADP	64.3 *	70.0 *
+AMP	97.5	98.2
+P_i	98.9	99.3
+PP_i	21.8 *	30.8 *
+Adenosine	98.7	99.5
+Adenine	101.0	100.5
+1,10-Phenanthroline	99.2	103.1
+Diethyl dithiocarbamate	101.1	100.8
+EDTA	101.9	99.0
+EGTA	97.8	94.0
+EDTA + PP_i	22.0 *	32.2 *

* $P < 0.001$.

study. As shown in Table I, ATP, ADP and PP_i inactivated both enzymes; the relative inactivation of phosphoprotein phosphatase I being greater than that of phosphoprotein phosphatase II. PP_i was the most potent inactivator for these enzymes. There was no significant difference between the inactivating effects of ATP and ADP. Other compounds such as AMP, adenosine, adenine, P_i, EDTA, EGTA, 1,10-phenanthroline and diethyl dithiocarbamate showed no effect on both enzymes. EDTA in combination with PP_i did not increase the effect of PP_i. Furthermore, if the enzymes were first preincubated with metal-chelating agents followed by PP_i, no enhanced effect of PP_i was observed with either enzymes. Since PP_i showed the maximum inactivation, this compound was used for all of the following experiments involving inactivation of these two enzymes.

Inactivation of rabbit liver phosphoprotein phosphatases by PP_i

The inactivation of liver phosphoprotein phosphatase I and phosphoprotein phosphatase II by 0.25 mM PP_i as a function of preincubation time was further determined. A 50% inactivation of phosphoprotein phosphatase I was observed in about 1 min of preincubation whereas similar inactivation of phosphatase II required approx. 8 min preincubation. After 30 min of preincubation, both enzymes showed 90% inactivation; maximum inactivation of approx. 97% was reached in 90 min. The rate of inactivation of both enzymes by PP_i was not altered with the addition of 0.5 mM EDTA in the preincubation mixture. There was no difference in the rate of inactivation, irrespective of the substrate employed in the assay.

The effect of varying concentrations of PP_i on inactivation of liver phosphoprotein phosphatases is shown in Fig. 1. Phosphoprotein phosphatase I was inactivated 50% by 0.05 mM PP_i in a preincubation time of 15 min, whereas 2—3 times higher concentration was required for 50% inactivation of phosphoprotein phosphatase II. As a matter of fact, 0.05 mM concentration of PP_i

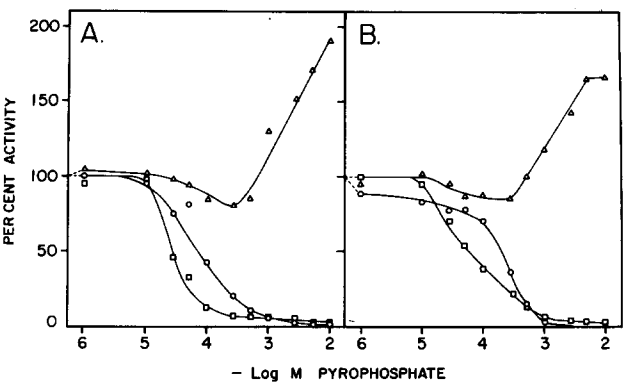

Fig. 1. Inactivation of purified rabbit liver phosphoprotein phosphatases by varying concentrations of inorganic pyrophosphate. Phosphoprotein phosphatase I (A) or phosphoprotein phosphatase II (B) was preincubated at 30°C with varying concentrations of PP_i and after preincubation of 15 min, 5-µl aliquots were withdrawn and diluted with 250 µl 100 mM Tris-HCl, pH 7.5, containing 5% sucrose, 0.5 mM dithiothreitol, and 1 mg/ml bovine serum albumin. Phosphatase activity was determined in diluted samples using phosphorylase *a* (○———○), casein (□———□), or histone (△———△) substrate. Phosphatase activity with no preincubation was taken as 100% with each substrate.

which inactivated 50% of phosphatase I has no significant effect on the inactivation of phosphatase II. The maximum inactivation by higher concentrations of PP_i (more than 0.05 mM for phosphatase I and more than 0.1 mM for phosphatase II) was observed to be approx. 97%. The maximum inactivation by lower concentrations of PP_i could not be precisely determined due to enzyme unstability with prolonged preincubation times. The activities of both phosphatases decreased similarly when assayed with phosphorylase *a* or phosphorylated casein as substrates. However, with phosphorylated histone as the substrate, inactivation of phosphatases was biphasic. At low concentrations of PP_i, a similar pattern of inactivation was observed while at higher concentrations (more than 0.25 mM), an apparent decrease in inactivation was noticed. Since the preincubated samples were assayed after a 50-fold dilution, the possibility that the carry-over PP_i caused an activation of phosphatases with histone was examined. For this, all preincubated samples were dialyzed to remove excessive amounts of PP_i and then were assayed for phosphatase activity with all three substrates. With dialyzed, preincubated samples, the inactivation rate with all three substrates was similar and identical to the inactivation rate observed with phosphorylase *a* and phosphorylated casein substrates for diluted samples. These results suggest that biphasic enzyme inactivation curves observed with histone substrate were probably due to an interaction of histone with PP_i during the assay.

Effect of PP_i on phosphoprotein-phosphatase activity

To confirm that the interaction between PP_i and histone causes an activation of enzyme activity, a detailed study on the effects of PP_i addition during the assay was undertaken. For comparison, the effect of PP_i on phosphatase activity with two other substrates, phosphorylase *a* and phosphorylated casein, was also examined. As can be seen in Fig. 2, with phosphorylase *a* and phosphorylated casein as substrates, a concentration-dependent inhibition of both phosphatases was observed. The percent inhibition was slightly greater with phos-

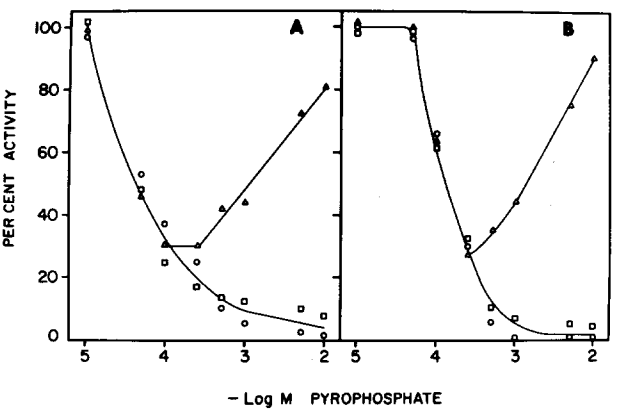

Fig. 2. Effect of varying concentrations of PP_i added during the assay on activities of purified rabbit liver phosphoprotein phosphatase I (A) and phosphoprotein phosphatase II (B). Substrates used are phosphorylase *a* (○———○), casein (□———□) and histone (△———△). Phosphatase activity with each substrate without pyrophosphate addition was taken as 100%.

phorylated casein than with phosphorylase *a*. With phosphorylated histone as the substrate, the effect of PP_i on the activities of both phosphoprotein phosphatases was different. Low concentrations of PP_i (less than 0.25 mM) showed slight inhibition, whereas higher concentrations resulted in enzyme activation. It should be pointed out, however, that with this substrate, PP_i showed almost identical effects with both phosphatases. All the results reported on the effect of PP_i on phosphatase activity with histone as the substrate were determined using 0.5 mg/ml histone in the assay mixture. When this concentration of histone was increased to 1 mg/ml in the assay, the inhibition of phosphatase activity by PP_i was observed up to a concentration of 1 mM. Concentrations higher than 1 mM PP_i again caused an activation of the enzyme but it was less than that observed with 0.5 mg/ml of histone. It can easily be deduced from these data that the percent inhibition or activation of phosphatase activity by PP_i with histone substrate is dependent on the concentration of PP_i and histone in the assay mixture.

In order to determine whether the interaction of histone and PP_i was specific, the effect of P_i on phosphatase activity with all three substrates was also determined. As was the case with PP_i, P_i inhibited phosphatase activity with phosphorylase *a* and phosphorylated casein as substrate (Fig. 3). With histone as the substrate, P_i activated the enzyme; maximum activation being observed between 1—5 mM P_i with 0.5 mg/ml histone in the assay. The exact interaction of P_i with histone was also dependent on their concentration in the assay.

The results reported in Figs. 2 and 3 with histone substrate were due to an interaction of P_i or PP_i with histone during the assay and not due to any artifact created in the precipitation step after the assay. Furthermore, blank values with histone substrate were not changed in the absence or presence of P_i and PP_i.

Reactivation of phosphatases by cobalt

It was previously shown that PP_i-inactivated phosphoprotein phosphatase II can be reactivated by a further preincubation of enzyme with Co^{2+} [14]. A

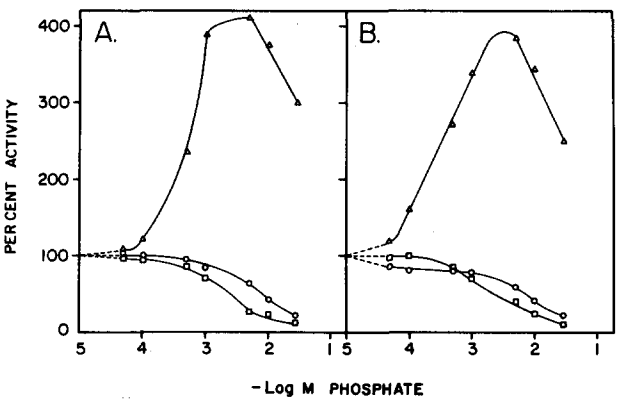

Fig. 3. Effect of varying concentrations of P_i added during the assay on activities of purified rabbit liver phosphoprotein phosphatase I (A) and phosphoprotein phosphatase II (B). Substrates used are phosphorylase *a* (○———○), casein (□———□) and histone (△———△). Phosphatase activity with each substrate without phosphate addition was taken as 100%.

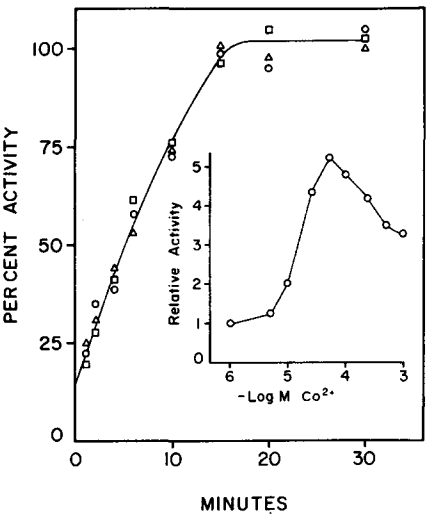

Fig. 4. Reactivation of PP_i-inactivated rabbit liver phosphoprotein phosphatase II by Co^{2+}. The inactivation of phosphoprotein phosphatase II was carried out as described in Fig. 1. The diluted PP_i-inactivated phosphoprotein phosphatase II was further preincubated with 0.25 mM Co^{2+} and at a desired time an aliquot was withdrawn and assayed for phosphatase activity with phosphorylase *a* (○———○), casein (□———□) or histone (△———△) as substrates. Phosphatase activity with no preincubation was taken as 100% with each substrate. Inset: optimal concentration of Co^{2+} for the reactivation of PP_i-inactivated rabbit liver phosphoprotein phosphatase II.

detailed investigation on reactivation of phosphatase by Co^{2+} was undertaken using both purified phosphatases and by employing all three substrates in the assay. The reactivation of PP_i-inactivated phosphoprotein phosphatase II is shown in Fig. 4. With 0.25 mM Co^{2+}, maximum reactivation was observed in 15 min of preincubation. No difference was observed among three substrates. The optimal concentration of Co^{2+} required for maximal activation was 25—50 μM (Fig. 4 inset). Similar results on Co^{2+} reactivation were obtained when PP_i-inactivated phosphoprotein phosphatase I was used in experiments otherwise identical with those of Fig. 4. In essence, the cobalt reactivation was identical with both phosphoprotein phosphatases.

Specificity of Co^{2+} for the activation of phosphatase

It was shown previously with phosphoprotein phosphatase II that the reactivation of the enzymes with Co^{2+} was very specific, and of several metal ions tested, only Mn^{2+} was capable of restoring activity [14]. The specificity of Co^{2+} was examined for the reactivation of both phosphoprotein phosphatases inactivated with PP_i. For this purpose, phosphoprotein phosphatases were preincubated with PP_i for inactivation, dialyzed to remove excess PP_i and then reactivated with different metal ions. Reactivation was only observed with Co^{2+} and Mn^{2+}. Although both of these ions completely reactivated the enzyme, Mn^{2+} required ten times higher concentration than Co^{2+} for this process. Other metal ions including Mg^{2+}, Ca^{2+}, Fe^{2+}, Ba^{2+}, Cd^{2+}, Cu^{2+}, Ni^{2+}, Hg^{2+}, Sn^{2+}, and Zn^{2+} did not reactivate the enzyme.

Requirement of dithiothreitol in inactivation-reactivation process

Since all buffers used in this study contained dithiothreitol, it was of interest to determine whether the reducing agent was required for inactivation-reactivation process. For the inactivation process, no difference was observed whether the preincubation of phosphatases was performed in the absence or presence of 0.5 mM dithiothreitol. However, 0.5—5 mM dithiothreitol was an absolute requirement for the reactivation process. No reactivation was observed with either 0.10 mM Co^{2+} or 0.5 mM Mn^{2+} if dithiothreitol was omitted during the reactivation process. Although other reducing agents were not examined in this study, the results obtained with dithiothreitol would indicate that sulfhydryl groups are involved in the reactivation process.

Mechanism for inactivation and reactivation of the enzyme

In order to determine whether inactivation of the enzyme by ATP, ADP or PP_i was due to chelation of a tightly-bound metal ion, active enzyme phosphoprotein phosphatase I and II were preincubated with 0.25 mM [γ-^{32}P]ATP and the bound radioactivity determined. Although no quantitative calculations were made, the enzyme bound labelled ATP. After the removal of excessive [γ-^{32}P]ATP by dialysis, the inactivated enzyme was then incubated with Co^{2+}. This treatment reactivated the enzyme without removing any radioactivity, suggesting that reactivation by Co^{2+} did not require the removal of bound ATP from the enzyme. The reactivated enzyme can be dialyzed to remove excess Co^{2+} and inactivated again by further preincubation with ATP, ADP or PP_i.

Discussion

It was previously considered to determine whether the two homogeneous phosphoprotein phosphatases purified from rabbit liver represent different enzymes [9,33]. Characterization of their properties suggested that it was so. Phosphoprotein phosphatase II was 5—10-fold more active than phosphoprotein phosphatase I with phosphorylase *a*, glycogen synthase D and phosphorylated histone are substrates [9]. With casein, the activity of phosphoprotein phosphatase I was higher than that of phosphoprotein phosphatase II. The K_m values for all substrates with phosphoprotein phosphatase I were lower than those of phosphoprotein phosphatase II. The two enzymes showed different mobilities on SDS-polyacrylamide gels [9]. Furthermore, the inactivation of phosphoprotein phosphatase I by PP_i requires relatively low concentration of this metabolite and less preincubation time when compared to the inactivation of phosphoprotein phosphatase II (Fig. 1). Such results would further strengthen the view that the two phosphatases are distinct proteins. Yet, it is still possible that phosphoprotein phosphatase I might represent a proteolytic product or some other modified form of phosphoprotein phosphatase II, since no absolute method is available for ruling this out.

In the past several years, attempts have been made to determine whether phosphorylase phosphatase (or phosphoprotein phosphatase) in mammalian tissues exists in active and inactive forms and whether the interconversion process involves phosphorylation-dephosphorylation of the enzyme. Work so far has revealed that this enzyme from skeletal muscle [30—32], adrenal cortex

[34], heart [23] and liver [14,24,35—37] can be inactivated by ATP or its degradation products. Among ATP degradation products examined, PP_i has been found to be the most potent inactivating metabolite [14,23,24]. The inactive enzyme can be reactivated by divalent cations such as Mn^{2+} or Co^{2+}. Earlier studies with partially-purified enzymes from pigeon breast muscle [30], adrenal cortex [34] and dog liver [35] suggested that reactivation was by ATP + Mg^{2+} alone, but no such reactivation was observed with purified heart and liver enzymes [14,23,24]. The results reported in this manuscript show inactivation of two homogeneous rabbit liver phosphoprotein phosphatases by ATP, ADP and PP_i, and reactivation by Co^{2+} and Mn^{2+}. Furthermore, it was also shown that the effect was on the enzyme and not on its substrates, since activities assayed with different substrates gave identical inactivation and reactivation results. Although all of these reports have suggested the interconversion of phosphoprotein phosphatase between active and inactive forms, no evidence has been found to suggest that this type of interconversion takes place by any covalent modification of the enzyme. In fact, the data suggest that covalent modification such as phosphorylation-dephosphorylation is not involved in this interconversion. First, the inactivation of the enzyme by ATP does not require any divalent cations, a necessary requirement for protein kinase-mediated phosphorylation reactions and secondly, the purified phosphoprotein phosphatases, which are free of protein kinase activity, undergo inactivation-reactivation without any exogenously added protein kinase [14, 23]. It was previously suggested that the enzyme in skeletal muscle [31,32] and heart [23] might be a metallo-enzyme containing a tightly bound divalent cation in the active site. ATP, due to its structural similarity to the phosphoprotein substrate and its ability to chelate metal ions, might easily enter the metal ion site and release it from that site. The results presented in this study with metal-chelating agents, as well as experiments dealing with binding of labelled ATP would not suggest that the ATP, ADP or PP_i effect is due to chelation of metal ions. The cobalt effect for the reactivation of the enzyme on the other hand, is also not due to removal of ATP, ADP or PP_i from the enzyme.

The specificity of divalent cations for reactivation of liver phosphoprotein phosphatases is intriguing. Although Co^{2+} and Mn^{2+} are the only two metal ions which can reactivate the purified enzymes, their involvement in vivo is difficult to understand from this study. The total concentration of Co^{2+} and Mn^{2+} in bovine liver was found to be 2 and 20 μM, respectively [38] and most of these ions are not in free but in bound form. The absolute requirement of dithiothreitol for reactivation process suggests that the enzyme must be in the reduced form before it can be reactivated. Recently, Shimazu et. al [39] reported inactivation of phosphorylase phosphatase by oxidized glutathione disulfide. These results should be considered in the light of oxidation-reduction of the enzyme which might play an important role in the regulation of enzyme activity in this type of interconversion.

The exact mechanism for the activation of phosphoprotein phosphatases by PP_i and P_i with histone as the substrate is not known. It is possible, however, that negative charge compounds would negate the positive charges on histone and probably make it a better substrate for phosphoprotein phosphatase. What-

ever the mechanism for this activation might be one should be careful when using negatively-charged compounds with histone substrate for studying the regulation of phosphoprotein phosphatase or any other enzyme which employs histone in the assay mixtures.

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