Biochimica et Biophysica Acta, 485 (1977) 379—390

BBA 68294

SOME PROPERTIES OF PURIFIED PHOSPHOPROTEIN PHOPHATASES FROM RABBIT LIVER

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(Received April 12th, 1977)

Summary

The activity of two purified homogeneous phosphoprotein phosphatases (types P I and P II) (phosphoprotein phosphohydrolase, EC 3.1.3.16) from rabbit liver (Khandelwal, R.L., Vandenheede, J.R., and Krebs, E.G. (1976) J. Biol. Chem. 251, 4850—4858) were examined in the presence of divalent cations, P_i, PP_i, nucleotides, glycolytic intermediates and a number of other compounds using phosphorylase *a*, glycogen synthase D and phosphorylated histone as substrates. Enzyme activities were usually inhibited by divalent cations with all substrates; the inhibition being more pronounced with phosphorylase *a*. Zn^{2+} was the most potent inhibitor among the divalent cations tested. The enzyme was competitively inhibited by PP_i (K_i = 0.1 mM for P I and 0.3 mM for P II), P_i (K_i = 15 mM for P I and 19.8 mM for P II) and *p*-nitrophenyl phosphate (K_i = 1 mM and 1.4 mM for P I and P II, respectively) employing phosphorylase *a* as the substrate. These compounds along with a number of others (Na_2SO_4, citrate, NaF and EDTA) also inhibited the enzyme activity with the other two substrates. Severe inhibition of the enzyme was also observed in the presence of the adenine and uridine nucleotides; monophosphate nucleotides being more inhibitory with phosphorylase *a*, whereas the di- and triphosphate nucleotides showed more inhibition with glycogen synthase D and phosphorylated histone. Cyclic AMP had no significant effect on enzyme activity with all the substrates tested. Phosphorylated metabolites did not show any marked effect on the enzyme activity with phosphorylase *a* as the substrate.

Introduction

Several studies on the specificity and regulation of phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) in crude extracts, partially purified and purified preparations of different tissues have appeared in

recent years [1—15]. In spite of all this work, the role of divalent cations in the regulation of this enzyme remains ambiguous primarily because this enzyme exists in multiple molecular forms. Some molecular forms of the enzyme are completely dependent on divalent cations for their activity whereas the other forms are either independent of or are only slightly stimulated by these metal ions [13—15]. Furthermore, the divalent cation effects vary with the substrate employed [1,5,14]. The role of nucleotides and glycolytic intermediates in the regulation of phosphoprotein phosphatase has not been a subject of extensive work.

Several investigators have observed that phosphoprotein phosphatase in crude extracts exists in multiple molecular forms [6,7,9,13,14] and the molecular weights vary from 54 500 to 500 000 [6,9,13,14]. However, the molecular weights of purified homogeneous enzymes in liver [11,12] and in skeletal muscle [4] are between 30 500 and 34 500. In 1975, Brandt et al. [16] reported that the interaction of purified phosphatase (M_r = 34 000) with a heat-stable protein inhibitor of phosphatase converted the low molecular weight purified enzyme into a multiple molecular form enzyme (M_r = 50 000—250 000). The possibility that high molecular weight forms are associated forms of low molecular weight catalytic subunit and some regulatory subunit presumably inhibitor protein has been extended by some workers [4,7,16].

We have recently purified two homogeneous low molecular weight forms (or presumably catalytic subunit) of phosphoprotein phosphatase from rabbit liver [11]. The enzyme was recovered in two distinct peaks after chromatography on Sepharose-4B-histone. Both of these peaks were able to dephosphorylate a number of phosphoproteins employed in this study, including phosphorylase *a*, glycogen synthase D and phosphorylated histone. The present communication reports the effect of divalent cations, various nucleotides and glycolytic intermediates, and several other compounds on the activity of both purified phosphoprotein phosphatase I and II using the above three substrates.

Experimental Procedures

Materials

Crystalline rabbit muscle phosphorylase *b* was isolated as described by Fischer and Krebs [17]. Skeletal muscle phosphorylase kinase, isolated according to the method of Hayakawa et al. [18], was a kind gift from Dr. J.H. Wang and Mr. T. Singh, Department of Biochemistry, University of Manitoba. Rabbit skeletal muscle glycogen synthase was isolated by the method of Soderling et al. [19]. The catalytic subunit of cyclic AMP-dependent protein kinase, isolated as described by Beavo et al. [20], was a generous gift from Drs. P.J. Bechtel and E.G. Krebs, University of California, Davis. Bovine serum albumin, histone II-A, dithiothreitol, *p*-nitrophenyl phosphate, and all the nucleotides and glycolytic intermediates used in this study were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. CNBr-activated Sepharose-4B, Sephadex G-75 and DEAE-Sephadex A-50 were products of Pharmacia. [γ-^{32}P]ATP was obtained from ICN, Irvine California. PCS (Phase-combining system) solvent for liquid scintillation was a product of Amersham/Searle. All other chemicals were of reagent grade.

Purification of phosphoprotein phosphatase from rabbit liver

The detailed procedure for the purification of phosphoprotein phosphatase from rabbit liver has been previously described [11]. Briefly, the livers were homogenized in a Waring blendor with 4 vols. 20 mM Tris (pH 7.4) containing 4 mM EDTA, 0.5 mM dithiothreitol and 0.1 mM phenylmethane sulfonyl fluoride. The 8000 × *g* supernatant obtained after centrifugation was adjusted to pH 5.2 with 1 M acetic acid and the pellet collected by centrifugation. The acid pellet was suspended in the above buffer and centrifuged at 200 000 × *g* for 4 h. The high speed clear supernatant was adsorbed on a column of DEAE-Sephadex A-50 and the enzyme was eluted with a NaCl linear gradient. The fractions containing the phosphatase activity were pooled and stored at 4° for 24 hours. After this period, the enzyme was precipitated with 50% saturated ammonium sulfate and the dissolved pellet was applied to a column of Sephadex G-75. The fractions containing enzyme activity in the retarded fraction of this column were pooled and applied to a column of Sepharose-histone. The enzyme activity was eluted from this last column with a NaCl linear gradient. The phosphoprotein phosphatase activity appeared in two distinct peaks. These two peaks were tentatively designated as phosphoprotein phosphatase I and phosphoprotein phosphatase II in the order of their elution from the Sepharose-histone column. This nomenclature has been used for these two peaks throughout this paper.

Preparation of ^{32}P-labelled substrates

The preparation of all ^{32}P-labelled substrates has been previously described in detail [11]. 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. [21]. ^{32}P-labeled glycogen synthase D was prepared by incubating purified muscle glycogen synthase I (2 mg/ml) with 0.2 mM [γ-^{32}P]ATP, 10 mM Mg^{2+} and 20 μg/ml purified catalytic subunit of protein kinase in the presence of 50 mM NaF at 30°C. After 2 h, the enzyme was precipitated with 50% saturated ammonium sulfate and the mixture centrifuged. The pellet was dissolved in the minimal volume of 50 mM Tris buffer (pH 7.2), containing 10% sucrose, 15 mM β-mercaptoethanol and 2 mM EDTA and passed through a small column of Sephadex G-75 to remove the catalytic subunit of protein kinase. Under these conditions, 1.7—2.0 mol phosphate were incorporated per subunit (M_r = 90 000) of synthase. ^{32}P-histone II-A was prepared essentially according to the method of Meisler and Langan [22]. The 20% trichloroacetic acid-insoluble pellet was washed once with water and then dissolved in a small volume of 25 mM 2[*N*-morpholino]ethane sulfonic acid buffer, pH 6.9. The dissolved suspension was first dialyzed against the same buffer and then dialyzed against 10 mM Tris, pH 7.0. The [^{32}P]histone contained 18 nmol of bound ^{32}P per mg of histone. The protein substrates, except phosphorylase *a*, were stored at —20°C; 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 [11]. Reaction mixtures for the dephosphorylation of [^{32}P]phosphorylase *a* contained 50 mM Tris (pH 7.4), 1 mM

caffeine, 2 mg/ml phosphorylase *a*, 0.5 mM dithiothreitol and phosphoprotein phosphatase preparation in a total volume of 50 μl. With [^{32}P]glycogen synthase D as substrate, reaction mixtures contained 50 mM Tris (pH 7.2), 2 mg/ml bovine serum albumin, 0.3 mg/ml glycogen synthase, 0.5 mM dithiothreitol, 10 mM $MgCl_2$ and phosphatase in a total volume of 50 μl. Reaction mixtures for the dephosphorylation of [^{32}P]histone contained 50 mM Tris (pH 7.0), [^{32}P]histone, 2 mg/ml, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 M KCl and phosphoprotein phosphatase in a total volume of 50 μl. 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 of 10% trichloroacetic acid for all substrates except [^{32}P]histone. In this instance, the reactions were stopped with 0.2 ml of 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 at 750 × *g* for 10 min. Aliquots of the clear supernatants were then counted with 5 ml of PCS 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 substracted from all the assays. One unit of phosphoprotein phosphatase was defined as that amount of enzyme released 1 nmol of 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. [23] 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 employed in this study.

Results

Effect of divalent cations

In previous studies by other investigators, the activity of partially purified phosphatase from skeletal muscle [1,14] and heart [5] is stimulated by Mg^{2+}, Co^{2+} and Mn^{2+} with glycogen synthase D or phosphorylated histone as substrates. However, these metal ions inhibited the activity of the same enzyme when phosphorylase *a* was the substrate. In order to clarify the roles of these ions as well as those of Ca^{2+} and Zn^{2+}, their effects on the activity of the homogeneous liver phosphoprotein phosphatase I and II [11] were determined with all these substrates. Fig. 1 shows the effects of added Mg^{2+}, Mn^{2+}, Ca^{2+}, Co^{2+} and Zn^{2+} on the activity of both purified phosphoprotein phosphatase I and II with phosphorylase *a* as the substrate. It is seen that all the cations inhibit the activity of both phosphoprotein phosphatases I and II non-competitively. Zn^{2+} was the most potent inhibitor followed by Co^{2+}, Mn^{2+}, Mg^{2+} and Ca^{2+}. There were no significant differences between the two phosphatases in the K_i values for these divalent cations (Table I).

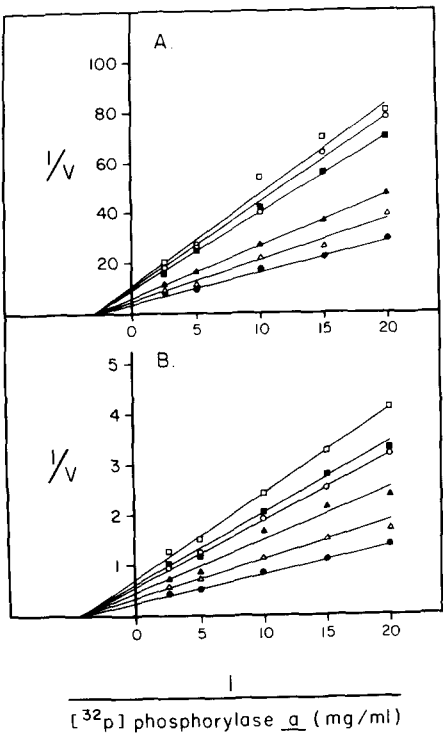

Fig. 1. Inhibition of dephosphorylation of [^{32}P]phosphorylase *a* by several divalent cations using rabbit liver phosphoprotein phosphatase I (A) and phosphoprotein phosphatase II (B). Double reciprocal plots of $1/[^{32}P]$phosphorylase *a* versus $1/v$ with no addition (●———●); Mg^{2+}, 1 mM (△———△); Zn^{2+}, 0.1 mM (○———○); Co^{2+}, 1 mM (■———■); and Ca^{2+}, 10 mM (□———□). All divalent cations were added as their chloride salts.

With phospho-histone as substrate the results were very similar. The cations inhibited both I and II phosphoprotein phosphatases noncompetitively and the relative order of inhibition was the same with this substrate. However, the concentration of cations required to effect a similar degree of inhibition was much higher with the exception of Zn^{2+}. The K_i values are given in Table I.

TABLE I

INHIBITION CONSTANTS FOR THE EFFECTS OF VARIOUS DIVALENT CATIONS ON PURIFIED PHOSPHOPROTEIN PHOSPHATASE FROM RABBIT LIVER

Divalent cations *	Phosphorylase *a* as the substrate		Phosphorylated histone as the substrate	
	Phosphoprotein phosphatase I (mM)	Phosphoprotein phosphatase II (mM)	Phosphoprotein phosphatase I (mM)	Phosphoprotein phosphatase II (mM)
Mg^{2+}	3.2	3.1	N.E. **	N.E.
Mn^{2+}	1.9	2.2	12.2	10.1
Ca^{2+}	5.1	3.7	33.0	16.0
Co^{2+}	0.7	1.0	11.8	3.0
Zn^{2+}	0.1	0.1	0.1	0.1

* All divalent cations were added as their chloride salts.

** N.E. = no effect.

TABLE II

EFFECT OF VARIOUS DIVALENT CATIONS ON THE ACTIVITY OF PURIFIED PHOSPHOPROTEIN PHOSPHATASE II FROM RABBIT LIVER USING GLYCOGEN SYNTHASE AS THE SUBSTRATE

Divalent cations *	Concn. (mM)	Activity (% of control)
Mg^{2+}	1	102.6
	10	82.6
Mn^{2+}	1	110.7
	10	97.7
Ca^{2+}	1	107.4
	10	67.3
Co^{2+}	1	133.4
	10	118.7
Zn^{2+}	0.1	76.7
	1.0	7.7
	10.0	2.0

* All divalent cations were added as their chloride salts.

The effect of divalent cations on the activity of phosphoprotein phosphatase II with substrate glycogen synthase D was also determined (Table II). At 1-mM concentration, all divalent cations, except $ZnCl_2$, either showed no inhibition or a little stimulation. At this concentration Zn^{2+} inhibition was about 92%. At a higher concentration, Mg^{2+}, Ca^{2+} and Zn^{2+} inhibited the activity of this phosphatase; the inhibition with Zn^{2+} being the most severe. Co^{2+} slightly stimulated the activity whereas Mn^{2+} showed no effect. A detailed study on the effects of these divalent cations with this substrate and enzyme phosphoprotein phosphatase I has not yet been done but in one experiment, the effect of 10 mM Mg^{2+} was similar to that obtained with phosphoprotein phosphatase II.

Effect of other compounds on liver phosphoprotein phosphatase

The effect of a number of compounds on the activity of phosphoprotein phosphatase II with phosphorylase *a*, phospho-histone and glycogen synthase D substrates is shown in Table III. All of these compounds either did not show any effect or inhibited the enzyme activity. The inhibition by P_i and PP_i on the enzyme activity were significantly different with various substrates employed. The enzyme activity in the presence of 10 mM P_i with substrate phosphorylase *a* was about 60% (or 40% inhibition) as compared to the control whereas the phosphatase activity with glycogen synthase D and phosphohistone was about 30% and 40%, respectively. On the other hand, in the presence of 1 mM PP_i, the enzyme activity was lowest with phosphorylase *a* compared to the activity with the other two substrates glycogen synthase D and phospho-histone. Among the other compounds, Na_2SO_4 (10 mM), p-nitrophenyl phosphate (2 mM), citrate (1 and 10 mM), NaF (10 mM), and glycogen (1 and 10 mg/ml) also showed differences with these three substrates. EDTA, at a concentration of 10 mM, inhibited the enzyme activity with all substrates whereas this compound at 1 mM concentration and EGTA at 1 and 10 mM concentration showed no significant effect.

TABLE III

EFFECT OF VARIOUS COMPOUNDS ON THE ACTIVITY (IN % OF CONTROL) OF PHOSPHOPROTEIN PHOSPHATASE II FROM RABBIT LIVER

Compounds	Concn. (mM)	Substrates		
		[^{32}P]phosphorylase *a*	[^{32}P]histone	^{32}P-labeled glycogen synthase D
Glucose	10	91.3	99.8	108.4
	100	86.2	99.2	103.7
Glc-6-*P*	1	80.0	93.0	100.5
	5	78.1	85.0	82.3
P_i	10	60.5	41.3	31.0
PP_i	1	24.2	71.7	61.3
Na_2SO_4	10	58.4	77.2	78.9
p-Nitrophenylphosphate	2	63.5	76.5	55.0
Citrate	1	80.8	93.3	105.3
	10	43.5	61.3	52.6
NaF	10	47.7	70.5	58.5
NaCl	10	102.0	N.D. *	98.3
	100	50.0	N.D.	54.8
EDTA	1	99.5	105.8	101.4
	10	72.3	73.5	57.0
EGTA	1	93.6	101.7	110.0
	10	94.7	102.2	101.2
Glycogen	1 mg/ml	84.8	107.5	111.8
	10 mg/ml	88.9	101.7	108.7

* N.D. = Not determined because all assays with this substrate were done in the presence of 100 mM KCl.

The kinetics of phosphoprotein phosphatase II inhibition by PP_i and *p*-nitrophenyl phosphate was examined using phosphorylase *a* as the substrate (Fig. 2). It is seen that both of these compounds competitively inhibited the enzyme activity. Results with phosphoprotein phosphatase I were almost identical (not shown). The inhibition by P_i employing phosphorylase *a* and both phosphatase was also competitive (results not shown). The K_m values are given in Table IV which indicate that PP_i was the most potent inhibitor for both enzymes.

Effect of nucleotides

The effect of adenine and uridine nucleotides on phosphoprotein phosphatase II is shown in Table V. Among adenine nucleotides, AMP inhibited the enzyme activity relatively more with substrate phosphorylase *a* whereas ADP and ATP showed more inhibition with the other two substrates. Cyclic AMP had no significant effect with any substrate. Uridine nucleotides, much less inhibitory than adenine, were either inhibitory or did not show any significant effect with the substrates. As was the case with AMP, UMP also showed more inhibition with substrate phosphorylase *a*. UDPglucose had no effect on phosphatase II activity with phosphorylase *a* or with glycogen synthase D.

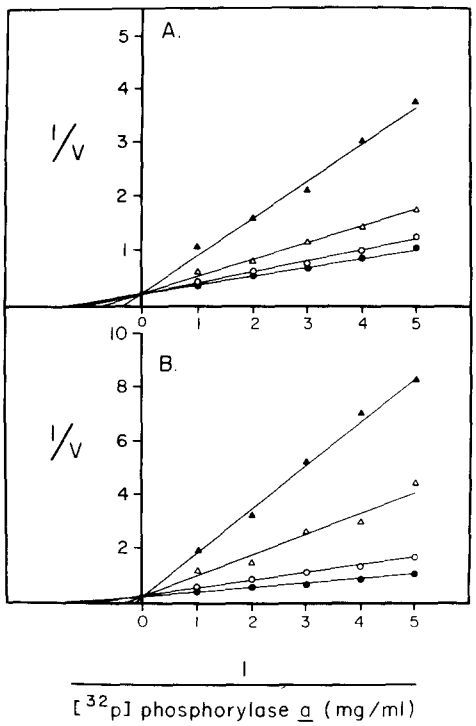

Fig. 2. Inhibition of dephosphorylation of [^{32}P]phosphorylase *a* by *p*-nitrophenyl phosphate (A) and by PP_i (B) using rabbit liver phosphoprotein phosphatase II. A, Double reciprocal plots of $1/([^{32}P]$phosphorylase *a*) vs. $\frac{1}{v}$ with 1 mM (○———○); 2 mM (△———△); and 4 mM (▲———▲) of *p*-nitrophenyl phosphate. B, double reciprocal plots of $1/([^{32}P]$phosphorylase *a*) vs. $\frac{1}{v}$ with 0.5 mM (○———○); 1 mM (△———△) and 2 mM (▲———▲) of PP_i. Controls without inhibitor are indicated by filled circles in both cases.

Effect of phosphorylated metabolites

In 1945, Cori and Cori [24] observed the inhibitory effect of a number of phosphorylated metabolites on the activity of a partially purified phosphorylase phosphatase from skeletal muscle. Recently, Martensen et al. [25] using a more purified phosphatase preparation from the same tissue reported that Glc-6-*P* activates this enzyme. In our studies, Glc-6-*P* inhibited the activity of liver phosphatase with all three substrates (Table III). The effects of other phos-

TABLE IV

INHIBITION CONSTANTS FOR P_i, PP_i AND *p*-NITROPHENYLPHOSPHATE FOR PURIFIED PHOSPHOPROTEIN PHOSPHATASE FROM RABBIT LIVER WITH PHOSPHORYLASE *a* AS THE SUBSTRATE

	Phosphoprotein phosphatase I (mM)	Phosphoprotein phosphatase II (mM)
P_i	15.0	19.8
PP_i	0.3	0.1
p-Nitrophenylphosphate	1.4	1.0

TABLE V

EFFECT OF VARIOUS NUCLEOTIDES ON THE ACTIVITY (IN % OF CONTROL) OF PHOSPHOPROTEIN PHOSPHATASE II FROM RABBIT LIVER

Nucleotides	Concn. (mM)	Substrates		
		[^{32}P]phosphorylase *a*	[^{32}P]histone	^{32}P-labeled glycogen synthase D
ATP	0.1	89.7	101.2	114.6
	1.0	56.8	55.0	33.1
ADP	0.1	87.9	97.4	118.9
	1.0	55.0	37.1	26.8
AMP	0.1	59.8	101.7	106.8
	1.0	14.0	88.0	96.0
Cyclic AMP	0.1	85.2	97.2	113.8
	1.0	86.4	103.8	107.6
UTP	0.1	81.1	100	113.9
	1.0	76.4	87.7	101.2
UDP	0.1	88.6	103.3	104.6
	1.0	78.1	91.0	110.8
UMP	0.1	85.0	97.5	108.4
	1.0	57.7	79.8	86.2
UDPglucose	0.2	117.1	N.D. *	106.5
	2.0	107.9	N.D.	101.2

* N.D. = Not determined using this substrate.

TABLE VI

EFFECT OF VARIOUS PHOSPHORYLATED METABOLITES (2 mM) ON THE ACTIVITY OF PHOSPHOPROTEIN PHOSPHATASE II FROM RABBIT LIVER BY USING ^{32}P-LABELLED PHOSPHORYLASE *a* AS THE SUBSTRATE

Metabolites	Activity (% of control)
Glc-1,6-P_2	108.6
Gal-1-*P*	112.7
Gal-6-*P*	113.4
Fru-1-*P*	109.3
Fru-6-*P*	105.1
Fru-1,6-P_2	117.3
Rib-5-*P*	112.1
Ara-5-*P*	117.4
Glyceraldehyde-3-*P*	88.9
NH_2-Glu-*P*	116,5

phorylated metabolites on the activity of phosphoprotein phosphatase II with phosphorylase *a* are shown in Table VI. As shown in this table, all compounds tested were without any marked effect, hence they were not further examined using the other two substrates.

Discussion

As early as 1945, Cori and Cori [24] observed that the presence of Mn^{2+} (0.5 mM) was necessary for the maximum activity of a partially purified skele-

tal muscle phosphorylase phosphatase. The same concentration of Mg^{2+} inhibited the enzyme whereas Co^{2+} showed no effect. In 1955, Keller and Cori [26] partially purified the same enzyme and reported that this enzyme did not require Mn^{2+} for its full activity. Mg^{2+} was again inhibitory. In 1972, Kato and Bishop [1] partially purified a glycogen synthase phosphatase from skeletal muscle and showed that the enzyme activity was stimulated by Mn^{2+}, Ca^{2+} and Mg^{2+}. Kato and Sato [14] later in 1974 separated the activity of the same enzyme into two distinct peaks on Sephadex G-150 and demonstrated that the activity of peak I enzyme was not affected by divalent cations whereas peak II enzyme was totally dependent on Mn^{2+} or Co^{2+} for activity. Similarly, Nakai and Thomas [5] studied the effect of divalent cations on a partially purified enzyme from bovine heart which dephosphorylated not only glycogen synthase D but also a number of other phosphoproteins including phosphorylase *a* and phosphorylated histone. The activity of this enzyme was stimulated by Mg^{2+}, Ca^{2+} or Mn^{2+} with glycogen synthase D and phospho-histone as substrates but was inhibited with phosphorylase *a* as the substrate. Kato et al. [27] and Kobayashi et al. [7] observed the activation of skeletal muscle phosphoprotein phosphatase by divalent ions without any change of molecular size with phosphorylase *a* and phospho-histone as substrates. A partially purified phosphoprotamine phosphatase from adrenal cortex [13] was stimulated by Mn^{2+}, unaffected by Mg^{2+} and Ca^{2+} and completely inhibited by Zn^{2+}, Co^{2+}, Cu^{2+} and Fe^{2+}. Purified liver phosphorylase phosphatase was also inhibited by Ca^{2+}, Mg^{2+} and Mn^{2+} [12].

All the confusion about the requirement of divalent cations for phosphoprotein phosphatase activity probably arises because of the use of different enzyme preparations of uncertain purities. The availability of homogeneous purified phosphoprotein phosphatase from rabbit liver [11] prompted examination of the effect of several divalent cations on the activity of this enzyme using a number of phosphoprotein substrates. With the exception of the effect of Co^{2+} and Mn^{2+} on the dephosphorylation of glycogen synthase D and Mg^{2+} on phosphorylated histone, the activity of liver phosphoprotein phosphatase was inhibited by all the cations tested with glycogen synthase D, phosphorylase *a* and phosphorylated histone as substrates. However, the degree of inhibition was variable depending on the substrate employed; being greatest with phosphorylase *a*. Although it is clear from the present study that the activity of phosphoprotein phosphatase is influenced to varying degrees by these divalent cations with the choice of substrate employed, whether the conflicting results obtained by other investigators are due to the difference in different extent of purity of preparations employed in those studies with an unknown protein factor(s) such as inhibitor protein [16] is not well understood. This possibility is presently under investigation.

Martensen et al. [28] reported that divalent metal ions inhibits skeletal muscle phosphorylase phosphatase by binding to an enzyme site which is presumed to be the one that interacts with the arginyl function of the substrate and not to the catalytic site. The non-competitive inhibition by divalent cations observed in the present study would also suggest that liver phosphoprotein phosphatases are also inhibited by the cations by binding to a site on the enzyme other than the catalytic site. As the cations showed a varying degree

of inhibition with different substrates employed (Fig. 1, Tables I and II), it is suggested that substrates also play a role in the inhibition of liver phosphoprotein phosphatases activity by divalent cations.

In the present study it was observed that PP_i was a strong inhibitor of liver phosphoprotein phosphatase having a K_i of 0.3 and 0.1 mM with phosphoprotein phosphatase I and II, respectively. The K_i values for PP_i were about 1/100 of the values obtained for P_i for both of these phosphatases (Table IV). These results were different from those of Kato and Bishop [1] who showed that both P_i and PP_i inhibited muscle glycogen synthase phosphatase with a similar K_i value of 0.2 mM. Martensen et al. [28] studied the effect of only P_i on muscle phosphorylase phosphatase and found that it inhibited the enzyme with a K_i value of 8 mM. As has been suggested for the skeletal muscle enzyme by Martensen et al. [28], competitive inhibition by P_i and PP_i of liver phosphatases would indicate that these compounds inhibit the enzyme activity by binding to the catalytic site of the enzyme. The inhibition of skeletal muscle phosphatase by P_i [29] and of heart phosphatase by P_i and PP_i [5] has also been previously reported. Although other metabolites, especially phosphorylated metabolites, have been shown to regulate partially purified phosphatases [5,12, 24,25,30–32], no such regulation was observed with our purified homogeneous phosphatases.

Purified liver phosphoprotein phosphatase was inhibited by all adenine and uridine nucleotides examined in this study (Table V). The two monophosphate nucleotides AMP and UMP were more inhibitory with substrate phosphorylase *a* whereas the di- and trinucleotides were more inhibitory with glycogen synthase D. The similar type of inhibition by several of these nucleotides has been reported for phosphoprotein phosphatase from adrenal cortex [13], liver [12, 30,31], skeletal muscle [24,27–29,32,33] and heart [5]. Martensen et al. [28] have examined the effect of AMP on muscle phosphorylase phosphatase in more detail and found that the inhibition by this nucleotide was by binding to substrate phosphorylase *a* rather than on the enzyme.

It is obvious from the present study on the effectors of purified phosphoprotein phosphatases from rabbit liver that the purified enzymes behave differently from the previously reported phosphatase activity in crude tissue extracts or in partially purified forms. As has been suggested before [4,7,16], if the high molecular weight forms are actually associated forms of the low molecular weight enzyme (or probably a catalytic subunit) with some regulatory subunit, one has to wait till the identification and purification of regulatory subunit(s) to understand the precise mechanism for the regulation of phosphoprotein phosphatases in liver and other tissues. However, if high molecular weight forms are the aggregates of the low molecular weight forms, future research may reveal whether these forms undergo aggregation and deaggregation and hence regulate the enzyme activity.

Acknowledgements

I am grateful to Mr. S.M. Zinman for excellent technical assistance. The criticisms and suggestions of Drs. E.T. Pritchard and C. Dawes during the preparation of this manuscript is appreciated. This study was supported by an operat-

ing grant from the Medical Research Council of Canada (MA-5633). The author is a MRC scholar.

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