Biochimica et Biophysica Acta, 566 (1979) 49—61
© Elsevier/North-Holland Biomedical Press

BBA 68631

NUCLEAR PHOSPHOPROTEIN PHOSPHATASE FROM CALF LIVER

MING W. CHOU *, ENG L. TAN and CHUNG S. YANG **

Department of Biochemistry, CMDNJ-New Jersey Medical School, Newark NJ, 07103 (U.S.A.)

(Received May 31st, 1978)

Key words: Phosphoprotein phosphatase; Histone; (Nuclear, Calf liver)

Summary

Calf liver nuclear phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) has been purified approx. 850-fold. The enzyme has a mol. wt. of 34 000 as determined by SDS-polyacrylamide gel electrophoresis. The purified enzyme has a pH optimum between 7.0 and 7.5 with phosphophosphorylase, phosphohistones f_1 and f_{2b}, and phosphoprotamine as substrates. The enzyme activity towards these substrates follows the order, phosphophosphorylase > phosphohistone f_1 > phosphohistone f_{2b} > phosphoprotamine. The K_m values toward phosphophosphorylase and phosphohistone f_1 are 17 and 28 μM phosphate, respectively. Dephosphorylated histone f_1 and orthophosphate are competitive inhibitors of the enzyme with respective K_i values of 11 μM and 4.1 mM. NaCl and divalent metal ions inhibit the enzyme but $CaCl_2$ is slightly stimulatory. It appears that metal ion inhibition occurs at two sites, one on the enzyme and the other on the substrate. The enzyme is also inhibited by NaF and EDTA. Nucleotides bearing the pyrophosphate structure are potent inhibitors of the enzyme while mononucleotides are slightly inhibitory. DNA and other polyions also inhibit the enzyme. The enzyme appears to require free sulfhydryl groups for activity since it is inhibited by *N*-ethylmaleimide and *p*-hydroxymercuribenzoate; the latter inhibition can be reversed by mercaptoethanol and dithiothreitol.

Introduction

The phosphorylation and dephosphorylation of proteins catalyzed by protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) and phosphoprotein

* Present address: Department of Pharmacology, Uniformed Service University of the Health Sciences, Bethesda, MD 20014, U.S.A.
** To whom correspondence should be addressed.

phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) have been shown to be important regulatory mechanisms of enzyme activity [1—4], membrane function [5,6] and other activities [7,8]. Most of the knowledge on the process of phosphorylation and dephosphorylation was obtained from studies of the cytosol enzymes involved in glycogen metabolism [9—12]. The properties of the cyclic AMP-dependent protein kinase have been delineated extensively [13]; but those of the phosphoprotein phosphatase are not well understood. This is especially true in the case of nuclear phosphoprotein phosphatase.

Since phosphorylation and dephosphorylation have been shown to be important in the regulation of gene activity, the elucidation of the properties of nuclear phosphoprotein phosphatase is of great current interest. Meisler and Langan [14] have shown that the nuclear enzyme accounts for about 4% of the phosphoprotein phosphatase activity of rat liver cells and its properties are similar to those of the cytosol phosphatase. Previously we have studied and compared the properties of the nuclear and cytosol enzymes [15,16]. The activity of the cytosol phosphatase was stimulated several fold by salts, divalent cations and ATP; but the nuclear enzyme was less responsive to these factors. After treating the enzyme with ethanol, however, the stimulatory effect was abolished and the properties of the two enzymes became similar. The ethanol treatment also increased the activity of both phosphoprotein phosphatases and they were then each eluted in a single peak in DEAE-cellulose chromatography. We have purified the nuclear phosphoprotein phosphatase from calf liver and report here the purification and some properties of this enzyme.

Materials and Methods

Materials. Calf thymus histones f_1 and f_{2b} (Sigma Type V-S and VII-S, respectively), phosphorylase *b* (from rabbit muscle), protamine (from salmon sperm), bovine serum albumin, myoglobin, carbonic anhydrase, cytochrome *c*, calf thymus DNA, polyphosphate, heparin, polylysine, dithiothreitol, ATP, GTP, ADP, GDP, AMP and cyclic AMP were purchased from Sigma Chemical Co. Ultrogel AcA-54 and ampholine were obtained from LKB Instruments, Inc. Sephadex G-75 (Superfine) and hexanediamine-Sepharose 4B were purchased from Pharmacia. [γ-^{32}P]ATP with specific activities of 15—30 Ci/mmol was obtained from New England Nuclear.

Isolation of calf liver nuclei. Fresh calf liver was freed of its connective tissue, chopped into small pieces, rinsed with saline and minced in a meat grinder. The minced liver was then homogenized with 3 vols. (v/w) homogenizing buffer (0.25 M sucrose, 25 mM KCl, 10 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4) in a Potter-Elvehjem type homogenizer. The homogenate was filtered successively through 2 and 6 layers of cheesecloth. The filtrate was centrifuged at 700 $\times g$ for 10 min. The precipitate was resuspended in the homogenizing buffer containing 0.5% (v/v) Triton X-100 and the mixture was centrifuged at 700 $\times g$ for 10 min. After repeating this procedure twice, the pellet was washed 3—5 times with the homogenizing buffer until no phosphoprotein phosphatase activity was detected in the supernatant fluid. The nuclear pellet was then examined

under a light microscope. The nuclear preparation and all subsequent steps were carried out at 4°C unless otherwise stated.

Assay of phosphoprotein phosphatase activity. The assay of phosphoprotein phosphatase was carried out as described previously [15]. The enzyme was incubated with ^{32}P-labeled phosphoprotein substrate in a total vol. of 50 μl at 30°C for 10 min. The reaction mixture comprised 50 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol and radioactive phosphoprotein. The reaction was initiated by the addition of the enzyme at a quantity that was within the linear range of the velocity vs. enzyme plot, i.e. less than 20% of substrate phosphate hydrolyzed. Following incubation, a 30 μl aliquot was spotted on a strip of Whatman 31 ET chromatography paper (2 × 22 cm) and developed by descending chromatography with 5% trichloroacetic acid to separate the product, [^{32}P]orthophosphate, from the substrate. The solvent front region of the paper strip which contained [^{32}P]orthophosphate was cut, washed with diethyl ether, dried and counted in a liquid scintillation counter. One unit of phosphatase activity corresponded to the release of 1 pmol orthophosphate from the [^{32}P]-phosphoprotein substrate per min. The specific activity is defined as units per mg protein.

Preparation of ^{32}P-labeled substrates. The phosphoprotein substrates used in this study were phosphohistones f_1 and f_{2b}, phosphorylated phosphorylase, and phosphoprotamine. They were phosphorylated with partially purified protein kinase. The protein kinase was isolated from calf liver cytosol by $(NH_4)_2SO_4$ fractionation (30—55% saturation) and DEAE-cellulose column chromatography. Two protein kinase fractions were resolved by the DEAE-cellulose step. Protein kinase I was eluted with 0.15 M NaCl in buffer A (10 mM Tris-HCl (pH 7.4), 1 mM β-mercaptoethanol, and 0.1 mM EDTA) and protein kinase II was eluted with 0.25 M NaCl in Buffer A. The protein kinase II fraction had a higher specific activity than protein kinase I but it also contained phosphoprotein phosphatase activity. Protein kinase I, which did not contain any detectable amount of phosphatase activity and did not undergo autophosphorylation in the presence of ATP, was used for the preparation of phosphoprotein substrates.

^{32}P-Labeled phosphoproteins were prepared according to the method of Meisler and Langan [14]. Histone or protamine was phosphorylated by incubation with [γ-^{32}P]ATP (200—500 cpm/pmol), Mg^{2+} and protein kinase I at 37°C for 2 h. The enzyme was precipitated by adding trichloroacetic acid to 2%. The phosphorylated histone or protamine was recovered from the supernatant by the addition of trichloroacetic acid to a final concentration of 25%. The precipitated histone or protamine was dissolved in distilled water, reprecipitated with 25% trichloroacetic acid and washed twice with ethanol/diethyl ether (1 : 4, v/v) and 0.1 N HCl in ethanol/diethyl ether (1 : 4, v/v). The final product was dissolved in water and dialyzed against distilled water overnight and stored at —20°C.

Phosphorylase *b* was phosphorylated by a similar procedure. The [^{32}P]phosphophosphorylase was precipitated by $(NH_4)_2SO_4$ at 50% saturation and then subjected to Ultrogel AcA-34 gel filtration chromatography. The [^{32}P]phosphophosphorylase was eluted in the void volume using Buffer A as the eluant. It was then precipitated with $(NH_4)_2SO_4$, dissolved in Buffer A and dialyzed

against the same buffer overnight. The product contained 0.5 nmol phosphorous per nmol phosphorylase subunit.

Purification of calf liver nuclear phosphatase. The purified calf liver nuclei were extracted by homogenizing with Buffer A containing 0.4 M NaCl. The extract was centrifuged at 105 000 × *g* for 60 min. To the clear 0.4 M NaCl extract, solid $(NH_4)_2SO_4$ was added to 70% saturation and the mixture was centrifuged at 16 000 × *g* for 10 min. The pellet was dissolved in a minimum volume of Buffer A and dialyzed against the same buffer. The dialysate was poured directly into 5 vols. ethanol at room temperature and centrifuged immediately at 10 000 × *g* for 5 min at 4°C. The enzyme was extracted from the precipitate twice by homogenizing the pellets in Buffer A and centrifugation at 10 000 × *g* for 10 min. The two extracts were combined and dialyzed against Buffer A.

The dialyzed sample was applied to a DEAE-cellulose (Whatman DE-52) column (1.5 × 30 cm) preequilibrated with 0.1 M NaCl in Buffer A. The column was washed with 50 ml equilibration buffer and eluted with a linear gradient (0.1—0.4 M NaCl) in 200 ml Buffer A. The active fractions were pooled and dialyzed against 2 l Buffer A for at least 4 h. The dialysate was rechromatographed on a DEAE-cellulose column (0.9 × 18 cm). The enzyme fraction was transferred to a dialysis tubing and concentrated by covering it with solid polyethylene glycol ($M_r \approx 20\,000$). The concentrated sample was applied on an Ultrogel AcA-54 gel filtration column (1.5 × 87 cm) and eluted with 0.1 M NaCl in Buffer A. The enzyme was pooled and dialyzed against Buffer A.

The dialyzed sample was chromatographed on a hexanediamine-Sepharose 4B column (0.9 × 28 cm) preequilibrated with Buffer A. The column was eluted with a linear gradient (0—0.3 M NaCl) in 200 ml Buffer A. The enzyme was pooled, dialyzed and rechromatographed on the same hexanediamine-Sepharose column. It was then chromatographed by an Ultrogel AcA-54 column followed by another hexanediamine-Sepharose 4B column. The enzyme was pooled, dialyzed against 50% glycerol in Buffer A, and stored at —90°C. The activity of the enzyme remained unchanged when it was stored in 50% glycerol in Buffer A at —90°C for 2 months.

Preparation of dephosphorylated histone f_1. The commercial histone f_1 contained approx. 12.5 nmol endogenous phosphate/mg protein as determined by the method of Meisler and Langan [14]. Most of the endogenous phosphate could be removed by incubating with phosphoprotein phosphatase. The enzyme could be separated from the dephosphorylated histone by the addition of trichloroacetic acid to 2%. The dephosphorylated histone was recovered from the supernatant by bringing the concentration of trichloroacetic acid to 25%.

SDS polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was carried out by the method of Weber and Osborn [17] in 10% acrylamide gels containing 0.1% sodium dodecyl sulfate. Bovine serum albumin, ovalbumin, carbonic anhydrase, chymotrypsinogen, myoglobin and cytochrome *c* were used as standards.

Protein determination. The protein content was measured by the spectrophotometric method of Ehresmann et al. [18], by measuring the absorbance

difference at 228.5 and 234.5 nm with bovine serum albumin as standard. The results obtained were comparable to the colorimetric method of Lowry et al. [19].

Results

Purification of nuclear phosphoprotein phosphatase. The results of the purification are summarized in Table I. The ethanol treatment step was found to be the most effective step which increased the specific activity by 7 fold and the total activity by 2 fold. Each column chromatography with DEAE-cellulose, Ultrogel, or hexanediamine gave an approx. 2-fold purification, with 50% yields. The elution profiles of the first two and last two steps are shown in Figs. 1 and 2, respectively. SDS-polyacrylamide gel electrophoresis of the enzyme showed a protein band with a mol. wt. of 34 000. In addition to the enzyme band, three faint impurity bands were detected. These bands had higher mobilities than the enzyme and the total amount of these proteins was less than 10% of the enzyme protein as determined by densitometry.

An overall 845-fold purification with a yield of 1.7% in enzyme activity was achieved by this procedure. During the course of the investigation, we have tried to vary the conditions of each step, to use different combinations of the steps, and to use additional methods such as histone-Sepharose 4B column for the purification. The sequence shown in Table I represents the procedure that afforded the best results. We had to use some of the chromatography steps more than once to increase the purity of the enzyme and a total of ten steps in the purification procedure.

Substrate specificity. The enzyme had very low alkaline phosphatase activity when *p*-nitrophenyl phosphate was used as substrate. Three enzyme prepara-

TABLE I

PURIFICATION OF CALF LIVER NUCLEAR PHOSPHOPROTEIN PHOSPHATASE

The starting material for preparation of nuclear phosphoprotein phosphatase was 200 g of nuclei isolated from 2500 g fresh calf liver. The phosphatase activity was determined at each stage of purification using phosphohistone f_1 as the substrate.

Procedures	Total protein (mg)	Total activity (units × 10^3)	Specific activity (units/mg protein)	Yield (%)	Purification (-fold)
0.4 M NaCl extraction	15 051	2166	144	100	1
$(NH_4)_2SO_4$ precipitation	7 071	1296	184	60	1.3
Ethanol treatment	1 804	2460	1 364	114	9.5
1st DEAE-cellulose column	240	816	3 400	38	23.6
2nd DEAE-cellulose column	77	1158	5 012	18	34.8
1st Ultrogel AcA-54 gelfiltration	11	172	15 544	7.9	108
1st hexanediamine-Sepharose 4B column	3.5	104	28 714	4.8	206
2nd hexanediamine-Sepharose 4B column	1.2	86	72 084	4.0	500
2nd Ultrogel AcA-54 gelfiltration	0.7	76	108 572	3.5	754
3rd hexanediamine-Sepharose 4B column	0.3	36	121 666	1.7	845

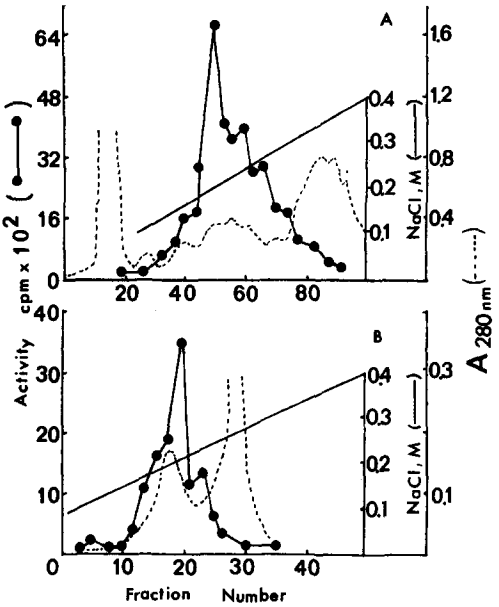


Fig. 1. DEAE-cellulose column chromatography of calf liver nuclear phosphatase. (A) The nuclear enzyme preparation after ethanol treatment was applied to a DEAE-cellulose column (1.5 X 30 cm) and eluted with a linear gradient of 0.1—0.4 M NaCl in 200 ml of Buffer A (2.7-ml fractions). A_{280nm} is in an arbitrary unit. (b) The enzyme was pooled and rechromatographed on another DEAE-cellulose column.

tions at the stages of $(NH_4)_2SO_4$ precipitation, ethanol treatment and second hexanediamine-Sepharose 4B column chromatography, were tested for alkaline phosphatase activity. The enzyme activities in these three preparations toward *p*-nitrophenyl phosphate was 7.4%, 0.75% and 0.04%, respectively, of the activity toward phosphohistone f_1.

The purified nuclear phosphoprotein phosphatase had a broad substrate spe-

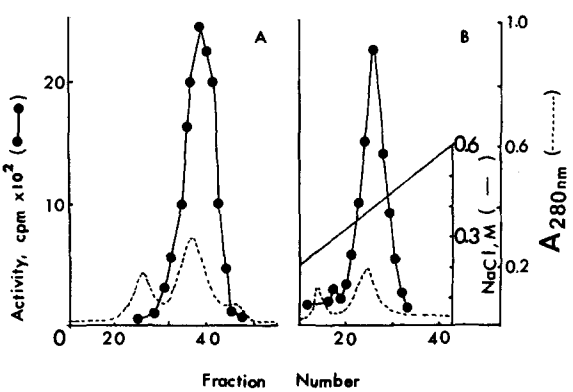
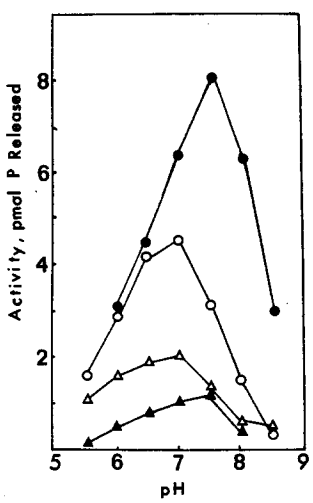


Fig. 2. Final steps of the purification of nuclear phosphoprotein phosphatase. (A) Nuclear phosphatase was rechromatographed on an Ultrogel AcA-54 column (1.5 X 87 cm) equilibrated and eluted with 0.1 M NaCl in Buffer A (3-ml fractions). (B) The resulting enzyme was chromatographed on a hexanediamine-Sepharose 4B column (0.9 X 28 cm). The enzyme was eluted with a linear gradient of 0—0.6 M NaCl in 140 ml Buffer A containing 10% (v/v) glycerol (2.7-ml fractions).

cificity toward phosphoproteins. It catalyzed the dephosphorylation reaction of various phosphoproteins, such as phosphohistones f_1 and f_{2b}, phosphophosphorylase, and phosphoprotamine with specific activities of 115 500, 43 750, 170 750, and 32 500 units/mg protein, respectively. The K_m values toward phosphohistone f_1 and phosphophosphorylase were 28 and 17 μM P_i, respectively.

The enzyme was also able to dephosphorylate the non-histone chromosomal proteins which were phosphorylated by protein kinases in vitro. However, it was found that only a small portion (less than 10%) of the incorporated phosphate could be hydrolyzed by the nuclear phosphatase. The molecular basis for this phenomenon remains to be elucidated.

Effect of pH on the nuclear phosphoprotein phosphatase activity. The pH optimum of the nuclear enzyme with phosphorylase, phosphohistones f_1 and f_{2b} and phosphoprotamine as substrates was between pH 7.0 and 7.5 (Fig. 3). The decline in activity at pH 6.0 and 8.5 was not due to the irreversible denaturation of the enzyme. When the enzyme was preincubated separately in buffers at pH 6.0 and 8.5 and the activity was assayed following adjustment to pH 7.4, there was no significant decrease in the enzyme activity. The histones and protamines are basic proteins having isoelectric points between pH 10 and 12, whereas phosphorylase has an isoelectric point of about 6. Thus, the former group of proteins bear positive charges even when phosphorylated and the phosphophosphorylase bears negative charges in the optimal pH range for the phosphatase activities with all substrates. The observed pH profile of activity appears to be due to the ionization of the amino acid residues on the enzyme



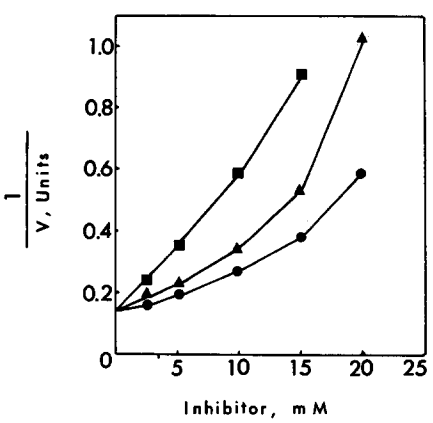


Fig. 3. Effect of pH on the activity of nuclear phosphoprotein phosphatase. The buffer consisted of 50 mM Tris and 50 mM imidazole. The enzyme activity was assayed at various pH points using [^{32}P]phosphohistone f_1 (○———○), [^{32}P]phosphohistone f_{2b} (△———△), [^{32}P]phosphophosphorylase (●———●) or [^{32}P]phosphoprotamine (▲———▲) as the substrate.

Fig. 4. Dixon plot of purified nuclear phosphoprotein phosphatase with phosphohistone f_1 as substrate. Experiments were performed in the presence of Mg^{2+} (●———●), Mn^{2+} (▲———▲) or orthophosphate (■———■). The conditions for these assays are described in Table II.

or on the enzyme binding sites of the substrates, and not to the gross charge that the substrates bear.

Product inhibition. When phosphohistone f_1 was used as the substrate for the assay of nuclear phosphatase, dephosphorylated histone f_1 and orthophosphate are the products of the dephosphorylation reaction. Histone f_1 was found to be an inhibitor of the purified nuclear phosphatase. The inhibition was not due to the effect of isotope dilution, since the activity of the enzyme inhibited by unlabeled histone was much lower than that predicted by isotope dilution. Dephosphorylated histone f_1 which was obtained by treating the commercial histone f_1 with phosphatase, was even more effective than the untreated histone f_1 in inhibiting the nuclear phosphatase activity.

The dephosphorylated histone f_1 and orthophosphate were competitive inhibitors when phosphohistone f_1 was used as the substrate. The K_i values of the dephosphorylated histone f_1 and orthophosphate are 11 μM and 4.1 mM, respectively.

Effects of divalent cations and salt. It was found previously that divalent metal ions, ATP, and NaCl stimulated the crude and partially purified phosphoprotein phosphatase, especially the cytosol enzyme [15]. However, the purified nuclear phosphatase was inhibited by NaCl and divalent ions (Table II). Ca^{2+} was an exception and slightly stimulated the purified enzyme (Table II). Among the divalent cations, Zn^{2+} and Co^{2+} were more inhibitory than Mn^{2+} and Mg^{2+}. NaCl was inhibitory only at high concentrations. The data in Tables II and III also indicate that the inhibition pattern observed with phosphohistone f_1 was similar to those found with phosphohistone f_{2b} as the substrate. In order to understand the mode of inhibition by Mg^{2+} and Mn^{2+}, the data were analyzed by Dixon plots (Fig. 4). The non-linear convex plots for these two cations suggest that the metal ions can act on at least two sites, one on the enzyme and the other on the substrate [20]. The Dixon plot for orthophos-

TABLE II

EFFECT OF METAL ION OR SALT ON PURIFIED CALF LIVER NUCLEAR PHOSPHOPROTEIN PHOSPHATASE ACTIVITY

Purified nuclear phosphoprotein phosphatase was assayed in triplicate in the presence of the divalent metal ion or NaCl at the concentrations indicated. The enzyme was dialyzed against 50% (v/v) glycerol in Buffer A and then diluted with Buffer A. Two substrates, phosphohistone f_1 and f_{2b} were used.

Salt	Concn. (mM)	Relative activity (%)	
		Phosphohistone f_1	Phosphohistone f_{2b}
None		100	100
$MgCl_2$	5	80	87
	30	15	39
$MnCl_2$	5	65	79
	30	8	38
$CoCl_2$	2	25	10
	10	18	7
$ZnCl_2$	1	9	4
$CaCl_2$	5	134	108
	20	107	89
NaCl	100	26	40

TABLE III

EFFECT OF NUCLEOTIDE ON PURIFIED CALF LIVER NUCLEAR PHOSPHOPROTEIN PHOSPHATASE ACTIVITY

Purified nuclear phosphoprotein phosphatase was assayed in the presence of nucleotide, orthophosphate, and pyrophosphate at the concentrations indicated. The conditions of assay are as described in Table II.

Nucleotide	Concn. (mM)	Relative activity (%)	
		Phosphohistone f_1	Phosphohistone f_{2b}
None		100	100
Cyclic AMP	$1 \cdot 10^{-4}$	113	109
	$1 \cdot 10^{-3}$	105	105
	$1 \cdot 10^{-2}$	95	90
	$1 \cdot 10^{-1}$	84	74
Cyclic GMP	$1 \cdot 10^{-4}$	114	100
	$1 \cdot 10^{-1}$	93	114
AMP	1	95	—
ATP	1	20	18
	4	0	0
GTP	1	6	2
	4	0	0
ADP	$5 \cdot 10^{-1}$	3	—
	3	0	—
Pyrophosphate	$1 \cdot 10^{-1}$	44	—
	1	4	—
Orthophosphate	1	90	95
	5	47	46

phate, a competitive inhibitor of the nuclear phosphoprotein phosphatase, was approximately linear (Fig. 4).

Effect of nucleotide, DNA and polyions. Since cyclic AMP is known to activate protein kinase, it is of great interest to investigate whether cyclic nucleotides can activate or inhibit the activity of phosphoprotein phosphatase. As shown in Table III, low concentrations of cyclic AMP and cyclic GMP had little effect on the phosphatase activity, and high concentrations of these cyclic nucleotides inhibited the activity. These data were obtained from one experiment; the values represent the average of triplicate assays. The experiments were repeated at least five times with the same and other enzyme preparations. The slight stimulatory effect of cyclic AMP and cyclic GMP was not reproduced in all experiments and is believed not to be of any significance. At physiological concentrations, these cyclic nucleotides also did not have any significant effect on the activities of nuclear and cytosol phosphoprotein phosphatases at different degrees of purity.

The effects of other nucleotides are also shown in Table III. ATP, ADP, and GTP were more potent inhibitors of the enzyme than AMP which showed little inhibition at 1 mM. Since pyrophosphate caused a similar inhibition to that exerted by ATP, GTP, and ADP (Table III), it is likely that the inhibitory action of these nucleotides is due to the pyrophosphate structure of the trinucleotides and dinucleotides. Orthophosphate, a competitive inhibitor of phosphatase, was less inhibitory than pyrophosphate and caused a 47% inhibition at 5 mM.

Since histones are bound to DNA in the cell nucleus, it is possible that DNA

may affect the phosphatase activity when phosphohistone is used as the substrate. Fig. 5A shows that DNA inhibited the purified phosphatase activity. The result is different from that of Nakai and Glinsmann [21] who showed that DNA, when present in a 3-time excess of histone, stimulated the histone phosphatase activity of rabbit muscle cytosol. The discrepancy is probably due to the different sources and different degrees of purity of the enzyme. The inhibition by DNA appeared to be non-specific since some other polyanions such as polyphosphate and heparin or polycations such as polylysine also inhibited the reaction (Fig. 5B).

Effects of EDTA and fluoride. EDTA is a commonly used metal ion chelator. When 1 mM EDTA was added to the assay mixture, the phosphatase activity toward phosphohistone f_1 was stimulated slightly (by about 10%), possibly due to the removal of heavy metal ion contaminants. At 20 mM, EDTA inhibited the phosphatase activity by 40%. This may be due to the polyionic structure of EDTA rather than its metal-chelating properties. F^- is known to inhibit enzymes that require Mg^{2+}, Ca^{2+} or other metal ions for activity. The purified nuclear phosphatase was inhibited 45% by 5 mM NaF and about 80% by 20 mM NaF when either phosphohistone f_1 or phosphohistone f_{2b} were used as the substrates. Since the mechanism of fluoride inhibition is not

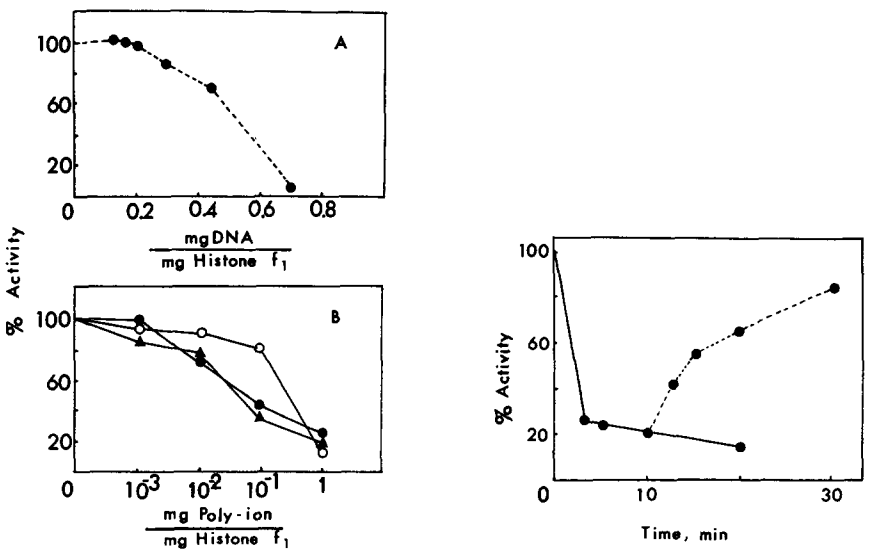


Fig. 5. The effect of DNA and polyions on the dephosphorylation of phosphohistone f_1. 0.3 μg purified nuclear phosphoprotein phosphatase was added to a DNA solution containing 15 μg phosphohistone f_1 and varying amounts of DNA or polyions. (A) The effect of increasing amounts of DNA. (B) Effects of increasing amounts of heparin (●——●), polylysine (▲——▲) or polyphosphate (○——○).

Fig. 6. Inhibition of nuclear phosphoprotein phosphatase by *p*-hydroxymercuribenzoate and the reversal of the inhibition by β-mercaptoethanol. 25.7 μg purified nuclear phosphatase was preincubated with 0.1 mM *p*-hydroxymercuribenzoate at 30°C in 1 ml 10 mM Tris-HCl (pH 7.4). At the times indicated, aliquots were removed and assayed for phosphatase activity. The preincubation and assay mixtures did no contain dithiothreitol. After 10 min preincubation, 40 mM β-mercaptoethanol was added to the modified enzyme and the phosphatase activity tested at various times thereafter. ———, decrease in activity; - - - - - -, recovery of enzyme activity.

known, the result cannot be used as evidence for the metal ion requirement of the nuclear phosphoprotein phosphatase.

The role of sulfhydryl groups in catalysis. The phosphatase activity, assayed with phosphohistone f_1 as substrate, was inhibited by *N*-ethylmaleimide; at 0.1 mM, it inhibited the activity by 30% and at 1 mM by 43%. *N*-Ethylmaleimide is known to react with the sulfhydryl groups along with other groups on the proteins. The phosphatase activity was also inhibited by a specific sulfhydryl group modifying reagent, *p*-hydroxymercuribenzoate (Fig. 6). The inhibition can be reversed by the addition of mercaptoethanol (Fig. 6) or dithiothreitol to the enzyme. The result suggests that sulfhydryl groups are important to the phosphatase activity.

Discussion

In the present work, the isolated calf liver nuclei were first washed with a buffer containing 0.5% Triton X-100 and then further washed with a buffer until no phosphoprotein phosphatase activity was detected in the wash. The nuclear phosphatase studied is therefore of nuclear origin and not due to cytosol contamination. Initially, we followed the purification procedure of Brandt et al. [22]. Later, modifications and additional steps had to be employed to increase the purity, yet the purity and yield were lower than those reported for the purification of phosphorylase phosphatase from rabbit liver [22]. It appears that some nuclear acidic proteins have properties similar to the phosphatase and are difficult to separate from the latter. It is also possible that during the purification, the phosphatase is gradually denatured leading to enzyme preparations with impurities and low specific activities. The use of a protease inhibitor, phenylmethanesulfonylfluoride, did not improve the results.

The purified nuclear phosphatase has a mol. wt. of 34 000 and is active toward phosphorylated histones, phosphorylase, and protamine. The enzyme activity is optimal between pH 7.0 and 7.5. These properties are similar to those of the cytosol phosphatase of calf liver reported previously [15] and observed during the present investigation. The calf liver enzyme is also similar to the phosphorylase phosphatase isolated from rabbit liver [22,23] and protein kinase phosphatase from bovine cardiac muscle [24] with respect to the above mentioned aspects. With phosphophosphorylase or phosphohistone as a substrate, the activity of the purified calf liver nuclear phosphoprotein phosphatase is lower than that of the cytosol enzyme from rabbit liver [22,23]. This may be due to the following reasons. (a) The nuclear enzyme purified in this work is not the same as the cytosol phosphorylase phosphatase of rabbit liver. (b) The nuclear phosphatase preparation contains denatured enzymes. (c) The phosphoprotein substrates used in this work are different from those used by Brandt et al. [22] and Khadelwal et al. [23].

We have shown that both histone and inorganic phosphate are competitive inhibitors of nuclear phosphoprotein phosphatase. The observation is important in understanding the K_m of the phosphatase. It is known that there is one phosphorylation site (serine) on histone f_1 for the protein kinase [25]. Since the histone f_1 was only phosphorylated to a level of 0.85 mol/mol histone, 15% of the nonphosphorylated histone should serve as an inhibitor; hence the true

K_m value should be lower than the presently observed 28 μM P_i for phosphohistone f_1. The product inhibition may play a role in affecting the phosphatase activity in vivo.

In studying the effects of metal ions on the nuclear phosphatase activity, the enzyme had been previously dialyzed against a buffer containing 0.1 mM EDTA and the enzymic activity was not stimulated by the added metal ions (Table III). There appears to be no metal ion requirement for the enzyme. Nevertheless, such a requirement cannot be ruled out because tightly bound metal ions may not be removed by the dialysis. The observed inhibitions with EDTA and NaF may be related to their metal ion-binding properties but do not provide firm evidence of the metal ion requirement of the enzyme. Most divalent metal ions, which were shown not to inhibit the phosphatase activities of nuclear and cytosol enzymes of lower purities [15], inhibit the activity of the purified nuclear phosphoprotein phosphatase (Table II). The detailed mechanism of inhibition remains to be elucidated. Among the divalent ions examined, Ca^{2+} is the only one that stimulates the activity (by 30%) of the nuclear phosphatase (Table II). Similar stimulations were also observed with cytosol phosphatase either before or after the ethanol treatment. The physiological importance of this stimulation is not known.

Brandt et al. [26] have found that when phosphorylase phosphatase was treated with ethanol. the enzyme activity was increased and the enzyme was converted from multimolecular weight forms to a single low molecular weight form of 32 000. Based on these observations, they postulated that the multiple forms of the enzyme were due to the binding of 'regulatory subunits' to a 'catalytic subunit'. This hypothesis is consistent with most of the existing data, but direct evidence for the presence of the regulatory subunit is lacking. Alternatively, it is possible that the nuclear phosphoprotein phosphatase is a polypeptide with a mol. wt. of 34 000, and the multiple molecular weight forms observed are due to the binding of this polypeptide to different proteins. The binding also inhibits the phosphatase activity. If we accept the second hypothesis, then the nuclear phosphoprotein phosphatase appears not to have an elegant regulatory mechanism comparable to that of the protein kinase. It may be regulated to a certain extent by metal ions, ATP, or its products through product inhibition.

Acknowledgment

This work was supported by National Institutes of Health Grants GM-23164 and CA-16788.

References

1 Delange, R.J., Kemp, R.G., Riley, W.D., Cooper, R.A. and Krebs, E.G. (1968) J. Biol. Chem. 243, 2200—2208
2 Kishimoto, A., Tukai, Y. and Nishizuka, T. (1977) J. Biol. Chem. 252, 7449—7452
3 Corbin, J.D., Reimann, E.M., Walsh, D.A. and Krebs, E.G. (1970) J. Biol. Chem. 245, 4849—4851
4 Abita, J.P., Milstein, S., Cheng, N. and Kaufman, S. (1976) J. Biol. Chem. 251, 5310—5314
5 Walton, K.G., Delorenzo, R.J., Curran, P.F. and Greengard, P. (1975) J. Gen. Physiol. 65, 153—177
6 Layne, D.D. and Najjar, V.A. (1977) Arch. Biochem. Biophys. 180, 328—333
7 Rubin, C.S. and Rosen, O.M. (1975) Annu. Rev. Biochem. 44, 831—887

8 Kleinsmith, L.J. (1974) in Acidic Protein of the Nucleus (Cameron, I.L. and Jeter, J.R., Jr., eds.), pp. 103—135, Academic Press, New York
9 Abe, N. and Tsuiki, S. (1974) Biochim. Biophys. Acta 350, 383—391
10 Kato, K. and Bishop. J.S. (1972) J. Biol. Chem. 247, 7420—7429
11 Kobayashi, M., Kato, K. and Sato, S. (1975) Biochim. Biophys. Acta 377, 343—355
12 Krebs, E.G. and Fischer, E.H. (1960) Ann. N.Y. Acad. Sci. 88, 378—384
13 Krebs, E.G. (1972) Curr. Topics Cell Regln. 5, 99—128
14 Meisler, M.H. and Langan, T.A. (1969) J. Biol. Chem. 244, 4961—4968
15 Chou, M.W., Tan, E.L. and Yang, C.S. (1978) Int. J. Biochem. 9, 27—32
16 Tan, E.L., Levine, M.A., Yang. C.S. and Li, H.L. (1975) Int. J. Biochem. 7, 21—26
17 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
18 Ehresmann, B., Imbault, P. and Weil, H.J. (1973) Anal. Biochem. 54, 454—463
19 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J.Biol. Chem. 193, 265—275
20 Martensen, T.M., Brotherton, J.E. and Graves, D.J. (1973) J. Biol. Chem. 248, 8323—8328
21 Nakai, C. and Glinsmann, W. (1977) Fed. Proc. 36, Abstract 2835
22 Brandt, H., Capulong, Z.L. and Lee, E.Y.C. (1975) J. Biol. Chem. 250, 8038—8041
23 Khadelwal, R.L., Vandenheede, J.R. and Krebs, E.G. (1976) J. Biol. Chem. 251, 4850—4858
24 Chou, C.K., Aljano, J. and Rosen, O.M. (1977) J. Biol. Chem. 252, 2855—2859
25 Shlyapnikov, S.V., Arutyunyan, A.A., Kurochkin, S.N., Memelova, L.V., Nesterova, M.V., Saschenko, L.P. and Severin, E.S. (1975) FEBS Lett. 53, 316—319
26 Brandt, H., Killilea, S.D. and Lee, E.Y.C. (1974) Biochem. Biophys. Res. Commun. 61, 598—604