CHEMICALLY PHOSPHORYLATED PROTAMINE: A NEW SUBSTRATE FOR THE STUDY OF PHOSPHOPROTEIN PHOSPHATASE ACTIVITY.

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Summary: A method for the chemical phosphorylation of seryl residues in protamine has been developed. Both non-radioactive and [^{33}P]phosphoprotamine have been prepared and characterized. Phosphoprotein phosphatases from a variety of mammalian tissues release inorganic phosphate from phosphoprotamine. Chemically phosphorylated protamine is a useful new substrate for the study of these phosphoprotein phosphatases.

INTRODUCTION

A number of enzymes are known to exist in two interconvertible forms which differ in their degree of phosphorylation (1,2). Interconversion of these forms by phosphorylation and dephosphorylation is catalyzed by protein kinases and phosphoprotein phosphatases, respectively. The enzymes regulated by phosphorylation and dephosphorylation include glycogen synthase, glycogen phosphorylase, phosphorylase kinase, and triglyceride lipase. The phosphorylation of these enzymes and of other proteins (including protamine, histone, and casein) by protein kinases is markedly stimulated by cAMP. cAMP-dependent protein kinases have been isolated and characterized from many tissues (3,4).

Although the regulation of protein phosphorylation by cAMP has been extensively studied, the role of dephosphorylation in the regulation of enzyme activity has been relatively ignored. The study of phosphoprotein phosphatases has been hampered by the lack of convenient assays for this enzyme. Previous investigators

Abbreviations:

DMSO, dimethyl sulfoxide; cAMP, cyclic adenosine 3',5'-monophosphate.

have studied phosphoprotein phosphatase activity either by measuring the release of P_i from enzymatically phosphorylated phosphoproteins (5,6), or else have studied the changes in the activity of enzymes such as glycogen phosphorylase or glycogen synthase which result from their dephosphorylation (7,8).

We have developed a new assay for phosphoprotein phosphatase activity, which is based on the release of P_i from chemically phosphorylated phosphoprotamine. In this communication, we describe the phosphorylation and characterization of phosphoprotamine, and we report the tissue distribution of phosphoprotein phosphatase activity which releases P_i from this substrate.

METHODS

Materials: Protamine chloride was purchased from Sigma. $H_3{}^{33}PO_4$ was obtained from New England Nuclear Corp. All other chemicals were reagent grade. DMSO was redistilled and stored over CaH_2 before use; triethylamine was also stored over CaH_2 before use.

Chemical assays: Protein was determined by the method of Lowry et al. (9), using bovine serum albumin as the standard. When protamine was determined by this method, the estimated protein concentration was multiplied by 1.7 in order to correct for the low color yield of protamine in this assay. Inorganic phosphate was determined by the method of Ames and Dubin (10). Alkali-labile phosphate was estimated after incubation for 15 minutes at 100°C in 1N NaOH, and acid-labile phosphate after incubation for 15 minutes at 100°C in 1N H_2SO_4.

Preparation of phosphoprotamine: To 6 ml DMSO, containing 20 mg protamine chloride/ml, are added 15 µl 85% H_3PO_4 (14.7M), 150 µl triethylamine (7.2M), and 150 µl trichloroacetonitrile (10.0M). Upon addition of the trichloroacetonitrile, the coupling reagent, the reaction mixture is incubated in a 40 ml conical glass tube for 30 minutes at 37°C, and is then rapidly chilled by the addition of 30 ml ice-cold ethanol. Phosphoprotamine is allowed to precipitate for 15 minutes at 0°C, and is then collected by centrifugation. The precipitate is dissolved in 5 ml 1N H_2SO_4, and incubated for 30 minutes at 30°C. The solution is again chilled, 20 ml of cold ethanol is added, and the precipitate again collected by

centrifugation. The precipitated phosphoprotamine is washed once with ethanol: ether, 1:4, containing 0.1 N HCl, and once with ethanol:ether, 1:4. The pellet obtained after centrifugation is then dissolved in water and purified by passage over two 3 ml columns of Bio-Rad AG1-X8 Cl^- anion exchange resin, which was equilibrated and eluted with water.

Preparation of [^{33}P]phosphoprotamine: To 6 ml DMSO, containing 20 mg protamine chloride/ml, are added 10-50 µl $H_3{}^{33}PO_4$ (10 mCi/ml), 10 µl 0.85% H_3PO_4 in DMSO, 50 µl triethylamine, and 50 µl trichloroacetonitrile, and the reaction mixture is incubated for 30 minutes at 37°C. The reaction is terminated by the addition of 20 µl 85% H_3PO_4, 2 ml ethanol, and 20 ml anhydrous ether, and the [^{33}P]phosphoprotamine is isolated as described for the preparation of phosphoprotamine. [^{33}P]phosphoserine in the [^{33}P]phosphoprotamine was identified by the method of Ahmed and Judah (11).

Phosphoprotein phosphatase assay: Phosphoprotein phosphatase activity was measured by the release of $^{33}P_i$ from [^{33}P]phosphoprotamine. The enzyme was incubated with [^{33}P]phosphoprotamine, containing 0.1 mM alkali-labile phosphate, with a specific activity of 100-300 cpm/nmole, in a final volume of 380 µl. The incubation with carried out for 30 minutes at 37°C, in a buffer containing 50 mM Tris-HCl, 2 mM $MnCl_2$, 1 mM dithiothreitol, and 400 mM NaCl, pH 7.4. The reaction was terminated by the addition of 25 µl of 0.1M silicotungstic acid in 0.1N H_2SO_4. 100 µl of 5% $(NH_4)_6Mo_7O_{24}.4H_2O$ in 4N H_2SO_4 and 0.5 ml isobutanol:benzene, 1:1, were added, and the mixture was agitated vigorously for 30 seconds and then centrifuged. The radioactivity in duplicate 0.1 ml aliquots of the upper (organic) phase was counted in a gas-flow radiation counter. One unit of protein phosphatase activity was defined as the amount of enzyme that catalyzes the release of 1 nmole of P_i from phosphoprotamine per minute.

Animals: Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories, and were killed by decapitation. The tissues were homogenized in a buffer containing 50 mM Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol, 5 mM $MnCl_2$, 50 mM KCl, and 10% glycerol, pH 7.4, 3-10 ml per gm wet weight, in a motor-driven

TABLE 1

PROPERTIES OF SYNTHETIC PHOSPHOPROTAMINE

	Protein Added	Phosphate Added	Protein Recovered	Phosphate Recovered		Phosphate / Protein
				Alkali-labile	Acid-labile	
	(mg)	(μmoles)	(mg)	(μmoles)	(μmoles)	mole/mole
Non-radioactive Phosphoprotamine	120	221	92	21.4	1.4	1.14
	(mg)	(μCi)		(μCi)		
[^{33}P]phospho-protamine	120	200	———	32	———	———

Table 1. Properties of chemically phosphorylated protamine.

Nonradioactive and [^{33}P]phosphoprotamine were prepared as described in the text. Free, alkali-labile, and acid-labile phosphate were determined; the values given for alkali-labile and acid-labile phosphate were corrected by subtraction of the free phosphate content. (——) means not determined.

Teflon-glass homogenizer. The crude homogenates and the supernatants obtained after centrifugation for one hour at 105,000 x g were assayed for phosphoprotein phosphatase activity and for protein.

RESULTS

Characterization of Phosphoprotamine: Salmon sperm protamine was phosphorylated according to the procedure described in Methods. The properties of typical preparations of phosphoprotamine are presented in Table 1. Preparations of non-radioactive phosphoprotamine contained approximately 1 mole of alkali-labile phosphate per mole of protein. Less than 10% of the phosphate in the phospho-protamine preparations was free or acid-labile. Protamine was phosphorylated with $^{33}P_i$ under conditions that maximize the incorporation of radioactivity into protein. Table 1 also shows the properties of typical preparations of [^{33}P]phos-phoprotamine. The alkali-labile phosphate in [^{33}P]phosphoprotamine was identified as phosphoserine by partial acid hydrolysis and ion-exchange chromatography

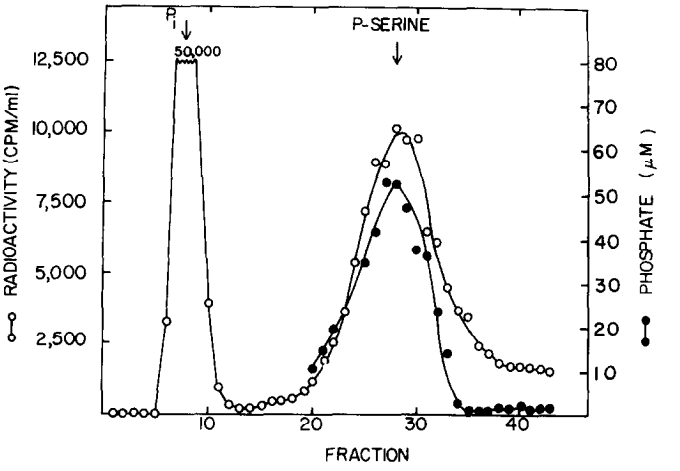

Figure 1. Identification of [^{33}P]phosphoserine in [^{33}P]phosphoprotamine.

A sample of [^{33}P]phosphoprotamine, containing about 6x10^5 cpm, was hydrolyzed for 8 hours at 100°C in 2M HCl, in a sealed tube in the presence of 5 μmoles of carrier phosphoserine. After hydrolysis, the sample was lyophilized, dissolved in 0.5 ml of 0.05M HCl, and applied to a 1.1 x 15 cm column of Dowex 50 H$^+$, 8X, 100-200 mesh, which had been equilibrated with 0.05M HCl. The column was eluted with 0.05M HCl. One ml fractions were collected and were assayed for radioactivity and for alkali-labile phosphate.

(Fig. 1). Preparations of non-radioactive and of [^{33}P]phosphoprotamine were mixed to yield a substrate with the desired concentration of alkali-labile phosphate and specific activity.

Tissue distribution of phosphoprotein phosphatase activity: The distribution of phosphoprotein phosphatase activity in crude homogenates and in 105,000 x g supernatants in a variety of rat organs is listed in Table 2. There is a five-fold difference in specific activity of phosphoprotein phosphatase in crude homogenates, with brain and intestine containing the highest specific activity, and pancreas containing the lowest. There was no consistent distribution of phosphoprotein phosphatase activities between particulate and soluble fractions. Virtually all the phosphatase activity in pancreas is present in the soluble fraction, whereas more than 80% of the phosphatase activity in brain, in skeletal muscle, and in stomach, was present in the insoluble fraction. Other tissues exhibited an approximately equal distribution between soluble and particulate

TABLE 2

TISSUE DISTRIBUTION OF PROTEIN PHOSPHATASE

Tissue	Protein Phosphatase in Crude Homogenate	Protein Phosphatase in 105,000xg Supernatants	Activity in Cytoplasmic Fraction
	(units/mg)	(units/mg)	(%)
Adrenal	0.50	0.74	44
Brain	2.02	0.84	16
Heart	0.76	0.50	51
Intestine	1.73	0.90	45
Kidney	0.90	0.87	62
Liver	1.04	0.40	38
Lung	0.86	0.40	44
Pancreas	0.38	0.57	100
Skeletal muscle	0.63	0.23	18
Spleen	0.79	0.74	79
Stomach	0.87	0.76	17
Testis	1.27	1.36	60

Table 2. Tissue distribution of protein phosphatase activity.

Tissues were obtained from 250-350 gm male Sprague-Dawley rats, and were homogenized and assayed as described in the text.

phosphoprotein phosphatase activity (38%-62% soluble).

DISCUSSION

We have developed a new method for the chemical phosphorylation of seryl residues in proteins. Our method, which utilizes thichloroacetonitrile as a coupling agent and anhydrous DMSO as the solvent, is based on the methods developed by Symons for nucleotide synthesis (12). We have used this method to

phosphorylate seryl residues in salmon sperm protamine. Dixon and his colleagues have shown that newly synthesized protamine is phosphorylated *in vivo* (13,14), and that protamine serves as a good substrate for cAMP dependent protein kinases *in vitro* (15). Protamines are a family of closely related basic polypeptides. Typically, protamines are composed of about 30 amino acyl residues. Of these, about 20 are arginyl, 3 or 4 are seryl, and the remainder are neutral amino acyl residues (16). The seryl residues are the only amino acids in protamine that would be expected to form alkali-labile phosphorylated derivatives. Phosphorylated protamine prepared by our methods contains about 1 mole of alkali-labile phosphate per mole of protein. A portion of the alkali-labile phosphate can be identified as phosphoserine after partial acid hydrolysis of the phosphoprotamine. Although the methods for the detection of phosphoserine are not quantitative, it is likely that all of the alkali-labile phosphate in phosphoprotamine is in phosphoseryl residues. The methods that we used to phosphorylate protamine would not be expected to hydrolyze peptide bonds, or to cause any other covalent changes in protein structure. Chemically phosphorylated protamine retains its macromolecular form, since it emerges in the void volume during gel filtration on Sephadex G-10 (data not shown).

We have not systematically investigated all of the parameters which might affect protein phosphorylation. However, we have found that it is important to perform the phosphorylation under anhydrous conditions. The addition of water to the reaction mixture greatly decreases the incorporation of phosphate into protein. Maximal protein phosphorylation is observed after ten to thirty minutes of incubation Longer incubation times resulted in much lower recoveries of protein. During the phosphorylation reaction, there is considerable incorporation of phosphate into acid-labile (presumably phosphoarginyl) linkages. The acid-labile phosphate can be removed by mild acid treatment of the phosphoprotein. The chemical phosphorylation of protamine is easier and cheaper than is enzymatic phosphorylation, and yields a substrate that is susceptible to hydrolysis by phosphoprotein phosphatases. Since protamine itself is heterogeneous, and since purified protamine fractions contain

several seryl residues, it is likely that the phosphoprotamine is also heterogeneous. It might be useful in the future to purify homogeneous fractions of phosphoprotamine.

We have used chemically phosphorylated protamine as a substrate for the assay of phosphoprotein phosphatase activity. The tissue distribution of phosphatase activity against this substrate is similar to that reported by Maeno and Greengard for phosphoprotein phosphatase activity against enzymatically phosphorylated protamine (6). It is likely that the same enzymes hydrolyze chemically phosphorylated and enzymatically phosphorylated protamine. Chemically phosphorylated protamine is a useful new substrate for the study of these enzymes.

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