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PHOSPHOPROTEIN PHOSPHATASES FOR MYELIN BASIC PROTEIN IN MYELIN AND CYTOSOL FRACTIONS OF BRAIN

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Summary

Phosphoprotein phosphatase (phosphoprotein phosphohydrolase EC 3.1.3.16) activity for myelin basic protein was found to be present in the myelin fraction of rat brain. The enzyme activity was in a latent form and solubilized by 0.2% Triton X-100 treatment with about 50% increase of activity. The cytosol fraction from bovine brain also had phosphoprotein phosphatase activity for myelin basic protein, which was resolved into at least two peaks of activity on DEAE-cellulose column chromatography. Myelin basic protein was the best substrate for both the solubilized myelin fraction and the cytosol enzymes among the substrate proteins tested. The K_m values of the solubilized myelin fraction were 4.2 μM for myelin basic protein, 7.4 μM for arginine-rich histone, 8.0 μM for histone mixture and 14.3 μM for protamine, respectively.

Introduction

After the discovery of adenosine 3′,5′-monophosphate (cyclic AMP)-dependent protein kinases in muscle [1], liver [2] and brain [3], this class of protein kinases has been found in many other tissues from a wide variety of species throughout the animal kingdom [4]. It has been proposed that the diverse actions of cyclic AMP may be mediated through the activation of such protein kinases [4,5]. According to this hypothesis, the dephosphorylation process is of importance in regulating the physiological effects of cyclic AMP. Phosphoprotein phosphatase capable of releasing orthophosphate from phosphorylated protein has been reported in several tissues [6—14].

Myelin basic protein has been found to serve as good substrate for exogenous and endogenous cyclic AMP-dependent protein kinases [15—18]. In addition, the protein has been shown to be phosphorylated in vivo by injection

of orthophosphate into the ventricle of rat brain, and to exist originally in the phosphorylated form [17,18]. Incubation of the myelin fraction with ATP resulted in its phosphorylation, followed by release of the incorporated phosphate. These results suggest the presence of both protein kinase and phosphoprotein phosphatase in the tissue. The cyclic AMP-dependent protein kinase in myelin of rat brain has been characterized and its properties compared with those of the brain cytosol enzyme [19]. This communication reports the presence of a phosphoprotein phosphatase activity in the myelin and cytosol fractions of brain, which catalyzes the release of phosphate from phosphorylated myelin basic protein.

Materials and Methods

Materials

Bovine brain was obtained from a local slaughter house and stored at −20° C until use. Histone mixture (calf thymus) and arginine-rich histone were obtained from Schwarz-Mann. Protamine was purchased from Sigma. Myelin basic protein was prepared from bovine brain according to the method of Oshiro and Eylar [20]. The protein preparation, which showed one major single band on analytical disc gel electrophoresis by the method of Reisfeld et al. [21] was used in the following studies. Cyclic AMP was obtained from Kohjin Co. ^{32}P-labelled Orthophosphate (carrier free) was purchased from Japan Radioisotope Association.

Preparation of phosphorylated substrate

Phosphorylation of myelin basic protein, arginine-rich histone, histone mixture and protamine was accomplished by incubation with cyclic AMP-dependent protein kinase, which was prepared from bovine brain as described previously [22] to the stage of DEAE-cellulose column chromatography. A typical reaction mixture for phosphorylation of protein contained 5 mg of the substrate protein, 350 μg of protein kinase, 250 μmol of sodium acetate buffer, pH 6.0, 50 μmol of magnesium acetate, 5 nmol of cyclic AMP, 5 μmol of [γ-^{32}P]ATP (8 to 15 · 10^6 cpm) and 1.5 μmol of ethylene glycol bis (β-aminoethyl ether)-*N*,*N*'-tetraacetic acid in a total volume of 5.0 ml. The incubation was carried out at 30° C for 24 h. The phosphorylation reaction was terminated by the addition of 1.25 ml of 100% trichloroacetic acid. The resulting precipitate was collected by centrifugation, washed twice by dissolving in water and reprecipitating with 20% trichloroacetic acid, and dialyzed against distilled water for 14 h with two changes of water. The incorporated phosphate into substrate protein was 47.4, 30.5, 28.7 and 6.6 nmol per mg protein for myelin basic protein, protamine, histone mixture and arginine-rich histone, respectively. The phosphate incorporation was negligible in the sample of the enzyme preparation incubated without substrate.

Assay for protein phosphatase activity

Phosphoprotein phosphatase activity was assayed by measuring the release of radioactive orthophosphate from ^{32}P-labeled protein. Unless otherwise specified, the reaction mixture contained, in a total volume of 0.1 ml, 10 μmol

of Tris/HCl buffer, pH 7.5, 0.1 μmol of dithiothreitol, 2.37 nmol of phosphorylated myelin basic protein as phosphate (50 μg of protein) and the indicated amount of enzyme protein. The incubation was performed at 30° C for 10 min and terminated by the addition of 0.4 ml of 25% trichloroacetic acid. After the addition of 0.1 ml of 0.625% bovine serum albumin as a carrier for the precipitation, protein was removed by centrifugation. ^{32}P-labelled orthophosphate in the supernatant was measured by the modified method of Meisler and Langan [12], and Maeno and Greengard [14]. To 0.4 ml of deproteinized supernatant were added 0.05 ml of 10^{-2} M KH_2PO_4 and 0.15 ml of 5% ammonium molybdate in 0.5 M H_2SO_4. The resulting phosphomolybdate complex was extracted with 1.0 ml of a mixture of isobutyl alcohol and benzene (1 : 1), and the radioactivity of the extract was determined by a liquid scintillation spectrometer of Packard Model 3310. The amount of phosphate released from the substrate protein calculated from the specific activity of the radioactive ATP used as precursor in the protein phosphorylation reaction. All of the assays were performed more than twice in duplicate determinations. The values presented are means of duplicate samples, the difference between each sample being less than 5%.

Solubilization of phosphoprotein phosphatase from myelin fraction

Male Sprague Dawley rats (4 weeks old, weighing about 80 g each) were sacrificed by decapitation under ether anesthesia. Myelin fraction was prepared from cerebrum according to the method of Uyemura et al. [23] with a slight modification [18]. The tightly packed white pellet of myelin fraction obtained was suspended in 10 mM Tris/HCl buffer, pH 7.5, at a concentration of 2.6 mg protein per ml. This suspension was designated as untreated myelin fraction. A typical experiment of the solubilization of phosphoprotein phosphatase activity from myelin fraction was carried out as follows. 1 ml of untreated myelin fraction was stirred in a final concentration of 0.2% Triton X-100 for 30 min in an ice bath, and then centrifuged at 22 000 × *g* for 15 min. The supernatant which was designated as Triton-treated supernatant I was decanted. The precipitate was suspended in 1 ml of 10 mM Tris/HCl buffer, pH 7.5, containing Triton X-100 at a final concentration of 0.2% and stirred at 0° C for 30 min. The suspension was centrifuged as described above. The solubilization procedure with 0.2% Triton X-100 in 10 mM Tris/HCl buffer, pH 7.5, was repeated. The supernatant solutions which were obtained by the second and third treatment with Triton X-100 were designated as Triton-treated supernatants II and III, respectively. The precipitate obtained by the final centrifugation was suspended in 1 ml of 10 mM Tris/HCl buffer, pH 7.5, and designated as Triton-treated precipitate. Triton X-100 itself had no effect on the activity of the cytosol phosphoprotein phosphatase from bovine brain at a final concentration of 0.04 to 0.08% in the reaction mixture.

Preparation of cytosol phosphoprotein phosphatase from bovine brain

All procedures were carried out at 0—4° C. Cerebral cortex of bovine brain (4 g) was thawed, cut into small pieces with scissors, and homogenized with 3 vol. of 10 mM Tris/HCl buffer, pH 7.5, in a Waring blendor. The homogenate was centrifuged at 105 400 × *g* for 1 h in an RP 40 A rotor of Hitachi ultra-

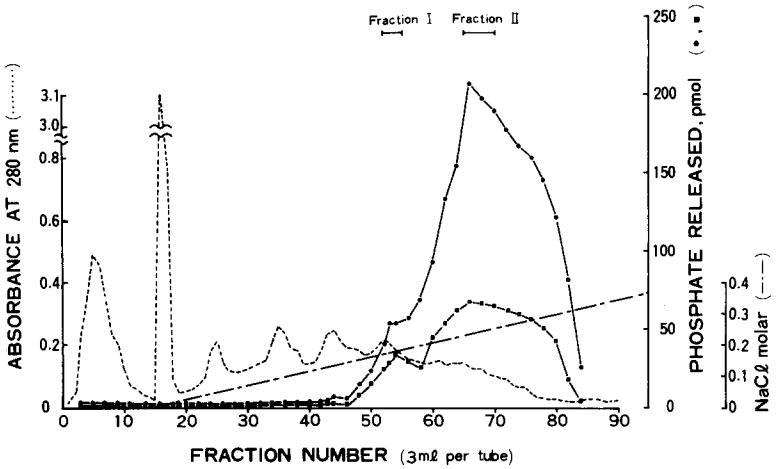

Fig. 1. Chromatography of bovine brain phosphoprotein phosphatase activity on DEAE-cellulose column. Experimental details were as described in the text. An aliquot (30 μl) from each fraction was assayed for phosphoprotein phosphatase activity with 11.8 μM (as phosphate) of myelin basic protein (●——●) and 11.6 μM (as phosphate) of arginine-rich histone (■——■) as substrate. Other incubation conditions were as described in the text.

centrifuge. The supernatant (9.0 ml) containing 51.3 mg protein was applied to a 1.3 × 5.0 cm column of DEAE-cellulose which had been equilibrated with 10 mM Tris/HCl buffer, pH 7.5. The column was washed with 36 ml of the same buffer, and then the enzyme protein was eluted with a linear gradient of sodium chloride (0—0.35 M) in 10 mM Tris/HCl buffer, pH 7.5, in a total volume of 240 ml. The phosphoprotein phosphatase activity was resolved into two distinct peaks of activity, one of which (Fraction I) was eluted with about 0.18 M NaCl and the other one (Fraction II) eluted with about 0.24 M NaCl (Fig. 1). The second peak may contain another peak of activity, as it had a shoulder of activity around fraction 76 (Fig. 1). In addition to myelin basic protein and arginine-rich histone, protamine was also used as substrate (data not shown). The enzyme activity was 16.0 pmol at the first peak (fraction 54) and 19.4 pmol at the second peak (fraction 66) after incubation for 10 min under the conditions shown in Fig. 1, using 11.5 μM (as phosphate) of protamine as substrate. The chromatographic pattern of activity with protamine was similar to that with arginine-rich histone. The active fractions of each peak were collected and dialyzed against 10 mM Tris/HCl buffer, pH 7.5, for 14 h with a change of the buffer.

Other methods

[γ-^{32}P]ATP was prepared by the method of Post and Sen [24]. Protein was measured by the method of Lowry et al. [25], with bovine serum albumin as the protein standard.

Results

Effect of Triton X-100 on the solubilization of phosphoprotein phosphatase from myelin fraction

A typical experiment of the solubilization of phosphoprotein phosphatase

TABLE I

EFFECT OF TRITON X-100 TREATMENT ON THE SOLUBILIZATION OF PHOSPHOPROTEIN PHOSPHATASE ACTIVITY FROM MYELIN FRACTION OF RAT BRAIN

Each assay contained 22 μg of untreated myelin fraction, 5.2, 1.9 and 1.5 μg of Triton-treated supernatant I, II and III, respectively, and 12.2 μg of Triton-treated precipitate as protein, as indicated. Myelin basic protein (23.7 μM as phosphate) was used as substrate. Other incubation conditions were as described in the text. Each value of phosphoprotein phosphatase activity represents the mean of duplicate determinations.

Fraction	Phospho-protein phosphatase activity (pmol/min)	Specific activity (nmol/mg protein)
Untreated myelin fraction	53.0	2.4
Triton-treated		
Supernatant I	50.3	9.7
Supernatant II	14.2	7.5
Supernatant III	5.9	3.9
Precipitate	9.5	0.8

activity achieved with 0.2% Triton X-100 is shown in Table I. The sum of the activity of the supernatant and precipitate exceeded that of untreated myelin fraction after Triton X-100 treatment. The value of the total activity was about 151% of untreated myelin fraction activity, of which 133 and 18% were derived from the supernatant and precipitate, respectively. The specific activity of

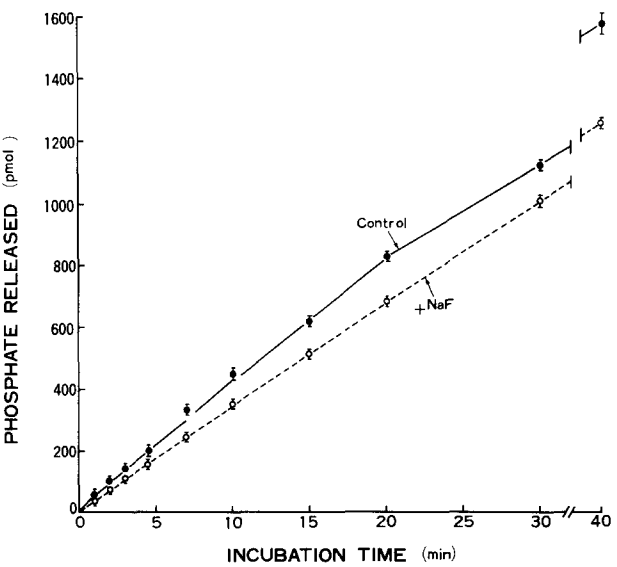

Fig. 2. Effect of incubation time on the phosphoprotein phosphatase activity of Triton-treated supernatant I. Each assay contained 5.2 μg of Triton-treated supernatant I and 2.37 nmol (as phosphate) of myelin basic protein as substrate. The values of activity were corrected for 4.8 and 4.7 pmol determined in the absence and presence of added NaF at zero time, respectively. Other incubation conditions were as described in the text, except for the variation in incubation time and the addition of 10 mM NaF as indicated. Each point represents the mean of duplicate determinations. The vertical bars represent the range of the values of duplicate determinations.

Triton-treated supernatant I was about 4 times higher than that of untreated myelin fraction (Table I).

Effect of incubation time on phosphoprotein phosphatase activity

The amount of phosphate released in the presence of Triton-treated supernatant I was proportional to the reaction time for 20 min and still increased up to 40 min (Fig. 2). In the presence of 10 mM NaF, the rate of phosphate release was slightly inhibited (Fig. 2). The percentage of inhibition was about 22% after 10 min incubation.

Effect of protein concentration of the solubilized myelin fraction on activity

The amount of phosphate released in the presence of Triton-treated supernatant I was proportional to about 104 μg of protein per ml of reaction mixture, which was the largest amount tested.

Ability of various proteins to serve as substrate for the solubilized myelin fraction and the cytosol enzymes

Several proteins including myelin basic protein, arginine-rich histone, histone mixture and protamine were compared in the ability to serve as substrate for Triton-treated supernatant I, and the cytosol phosphoprotein phosphatases Fraction I and II (Table II). Myelin basic protein was the best substrate for the three preparations, followed by arginine-rich histone, histone mixture and protamine. The order of effectiveness of the substrates tested was identical for these three preparations.

The effect of varying concentrations of substrate protein on the activity of Triton-treated supernatant I was examined. From the double-reciprocal plots of the data, the apparent K_m value of each substrate for the preparation was

TABLE II

COMPARISON OF VARIOUS PROTEINS AS SUBSTRATE FOR PHOSPHOPROTEIN PHOSPHATASE ACTIVITY OF THE SOLUBILIZED MYELIN FRACTION FROM RAT BRAIN AND FOR THE CYTOSOL ENZYMES FROM BOVINE BRAIN

Each assay contained 5.2 μg of Triton-treated supernatant I, and 8.0 and 5.8 μg of the cytosol enzymes Fraction I and II as protein, respectively, as indicated. Other incubation conditions were as described in the text, except for the addition of the kind and amount of substrate indicated. Each value for phosphate released was corrected for that determined at zero time by using the indicated substrate, and represents the mean of duplicate determinations.

^{32}P-labelled substrate	Substrate concn as phosphate (μM) (μg of protein)	Phosphate released (pmol/min)		
		Triton-treated supernatant I	Cytosol enzymes	
			Fraction I	Fraction II
Myelin basic protein	5.9 (12.5)	24.3	5.2	9.3
	23.7 (50)	37.7	8.1	17.0
Arginine-rich histone	3.3 (50)	10.9	3.7	5.8
	11.6 (175)	15.6	5.3	8.3
Histone mixture	7.2 (25)	7.6	1.9	4.5
	25.1 (87.5)	11.0	5.1	6.6
Protamine	7.6 (25)	5.1	2.0	3.6
	26.7 (87.5)	6.5	5.0	5.3

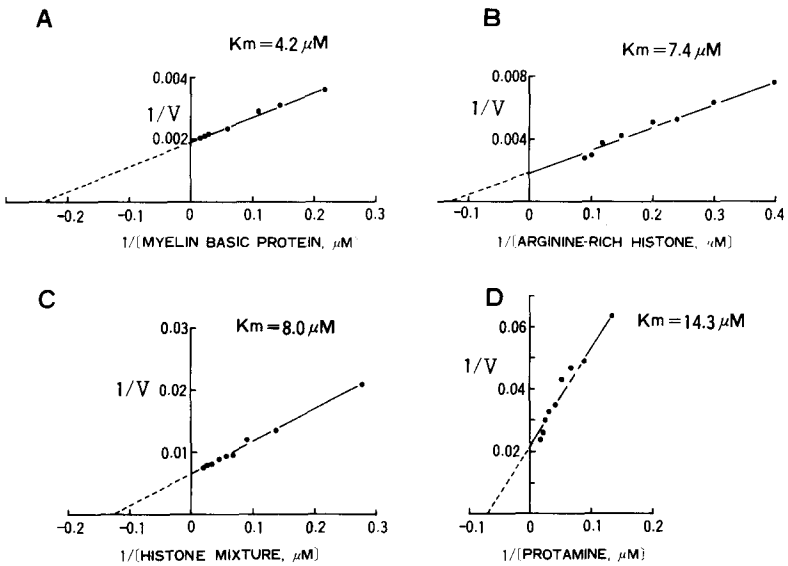

Fig. 3. Lineweaver-Burk plots of phosphoprotein phosphatase activity of Triton-treated supernatant I with respect to various substrates. Each assay contained 5.2 μg of Triton-treated supernatant I and various amounts of each substrate protein as indicated. Other incubation conditions were as described in the text. Each value of activity represents the mean of duplicate determinations. (A) Myelin basic protein. (B) Arginine-rich histone. (C) Histone mixture. (D) Protamine.

determined to be 4.2 μM for myelin basic protein, 7.4 μM for arginine-rich histone, 8.0 μM for histone mixture and 14.3 μM for protamine, respectively (Fig. 3).

The K_m values of myelin basic protein for the cytosol enzymes Fraction I and II were determined to be 4.2 and 7.5 μM, respectively.

Discussion

The results described in this communication indicate that not only myelin fraction but also cytosol fraction of brain contains phosphoprotein phosphatases active with myelin basic protein. The enzyme preparations were also active in dephosphorylating arginine-rich histone, histone mixture and protamine, although myelin basic protein was the best substrate among the proteins tested. In association with evidence reported previously [19], the results indicate the presence of both protein kinase and phosphoprotein phosphatase in myelin of brain, which are responsible for the phosphorylation and dephosphorylation of myelin basic protein. This provides a means for regulation of the phosphate content of myelin basic protein. The physiological events in myelin, which bring about the phosphorylation or dephosphorylation of the protein, and their significance remain to be solved at the moment. One possibility is that phosphorylation or dephosphorylation of the protein leads to altering the interaction between myelin basic protein and lipid.

The phosphoprotein phosphatase activity of the solubilized myelin fraction was slightly inhibited with 10 mM NaF. However, this inhibition seemed to be insufficient to account for the increase of the endogenous phosphoryla-

tion determined in the presence of NaF [19]. It is more likely that the increase of phosphate incorporation observed in the presence of NaF is mainly due to the inhibition of ATPase activity.

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