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SOLUBILIZATION AND PARTIAL CHARACTERIZATION OF A PHOSPHOPROTEIN PHOSPHATASE FROM HUMAN MYELIN

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Summary

The phosphoprotein phosphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) solubilized from human central nervous system myelin has been shown to possess a comparatively high degree of specificity towards myelin basic protein, a constituent of the membrane and most likely its natural substrate, rather than the mixed histones. The enzyme has a pH optimum of 7.5. Hydrolysis of both the substrates is stimulated by dithiothreitol and is almost completely dependent upon the presence of divalent metal ions. The maximum rate of dephosphorylation of basic protein is attained in the presence of 125 μM Mn^{2+} whereas a much higher concentration of Mg^{2+} (50—100 mM) is required for the optimal dephosphorylation of histones.

The dephosphorylation of basic protein was also stimulated by Triton X-100 (0.15%, v/v) and was shown to result from a 3-fold increase in the V of the reaction catalyzed by the phosphatase. The apparent K_m values for basic protein and histones were unaffected by the presence of Triton X-100 and were found to be approx. 1 and approx. 160 μM, respectively. Under optimal conditions of assay, the phosphatase cleaved approx. 32 and approx. 0.7 nmol of orthophosphate $\cdot$ min^{-1} $\cdot$ mg^{-1} of protein from basic protein and histones, respectively.

Introduction

The interconversion of enzymes into their active and inactive forms by phosphorylation and dephosphorylation is now established to be an important mechanism for the regulation of enzyme activity [1,2]. The phosphorylation of proteins is mediated by (a) cyclic nucleotide-stimulated protein kinases and (b) by those protein kinases whose possible regulatory mechanisms are not yet

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understood [3—5]. It has been proposed that in eukaryotes the various cellular effects elicited by cyclic AMP, whose levels are hormonally controlled, may solely result from the activation of protein kinases by this nucleotide [6]. These protein kinases have been found both in the soluble and particulate fractions of the cell [7,8]. The cyclic AMP-dependent phosphorylation of membranous proteins by membrane-associated kinases has also been reported in a variety of systems [4,9,10]. In addition a rapid turnover of protein-bound phosphorylserine has been observed in several systems suggesting that the kinase and phosphatase activities associated with membranes may provide an enzymatic basis of physiologic significance for controlling the phosphate content of membranous proteins [11—14].

Myelin isolated from rat, and human central nervous system has been shown to contain protein kinase(s) which phosphorylates the endogenous basic (A-1) protein of this membrane [14—17]. Evidence has also been presented which suggests that rat central nervous system myelin contains both the cyclic AMP-dependent and -independent protein kinases [18]. The results of in vivo and in vitro studies have provided evidence for the existence of an additional system in rat myelin that dephosphorylates the phosphorylated basic protein [15,19]. However, at present relatively little is known about the nature and properties of particulate bound and more specifically about the human myelin-associated kinase(s) as well as phosphoprotein phosphatase(s). This report describes solubilization of a phosphoprotein posphatase (phosphoprotein phosphohydrolase, EC 3.1.3.16) from human central nervous system myelin into aqueous buffers. The data given herein show that human myelin-associated phosphatase is dependent upon divalent metal ions for catalysis, is activated by Triton X-100 and dithiothreitol, and results of the comparative kinetic studies show that it is specific for basic protein.

Materials and Methods

Materials

Mixed histones (calf thymus), ATP, ADP, AMP and cyclic AMP (free acid and its dibutyryl derivative), cardiac muscle protein kinase and Triton X-100 were purchased from Sigma Chemical Co., radioactive [γ-^{32}P]ATP (30 Ci/mmol) from ICN, ultrapure sucrose from Schwartz/Mann, and sodium dodecyl sulfate (SDS) (Sequenal grade) from Pierce Chemical Co. All other chemicals were of the highest purity, commercially available.

Methods

Isolation of myelin. Human myelin was isolated from the corpus callosum by the density gradient technique of Autilio et al. [20] using a zonal rotor modification [21]. The myelin thus obtained was osmotically shocked and repeatedly washed with water prior to rapid freezing, or after washing, it was lyophilized and then held at −20°C.

Isolation of basic protein. The basic protein was purified from human lyophilized myelin essentially by the previously published procedure [22]. Each preparation of basic protein exhibited one major protein band of $M_r \cong 19\,000$ upon SDS gel electrophoresis [23]. Similarly, at pH 4.5 [24], a single protein

band was observed upon polyacrylamide gel electrophoresis.

Preparation of acetone powder of myelin. Frozen myelin (1 g) was suspended in 5 ml of pre-cooled acetone (−20°C) with rapid mixing using a glass rod. The suspension was centrifuged immediately at 23 000 × *g* for 10 min. This procedure was repeated twice more followed by a similar treatment of the pellet with cold anhydrous ether. The pellet obtained was desiccated to remove traces of acetone and ether and stored over a dessicant at −20°C under vacuum.

Extraction of phosphoprotein phosphatase from myelin acetone powder. Myelin acetone powder (approx. 200 mg) was suspended in 2 ml of 10 or 50 mM Tris · HCl buffer (pH 7.5) with or without Triton X-100 and the suspension was homogenized in a Potter-Elvehjem type homogenizer for 5 min at 4°C. The resulting homogenate was centrifuged at 100 000 × *g* for 1 h at 0°C. The supernatant solution contained 0.2—0.4 mg of protein/ml and served as the source for phosphoprotein phosphatase. Frequently, aliquots of the enzyme were frozen in an acetone/solid CO_2 bath and held at −70°C. Under these conditions the enzyme retained most of its activity when stored for up to 3 weeks.

Preparation of substrate. Phosphorylation of mixed histones and basic protein by cyclic AMP-dependent protein kinase: Phosphorylation of mixed histones and basic protein was carried out essentially by the procedure of Meisler and Langan [25] using cyclic AMP-dependent protein kinase of bovine cardiac muscle origin obtained through a commercial source. Mixed histones (10 mg) or basic protein (4.0 mg) were phosphorylated in the presence of 100 μmol sodium acetate (pH 6.0)/20 μmol NaF/5 μmol theophylline/20 μmol magnesium acetate/600 nmol ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*-tetraacetic acid/20 nmol cyclic AMP and 350 μg of bovine cardiac muscle protein kinase contained in 2 ml. The reaction was started by the addition of 400 nmol of [γ-^{32}P]ATP ($4 \cdot 10^7$—$10 \cdot 10^7$ cpm) and incubated at 30°C for 24 h. The reaction was terminated by addition of 0.67 ml of cold trichloroacetic acid (100%, w/v). The precipitated protein was separated by centrifugation at 3000 × *g* at 4°C for 10 min. It was dissolved in 2 ml of water and reprecipitated by the addition of trichloroacetic acid. This procedure was repeated three more times. Finally, the pellet was washed twice with cold ethanol/ether (1 : 4, v/v) and dissolved in 1 ml of 10 mM Tris · HCl (pH 7.5) followed by extensive dialysis against the same buffer until the radioactivity in the diffusate reached background levels.

From the radioactivity incorporated, it was determined that the phosphate content of the basic protein varied from 39 to 52 nmol/mg of protein whereas mixed histones contained 23—24.5 nmol of $^{32}PO_4^{3-}$ per mg. In both the cases, the incorporated phosphate was found to be insensitive to acid or hydroxylamine treatments. However, it was quantitatively released with NaOH suggesting it is in a phosphoester linkage as shown previously in the case of phosphohistones by Meisler and Langan [25] and by Beavo et al. [26]. The phosphorylated amino acid residues were identified as serine and threonine [25,26]. Furthermore, chloroform/methanol (2 : 1, v/v) and chloroform/methanol/Triton X-100 (2 : 1 : 0.2%, v/v) failed to release (<1%) the incorporated radioactivity from basic protein. The purity of the phosphorylated basic protein was evaluated by polyacrylamide gel electrophoresis at pH 4.5 [24] and also in the presence of SDS [23].

Phosphoprotein phosphatase assay. The phosphoprotein phosphatase assay

was carried out by a modified method of Maeno and Greengard [12]. The reaction mixture (200 μl) contained the following components: 10 μmol Tris · HCl (pH 7.5)/1 μmol dithiothreitol/basic protein or mixed histones with or without metal ions and enzyme protein as shown under appropriate legends. The reaction was started by the addition of substrate and incubated at 37°C in a shaking water bath. The reaction was terminated by the addition of 0.1 ml of 80 mM silicotungstic acid in 0.005 M H_2SO_4. Following the addition of 0.4 mg of bovine serum albumin and 0.2 μmol of orthophosphate, the precipitated protein was removed by centrifugation and the $^{32}PO_4^{3-}$ released was converted to the phosphomolybdate complex which was extracted as described by Plaut [27]. Radioactivity of the extract was quantitated by scintillation spectrometry [28].

Assays were performed under conditions where dephosphorylation of the phosphoproteins was linear with respect to enzymic protein concentration and time. Phosphate associated with proteins employed as the substrate prior to phosphorylation has been neglected. Orthophosphate released by the action of phosphatase was quantitated from the specific radioactivity of [γ-^{32}P]ATP employed during the phosphorylation reaction. One unit of enzymatic activity is equal to the release of 1 μmol of orthophosphate per min at 37°C and specific activity is units/mg of enzymic protein.

Protein was determined by the method of Lowry et al. [29] or by the method of Wang and Smith [30] when the samples contained Triton X-100, with crystalline bovine serum albumin as the standard.

Results

Solubilization of phosphoprotein phosphatase from human central nervous system myelin

Although phosphoprotein phosphatase activity can be readily demonstrated in whole myelin suspensions, many attempts made to solubilize this activity (e.g. with 0.1 M NaCl, NH_4Cl, KBr, EDTA or 10—50 mM Tris · HCl, pH 7.5) were unsuccessful. Because of the inherent problem of quantitatively removing detergents from proteins, alternate methods were sought that might allow solubilization of phosphoprotein phosphatase from myelin without the use of detergents. In preliminary experiments whole myelin and myelin acetone powder were separately suspended in a buffer (10 mM Tris · HCl, pH 7.5) supplemented and unsupplemented with 0.2% Triton X-100 and potential of the resulting suspensions to dephosphorylate phosphohistones was evaluated. The phosphatase activity of whole myelin was greatly enhanced (approx. 6-fold) by its incubation with Triton X-100. However, the same detergent concentration elicited a marginal stimulatory effect on the phosphatase activity of myelin acetone powder.

Based upon the above observations, a more detailed study was undertaken to evaluate (a) whether Triton was solubilizing phosphatase from whole myelin and myelin acetone powders or whether it was facilitating expression of the "masked" membrane associated activity [12] and (b) to quantitate whether the total phosphatase activity directed towards basic protein and histones of whole myelin was retained and could be quantitatively extracted from the acetone

powders of myelin. To answer these questions, myelin and myelin acetone powders were separately extracted with Tris buffer supplemented and unsupplemented with Triton X-100 and centrifuged at 100 000 × *g*. The supernatant solutions were assayed in the presence and absence of the detergent for their ability to dephosphorylate basic protein and histones. The results of these comparative studies (Table I) show that irrespective of the substrate, myelin acetone powders contain almost the same amounts of extractable phosphatase as obtained from whole myelin extracted with Tris buffer containing Triton X-100. Therefore, partial delipidation of myelin with acetone does not appear to adversely affect the phosphoprotein phosphatase activity associated with myelin. In addition, it is seen that Triton X-100 does not only solubilize the phosphatase but that it also exerts a stimulatory effect on the solubilized enzyme (also see below).

The results presented in Table I also show that phosphoprotein phosphatase is readily solubilized from myelin acetone powders with buffers which do not contain the detergent. In comparison with the levels solubilized from myelin acetone powder with buffer containing detergent, 10 mM Tris · HCl (pH 7.5) extracted approx. 50% of the phosphatase activity whereas quantitative solubilization was achieved with a buffer of higher ionic strength (50 mM). Furthermore, these extracts contain about 3—4-fold greater phosphatase activity with basic protein than with histones as the substrate.

TABLE I

EXTRACTION OF PHOSPHOPROTEIN PHOSPHATASE FROM MYELIN AND MEYLIN ACETONE POWDERS WITH Tris · HCl BUFFER SUPPLEMENTED AND UNSUPPLEMENTED WITH TRITON X-100

Whole myelin and myelin acetone powders were separately suspended (100 mg/ml) in 10 mM or 50 mM Tris · HCl (pH 7.5) with or without 0.2% Triton X-100. Each suspension was homogenized at 4°C for 5 min and centrifuged at 100 000 × *g* for 1 h at 0°C. The supernatant solutions were tested for phosphatase in the presence and absence of 0.2% (v/v) Triton X-100. The assays were conducted in 0.2 ml of the reaction mixture containing: 10 μmol Tris · HCl buffer, pH 7.5, 1 μmol dithiothreitol, 25 nmol $MnCl_2$ (basic protein as the substrate) or 12 μmol $MgCl_2$, (histones as the substrate) and 0.25 μg enzymic protein. When assayed in the presence of Triton X-100, an aliquot of the enzyme (0.5 μg of protein) was added to the reaction mixture containing 0.2% (v/v) Triton X-100 and incubated at 37°C for 30 min. Reactions were started by the addition of substrates (basic protein, 16 μg = 0.95 nmol of $^{32}PO_4^{3-}$ or histones, 52 μg = 1.3 nmol of $^{32}PO_4^{3-}$) and incubated at 37°C for 5 min (basic protein) or 20 min (histones), respectively. After terminating the reaction, released orthophosphate was quantitated as given under Methods.

Numbers in parentheses represent munits of phosphatase extracted with 50 mM Tris · HCl, pH 7.5. — and + denote without and with Triton during extraction.

Substrate	Triton during assay	Phosphatase activity (munits/g wt weight myelin)			
		Whole myelin		Myelin acetone powder	
		—	+	—	+
Basic protein	—	0.0	10.5	5.1 (10.9)	14.0
	+	0.0	18.5	7.7 (17.3)	15.0
Histones	—	0.0	3.8	1.5	3.6
	+	0.0	4.2	1.3	3.5

Characterization of phosphoprotein phosphatase solubilized from myelin acetone powders

Despite the fact that higher amounts of the enzyme could be extracted from myelin acetone powders with either 50 mM Tris · HCl buffer or with 10 mM Tris · HCl (pH 7.5) containing 0.2% Triton X-100 (Table I), the studies summarized below were conducted on the enzyme present in the 100 000 × *g* supernatant solution obtained from myelin acetone powder extracted with 10 mM Tris · HCl. These preparations possessed higher specific activity than the enzyme extracted from whole myelin or myelin acetone powders with buffers of higher ionic strength with or without Triton X-100.

Characteristics of the phosphoprotein phosphatase assay

The rate of dephosphorylation catalyzed by the solubilized phosphatase was proportional to the enzymic protein concentration (approximately up to 4 μg per assay tube) and to the time of incubation up to 10 min with basic protein and approximately for 30 min with phosphohistones as the substrate (data not shown). Upon prolonged incubation (24 h at 37°C), 80—85% of the protein-associated phosphate was released by the phosphatase. In addition the phosphatase activity was markedly stimulated (approx. 10-fold) when assayed in the presence of 5 mM dithiothreitol. The results presented in Fig. 1 show that like other neuronal phosphoprotein phosphatases [12] the enzyme solubilized from

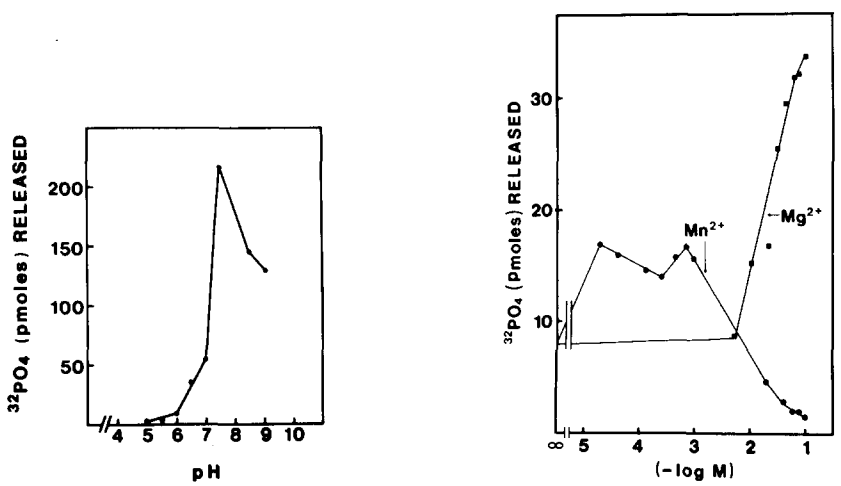

Fig. 1. pH dependence of phosphoprotein phosphatase activity solubilized from human myelin. The dephosphorylation of basic protein as a function of pH was monitored in an assay mixture (0.2 ml) containing 1 μmol dithiothreitol, 25 nmol $MnCl_2$, and 2.0 μg of the enzymic protein. The assays were conducted in the presence of buffers of varying pH; 50 μM acetate; pH 5.0—7.0; 50 μM Tris · HCl, pH 7.0—8.5 and 50 μM glycylglycine · HCl, pH 8.5—9.1. The assays were started by adding basic protein 15 μg = 0.58 nmol of $^{32}PO_4^{3-}$ and incubated for 5 min at 37°C. After termination of the reaction released phosphate was quantitated as given under Methods.

Fig. 2. Effect of Mg^{2+} and Mn^{2+} on phosphatase activity with phosphohistones as substrate. The assay mixture was similar to that described under Table I expect that the concentrations of Mg^{2+} (■———■) and Mn^{2+} (●———●) were varied as shown. The reactions were started by adding phosphohistones (52.5 μg = 1.2 nmol $^{32}PO_4^{3-}$) and the enzymic protein added equalled 2.2 μg.

human central nervous system myelin catalyzes optimal dephosphorylation of the phosphoprotein at pH 7.5.

It is interesting to note that the enzyme under investigation is essentially inactive when assayed in buffers (ethanolamine · HCl, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, morpholinopropane sulfonic acid, imidazole · HCl and Tris/maleate) other than Tris · HCl of similar molarity and pH.

Effect of metal ions on the activity of solubilized phosphoprotein phosphatase

The activity of certain phosphoprotein phosphatases is greatly enhanced by divalent metal ions [31,32]. Likewise human myelin-associated phosphatase was found to be stimulated by Mg^{2+} while catalyzing dephosphorylation of phosphohistones (data not shown). The results presented in Fig. 2 show that the enzyme solubilized from myelin retains its ability to be activated by Mg^{2+}. The maximum rate of dephosphorylation of histones is generally observed at Mg^{2+} concentration of 50—100 mM. However, when the rate of dephosphorylation of histones was monitored in the presence of Mn^{2+} (see below for its requirement in the dephosphorylation of basic protein), a somewhat different profile of activity was obtained. The highest activity representing about 40% of that obtained in the presence of optimal concentration of Mg^{2+} was observed at a Mn^{2+} concentration of 20 μM with minor variations as the concentration of Mn^{2+} was increased to 1 mM. The Mn^{2+} concentrations in excess of 1 mM led to a decrease in enzymatic activity in contrast to Mg^{2+} which did not decrease the rate of catalysis at a concentration of 100 mM, the highest concentration tested in the dephosphorylation of phosphohistones (Fig. 2).

Since the maximum dephosphorylation of phosphohistones catalyzed by the solubilized enzyme occurred at a relatively high concentration of Mg^{2+}, an attempt was made to determine whether the same or a different divalent metal ion was required for the optimal dephosphorylation of basic protein. In Table II, activity of the enzyme directed towards basic protein in the presence of varying concentrations of different metal ions is given. In independent experiments (Fig. 3) it was found that the optimal dephosphorylation of basic protein occurred at a Mn^{2+} concentration of 125 μM. Therefore, the phosphatase activity found with other metal ions has been compared with the activity determined in the presence of 125 μM Mn^{2+}, assumed as 100%.

From the data given in Table II it is clear that none of the metal ions tested can effectively substitute for Mn^{2+} in the dephosphorylation of basic protein by the solubilized phosphatase. In fact the residual activity of the dialyzed enzyme (Expt. 2) decreases when tested in the presence of Co^{2+} (Expt. 7) and is completely abolished in the presence of 0.125 mM Zn^{2+} (Expt. 8). The latter cation exerts a similar inhibitors effect when added to the reaction mixture containing optimal concentration of Mn^{2+} (Expt. 9).

Effect of Triton X-100 on the kinetics of the reaction catalyzed by phosphoprotein phosphatase

Non-ionic detergents are known to alter the activities of membrane-associated enzymes [33,34]. The data summarized in Table III show that the phosphatase activity present in the particle-free supernatant solutions derived from myelin acetone powder is activated by Triton X-100 when assayed with basic

TABLE II

EFFECT OF DIFFERENT METAL IONS ON THE ACTIVITY OF PHOSPHATASE WITH BASIC PROTEIN AS THE SUBSTRATE

The assays were carried out as given under Table I in the presence of different metal ions at the concentrations shown. Dephosphorylation was monitored in the presence of 1.13 μg of enzymic protein and concentration of the basic protein corresponded to 14.2 μg = 0.74 nmol $^{32}PO_4^{3-}$. The reactions were incubated for 5 min at 37°C and the released phosphate quantitated as given under Methods.

	Metal added	Concentration (mM)	Emzymatic activity (%)
1	$MnCl_2$	0.125	100
		2.0	81
		50.0	42
2	—	—	5
3	EDTA	2.0	0.9
4	$CaCl_2$	0.1	0
		2.0	22.9
5	$FeCl_3$	0.125	16.1
		2.0	0.33
6	$MgCl_2$	0.125	6.0
		2.0	8.9
7	$CoCl_2$	0.125	2.9
8	$ZnCl_2$	0.125	0
		2.0	0
9	$MnCl_2$ (0.125 mM) + $ZnCl_2$ (0.1 mM)		81.8
	$MnCl_2$ (0.125 mM) + $ZnCl_2$ (2.0 mM)		8.5

protein as the substrate. The maximum stimulation (approx. 2-fold) was observed at a detergent concentration of 0.15%. The phosphatase activity, however, was unaffected by the detergent with phosphohistones as the substrate. The specific activity of the solubilized phosphatase when determined under optimal conditions with respect to divalent metal ions, pH, dithiothreitol and

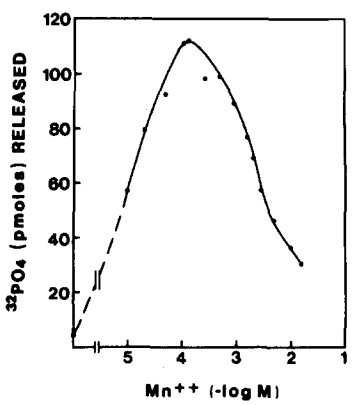

Fig. 3. Effect of Mn^{2+} on phosphatase activity with basic protein as substrate. The reaction mixture was identical to that given in Table I except that the concentration of Mn^{2+} was varied. The reactions were initiated by adding basic protein 6.5 μg = 0.2 nmol $^{32}PO_4^{3-}$. The rates of dephosphorylation were determined in the presence of 2.2 μg of enzymic protein for 5 min at 37°C.

TABLE III

EFFECT OF TRITON X-100 ON PHOSPHATASE ACTIVITY WITH HISTONES AND BASIC PROTEIN AS SUBSTRATES

The assays were conducted as given in Table I in the presence of varying concentrations of Triton X-100 as shown. During these assays, the concentration of phosphohistones was 52.5 μg = 2.32 nmol $^{32}PO_4^{3-}$ and of basic protein corresponded to 16.6 μg = 0.49 nmol $^{32}PO_4^{3-}$. Dephosphorylation of phosphohistones was monitored in the presence of 2.1 μg of enzymic protein and 60 mM $MgCl_2$ for 20 min. The dephosphorylation of basic protein was conducted in the presence of 0.48 μg of enzymic protein and 125 μM $MnCl_2$ for 5 min. Prior to addition of substrates, the reaction mixture was preincubated at 37°C for 10 min in the presence of varying concentrations of the detergent. Released phosphate was determined as given under Methods.

No.	Concentration of Triton X-100 (%, v/v)	Specific activity (munits/mg protein)	
		Histones	Basic protein
1	—	0.68	16.9
2	0.05	0.59	26.4
3	0.10	0.61	30.2
4	0.15	0.7	32.4
5	0.20	0.66	27.8

Triton X-100 corresponded to 30 and 0.7 nmol of phosphate released · min^{-1} · mg^{-1} of enzymic protein from basic protein and histones, respectively.

In Fig. 4 the effect of Triton X-100 on the kinetic parameters (K_m and V) of the reaction catalyzed by phosphoprotein phosphatase with basic protein (Fig. 4A) and phosphohistones (Fig. 4B) is depicted. With basic protein as the substrate, the V determined in the presence of Triton X-100 was 67, whereas it was approx. 23 in the absence of the detergent. The K_m value (approx. 1.2 μM) for the basic protein was unaffected by the detergent.

In contrast, Triton X-100 did neither affect the V (8.4) nor the K_m (167 μM) of the phosphatase with phosphohistones as the substrate.

Effect of certain substances on phosphoprotein phosphatase solubilized from myelin

The effect of different substances on the activity of solubilized phosphatase with basic protein as the substrate is given in Table IV. Addition of inorganic phosphate or fluoride at a concentration of 10 mM inhibited approx. 30% of the phosphatase activity. Whereas further increase in fluoride concentration did not appreciably alter the enzymatic activity, higher concentrations of phosphate (30 mM) almost totally abolished the phosphoprotein phosphatase activity. Other neuronal phosphatases are known to be inhibited by these compounds [12,19].

In comparison with inorganic phosphate and fluoride, lower concentrations of adenine nucleotides when included individually in the complete assay mix inhibited phosphoprotein phosphatase activity. However, addition of Mn^{2+} in excess of the nucleotide concentrations reversed the inhibition elicited by these compounds. Therefore, the inhibitory effect of adenosine phosphates is due to chelation of the divalent metal ion which has been shown to be required for catalysis. Furthermore, this explanation appears to be consistent with the findings that these nucleotides when included individually in the assays at a concen-

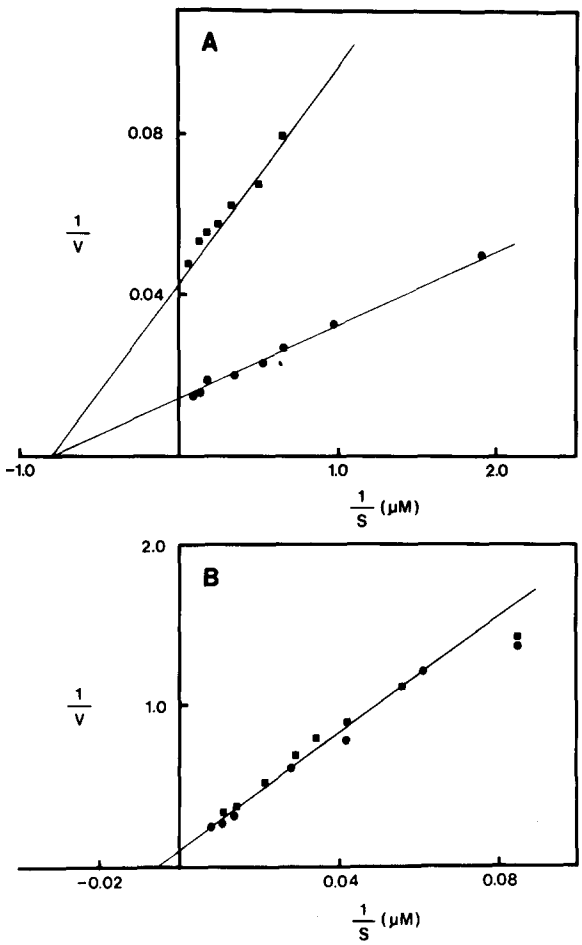

Fig. 4. Determination of V and K_m of phosphatase for basic protein and phosphohistones in the presence (●——●) and absence (■——■) of Triton X-100. (A) Basic protein. The reaction components were similar to those given in Table I except that the concentration of basic protein (1 nmol $^{32}PO_4^{3-}$ = 20 μg of protein) was varied as shown. The rates of dephosphorylation of basic protein were determined in the presence and absence of Triton X-100 (0.15%, v/v) with 0.1 μg of enzymic protein. Reactions were initiated by adding varying concentrations of basic protein and were incubated at 37° C for 5 min. The molecular weight of approx. 19 000 for basic protein has been employed to calculate the K_m. (B) Histones. The dephosphorylation of phosphohistones was conducted in the presence and absence of Triton X-100 (0.15%, v/v) with 0.5 μg of enzymic protein and varying concentrations of phosphohistones (1 nmol $^{32}PO_4^{3-}$ = 56 μg of protein). The assays were incubated for 20 min at 37°C. The molecular weight of 10 000 for phosphohistones has been assumed in calculating the K_m.

tration of 2 mM did not inhibit the activity of the enzyme with phosphohistones as the substrate which is determined in the presence of a relatively high concentration (50—100 mM) of Mg^{2+} (data not included).

Discussion

The results presented in the paper show that human central nervous system myelin contains phosphoprotein phosphatase which exhibits a high degree of specificity towards the basic protein. This phosphoprotein phosphatase appears

TABLE IV

EFFECT OF CERTAIN SUBSTANCES ON PHOSPHOPROTEIN PHOSPHATASE ACTIVITY

The reaction mixture in addition to containing components given in Table I was supplemented with the compounds shown below. Dephosphorylation of the substrate (basic protein 15.6 μg = 0.78 nmol $^{32}PO_4^{3-}$) was monitored in the presence of 0.1 μg of enzymic protein and the released phosphate was quantitated as given under Methods.

	Addition	Concentration (mM)	Enzymatic activity (%)
1	None	—	100
2	NaH_2PO_4	10	69
		15	9
		30	6
3	NaF	10	69
		30	59
		60	46
4	Phosphoserine	0.1	100
		2.0	100
5	Phosphothreonine	0.1	100
		2.0	100
6	*p*-Nitrophenylphosphate	0.1	100
		2.0	100
7	$\overline{5}$-AMP	0.1	100
8	ADP	0.1	81
9	ADP (0.1 mM) + $MnCl_2$ (0.2 mM)		100
10	ATP	0.1	80
11	ATP (0.1 mM) + $MnCl_2$ (0.2 mM)		100

to be firmly associated with the membrane since it resists solubilization under conditions (extraction with NaCl, NH_4Cl, KBr, EDTA, etc.) that are known to release proteins considered to be loosely associated with various membranes [33]. The enzyme, however, was readily extracted into aqueous buffers from acetone powders prepared from myelin. These observations indicate that partial delipidation, which most likely leads to disruption of the membrane, is an essential pre-requisite for solubilization of the phosphatase into aqueous buffers.

The human myelin-associated phosphoprotein phosphatase was essentially inactive in the absence of divalent cations. Because of an almost absolute requirement for divalent metal ions during catalysis, the enzyme solubilized from human myelin differs from phosphoprotein phosphatase(s) found associated with different particulate fractions derived from rat cerebral cortex [12]. These enzymes, like the phosphatase that has recently been solubilized from rat myelin, with Triton X-100, dephosphorylate a number of phosphoproteins in the absence of divalent metal ions [12,19]. However, in its ability to be stimulated by divalent metal ions, the phosphatase associated with human myelin resembles phosphoprotein phosphatases of bovine adrenal cortex and canine heart origins [31,32]. Therefore, activation of some phosphoprotein phos-

phatases by divalent metal ions (e.g. Mn^{2+}) and their inhibition by other cations (e.g. Zn^{2+}) may have physiologic significance [31].

It was interesting to note that activation of the solubilized phosphatase by Triton X-100 with basic protein as the substrate resulted from an increase in the V without affecting the K_m (Fig. 4A). In view of the recent findings that non-ionic detergents can stimulate lipid-dependent enzymes solubilized from membranes, it may be that Triton X-100 exerts a similar effect on the phosphatase that we have solubilized from partially delipidated myelin membrane [34]. For example, Triton X-100 may bind to the lipid-depleted enzyme which results in an alteration of the catalytic center activity of the phosphatase [35].

None of the organic phosphate compounds of low molecular weight (Table IV) inhibited the phosphatase activity. Therefore, it appears unlikely that the myelin-associated enzyme is a "non-specific" phosphatase. The results of comparative kinetic studies (Michaelis constants and V) clearly suggest that myelin-associated phosphatase is rather specific for the basic protein. This high degree of substrate specificity as compared to the other neuronal phosphoprotein phosphatases [12,19] may lie in the ability of the human myelin phosphatase to recognize more precisely the particular phosphoamino acid(s) in the covalent structure of basic protein, also a constituent of this membrane, than in protein(s) that are unrelated to myelin.

Based upon the differential effects of Triton X-100 and divalent metal ions on the phosphatase activity directed towards basic protein and histones, we have considered the possibility that the particle-free supernatant solution derived from myelin acetone powder may contain more than one phosphoprotein phosphatase. Limited amounts of enzymatic protein available have at present hampered further purification and possible resolution of these activities by conventional protein fractionation procedures. However, in preliminary attempts where the solubilized enzyme was electrophoresed in polyacrylamide gel columns at pH 7.5, greater than 85% of the recovered enzymatic activity was found in a gel segment that dephosphorylated both the substrates at rates that were proportional to those determined prior to electrophoresis *, thus suggesting that human myelin most likely contains a single major type of phosphatase which can dephosphorylate basic protein and other phosphoprotein(s) though at a markedly reduced rate.

To our knowledge this is the first report describing solubilization of myelin-associated phosphatase into aqueous buffers exhibiting a marked degree of specificity towards basic protein, most probably its natural substrate. As noted in Introduction, myelin isolated from different sources has been shown to contain protein kinase(s). Since other myelin proteins (Folch-Lees proteolipid and Wolfgram proteolipid) were not found to be phosphorylated by the endogenous protein kinase of human myelin (Yourist, J.E. and Ahmad, F., unpublished), the presence of protein kinase and phosphatase in this membrane may provide a mechanism with a potential to control preferentially the degree of phosphorylation of basic protein, a major constituent of myelin proteins. The

* The enzyme eluted from polyacrylamide gel segments is extremely labile and undergoes rapid inactivation ($t_{0.5} \approx 30$ min) at 4°C. A number of attempts made to stabilize the enzyme thus purified have so far been unsuccessful.

physiological significance of these covalent modifications of basic protein, that may occur within myelin, is not yet understood.

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