PHOSPHOPROTEIN PHOSPHATASE OF HUMAN CENTRAL NERVOUS SYSTEM MYELIN

Purification to apparent homogeneity of a low M_r phosphatase and characterization of the high M_r phosphatase

N. C. WU, J. J. MARTINEZ and Fazal AHMAD*
Papanicolaou Cancer Research Institute, PO Box 016188, Miami, FL 33101, USA

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1. Introduction

Phosphoprotein phosphatase (PPase) activity in brain tissue has been reported [1–4]. Properties of a PPase solubilized from human central nervous system myelin described were [5]. We now report on the presence of a high molecular weight form of the PPase (M_r 250 000) found in extracts prepared from delipidated myelin. This high M_r form of the enzyme has been converted to a small molecular weight species of M_r 46 000. The latter has been purified to apparent homogeneity. We report on the substrate specificity and activation by divalent cations of the high molecular weight PPase.

2. Materials and methods

2.1. *Materials*

Myelin basic protein was prepared from human brain by the method in [6]. Myelin was isolated from human brain as in [7]. Mixed histones, lysine-rich histones, arginine-rich histones, protamine, and cyclic AMP-dependent protein kinase were purchased from Sigma. [γ-^{32}P]ATP was purchased from New England Nuclear.

2.2. *Preparation of [^{32}P]phosphorylated substrates and assay of the phosphatase activity*

Phosphorylation of basic protein, histones, and protamine was mainly as in [8]. The [^{32}P]phosphoproteins used here contained 10–40 nmol alkali-labile phosphate/mg protein. The phosphatase was assayed by the modified method in [2]. The reaction mixture (100 μl) contained 50 mM Tris–HCl buffer (pH 7.5), 5 mM dithiothreitol, 4–6 μM [^{32}P]orthophosphate moiety in the respective phosphoproteins, 125 μM Mn^{2+}, and appropriate amounts of the phosphatase. After a 20 min incubation at 37°C, the reaction was stopped by adding 20 μl glacial acetic acid. Released orthophosphate was separated and quantitated as in [9]. Conditions for assays were selected so that the release of phosphate was proportional to the time of incubation and to the amount of enzyme protein added. One unit of phosphatase activity releases 1 nmol [^{32}P]phosphate/min from phosphoproteins.

2.3. *Isolation and purification of phosphoprotein phosphatase from human myelin*

Human myelin was stirred in 30 vol. ether/methanol (3:2, v/v) for 30 min at 4°C. After repeating the delipidation process twice, the suspension was centrifuged at 12 000 × *g* for 10 min, and the pellet was dried under nitrogen. The myelin powder was homogenized in 10 vol. 50 mM Tris–HCl buffer (pH 7.5) and centrifuged at 100 000 × *g* for 45 min. The supernatant solution contained the high-molecular form of the PPase. The PPase was precipitated from the supernatant solution on the addition of ethanol to 80%. This step was done at room temperature as in [10]. The resulting protein precipitate was collected by centrifugation, dissolved in a volume equal to that of the original extract in buffer A (50 mM Tris–HCl (pH 7.5), 20% glycerol, and 2 mM dithiothreitol) then 1.0 ml enzyme solution was chromatographed on a Bio-gel A-1.5 m column (1 × 30 cm) using buffer A at 4°C. Fractions with phosphatase activity were pooled and chromatographed on a DE-52 cellu-

* To whom correspondence should be addressed

lose column (0.7 X 10 cm) equilibrated with buffer A. The column was developed with a linear gradient of 0.1–0.4 M NaCl in buffer A. Phosphatase activity was found to elute from 0.2–0.3 M NaCl. The active fractions were pooled and concentrated to 0.5 ml by ultrafiltration using Amicon PM-10 membrane. Finally the enzyme solution was chromatographed on a Sephadex G-100 column (0.7 X 30 cm) using buffer A. Fractions containing PPase activity were pooled and concentrated as above.

Protein was determined as in [11]. Molecular weight of the phosphatase was estimated by sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis [12].

3. Results and discussion

3.1. *Properties of the low* M_r *phosphatase*

A relatively simple scheme for the purification to apparent homogeneity of myelin associated PPase is presented in table 1. After ethanol precipitation, the activity of most phosphorylase phosphatases has been reported to increase [10,13–16]. On the contrary, the activity of the PPase solubilized from myelin decreased by 45% on ethanol precipitation when assayed with phosphorylated basic protein or histones. A similar loss in activity has been observed during the purification of rat liver PPase [17]. As reported for other PPases, the high M_r form of the myelin PPase dissociated to a low M_r form by ethanol treatment at room temperature (fig.1). This form of the enzyme could then be purified to apparent homogeneity by the 3 additional steps given in table 1. When the enzyme purified through step 5, table 1 was subjected to SDS–polyacrylamide gel electrophoresis, it revealed a single protein band of

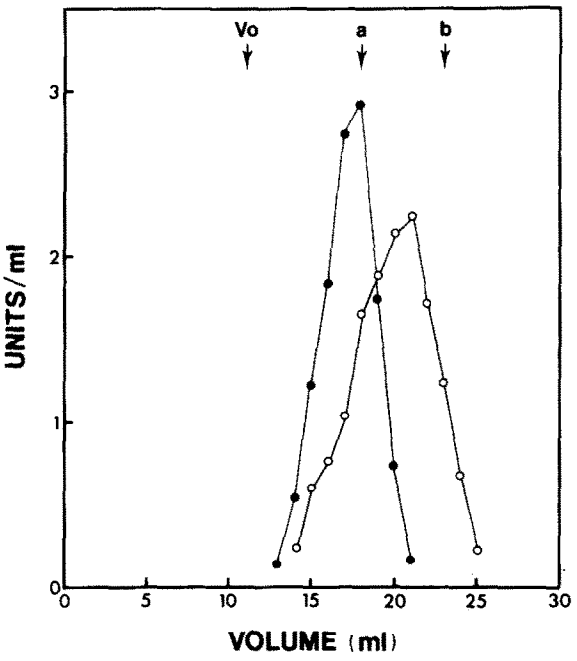

Fig.1. Chromatography of human myelin phosphoprotein phosphatase on Biogel A-1.5 m column before and after ethanol precipitation. Phosphoprotein phosphatase carried through purification Steps 1 (●——●) and 2 (○——○) of table 1 was separately chromatographed over a Biogel A-1.5 m column under conditions in the text. Phosphorylated human myelin basic protein was used to assay the phosphatase activity. The column was calibrated with proteins of known M_r: (a) beef liver catalase (250 000); (b) ovalbumin (43 000). The void volume (V_O) was determined by chromatographing blue dextran 2000.

M_r 46 000 (fig.2). The phosphatase activity emerged as a symmetrical peak from Sephadex G-100 column with an estimated M_r 50 000. Therefore these observations indicate that the low M_r phosphatase may be constituted by a single polypeptide chain.

Table 1
Purification of human myelin phosphoprotein phosphatase

Step	Total units	Units/mg protein	Yield(%)
1. 100 000 X *g* supernatant	13.2[a]	3.8	100
2. Extract of ethanol precipitate	7.1	5.9	54
3. Biogel A-1.5m	6.7	10.7	51
4. DE 52 cellulose	2.0	29.9	15
5. Sephadex G-100	0.7	14.6	5

[a] The number represents the total activity in 1 ml 100 000 X *g* supernatant

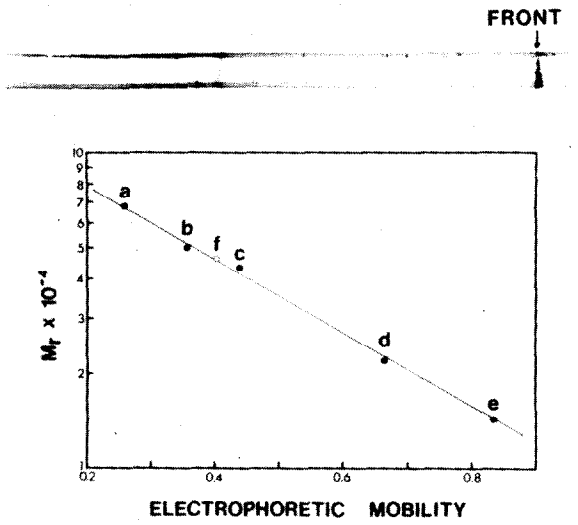

Fig.2. Estimation of the molecular weight of purified phosphatase by SDS–polyacrylamide gel electrophoresis. The standard curve was constructed using following proteins: (a) bovine serum albumin (68 000); (b) heavy chain of human γ-globulin (50 000); (c) ovalbumin (43 000); (d) light chain of human γ-globulin (22 000); (e) lysozyme (14 300). The PPase purified through step 5, table 1 when electrophoresed under identical conditions migrated to a position which corresponds to M_r 46 000.

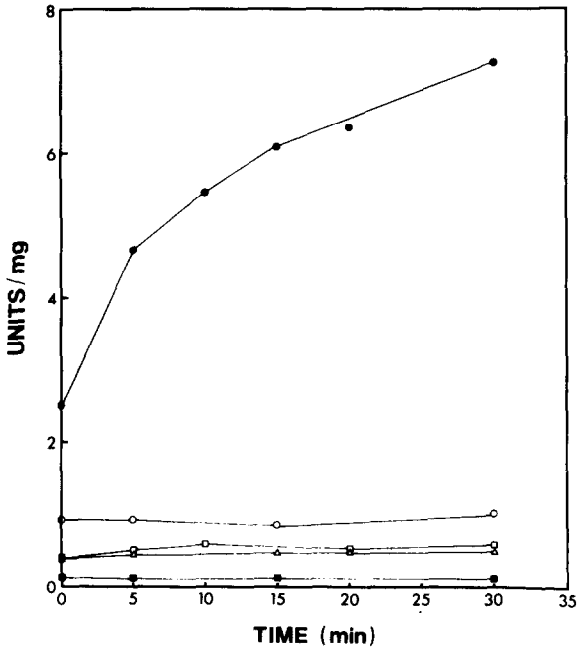

Fig.3. Time course of activation of the large molecular weight phosphoprotein phosphatase by Mn^{2+}. The myelin phosphatase (step 1 of table 1) was preincubated with 125 μM Mn^{2+} for the time intervals shown. The activity was then determined as described in the text with the following phosphoproteins: human myelin basic protein (●); protamine (○); mixed histones (□); arginine-rich histones (■); and lysine-rich histones (▲).

The optimal activity with basic protein was expressed in the presence of 125 μM Mn^{2+} and 5 mM dithiothreital. The enzyme was inactive when tested with *p*-nitrophenyl phosphate as the substrate.

The activity of the highly purified PPase is comparatively unstable. It has not yet been possible to stabilize the activity of the low M_r PPase. In comparison with the starting material the highly purified enzyme (step 5, table 1) showed only a 4-fold increase in its specific activity. However, due to the inherent instability of the purified PPase, both the specific activity as well as the yield of the final preparation are substantially underestimated.

3.2. *Substrate specificity and activation of the high M_r phosphatase by cations*

A number of phosphoprotein phosphatases possessing broad substrate specificity have been described. Some of these enzymes are activated by divalent cations. When the high M_r phosphatase was incubated with Mn^{2+} prior to the addition of the substrate, there was a marked activation (~4-fold) in the rate at which it abstracted phosphate from myelin basic protein (fig.3). For maximum activation a 30 min preincubation period in the presence of 125 μM Mn^{2+} was found to be optimal. This activation occurred only when basic protein served as the substrate since the rate of dephosphorylation was not enhanced when the enzyme was assayed with protamine, mixed histones, lysine-rich histones, or arginine-rich histones (fig.3).

We have explored the possibility whether activation of the high M_r PPase by Mn^{2+} involves its conversion to the low M_r species. The data obtained (not included) suggest that the molecular weight of the PPase does not change during its activation by divalent cation. Thus far only the high M_r PPase could be activated by divalent cations. Attempts to activate the low M_r PPase have not been successful. The mechanism by which the divalent cation stimulates high M_r phosphatase is not yet known. It is possible that the binding of cation may cause conformational change in the high M_r PPase thus facilitating recognition of the environment existing at the phos-

phate carrying site of the substrate. Such conformational change may not be necessary for binding of the low M_r PPase to its substrate.

Besides Mn^{2+}, a much higher concentration of Mg^{2+} (10 mM) activated the high M_r PPase ~4–5-fold. This activation was also observed only when the activity was assayed with phosphorylated basic protein.

Both Zn^{2+} and orthovanadate inhibited the PPase activity. These experiments were conducted with basic protein as the substrate. In the presence of 0.1, 0.5 and 1.0 mM Zn^{2+} the enzymatic activity was inhibited by 43, 72 and 80%, respectively. In the presence of 2, 10 and 50 μM orthovanadate, the loss in enzymatic activity corresponded to 63, 85 and 92%, respectively.

Like activation by Mn^{2+} and Mg^{2+}, the inhibition of PPase activity by Zn^{2+} was most noticeable with basic protein as the substrate. The PPase activity was not affected by Zn^{2+} when assayed with mixed histones, lysine-rich histones and arginine-rich histones.

These results indicate that the myelin-associated PPase preferentially abstracts phosphate from myelin basic protein, perhaps its natural substrate. Further, the activity of this PPase may be regulated by divalent cations.

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