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O-PHOSPHOSERINE PHOSPHATASE FROM BOVINE BRAIN AND KIDNEY

HIGH MOLECULAR WEIGHT FORMS OCCURRING DURING THE PURIFICATION

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SUMMARY

O-Phosphoserine phosphohydrolase (EC 3 1 3 3) from bovine brain and kidney was found to exist in several molecular forms, which can be separated by chromatography on Sephadex gels This is the consequence of a self-association process, that appears at any purification step when extracts containing the brain or kidney enzymes are precipitated with $(NH_4)_2SO_4$ at pH 7 5 but does not appear when other concentrating methods of concentration are used or if the $(NH_4)_2SO_4$ precipitations are made at pH 5 0 or lower

β-Mercaptoethanol prevents the formation of associated forms and dissociates them when they are present *N*-Ethylmaleimide also prevents their formation It is suggested that disulfide bonds are involved in this self-association process

From these observations, phosphoserine phosphatase from bovine kidney has been partially purified 170-fold in the unassociated form and 270-fold in the associated forms Some properties of these two species of enzyme are also compared in this paper

INTRODUCTION

Studies on the biosynthesis of serine from carbohydrate in animal tissues [1–4] and in bacteria [5, 6] have demonstrated that the hydrolysis of *O*-phosphoserine is achieved by a specific phosphatase (*O*-phosphoserine phosphohydrolase, EC 3 1 3 3) which also catalyzes a phosphoryl-group transfer from phosphoserine to serine [7] The enzyme has been partially purified from rat liver [8], chicken liver [7], rat brain [9], mouse brain [10, 11] and yeast [12] Its distribution in normal and neoplastic rat tissues [13] and its hormonal regulation in rat kidney and liver [14] have also been investigated, but because the pure enzyme has never been obtained, few data are available concerning the molecular properties and the identity of the dissociable groups at or near the active site

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In the course of preliminary experiments to obtain purified phosphoserine phosphatase from bovine brain, we have observed that dimers and higher-molecular-weight forms can arise through the formation of intermolecular disulfide bonds It is possible that such associations may explain the difficulties encountered in past efforts Indeed from these observations and from the fact that the kidney enzyme, which can be obtained in larger quantities than the brain enzyme, also is able to exist in high-molecular-weight forms, it has been possible to improve the previous procedures to purify phosphoserine phosphatase Some properties of the unassociated and associated forms of the kidney enzyme are also compared in this paper

METHODS

Materials

DL-Phosphoserine was obtained from Fluka, $(NH_4)_2SO_4$, Tris, $MgCl_2$ and β-mercaptoethanol from Merck, *N*-ethylmaleimide from Calbiochem, DEAE-cellulose anion-exchanger (capacity 0 75 mequiv /g) from Biorad, Sephadex G-100 and G-200 from Pharmacia All other chemicals were Reagent Grade, obtained from commercial sources Bovine brains and kidneys were obtained from the slaughterhouse

Enzyme assay

Enzymatic activity was measured at 37 °C in a system containing 60 mM Tris–succinate buffer (pH 6 55), 1 mM DL-phosphoserine, 5 mM $MgCl_2$ and a suitable amount of the brain or kidney enzymes (final volume 2 5 or 5 ml) At timed intervals, 1-ml aliquots were withdrawn and added to 0 5 ml of 15% trichloracetic acid After centrifugation, 1 ml of the supernatant was analyzed for inorganic phosphate by the method of Delsal and Manhouri [15] For the purified extracts the trichloracetic acid precipitation was not necessary

The reaction rates were a linear function of the enzyme concentration in all experiments and the rates were also linear with respect to time of incubation 1 enzyme unit is defined as the amount required to release 1 μmole of inorganic phosphate per 10 min The specific activity is the number of units per mg of protein

In order to rule out possible contaminations by non-specific phosphatases which also hydrolyze phosphoserine, the following controls were made (a) With unpurified extracts, released inorganic phosphate was measured in the absence and in the presence of 100 mM L-serine [14] which completely and specifically inhibits phosphoserine phosphatase In all cases, the contribution of non-specific phosphatases was negligible Besides no significant activity was found when DL-phosphoserine was replaced by *p*-nitrophenylphosphate which is the substrate routinely used in determinations of non-specific phosphatases activities (b) With purified extracts, suitable assays were made in order to verify the absence of non-specific phosphatases using *p*-nitrophenylphosphate as previously described [16] for bovine brain alkaline phosphatase, EC 3 1 3 1 and according to Tani et al [17] for acid phosphatase, EC 3 1 3 2

Analytical

Protein concentrations were determined by the method of Lowry et al [18] using bovine serum albumin as a standard

The molecular weight of the different forms of the enzyme was estimated by gel chromatography according to the method of Whitaker [19] A column of Sephadex G-200 (2 5 cm × 85 cm) was equilibrated with 0 05 M Tris–HCl buffer (pH 8 0) containing 0 1 M NaCl and calibrated at 4 °C with trypsin (23 800), ovalbumin (45 000), bovine serum albumin (67 000), *Escherichia coli* alkaline phosphatase (80 000) and human γ-globulin (160 000) Each protein (1–2 mg) was run separately with Blue Dextran dye marker and detected by its absorbance at 280 nm Phosphoserine phosphatase and *E coli* alkaline phosphatase were detected by their activity

In order to control the purity of our preparations, polyacrylamide gel electrophoresis were carried out with Tris–glycine buffer (pH 8 4), essentially as described by Davis [20] Electrophoresis on cellulose acetate strips (Cellogel) was used to localize the phosphoserine phosphatase activity Electrophoresis was performed at pH 8 6 (veronal buffer) or pH 5 6 (Tris–succinate buffer) A current of 2 mA per cm of strip-width was applied for 90 min After electrophoresis the strips were cut into 5-mm slices and each slice was placed in a test tube containing 0 8 ml of 1 25 mM DL-phosphoserine in 60 mM Tris–succinate buffer (pH 6 55) and 5 mM $MgCl_2$ After 60 min at 37 °C, each sample was analyzed for inorganic phosphate

RESULTS

Attempts to purify the phosphoserine phosphatase from bovine brain, evidence for a self-association process

A whole bovine brain (275 g) was homogenized at 2 °C with 550 ml of 10 mM Tris–HCl buffer (pH 7 5) containing 5 mM $MgCl_2$ After centrifugation at 30 000 × *g* for 30 min, the precipitate was again extracted with 275 ml of the same buffer and centrifuged The supernatant solutions of the first and second extraction were combined and subjected to exhaustive dialysis against Tris–HCl buffer (crude homogenate, Step 1) An acetone fractionation was carried out at −10 °C and the 30–45% (v/v) precipitate, which contained more than 85% of the phosphoserine phosphatase activity, was washed with cold acetone, dried in vacuo and kept at −20 °C until use (acetone powder, Step II) This acetone powder (1 g) was extracted by stirring at 2 °C with 150 ml of Tris–HCl buffer, centrifuged at 30 000 × *g* for 30 min and the supernatant solution was heated for 90 min at 52 °C No activity was lost and a significant amount of denatured proteins was obtained and eliminated by centrifugation (heat treatment, Step III) Further purification resulted when the supernatant was again heated for 60 min at 52 °C and pH 5 0 Acidification was achieved by adding 1 M acetic acid at 4 °C and the supernatant obtained after heating and centrifugation was brought back to pH 7 5 by addition of 1 M NaOH at 4 °C (acid heat treatment, Step IV) The specific activity of this unpurified enzymatic extract was 0 6 which only corresponds to a purification of about 10-fold compared with the specific activity of a crude homogenate

Attempts to purify further at this stage, by utilizing electrophoretic or chromatographic methods including DEAE-cellulose or CM-cellulose columns, all resulted in either large losses of activity or no increases in specific activity However, other methods including $(NH_4)_2SO_4$ precipitations combined with chromatographic separations on Sephadex gels, revealed the following self-association process

An extract from Step IV containing 15 enzyme units was subjected to an

$(NH_4)_2SO_4$ precipitation at pH 7 5 by addition of a saturated solution until 80% of saturation was obtained The precipitate formed was separated by centrifugation, dissolved in 2 ml of 10 mM Tris–HCl buffer (pH 7 5) containing 0 1 M NaCl and 5 mM $MgCl_2$, dialysed in a cellophane bag against frequent changes of Tris–HCl buffer and fractionated on a Sephadex G-100 column (1 5 cm × 85 cm) Two peaks of activity, one of which was not homogeneous, were readily obtained and the proportion of Peak I could be enhanced if successive $(NH_4)_2SO_4$ precipitations were made Results of a typical experiment are shown in Fig 1 Two peaks were also obtained when Peak II of a typical experiment was subjected to further $(NH_4)_2SO_4$ precipitations and chromatographed through the same Sephadex G-100 column Attempts to obtain Peak I alone were unsuccessfull

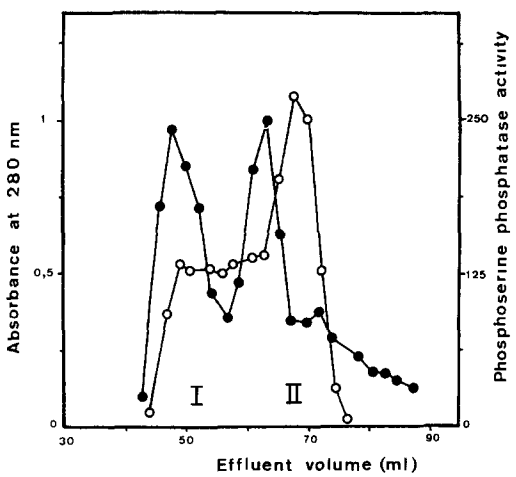

Fig 1 Typical elution profile of enzyme and protein when $(NH_4)_2SO_4$ precipitation of brain extract was made at pH 7 5 Elution was performed with 10 mM Tris–HCl buffer (pH 7 5) containing 0 1 M NaCl 2 5-ml fractions were collected ●—●, absorbance at 280 nm, ○—○, phosphoserine phosphatase activity (μg released P_i/h per ml effluent in the conditions described in "Enzyme assay")

In contrast, when $(NH_4)_2SO_4$ precipitations were made at pH 5 0 or lower, or when other methods were used to concentrate the extract from Step IV, including vacuum dialysis across collodion bags or ultrafiltration in Amicon cells, only one peak was present in the elution profile of the Sephadex G-100 column (Fig 2), which corresponded to Peak II of the above typical experiment

In brief, these results strongly suggest that a self-association process occurs during the $(NH_4)_2SO_4$ precipitation at pH 7 5 which produces an heterogeneous population of phosphoserine phosphatase molecules no matter what stage of purification has been reached

Attempts to characterize the present forms of phosphoserine phosphatase were made with an extract from Step IV which was subjected to three successive $(NH_4)_2SO_4$ precipitations at pH 7 5 in order to enhance the proportion of the heterogeneous Peak I The last precipitate was redissolved in a small volume of Tris buffer which was dialyzed against Tris buffer containing 0 1 M NaCl (pH 8 0) and applied to a column of Sephadex G-200 calibrated with protein markers (see Methods) From

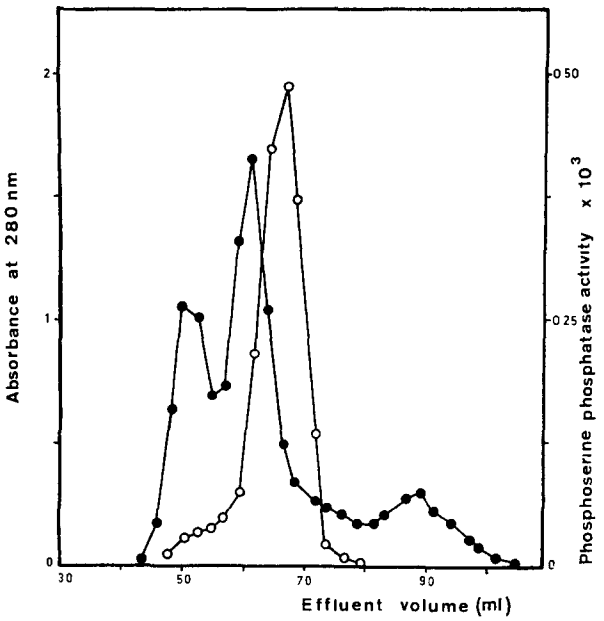

Fig 2 Elution profile of enzyme and proteins when $(NH_4)_2SO_4$ precipitation of brain extract was made at pH 4 5 Prior to the chromatography, the redissolved precipitate was equilibrated at pH 7 5 by dialysis The elution was performed as in Fig 1 ●—●, absorbance at 280 nm, ○—○, phosphoserine phosphatase activity (expressed as in Fig 1)

the profile indicated in Fig 3, the active fractions were separated into three parts (A, B and C) which were concentrated and passed again through the same column In this way, it was possible to characterize four forms corresponding to monomer (from Part C), dimer (from Part B), trimer and tetramer (from Part A) as judged by their apparent molecular weight (65 000, 125 000, 190 000, 260 000)

Mode of association of phosphoserine phosphatase molecules

Experiments to investigate the mode of association revealed that the high-molecular-weight forms probably arise through the formation of intermolecular disulfide bonds

When an extract from Step IV was subjected to $(NH_4)_2SO_4$ precipitation at pH 7 5 in the presence of 25 mM β-mercaptoethanol, only one form corresponding to a molecular weight of 65 000 was found in Sephadex gel chromatograms Additionally the high-molecular-weight forms occurring in the typical experiment were changed to the lower-molecular-weight form when the fractions of Peak I were treated by 25 mM β-mercaptoethanol and rechromatographed on the same Sephadex gel column Moreover, when an extract from Step IV was incubated with 0 75 mM *N*-ethylmaleimide at 25 °C for 15 min in Tris–HCl buffer (pH 6 8) prior to the $(NH_4)_2SO_4$ precipitation at pH 7 5, the enzymatic activity was approximatively halved but the formation of high-molecular-weight forms was prevented

Stability of the unassociated and self-associated forms of phosphoserine phosphatase

The associated forms including dimer, trimer and tetramer and perhaps higher

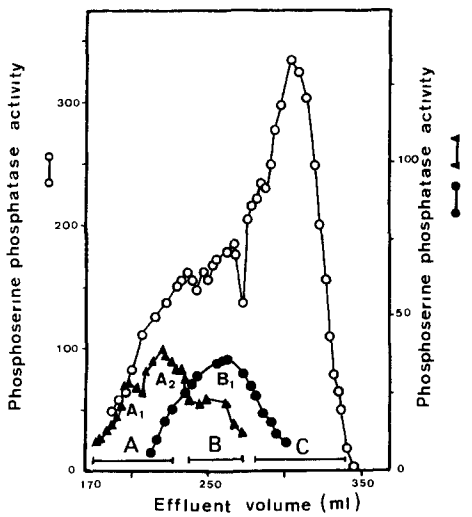

Fig 3 Elution profile of the bovine brain phosphoserine phosphatase activity (μg released P_i/h per ml effluent) on a Sephadex G-200 column (2 5 cm × 85 cm) carried out after three successive $(NH_4)_2SO_4$ precipitations at pH 7 5 of the extract from Step IV (acid heat treatment) (○—○) 5-ml fractions were collected As indicated in the text, the fractions corresponding to Parts A and B respectively were pooled, concentrated by vacuum dialysis and separately rechromatographed on the same column The profiles obtained from Parts A and B are indicated by ▲—▲ and ●—●, respectively Plotting V/V_0 as a function of log molecular weight of protein markers, the Peaks A_1 and A_2 corresponded to tetramer and trimer of phosphoserine phosphatase, B_1 to dimer and C to monomer

molecular forms exhibited the same heat and urea stability (3 h at 52 °C and 4 M urea, respectively) as the unassociated form and were stable at low pH (4 5) in the presence of Mg^{2+} The substrate did not promote dissociation in contrast to what was observed by Makinen [21] with aminopeptidase B Indeed, no change in the Sephadex G-100 profile was observed if the $(NH_4)_2SO_4$ extract was incubated during several hours with a saturating concentration of DL-phosphoserine ([S] $\gg K_m$)

In electrophoresis on cellulose acetate strips, the phosphoserine phosphatase activity of the two forms was lost when the experiments were carried out at pH 8 6 in veronal buffer However, it was possible to localize the activity if the electrophoresis were made at pH 5 6 in Tris–succinate buffer, which indicates that a mild acidity is more suitable for the two forms of phosphoserine phosphatase than an alkaline pH

Partial purification of phosphoserine phosphatase from bovine kidney

These findings suggested that some progress towards obtaining a pure phosphoserine phosphatase could be made Bovine kidney which contains much more phosphoserine phosphatase than bovine brain was chosen as a source of enzyme, after investigation to see if this enzyme ressembled brain enzyme is existing in several molecular forms

Preliminary assays, using different β-mercaptoethanol concentrations in order to protect the unassociated form of the enzyme during the purification, resulted in irreversible large losses of activity which occurred particularly in DEAE-cellulose experiments However, interesting results were obtained if the homogenization of

tissues, the acetone powder preparation, then a DEAE-cellulose chromatography and two consecutive filtrations on Sephadex G-100 were made at pH 5 6 in 10 mM Tris–citrate buffer containing 10 mM $MgCl_2$ Therefore, the three first steps of the purification (crude homogenate, acetone powder and heat treatment) were the same as those mentioned above for the brain enzyme except that Tris–HCl buffer (pH 7 5) was replaced by Tris–citrate buffer (pH 5 6) (pH 7 5 in the step of thermodenaturation at 52 °C for 90 min)

The heat treatment for 60 min at 52 °C and pH 5 0 made for the brain enzyme resulted in no increase in specific activity and was therefore omitted Subsequently, the supernatant from the third step was dialyzed at pH 5 6 and applied to a column (3 5 cm × 19 cm) of DEAE-cellulose equilibrated with the Tris–citrate buffer After washing, the column was developed by a 2000-ml linear gradient elution from 0 to 0 2 M NaCl in 10 mM Tris–citrate buffer containing 10 mM $MgCl_2$ (Fig 4) and the most active fractions containing the phosphoserine phosphatase were pooled and concentrated by ultrafiltration in an Amicon cell equipped with a PM 30 membrane In some cases, the concentration was achieved by vacuum dialysis across a collodion bag (Schleicher and Shull) (DEAE-cellulose, Step IV)

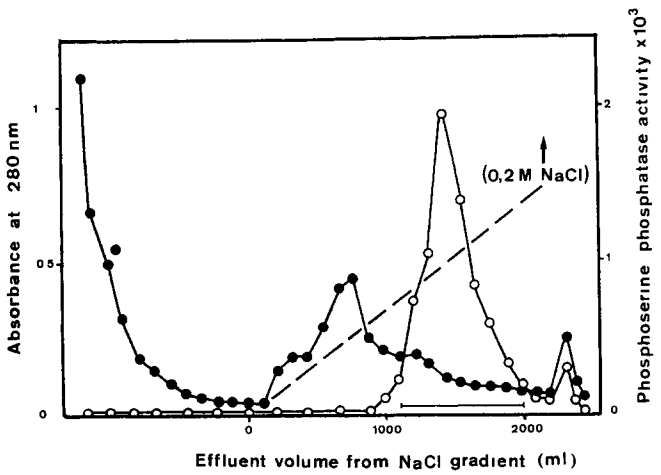

Fig 4 DEAE-cellulose anion-exchange chromatogram in the purification of the kidney enzyme ●—●, absorbance at 280 nm, ○—○, phosphoserine phosphatase activity (expressed as in Fig 1) The pooled fractions are indicated by |—|

In the next step, the concentrated extract was layered onto the surface of a 2 5 cm × 90 cm Sephadex G-100 column equilibrated with the Tris–citrate–Mg^{2+} buffer which was developed by gravity at a rate of 10–15 ml/h The most active fractions were pooled together (about 35 ml) concentrated by vacuum dialysis (Sephadex G-100, Step V) and passed again through the same column (Sephadex G-100, Step VI) (Fig 5)

In order to obtain the associated forms of the enzyme, the pooled active fractions from Step VI were subjected to five successive $(NH_4)_2SO_4$ precipitations at 67% and pH 7 5 The last precipitate obtained was dissolved and the solution was again

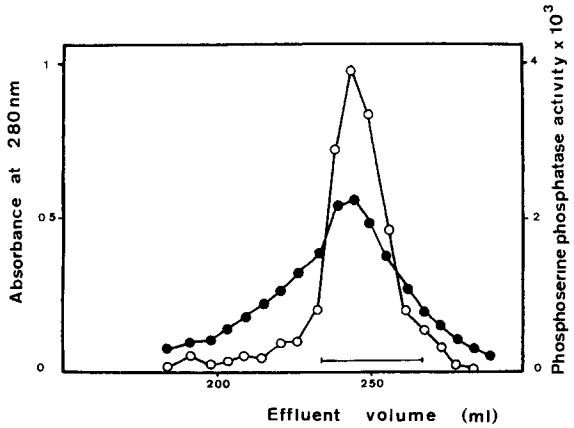

Fig 5 Gel filtration on Sephadex G-100 in the purification of the kidney enzyme (Step VI) Conditions are described in the text ●—●, absorbance at 280 nm, ○—○, phosphoserine phosphatase activity (expressed as in Fig 1) The pooled fractions are indicated by |—|

equilibrated at pH 5 6 by dialysis and passed through the same Sephadex G-100 column as above (Fig 6) The pooled fractions resulting from Peaks I and II were considered as the self-associated and unassociated forms respectively and were utilized in the kinetic experiments (self-association, Step VII)

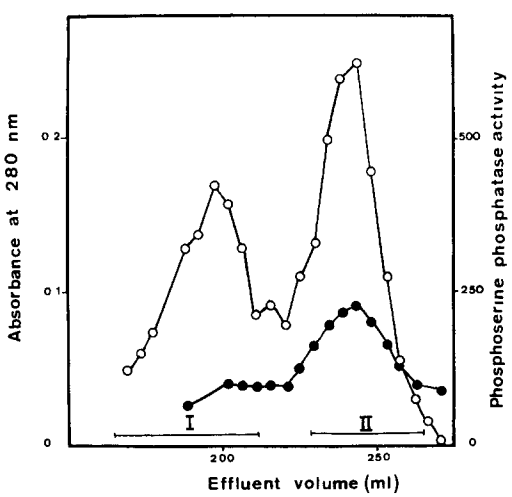

Fig 6 Separation on Sephadex G-100 of the unassociated and self-associated forms of bovine kidney, phosphoserine phosphatase See the text for conditions ●—●, absorbance at 280 nm, ○—○, phosphoserine phosphatase activity (expressed as in Fig 1)

The purification and recovery of a typical experiment are summarized in Table I In polyacrylamide gel electrophoresis, Fraction I exhibited two protein bands one of which, the most electronegative, was much more pronounced than the other Fraction II exhibited two major protein components and three minor (i e faint) bands Further purification of these two fractions was not attempted

TABLE I

SUMMARY OF PURIFICATION OF PHOSPHOSERINE PHOSPHATASE FROM BOVINE KIDNEY

Step		Protein (mg)	Enzyme units	Specific activity	Recovery (%)	Fold purification
I	(Crude homogenate)	45 000	4500	0 1	100	1
II	(Acetone powder)	6 000	3000	0 55	72	5 5
III	(Heat treatment)	4 200	2800	0 67	61	6 7
IV	(DEAE-cellulose)	145	1900	13	43	130
V	(Sephadex G-100)	35	560	16	13	160
VI	(Sephadex G-100)	23	400	17	10	170
VII	(Self-association)					
	Peak I	3 3	90	27	2	270
	Peak II	8 8	115	13	3	130

Some enzymatic properties of the unassociated and self-associated forms of the kidney enzyme

pH optimum and affinity for substrate The optimum pH for phosphoserine phosphatase activity is from 5 8 to 6 2 in the case of the unassociated form of the enzyme and 6 0 to 6 4 in the other case At pH 5 0 the activity of the unassociated form is 50% of its value at pH 6 0, whereas the associated forms are inactive at this pH When the pH is 6 1, the K_m of the unassociated form for DL-phosphoserine is 0 1 mM and that of the associated forms is 0 05 mM

Requirement for Mg^{2+} Dialyzed preparations of the two kinds of enzyme were inactive in the absence of Mg^{2+} The effect of $MgCl_2$ concentration on phosphatase activity of the two forms is shown in Fig 7 in which it can be seen that in comparison with the maximum of activity that can be obtained, the associated forms

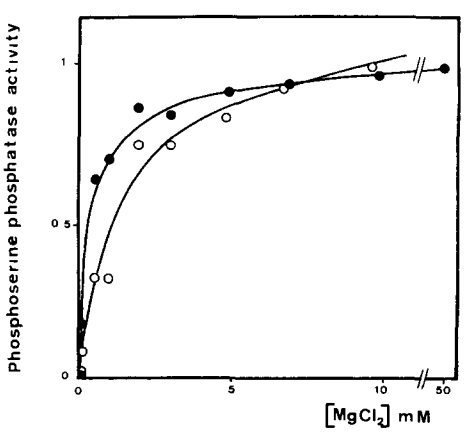

Fig 7 Variation of the relative rate of DL-phosphoserine hydrolysis as a function of $MgCl_2$ concentration ●—●, unassociated enzyme, ○—○, self-associated enzyme The enzyme preparation which contained Mg^{2+} was dialyzed for 48 h at least against frequent changes of 10 mM Tris–citrate buffer (pH 5 6) containing 0 1 M NaCl The value 1 was given to the maximum of activity which can be obtained

are significantly less active at low $MgCl_2$ concentrations than the other form It is also of interest to note that the unassociated form survives dialysis against Tris–citrate buffer in the absence of Mg^{2+}, whereas the associated forms loose 65% of their activity if the dialyzed preparations are left without Mg^{2+} during 6 days at 4 °C So, it is possible that Mg^{2+} serves to stabilize the self-associated structure of the enzyme

Alkylation by N-ethylmaleimide The two forms of the phosphoserine phosphatase are partially inactivated by *N*-ethylmaleimide indicating that –SH groups are present even in the associated forms (Fig 8) The fact that the inactivation kinetics of the unassociated phosphoserine phosphatase is a biphasic curve suggests that two

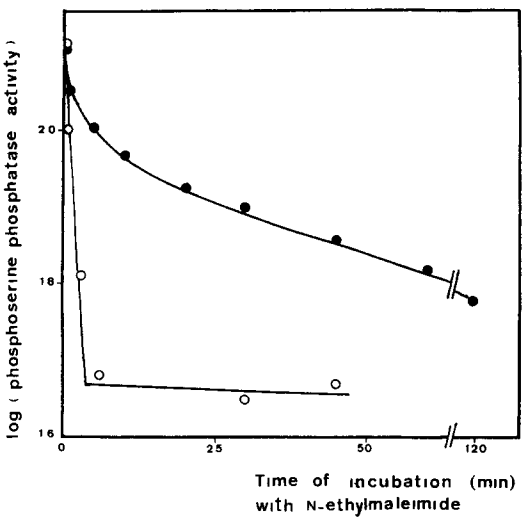

Fig 8 Kinetics of the reactions of inactivation by *N*-ethylmaleimide of unassociated (●—●) and self-associated (○—○) enzymes The enzymatic samples were incubated at 25 °C with 1 mM *N*-ethylmaleimide in 10 mM Tris–HCl buffer (pH 7 0) containing 5 mM $MgCl_2$ and at timed intervals aliquots were withdrawn which were assayed for phosphoserine phosphatase activity 1 min after stopping the inactivation reaction by the addition of an excess of β-mercaptoethanol (5 mM) In these conditions, the dissociating effect of β-mercaptoethanol on the self-associated forms was reduced as much as possible

kinds of –SH groups (fast and slow) are involved in the inactivation process whereas the associated forms only exhibit one kind of –SH groups reacting twice as fast as the most reactive groups of the unassociated enzyme So, the free –SH groups could be more exposed in the self-associated enzyme than in the unassociated enzyme indicating that an altered conformation is produced in the self-association process From the fact that the alkylation does not affect the Michaelis constant of either form, it is likely that the free –SH groups do not occur in the binding of the substrate to phosphoserine phosphatase

CONCLUSION

We have shown that depending on conditions, phosphoserine phosphatases from bovine brain and kidney are can be reversibly changed into several active mole-

cular forms In addition, the results are consistent with the view that the enzymes contain sulfhydryl groups which do not seem to play an essential role in the hydrolytic process as judged by the fact that the associated forms are only 25% less active than unassociated forms, the alkylation by *N*-ethylmaleimide has no effect on K_m of either form and the substrate does not promote dissociation According to Frieden [22], who published an extensive review on protein–protein interactions, it is conceivable that polymerizations through the formation of disulfide bonds may play an important role in metabolic regulation, but our results do not enable us to say if a sulfhydryl–disulfide interchange phenomenon can take place in phosphoserine phosphatase under physiological conditions

Technically, the formation of high-molecular-weight forms during the purification is a possible explanation of the difficulties which have been encountered in the past in attempts to obtain a pure phosphoserine phosphatase Bridgers [10] reported that the fractionation through DEAE-cellulose resulted in large losses of activity and we have ourselves observed the same effect with the bovine brain enzyme

However, taking into account our observations on the stability of the phosphoserine phosphatase and its ability to exist only in the unassociated form at a mildly acidic pH, it has been possible to obtain a relatively pure enzyme from bovine kidney It seems that the purification line described here is better than any other previously published

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