Purification, Properties, and Substrate Specificities of Phosphoprotein Phosphatase(s) from Rabbit Liver*

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The phosphoprotein phosphatase(s) acting on muscle phosphorylase a was purified from rabbit liver by acid precipitation, high speed centrifugation, chromatography on DEAE-Sephadex A-50, Sephadex G-75, and Sepharose-histone. Enzyme activity was recovered in the final step as two distinct peaks tentatively referred to as phosphoprotein phosphatases I and II. Each phosphatase showed a single broad band when examined by sodium dodecyl sulfate gel electrophoresis; the molecular weights derived by this method were approximately 30,500 for phosphoprotein phosphatase I and 34,000 for phosphoprotein phosphatase II. The $s_{20, w}$ value for each enzyme was 3.40. Using this value and values for the Stokes radii, the molecular weight for each enzyme was calculated to be 34,500.

Both phosphatases, in addition to catalyzing the conversion of phosphorylase a to b, also catalyzed the dephosphorylation of glycogen synthase D, activated phosphorylase kinase, phosphorylated histone, phosphorylated casein, and the phosphorylated inhibitory component of troponin (TN-I). The relative activities of the phosphatases with respect to phosphorylase a, glycogen synthase D, histone, and casein remained essentially constant throughout the purification. The activities of both phosphatases with different substrates decreased in parallel when they were denatured by incubation at 55° and 65°. The $K_m$ values of phosphoprotein phosphatase I for phosphorylase a, glycogen synthase D, histone, and casein were lower than the values obtained for phosphoprotein phosphatase II. With glycogen synthase D as substrate, each enzyme gave essentially the same $K_m$ value. Utilizing either enzyme, it was found that activity toward a given substrate was inhibited competitively by each of the alternative substrates. The results suggest that phosphoprotein phosphatases I and II are each active toward all of the substrates tested.

It is now recognized that an important mechanism for metabolic control is through the interconversion of enzymes between active and inactive forms as a result of their phospho-

EXPERIMENTAL PROCEDURES

Materials

Crystalline rabbit muscle phosphorylase b was isolated as described by Fischer and Krebs (14). Liver phosphorylase b was either kindly provided by Dr. Sidney Velick of the University of Utah or was isolated by the procedure of Wolf et al. (15). Skeletal muscle phosphorylase kinase was isolated as described previously (16). Glycogen synthase was isolated by the method of Soderling et al. (17). The catalytic subunit of cyclic AMP-dependent protein kinase was isolated as described previously (18). Catalase, fumarase, myoglobin, bovine serum albumin, histone II-A, dithiothreitol and p-nitrophenyl phosphate were products of Sigma Chemical Co. Horse radish peroxidase and ovalbumin were obtained from Worthington Chemical Co. and vitamin-free casein from Nutritional Biochemicals. Casein was par-

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tially dephosphorylated by heating at 100°C for 10 min at pH 9.5 before use (19). CNBr-activated Sepharose 4B, Sephadex G-75, and DEAE-Sephadex A-50 were products of Pharmacia. Bio-Gel P-300 was obtained from Bio-Rad. Carrier-free [32]P, and UDP-[14C]glucose were obtained from ICN, PCS (phase-combining system) solvent for liquid scintillation was a product of Amersham. All other chemicals were of reagent grade.

**Methods**

**Preparation of [γ-32P]ATP—Carrier-free [32]P,** in diluted HCl for the preparation of labeled ATP was obtained from ICN. [γ-32P]ATP was prepared according to the method of Glynn and Chappell (20) as modified by Walsh et al. (21).

**Preparation of-Sepharose—Histone—**Sepharose-histone was prepared as described by Neavilué and Kassell (22). Fifteen grams of CNBr-activated Sepharose-4B was suspended in 50 ml of cold 0.1 M NaHCO₃ and 50 ml of histone II-A solution (5 mg/ml in 0.1 M NaHCO₃) was then added. The mixture was stirred gently for 24 hours. The gel was washed with 750 ml of 0.1 M NaHCO₃, and 750 ml of 0.5 M NaCl adjusted to pH 9.2, and equilibrated with the desired buffer before use.

**Preparation of [32P]-labeled Substrates—**Rabbit skeletal muscle [32P]phosphorylase a was prepared from phosphorylase b using [γ-32P]ATP, Mg²⁺, and phosphorylase kinase as described by Krebs et al. (23). [γ-32P]-labeled glycogen synthase D was prepared by incubating purified muscle glycogen synthase I (2 mg/ml) with 0.2 mM [γ-32P]ATP, 10 mM Mg²⁺, and 20 µg/ml of purified catalytic subunit of protein kinase in the presence of 50 mM NaF at 30°C. After 2 hours the enzyme was precipitated at 50% saturated ammonium sulfate and the mixture centrifuged. The pellet was dissolved in the minimal volume of 50 mM Tris buffer (pH 7.2) containing 15% sucrose, 15 mM β-mercaptoethanol, and 2 mM EDTA, and passed through a small column of Sephadex G-75 to remove the catalytic subunit of protein kinase. Under these conditions, 1.7 to 2.0 mol of phosphate were incorporated per subunit (M₀ = 90,000) of synthase. [32P]-Histone II-A was prepared essentially according to the method of Meisler and Langm (24). The 20% trichloroacetic acid-insoluble pellet was washed once with water and then dissolved in a small volume of 25 mM Mes buffer, pH 6.9. The dissolved suspension was first dialyzed against the same buffer and then dialyzed against 5 mM Tris, pH 7.0. The [32P]-histone contained 20 nmol of bound [32P]/mg of histone. [32P]-casein was prepared using purified catalytic subunit of muscle protein kinase employing essentially the same procedure as used for making phosphorylase. Phosphorylated casein was precipitated by 5% trichloroacetic acid, washed with water, and suspended in 50 mM Tris buffer, pH 8.0. The dissolved casein was first dialyzed against this buffer and then dialyzed against 5 mM Tris, pH 7.0. The [32P]-casein contained 0.35 mol of bound [32P]/mol of casein (M₀ = 24,000). The [32P]-TN-I prepared using either catalytic subunit of cyclic AMP-dependent protein kinase or phosphorylase kinase as previously described (25), was a gift from Dr. James T. Stull, University of California, San Diego. The amount of bound [32P] per mol of TN-I (M₀ = 24,000) was 1.17 with protein kinase and 2.15 with phosphorylase kinase. All of phosphorylated protein substrates were also prepared using nonlabeled ATP by the above methods. The protein substrates, except phosphorylase a, were stored at -20°C; phosphorylase a was stored at 4°C.

**Enzyme Assays—**Phosphorylase a activity was determined by the method of Cori et al. (26). Glycogen synthase activity was determined by the incorporation of [14C]glucose from UDP-[14C]glucose into glycogen (27). Total synthase (I + D) and synthase I activities were determined in the presence of 10 mM glucose-6-P and 14 mM NaSO₄, respectively.

Phosphoprotein phosphatase activity was determined by the release of [32P] from [32P]-labeled substrates at 37°C. Reaction mixtures for the dephosphorylation of [32P]phosphorylase a contained 50 mM Tris (pH 7.4)/5 mM theophylline/2 mg/ml of phosphorylase a/0.5 mM dithiothreitol/phosphoprotein phosphatase preparation in a total volume of 50 µl. With [32P]-labeled glycogen synthase D as substrate, reaction mixtures contained 50 mM Tris (pH 7.2)/2 mg/ml of bovine serum albumin/0.3 mg/ml of glycogen synthase/0.5 mM dithiothreitol/10 mM MgCl₂/phosphatase in a total volume of 50 µl. Mixtures for dephosphorylation of [32P]-casein contained 50 mM Tris (pH 7.0)/[32P]-casein, 5 mg/ml/10 mM MgCl₂/0.5 mM dithiothreitol/phosphoprotein phosphatase in a total volume of 50 µl. With [32P]-histone as substrate, reaction mixtures were the same as for the dephosphorylation of [32P]-casein except that 0.1 M KCl was also added and [32P]-histone was used in place of casein. Phosphatase activity using phosphorylated TN-I as substrate was determined in a similar reaction mixture as for [32P]phosphorylase a except that [32P]phosphorylase a was replaced by [32P]-TN-I and was supplemented with 0.1 M KCl. In all cases, the reactions were started by the addition of phosphorylase phosphatase to the reaction mixture and terminated by the addition of 0.2 ml of 10% trichloroacetic acid for all substrates except [32P]-histone. In this instance, the reactions were stopped with 0.2 ml of 5% trichloroacetic acid containing 0.25% sodium tungstate. After storing at 4°C for 20 min, all tubes were centrifuged in a microfuge centrifuge for 10 min. Aliquots of the clear supernatants were then mixed with 5 ml of PCS solvent. In all cases the amount of phosphoprotein phosphatase added was such that less than 10% of the substrate was dephosphorylated in a 10-min period of incubation. Reaction rates were linear with time and proportional to the amount of enzyme under conditions in which no more than 20% of the protein substrate was dephosphorylated except with [32P]-casein. With [32P]-casein rates were linear only up to 10% of dephosphorylation.

**Analytical Methods—**Protein was determined by using a microbiur method of Ishihaki and Gill (29) with bovine serum albumin as the standard. In the last step of the enzyme purification procedure, protein was determined by measuring the optical density at 280 nm and arbitrarily assuming an absorbance index of 1.0 for 1.0 mg/ml of solution.

**Determination of Molecular Weights—**Molecular weights of proteins were determined either by the method of Weber and Osborn (30) employing 7.5% sodium dodecyl sulfate gels with phosphorylase b (M₀ = 92,500), bovine serum albumin (M₀ = 68,000), catalase (M₀ = 60,000), fumarase (M₀ = 49,000), ovalbumin (M₀ = 43,000), and myoglobin (M₀ = 17,200) as the marker proteins or by the method of Siegel and Monty (31) from sₓₓ,values and Stokes radii. sₓₓ,values were determined by sucrose density gradient according to the method of Martin and Ames (32) using a Beckman SW 40 rotor run at 39,000 rpm for 20 hours at 4°C. Linear sucrose density gradients from 5 to 15% sucrose in 100 mM Tris/0.5 mM dithiothreitol, pH 7.4, were employed. Approximately 5 µg of purified phosphatase together with marker proteins, muscle phosphorylase b (0.6 mg), peroxidase (0.125 mg), and myoglobin (0.250 mg) were applied to each gradient in a total volume of 100 µl. After the run, each gradient tube was fractionated and assayed for phosphatase activity as well as for the marker proteins. Peroxidase activity was measured by the guaiacol method as described by George (33). Phosphorylase b was identified either by determining the activity in the presence of 5′-AMP or by optical density of 280 nm. Stokes radii were determined by gel filtration using a column of Bio-Gel P-300 (1.5 × 86 cm). Void volume of the column was taken as the volume eluting before blue dextran. Twenty micrograms of purified phosphatase together with marker proteins, muscle phosphorylase b (3 mg), peroxidase (0.625 mg), myoglobin (1.25 mg), and catalase (0.8 mg) were applied to the column in a total volume of 0.3 ml. Column fractions were analyzed for phosphatase activity as well as marker proteins. Phosphatase activity was determined using phosphorylase a as substrate. Catalase activity was determined by the method of Chance and Maely (34). All other markers were assayed as outlined in the sucrose density studies.

**RESULTS**

**Preliminary Characterization of Assay System**

Employing crude liver acid precipitate fraction (see below) as a source of enzyme and [32P]phosphorylase a as the substrate, preliminary experiments were carried out to determine conditions for carrying out valid phosphoprotein phosphatase assays. With higher concentrations of enzyme (>0.2 mg) linear initial reaction rates were found only nearly 60% of the substrate had been dephosphorylated but with lower production.
amounts of extract (<0.1 mg) the period of linearity extended only up to 20% dephosphorylation of the substrate. Reactions were linear for 10 min with all of the protein concentration tested. When the specific activity of phosphatase was calculated using initial rates, a constant value was observed when less than 10 µg of protein was used in assay. With greater amounts of protein, a decrease in specific activity was obtained. These phenomena indicated the probable presence of interfering substances in the enzymes used. In assaying crude fractions, therefore, precautions were always taken to be certain that the amount of enzyme employed was in the range where rates were proportional to enzyme. With the highly purified phosphoprotein phosphatase(s), this problem was not encountered so long as attention was paid to the amount of substrate hydrolyzed (see “Methods”).

Purification of Liver Phosphoprotein Phosphatase(s)

Preparation of Crude Liver Extract—White rabbits were anesthetized deeply with sodium pentobarbital solution and the blood was drained from the jugular veins. The liver was removed immediately, packed in crushed ice, and cut into small pieces that were homogenized in a Waring Blendor at high speed for 1 min in 4 volumes of 20 mM Tris (pH 7.4) containing 1 mM EDTA/0.5 mM dithiothreitol/0.1 mM phenylmethylene sulfonyl fluoride (Buffer A). The homogenate was centrifuged at 8000 × g for 30 min and the supernatant solution was collected after filtering through glass wool and four layers of cheesecloth. This step and all subsequent ones were carried out at 4°.

Preparation of Acid Precipitate Fraction—Crude extracts prepared as described above were adjusted to pH 5.9 by the dropwise addition of 1 N acetic acid. The acidified extract was centrifuged at 8000 × g for 20 min and the supernatant solution discarded. The precipitate was suspended in Buffer A bringing it to approximately one-half the volume of the original extract and then homogenized with the help of a motor-driven Potter-Elvehjem Teflon-glass homogenizer. The mirky solution was then adjusted to pH 7.4 with 2 M Tris base.

High Speed Centrifugation—The acid precipitate fraction was centrifuged at 35,000 rpm for 4 hours using a No. 35 rotor in a Beckman L-265 B centrifuge. The clear supernatant, referred to as the “high speed supernatant fraction” and which contained most of the phosphoprotein phosphatase activity, was decanted and filtered through glass wool and four layers of cheesecloth. The heavy gray glyogen pellet and a loosely packed reddish brown pellet was discarded.

Ion Exchange Chromatography on DEAE-Sephadex—One volume of the high speed supernatant fraction was mixed with 1 volume of DEAE-Sephadex A-50 previously equilibrated in 100 mM Tris (pH 7.4) containing 5% sucrose and 0.5 mM dithiothreitol (Buffer B). After 30 min at 4°, the slurry was filtered through a sintered glass funnel. The filtrate was again mixed with 1 volume of DEAE-Sephadex and filtered through the same funnel. The DEAE-Sephadex was then washed twice using 2 volumes of Buffer B. Finally, it was suspended in the same buffer and introduced into a 2.5-cm diameter column and packed under a pressure of 30 to 40 cm of water. The enzyme was eluted from the column using a linear gradient from 0 to 0.5 M NaCl in Buffer B. Phosphoprotein phosphatase, determined using phosphorylase a as the substrate, was eluted as a broad peak (see Fig. 1, open circles). The major fractions containing the phosphatase activity were pooled and stored at 4° for 24 hours. During the storage, no significant loss in phosphatase activity was observed. This fraction had no detectable phosphorylase or glycerol synthase activity.

Sephadex G-75 Gel Filtration—The pooled DEAE-Sephadex fraction, after storage, was mixed with an equal volume of neutral saturated ammonium sulfate solution. After 30 min at 4°, the mixture was centrifuged at 15,000 × g for 30 min. The pellet was dissolved in Buffer B with gentle stirring. The sample was then applied to a Sephadex G-75 column (5 × 150 cm) previously equilibrated with Buffer B. More than 80% of the enzyme activity was present in the retarded fraction from this column, the rest being eluted in the void volume (Fig. 2). It should be noted, however, that if the pooled DEAE-Sephadex fraction was not stored at 4° for 24 hours prior to this gel filtration step, most of the enzyme was eluted in the void volume.2 For further purification only the retarded fractions were pooled and processed. The enzyme at this stage was very unstable, probably because of high dilution, and it was essential that the next step be carried out as soon as possible.

Sephrose-Histone Chromatography—The pooled G-75 fraction was applied to a Sephrose-histone column (1.5 × 10 cm) that had been equilibrated with Buffer B. The enzyme activity was eluted from this column with a linear gradient from 0 to 0.4 M NaCl in Buffer B. The phosphoprotein phosphatase activity appeared in two distinct peaks (Fig. 3, open circles). Each peak was pooled separately and bovine serum albumin was added to a concentration of 0.2 mg/ml. The fractions were then dialyzed against saturated ammonium sulfate using 1-cm tubing. The precipitate, which appeared in the dialysis tubes within 1 hour, was collected by centrifugation at 40,000 × g for 20 min and

2The higher molecular weight form or aggregate present at this step could also be dissociated by treating with 0.1% Triton X-100 or by freezing and thawing in the presence of 100 mM 2 mercaptoethanol without storage.
Phosphoprotein Phosphatase(s) was dissolved in 1 ml of Buffer B for each fraction. These solutions were then dialyzed against 1 liter of the same buffer for 4 hours. The dialyzed enzyme fractions were divided into 0.1-ml aliquots and stored at -70°C. In order to estimate the amount of enzymic protein present in these fractions, aliquots of the original pooled fractions were dialyzed against saturated ammonium sulfate without the addition of bovine serum albumin. The precipitates that formed were collected by centrifugation, dissolved in a small volume of Buffer B, dialyzed, and their optical density determined. An absorbance index at 280 nm of 1.0 was arbitrarily assumed for 1.0 mg/ml of protein.

Table I shows the extent of purification and yields at different stages in the preparation. It can be seen that there was a great loss of activity in the final step. This occurred primarily in concentrating and dialyzing the pooled fractions. If yields was calculated directly from the individual column fractions, a 3- to 4-fold higher recovery was obtained. Each enzyme fraction was very unstable when stored at -4°C or -70°C in the absence of bovine serum albumin. However, in the presence of bovine serum albumin at a concentration of at least 2 mg/ml the enzyme fractions were stable and could be stored for months at -70°C although each thawing and refreezing resulted in about a 10% loss of activity. After repeated freezing and thawing the enzyme(s) rapidly lost activity. Because of these properties the enzyme(s) were stored in small aliquots as noted above. All studies reported on purified phosphatases in this paper were performed using the pooled Sepharose-histone fractions, which were tentatively designated as phosphoprotein phosphatase I and phosphoprotein phosphatase II in the order of their elution from the Sepharose-histone column. The two fractions of phosphoprotein phosphatase were obtained consistently. Inclusion of phenylmethanesulfonyl fluoride, a proteolytic enzyme inhibitor, in the buffer during initial steps of the fractionation procedure had no effect. Nor were the relative amounts of the two peaks affected by different intervals of storage after the DEAE-Sephadex chromatography step.

**Purity of Phosphoprotein Phosphatase(s)**—Rigorous characterization of the purified phosphoprotein phosphatase fractions was not possible because of the low yields of protein. Nonetheless, the data that were obtained suggest that phosphoprotein phosphatases I and II were essentially homogeneous. On sodium dodecyl sulfate gel electrophoresis, each fraction migrated as a single diffuse band (Fig. 4). Each fraction was eluted as a single symmetrical activity peak when filtered through Bio-Gel P-300 (Fig. 5). In this experiment, the optical density readings were very low but coincided with enzyme activity. Similarly, the purified fraction behaved as single

**TABLE I**

**Purification of phosphoprotein phosphatase(s) from rabbit liver**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>31,000 mg</td>
<td>35,000 units</td>
<td>1.13 units/mg</td>
<td>100</td>
<td>%</td>
</tr>
<tr>
<td>Crude extract</td>
<td>19,700 mg</td>
<td>29,500 units</td>
<td>1.50 units/mg</td>
<td>84</td>
<td>%</td>
</tr>
<tr>
<td>Acid precipitate</td>
<td>12,400 mg</td>
<td>19,600 units</td>
<td>1.58 units/mg</td>
<td>56</td>
<td>%</td>
</tr>
<tr>
<td>High speed supernatant</td>
<td>12,400 mg</td>
<td>19,600 units</td>
<td>1.58 units/mg</td>
<td>56</td>
<td>%</td>
</tr>
<tr>
<td>DEAE-Sephadex eluate</td>
<td>3,200 mg</td>
<td>15,300 units</td>
<td>4.85 units/mg</td>
<td>44</td>
<td>%</td>
</tr>
<tr>
<td>Sepharose-G-75 eluate</td>
<td>11.5 mg</td>
<td>5,600 units</td>
<td>487 units/mg</td>
<td>16</td>
<td>%</td>
</tr>
<tr>
<td>Sepharose-histone eluate</td>
<td>0.43 mg</td>
<td>217 units</td>
<td>504 units/mg</td>
<td>451</td>
<td>%</td>
</tr>
<tr>
<td>Peak I</td>
<td>0.43 mg</td>
<td>217 units</td>
<td>504 units/mg</td>
<td>451</td>
<td>%</td>
</tr>
<tr>
<td>Peak II</td>
<td>0.12 mg</td>
<td>615 units</td>
<td>5,130 units/mg</td>
<td>4,610</td>
<td>%</td>
</tr>
</tbody>
</table>
Phosphoprotein Phosphatases I and II

Electrophoresis was carried out using 7.5\% gels with 50 mM phosphate buffer, pH 7.0, and 0.1\% sodium dodecyl sulfate. A, 10 \( \mu \)g of phosphoprotein phosphatase I; B, 10 \( \mu \)g of phosphoprotein phosphatase II; and C, 10 \( \mu \)g of each enzyme. Proteins were stained with 0.25\% Coomassie blue.

Molecular Weights—By the sodium dodecyl sulfate gel electrophoresis method the molecular weight of phosphoprotein phosphatase I was found to be 30,500 and that of phosphoprotein phosphatase II, 34,000. The sedimentation constant \( (s_{20,\text{w}}) \) for either enzyme, as determined by sucrose density gradient centrifugation, was 3.40. The sedimentation pattern for phosphoprotein phosphatase II is shown in Fig. 6A. The Stokes radius for each enzyme, as determined by gel filtration was 24 Å (Fig. 6B). From these data a molecular weight of 34,500 was calculated (31).

Substrate Specificities and pH Optima—Purified phosphoprotein phosphatase I and II were both able to catalyze the dephosphorylation of \( ^{32}P \)-labeled glycogen synthase D, \( ^{32}P \)-histone, and \( ^{32}P \)-casein in addition to acting on \( ^{32}P \) phosphorylase a. Phosphoprotein phosphatase II was also found to catalyze the dephosphorylation of \( ^{32}P \)-TN-I and \( ^{32}P \)-activated rabbit skeletal muscle phosphorylase kinase.\(^3\) Phosphoprotein phosphatase I was not tested with the latter two substrates. Other low molecular weight phosphate esters such as p-nitrophenyl phosphate, \( \beta \)-glycerophosphate, and inorganic pyrophosphate did not serve as substrates for either enzyme.

Valid enzyme assay conditions (described under "Methods") were established using the various substrates for phosphoprotein phosphatases I and II and their pH optima were then determined (Fig. 7). With \( ^{32}P \) phosphorylase a as the substrate, the optimum for either enzyme was between 7.2 and 8.4 (Fig. 7A). With \( ^{32}P \) glycogen synthase D as the substrate or with \( ^{32}P \)-casein, the optimal pH values were 7.2 and 6.8, respectively, for either enzyme (Fig. 7, B and C). With \( ^{32}P \)-histone, the optimal pH was between 5.6 and 6.8 for both enzymes (Fig. 7D). No pH optima were determined using \( ^{32}P \)-TN-I or \( ^{32}P \)-activated phosphorylase kinase.

Relative Phosphoprotein Phosphatase Activity toward Different Substrates at Stages in Preparation—In order to obtain information as to whether the two phosphoprotein phosphatases constituted the major enzymes in liver that would dephosphorylate the various substrates, and to support the concept that enzymes of wide specificity had in fact been isolated, fractions obtained at all stages of purification were examined for their activities with respect to four substrates. As

\(^3\) \( ^{32}P \)-activated phosphorylase kinase was prepared by autophosphorylation according to the method of Walsh et al. (21). This preparation had 1.5 mol of \( ^{32}P \) bound/mol of phosphorylase kinase \( (M = 1.3 \times 10^9) \).
shown in Table II, the relative activities toward \(^{32}P\)phosphorylase \(a\), \(^{32}P\)-labeled glycogen synthase \(D\), \(^{32}P\)-histone, and \(^{32}P\)-casein were relatively constant in all of the fractions of purification up to the last step. The relative activities with the different substrates changed for phosphoprotein phosphatase II. The reason for this is not clear and the data are not easy to interpret because of the great loss of total activity in the last step of purification. One possibility would be that an inhibitory substance acting differently with different substrates has been removed at this point. Relative activities toward the different substrates were also measured in the fractions that were discarded during the preparation. These were not appreciably different from those of the retained fractions. The activity profile with all four substrates was also determined for the DEAE-Sephadex chromatographic step (Fig. 1) and in the Sepharose-histone chromatographic step (Fig. 3). The different activity profiles more or less coincided in all cases, although a small peak with \(^{32}P\)phosphorylase \(a\) and \(^{32}P\)-casein on the ascending part and a small extra peak with \(^{32}P\)-histone and \(^{32}P\)-casein was present on the trailing part of the DEAE-Sephadex pattern. However, when the fractions of ascending part (Fractions 9 to 14) were pooled and rechromatographed on a second DEAE-Sephadex column, no evidence for a specific phosphorylase phosphatase or casein phosphatase was observed.

Kinetics Studies—The apparent \(K_m\) values of the liver phosphoprotein phosphatases for various substrates are shown in Table III. With phosphoprotein phosphatase I all of the \(K_m\) values were somewhat lower than those with phosphoprotein phosphatase II for all the substrates except \(^{32}P\)-labeled glycogen synthase \(D\). No significant difference was observed in the \(K_m\) values for this last substrate. \(K_m\) values of the liver phosphoprotein phosphatases for phosphorylase \(a\) and glycogen synthase \(D\) were within the range reported by others for skeletal muscle and heart phosphoprotein phosphatases (3, 4, 7). The \(K_m\) values of the liver phosphoprotein phosphatases for \(^{32}P\)-histone were similar to those reported for heart phosphoprotein phosphatase (7) when expressed in milligrams per ml of substrate but were 2- to 5-fold higher when expressed in terms of the molarity of \(^{32}P\)-substrate. It should be pointed out however, that the phosphorylated histone used in the present study contained about 5 times as much bound \(^{32}P\) as that used by Nakai and Thomas (7). The \(K_m\) value of liver phosphoprotein phosphatase II for \(^{32}P\)-TN-I was also similar to that reported for skeletal muscle phosphorylase phosphatase (4).

Further evidence that the two isolated phosphoprotein phosphatases represent entities responsible for the dephosphorylation of the various substrates was obtained by showing that the phosphorylated histone used in the present study contained about 5 times as much bound \(^{32}P\) as that used by Nakai and Thomas (7). The \(K_m\) value of liver phosphoprotein phosphatase II for \(^{32}P\)-TN-I was also similar to that reported for skeletal muscle phosphorylase phosphatase (4).

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Further evidence that the two isolated phosphoprotein phosphatases represent entities responsible for the dephosphorylation of the various substrates was obtained by showing that the phosphorylated histone used in the present study contained about 5 times as much bound \(^{32}P\) as that used by Nakai and Thomas (7). The \(K_m\) value of liver phosphoprotein phosphatase II for \(^{32}P\)-TN-I was also similar to that reported for skeletal muscle phosphorylase phosphatase (4).
that one substrate acts as an inhibitor of phosphatase action on another substrate. For example, the inhibition of dephosphorylation of \[^{32}P\]phosphorylase a by nonradioactive phosphorylated casein and phosphorylated histone is shown in Fig. 8 for both phosphoprotein phosphatases. In the first instance, simple competitive inhibition was found in that casein increased the $K_m$ values for phosphorylase a without any change in $V_{\text{max}}$ (Fig. 8, A and B). With phosphorylated histone as an inhibitor, simple competitive inhibition was not observed (Fig. 8, A and B) probably due to precipitation of negatively charged phosphorylase a by the positively charged histone. Nonetheless, no change in $V_{\text{max}}$ was observed. Nonradioactive phosphorylase a and phosphorylated casein served as competitive inhibitors for the dephosphorylation of \[^{32}P\]labeled glycogen synthase (Fig. 8A) and \[^{32}P\]-histone (Fig. 8B) by phosphoprotein phosphatase II. Nonradioactive phosphorylase a and phosphorylated histone also inhibited the dephosphorylation of \[^{32}P\]casein (Fig. 8C) by phosphoprotein phosphatase II. Linear inhibition kinetics were observed with phosphorylase a, but with phosphorylated histone as the inhibitor, an aberration in normal kinetics was again observed. Similar results were obtained when phosphoprotein phosphatase I was used in experiments otherwise identical with those of Fig. 9, A to C. Finally, the inhibition of dephosphorylation of radioactive \[^{32}P\]-TN-I, phosphorylated by phosphorylase kinase, by phosphorylase a was also found to be competitive (Fig. 8D). Similar results were obtained when \[^{32}P\]-TN-I phosphorylated by cyclic AMP-dependent protein kinase, was used for such a study (not illustrated).

**Heat Inactivation**—Although each of the phosphoprotein phosphatases showed a single band on sodium dodecyl sulfate gels, the bands were broad and could represent more than one species of enzyme in each case. In order to obtain further information on this point each enzyme was incubated at 55° and 65° to determine whether inactivation of phosphatase activities toward the different substrates fell in parallel with all four substrates as would be expected if all activities resided in single proteins. As shown in Fig. 10, this result was obtained for both phosphatases.

**Identification of Enzymes as Phosphoprotein Phosphatases**

Because all the assays used in the study involved the determination of trichloroacetic acid-soluble \[^{32}P\], it was important to determine whether all of the radioactivity detected was \[^{32}P\], and not acid-soluble \[^{32}P\]-labeled peptides released by proteases. Therefore, with \[^{32}P\]phosphorylase a and \[^{32}P\]-labeled glycogen synthase D as substrates dephosphorylation and activity changes were measured. As shown in Fig. 11, both phosphoprotein phosphatases catalyzed dephosphorylation as well as the anticipated changes in activity. The dephosphorylated substrates could then be rephosphorylated in the presence of ATP, Mg\(^2+\), and NaF using phosphorylase kinase for phosphorylase and protein kinase for glycogen synthase. The rephosphorylation reactions were again associated with changes in enzyme activity.

**DISCUSSION**

The preparation of liver phosphorylase phosphatase described in this paper is similar to that of Brandt et al. (10) in that it contains acid precipitation, ion exchange chromatography, and gel filtration steps. Other features of the preparation are unique, however, and it is noteworthy that the present method leads to the separation of two peaks of activity in the final step, whereas the method of Brandt et al. (10) led to the isolation of a single species of phosphoprotein phosphatase. Another difference in the two preparations is that in this study only a 5,000-fold enrichment of activity was required to achieve an apparently homogeneous pure enzyme, a 25,000-fold purification was necessary by their method. It is possible, however, that difficulties encountered in determining enzyme activity in crude fractions, or difficulties in the estimation of the protein...
was purified to apparent homogeneity and was shown to catalyze the dephosphorylation of phosphorylase a, glycogen synthase D, activated phosphorylase kinase, phosphorylated histone, and phosphorylated casein. Collectively, these and other observations suggest a lack of specificity, with respect to this type of catalyzed the dephosphorylation of phosphorylase a, glycogen synthase D, and phosphorylated histone as substrates (Fig. 7). All of the substrates behaved as competitive inhibitors for the phosphatase fractions isolated in this study. The phosphorylated substrates used in the present study were made using two different protein kinases. Thus, \(^{32P}\)TN-I and \(^{32P}\)-labeled glycogen synthase D were dephosphorylated using phosphoprotein phosphatase I (A and C) and phosphoprotein phosphatase II (B and D) and rephosphorylated with ATP, Mg\(^{2+}\), and phosphorylase kinase (for phosphorylase) or protein kinase (for glycogen synthase). The time for addition of the kinase and MgATP is indicated by the arrows. Phosphorylase a and glycogen synthase I and D + I activities (●) were determined as described under “Methods.” Phosphate content (○) of these two enzymes was determined by the method of Reimann et al. (10).

The recovery of two peaks of phosphoprotein phosphatase activity in the final step of the preparation was a consistent finding, but it is not known for certain whether the two fractions should be looked upon as representing different enzymes. Several observations suggest that it is so. Phosphoprotein phosphatase II was 5- to 10-fold more active than phosphoprotein phosphatase I with phosphorylase a, glycogen synthase D, and phosphorylated histone as substrates (Fig. 7). With casein, the activity of phosphoprotein phosphatase II was 50 to 100% higher than that of phosphoprotein phosphatase I. The \(K_m\) values of phosphoprotein phosphatase II for all substrates were lower than those of phosphoprotein phosphatase I (Table III). These different properties of the phosphatases, together with their different mobilities on sodium dodecyl sulfate gels, suggest that they are distinct proteins. However, it is still possible that phosphoprotein phosphatase I might be a proteolytic product of phosphoprotein phosphatase II, since no absolute method was available for ruling this out.

It has been known for several years that muscle glycogen synthase phosphatase, in addition to acting on glycogen synthase \(D\) can also catalyze the dephosphorylation of histone (3) and active phosphorylase kinase (5). Recently, it was observed that muscle phosphorylase phosphatase also catalyzes the dephosphorylation of TN-I (4). In 1974, Nakai and Thomas (7) reported that heart glycogen synthase phosphatase catalyzed the dephosphorylation of phosphorylase a, active phosphorylase kinase, phosphorylated histone, and phosphorylated casein. Collectively, these and other observations suggest a lack of specificity, with respect to this type of enzyme. It should be pointed out, however, that in all of these studies only partially purified enzymes were used and the possibility that more than one enzyme was catalyzing the dephosphorylation of the several substrates could not be ruled out. In the present study, liver phosphorylase phosphatase(s) was purified to apparent homogeneity and was shown to catalyze the dephosphorylation of phosphorylase a, glycogen synthase \(D\), activated phosphorylase kinase, phosphorylated histone, phosphorylated casein, and phosphorylated TN-I. The relative activities toward glycogen synthase \(D\), phosphorylase a, phosphorylated histone, and phosphorylated casein remained constant throughout the purification except in the very last step in which two fractions were obtained (Table II). All of the substrates behaved as competitive inhibitors for the dephosphorylation of other substrates (Figs. 8 and 9). All of these properties strongly support the concept that the dephosphorylation of all of the substrates is catalyzed by each of the phosphatase fractions isolated in this study.

The phosphorylated substrates used in the present study were made using two different protein kinases. Thus, \(^{32P}\)phosphorylase a was made using phosphorylase kinase and \(^{32P}\)-labeled glycogen synthase \(D\) using the cyclic AMP-dependent protein kinase, the enzymes generally accepted as catalyzing the in vivo phosphorylation of these enzymes. \(^{32P}\)-TN-I was made utilizing either phosphorylase kinase or the cyclic AMP-dependent protein kinase as the catalyst for the phosphorylation reaction. It is clear that the same phosphoprotein phosphatase(s) can dephosphorylate phosphorylated substrates irrespective of the type of protein kinase used in the phosphorylation reaction.

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