Rabbit Muscle Phosphorylase Phosphatase. 1. Purification and Chemical Properties†

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ABSTRACT: Rabbit muscle phosphorylase phosphatase (EC 3.1.3.17) has been purified 4- to 6000-fold by isolation of a glycogen–protein complex to which it is bound, disruption of the complex with α-amylase, and then affinity chromatography on a Sepharose–poly(L-lysine) column from which it is eluted with 6 M urea. The purified enzyme has a specific activity in excess of 1 μM of phosphorylase *a* converted min^{-1} mg^{-1} of protein; assuming a minimum mol wt of ca. 33 000 for the phosphatase, this corresponds to a turnover number of 30 mol of phosphorylase subunit coverted min^{-1} mol^{-1} of phosphatase. The purified phosphatase is free from phosphorylase *b* or *a*, phosphorylase kinase, protein kinase, or glycogen synthase activities. While it readily dephosphorylates phosphorylase *a* and troponin I, it is essentially inactive, at least in the absence of Mn^{2+} ions, on phosphorylase kinase and glycogen synthase, and totally inactive on low molecular weight esters such as *p*-nitrophenyl phosphate, phosphoserine, and various phosphoseryl peptides. It appears to be free of bound phosphate, carbohydrates, lipids, and nucleic acids; it has an isoelectric point around pH 5 and an optimum pH of activity of ca. 7.8; it displays an absolute requirement of SH compounds and is quantitatively, and sometimes irreversibly, inactivated by disulfides. The phosphatase is resistant to trypsin and chymotrypsin and to 8 M urea; it is stable in low concentrations of certain solvents or detergents. In contrast, it is unstable at pHs below 6.0. It is strongly inhibited by poly(L-lysine) at concentrations as low as 1 μM. While the active enzyme has a minimum mol wt of ca. 33 000, it displays a strong tendency to aggregate with loss of enzymatic activity, as observed in gel filtration, sucrose density gradients sedimentation, and sedimentation velocity or equilibrium experiments.

Skeletal muscle glycogen phosphorylase (EC 2.4.1.1) exists in two interconvertible forms, namely, active phosphorylase *a* and phosphorylase *b*, inactive unless AMP is present. Conversion of phosphorylase *b* to *a* requires Mg^{2+}-ATP and active phosphorylase kinase and is accompanied by incorporation of 1 molecule of phosphate per enzyme subunit; dephosphorylation is catalyzed by phosphorylase phosphatase (for a review see Fischer et al., 1971).

Various protein kinases, and rabbit skeletal muscle phosphorylase kinase in particular, have been extensively investigated with respect to both their structure and mode of regulation (for reviews see Hayakawa et al. (1973) and Cohen (1974)). By contrast, little information has been gained on protein phosphatases that carry out the reverse reactions. Among these, one of the first to be described is skeletal muscle phosphorylase phosphatase; however, some of its properties are, despite two decades of investigation, still little understood. While earlier work carried out both on intact animals and on in vitro systems indicated that control of muscle phosphorylase activity occurred preferentially through kinase activation rather than phosphatase inhibition (for reviews, see Danforth et al., 1962; Fischer et al., 1971), regulation of phosphatase activity in a glycogen particulate complex has been suggested (Haschke et al., 1970). Recently, evidence was provided that control of phosphatase activity may result from interaction of both the liver and muscle enzymes with regulatory proteins (Brandt et al., 1974, 1975; Huang and Glinsmann, 1975, 1976; Cohen et al., 1977).

Studies on phosphorylase phosphatase have been hampered greatly by the lack of a satisfactory purification procedure. This difficulty is due in part to the fact that rabbit skeletal muscle contains little phosphatase (at least 300 times less than phosphorylase) and purified preparations of the enzyme have a strong tendency to aggregate, and thus appear inhomogeneous on polyacrylamide gel electrophoresis (Hurd et al., 1966; Hurd, 1967; England et al., 1972). Brandt et al. (1975) described the isolation in homogeneous form of a phosphorylase phosphatase from rabbit liver similar in molecular weight and specific activity to the one described here. Antoniw et al. (1977) reported that a single enzyme termed "protein phosphatase-III" catalyzed the dephosphorylation of phosphorylase *a*, the β subunit of phosphorylase kinase, and the β_1 and β_2 forms of glycogen synthase.

The present article describes a convenient purification procedure for phosphorylase phosphatase yielding reasonable amounts of protein after a 4- to 6000-fold enrichment. Some of the properties of the purified enzyme are discussed. Preliminary accounts of this work were already presented (Gratecos and Fischer, 1973; Gratecos et al., 1974). In an accompanying paper, some kinetic and regulatory features of the enzyme are reported (Detwiler et al., 1977).

Materials and Methods

Phosphorylase *b*, three times crystallized, was prepared according to Fischer and Krebs (1958) and purified phosphorylase kinase was prepared according to Delange et al. (1968). Glycogen synthase phosphorylated at both of its sites of covalent modification was kindly provided by Dr. Tom Soderling, Vanderbilt University. Phosphorylase *a* was pre-

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pared according to Krebs et al. (1964) with slight modifications: after completion of the *b* to *a* conversion, the solution was acidified to pH 7.0 and the enzyme allowed to crystallize overnight at 0 °C. The crystals were centrifuged and redissolved at 30 °C in 0.05 M sodium glycerophosphate–0.05 M 2-mercaptoethanol buffer (pH 7.0) (with the help of a few drops of 5 M NaCl, if necessary), and the clear solution was dialyzed against the same buffer in the cold. Phosphorylase *a* crystallized during dialysis and the procedure was repeated once more. The third crystals were stored at 4 °C. Phosphorylase *a* labeled with ^{32}P was prepared by the same procedure (specific radioactivity approximately 4×10^5 cpm/mg) using [γ-^{32}P]ATP obtained by the method of Glynn and Chappell (1964).

Two buffer solutions were used, namely, buffer A consisting of 0.05 M Tris[1] and 0.05 M 2-mercaptoethanol (pH 7.5) and buffer B, made up of 0.05 M Tris, 0.5 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride (pH 7.2). Sepharose 4B–poly(L-lysine) was prepared according to Nevaldine and Kassell (1971). Fifty grams of CNBr-activated gel (Cuatrecasas et al., 1968) was suspended in 50 mL of cold 0.1 M $NaHCO_3$. A polylysine solution (50 mL, 5 mg/mL poly(L-lysine)·HBr, type VA, mol wt 20 000–30 000 from Sigma Chemical Corp.) in 0.1 M $NaHCO_3$ was added and the mixture was stirred at 4 °C for 24 h. The gel was washed with 1 L of 0.1 M $NaHCO_3$, then 1 L of 0.5 M NaCl adjusted to pH 9.2, and finally equilibrated with buffer B.

Sephadex G-100 or G-150 and Sepharose 4B were from Pharmacia Fine Chemicals Inc., DEAE-cellulose DE-52 was from Whatman, ampholytes for electrofocusing were from LKB, and dithiothreitol and α-amylase (*Bacillus subtilis* type 11A) were from Sigma. Urea solutions (8 M) were freshly prepared and freed from contaminating ions by adding 10 g/L of a mixed-bed resin (Amberlite MB-3 from Mallinckrodt); the suspension was stirred for 1–2 h and kept no longer than 24 h in the cold; it was filtered before use.

Enzyme Assay. Phosphorylase was assayed by the method of Hedrick and Fischer (1965) in the direction of glycogen synthesis. Phosphorylase phosphatase was assayed by Method I of Haschke et al. (1970) except that phosphatase dilutions were carried out in buffer A containing 2 mg/mL bovine serum albumin. The assay mixture usually contained 3 mg/mL phosphorylase *a* with a specific radioactivity adjusted to 5×10^4 cpm/mg (5×10^3 cpm/nmol of enzyme subunit). A unit of phosphatase activity is defined as that amount of enzyme liberating 1 nmol of $^{32}P_i$ per min at 30 °C under the assay conditions.

Polyacrylamide gel electrophoresis was performed in 7.5% acrylamide–0.11% *N,N'*-methylenebisacrylamide gels using the continuous Tris–glycine buffer system (pH 8.3) of Ornstein (1964) and Davis (1964), at a constant current of 2.3 mA/tube for 2–3 h; gels were stained with 0.25% Coomassie brilliant blue (Weber and Osborn, 1969). Identification of enzymatic activity was accomplished by incubating unstained gel slices with 0.1 mL of phosphorylase *a* solution for 1 h at 30 °C and then measuring the $^{32}P_i$ released.

Protein was determined by absorbancy at 280 nm using an arbitrary value $A_{1cm}^{1\%}$ of 10. Bound phosphate was analyzed by the method of Schaffer et al. (1953) after hydrolysis of 0.6 mg of protein in 60% perchloric acid at 190–200 °C for 2 h, as described by Nakamura (1952).

Sucrose gradient centrifugations were performed in linear 5–20% (w/v) gradients as described by Martin and Ames (1961) at 50 000 rpm for 12 h at 1 °C in a Beckman centrifuge equipped with an SW 50 rotor. Labeled protein markers (see below) were counted in a Tri-Carb Packard scintillation counter. Fractions from urea gradients were counted in a toluene-based scintillation fluid containing 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter of toluene.

^{14}C-Labeled Marker Proteins. Ovalbumin and γ-globulin were methylated with ^{14}C-labeled formaldehyde (specific radioactivity 10 nCi/nmol from Schwarz/Mann) followed by $NaBH_4$ reduction as described by Means and Feeney (1968). Specific radioactivities of 2×10^4 cpm/mg of protein were obtained; sedimentation coefficients were taken as 3.6 S for ovalbumin and 7.0 S for γ-globulin (Sober, 1970).

Results

Purification of Phosphorylase Phosphatase. The first three steps of the purification were carried out on 6 kg of rabbit skeletal muscle according to procedures describing the isolation of a protein–glycogen complex to which 30 to 50% of the initial phosphatase activity is bound. The glycogen particles also contain phosphorylase, phosphorylase kinase, protein kinase, glycogen synthase, and several other glycolytic enzymes, as well as a strong ATPase activity associated with elements of the sarcoplasmic reticulum (Krebs et al., 1964; Hurd, 1967; Meyer et al., 1970; Haschke et al., 1970). Phenylmethanesulfonyl fluoride was included at every step (even though the phosphatase itself is resistant to various proteolytic enzymes) to avoid the degradation of contaminating proteins and subsequent alteration in the behavior of the enzyme.

In order to disrupt the glycogen particles and solubilize the enzymes present, the pellet was suspended in 30 mL of buffer A per kg of muscle and incubated with protease-free crystalline α-amylase (10 μg/mL) at room temperature until digestion of glycogen was completed (usually 30 min) as monitored by precipitation with 10% trichloroacetic acid and 50% ethanol. The clarified suspension was diluted with 0.5 vol of buffer A, containing 1 mM EDTA and 0.1 M NaCl, and centrifuged at 80 000*g* for 90 min; the α-amylase treatment often resulted in a doubling of phosphatase activity.

Further purification of phosphorylase phosphatase relied on two properties of the enzyme: first, it is inhibited by low concentrations of poly(L-lysine) which suggested that it might bind rather specifically to the basic polypeptide; second, it is stable in 8 M urea. The amylase-treated solution was chromatographed on a Sepharose–polylysine column (see Materials and Methods). Phosphorylase kinase which is not retarded emerged at the void volume followed by phosphorylase and most other enzymes, while phosphorylase phosphatase was quantitatively retained. After washing out contaminating proteins, the phosphatase was eluted with 6 M urea (Figure 1). The step provides for a tenfold purification; the overall enrichment of the crude extract is ca. 500-fold.

The active fractions were collected, dialyzed for 1 h against buffer B at pH 7.5 to lower the urea concentration, and precipitated with 35% ammonium sulfate. The pellet was dialyzed overnight against the same buffer during which time a large precipitate of denatured protein developed; upon centrifugation, the phosphatase remained in the clear supernatant affording another tenfold purification. At this stage, the enzyme can be stored frozen for at least 1 month without loss of activity.

The phosphatase could also be eluted from the Sepharose–polylysine column with 1 M NaCl. In this case, no precipitation occurred upon dialysis and, contrary to the urea-treated

[1] Abbreviations used are: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; Nbs_2, 5,5'-dithiobis(2-nitrobenzoic acid).

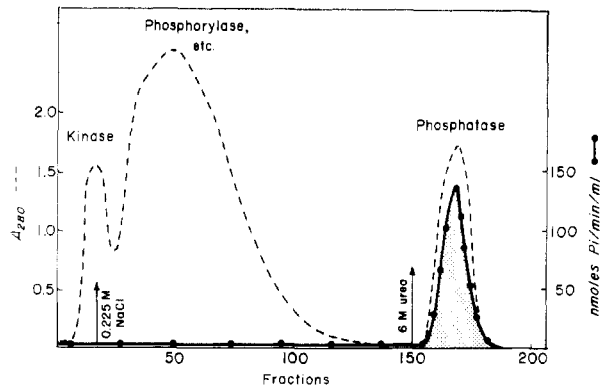

FIGURE 1: Elution profile of phosphorylase phosphatase from a 4 × 10 cm polylysine-Sepharose 4B column. The amylase supernatant solution (130 mL) contained ca. 25 mg of protein/mL, 60 000 phosphatase units total, in buffer A. The chromatography was run in buffer B. Elution was carried out first with 1.5 L of buffer B containing 0.225 M NaCl and 1 mM lysine and then in 1 L of buffer B containing 6 M urea. The flow rate was 150 mL/h; 12-mL fractions were collected.

TABLE I: Purification of Rabbit Muscle Phosphorylase Phosphatase from 6 kg of Fresh Rabbit Skeletal Muscle.[a]

Fraction	Total protein (g)[b]	Total units × 10^{-3}	Sp act. (nmol of P_i min^{-1} mg^{-1})	Yield (%)	Purification
Crude extract	453	114	0.25	100	1
pH 6.1 precipitate[c]	45	63	1.4	53	6
80 000*g* pellet[c]	18	50	3	46	11
Supernatant after α-amylase treatment	5	57	12	47	46
Sepharose-polylysine fraction	0.4	40	100	34	400
Removal of urea-denatured proteins	0.03	32	1000[d]	27	4000

[a] The values listed represent the average of nine preparations. [b] Determined by absorbancy at 280 nm assuming $A_{1cm}^{1\%} = 10$. [c] The first steps are described by Meyer et al. (1970). [d] The specific activities varied from 800 to 1500 nmol of P_i released min^{-1} mg^{-1}.

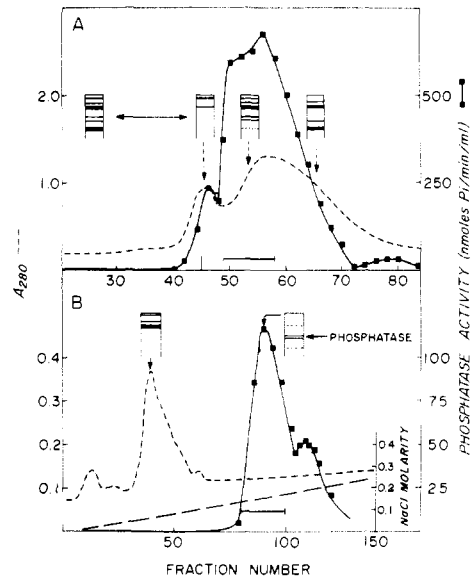

FIGURE 2: Further purification of phosphorylase phosphatase on Sephadex and DEAE-cellulose. (A) A sample obtained from a Sepharose-polylysine-urea column (25.5 mg; 25 000 units) was concentrated by precipitation with 60% ammonium sulfate and chromatographed on a Sephadex G-150 column (1.5 × 90 cm) equilibrated with buffer B containing 0.1 M NaCl (pH 7.5); 1-mL fractions were collected at a flow rate of 10 mL/h. Polyacrylamide gel electrophoresis patterns of the original sample (left gel) and of fractions taken along the chromatogram (indicated by vertical arrows) are schematized on the diagram. The horizontal arrow indicates the band corresponding to phosphatase activity determined on slices of the gel as described under Materials and Methods. (B) The active fractions pooled as indicated by the horizontal bar in A (12 mg; 11 500 units) were dialyzed against buffer B and chromatographed on a DEAE-cellulose column (1 × 10 cm) equilibrated in buffer B. A linear (0-0.35 M) NaCl gradient (150 mL/chamber) was initiated after the sample had penetrated the gel; 1.0-mL fractions were collected. Gel electrophoreses of the material under both peaks are shown. Phosphatase was pooled as indicated by the horizontal bar and concentrated on a 1-mL DEAE-cellulose column followed by elution with 0.4 M NaCl. After dialysis of the active fraction, 0.7 mg of protein containing 880 units was obtained. All gel patterns illustrated were obtained in the absence of dodecyl sulfate.

preparation, the enzyme emerged in an aggregated form in which it was particularly unstable and the solution eventually precipitated when kept in the cold or frozen.

The purification steps are summarized in Table I. Although the extent of purification of the muscle phosphatase is high compared to that of earlier preparations (e.g., Hurd, 1967), the purified enzyme still did not appear to be homogeneous as judged by polyacrylamide gel electrophoresis.

Many attempts were made to further purify the preparation; all approaches led to poor recoveries with essentially no increase in specific activity. For example, when samples obtained from Sepharose-polylysine-urea columns were chromatographed on Sephadex G-150, typical elution profiles as illustrated in Figure 2A were observed. Phosphatase activity emerged in several peaks corresponding to molecular weights ranging from 33 000 to 120 000 or more, emphasizing the tendency the purified enzyme has to aggregate; analysis by polyacrylamide gel electrophoresis showed that some of the bands originally present had been separated. When the material obtained by pooling the active fractions was further chromatographed on DEAE-cellulose, once more the enzyme activity emerged in two peaks displaying essentially no absorbance at 280 nm; they were preceded, however, by a large inactive fraction containing most of the ultraviolet (UV) absorbing material (Figure 2B).

Since lyophilization always led to considerable inactivation, the very dilute enzyme solutions obtained were concentrated by adsorption-desorption from 1-mL columns of DEAE-cellulose which preserved most of the activity. Polyacrylamide gel electrophoresis of the pooled purified fractions showed two major bands, both active. But again, the specific activity of the sample was no higher than that of the original solution obtained after polylysine-urea treatment.

Purified phosphatase was subjected to isoelectric focusing using ampholyte carriers from pH 3 to 10 (Wrigley, 1968); no protein band could be detected by precipitation with 5% trichloroacetic acid but activity measurements carried out on gel slices yielded a peak of activity corresponding to an isoelectric point of ca. 5.

Enzymatic Specificity and Purity. The phosphatase obtained after polylysine-urea treatment is essentially free of other enzymatic activities; no phosphorylase *b* or *a*, phosphorylase kinase, protein kinase, or glycogen synthase activity could be detected. The enzyme readily attacks phosphorylase *a* and various phosphopeptides derived from phosphorylase *a* (Nolan et al., 1964) and the phosphorylated I and T subunits of troponin (England et al., 1972). It does not attack low molecular weight esters such as *p*-nitrophenyl phosphate, seryl phosphate, or various phosphoseryl peptides (Graves et al., 1960) nor does it dephosphorylate a number of acidic phosphoproteins such as phosvitin or casein. Recently, Antoniw et

TABLE II: Stability of Phosphorylase Phosphatase.

Enzyme[a] and additives	Time of incubation	% residual act.
Control at pH 5.0	30 min	10
Control at 60 °C	15 min	50
Ethanol, 25% at −5 °C	3 h	100
Deoxycholate, 0.5%[b]	24 h	100
Tween 20, 40, and 80, 0.1%	24 h	100
Triton X-100, 0.5%[b]	24 h	100
Trypsin or chymotrypsin, 1% w/w at 30 °C	3 h	100
Pronase, 10% w/w at 30 °C	3 h	0
Dodecyl sulfate, 1%, or $CetMe_3NBr$, 0.05%,[c] or guanidine·HCl, 1 M	15 min	0
0.3 M perchloric acid, 0.3 M, or trichloroacetic acid, 10%	15 min	0

[a] Unless otherwise stated, all reactions were carried out with 2 mg of enzyme/mL in buffer A at pH 7.5, 4 °C. At 0.1 mg/mL in glass tubes, the enzyme loses 80% activity in 60 min. [b] In the presence of 0.5 mM dithiothreitol. [c] $CetMe_3NBr$, cetyltrimethylammonium bromide.

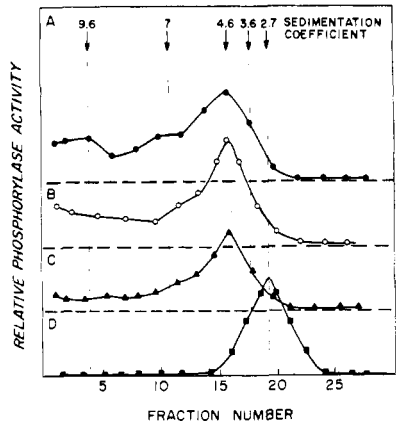

FIGURE 3: Effects of dilution and 6 M urea on the sedimentation properties of phosphorylase phosphatase. Samples obtained after Sepharose-polylysine chromatography were layered on sucrose gradients prepared in buffer B, in the presence or absence of 6 M urea, and centrifugation was carried out at 1 °C for 12 h. ^{14}C-Labeled ovalbumin and γ-globulin were included as markers: (A) (●) undiluted sample (20 mg/mL); (B) (○) 1:5 dilution (4 mg/mL); (C) (△) 1:40 dilution (0.5 mg/mL); (D) (□) undiluted sample in 6 M urea. Sedimentation coefficients are indicated by arrows.

al. (1977) reported that the dephosphorylation of phosphorylase *a*, the β subunit of phosphorylase kinase, and glycogen synthase β_1 and β_2 was catalyzed by a single protein fraction. The purified material described herein was essentially inactive on phosphorylase kinase phosphorylated on both its α and β subunits and on doubly phosphorylated glycogen synthase (Soderling, 1976) at least in the absence of Mn^{2+} ions.

Chemical Properties. Analyses for nucleic acids, carbohydrates by the phenol–sulfonic acid test (Dubois et al., 1956) or the anthrone reagent (Spiro, 1966), and lipids and bound phosphate (Schaffer et al., 1953) were negative under conditions where the presence of 1 mol of any of those compounds per mol of enzyme would have given a clear positive response. No amino sugar could be detected on the amino acid analyzer.

No requirement for divalent cations for activity or stability could be demonstrated; at most, the enzyme showed a 20% inhibition by 10 mM Ca^{2+} or Mg^{2+} probably due to interaction with the substrate rather than with the phosphatase itself.

pH Optimum. Both the amylase supernatant and the polylysine–urea fractions gave identical pH–activity profiles with a broad optimum around pH 7.8 and half-maximum activities at pH 6.5 and 8.7, suggesting that the enzyme had not been affected by the urea treatment.

Stability. The stability of the phosphatase under different conditions is reported in Table II. Dilute solutions of the purified enzyme adsorb on glass surfaces and are irreversibly inactivated; maximum stability and activity were achieved in Teflon tubes in the presence of 2 mg/mL bovine serum albumin. It must be pointed out that below pH 5.0, or in the presence of 0.3 M perchloric acid or 10% trichloroacetic acid, the enzyme precipitates but redissolves readily by simple neutralization of the inactive suspensions without, however, restoration of activity. Finally, it is interesting to note the stability of the phosphatase in the presence of low concentrations of solvents or detergents, and its remarkable resistance to proteolysis.

Effectors. Inhibition by poly(L-lysine) is immediate, and does not affect the time course of the reaction which remains linear until 50% of phosphorylase *a* has been consumed. It does vary, however, with the molecular weight of the basic polypeptide with 80% inhibition afforded by 4 μM polylysine of mol wt in the range of 15 000–30 000 ($K_i \simeq 1$ μM) and only half as much when the mol wt is 1000 to 5000. By contrast, lysine, arginine, cadaverine, spermidine, or tosyl-L-lysine chloromethyl ketone in millimolar concentrations did not affect the activity of the enzyme suggesting that the polylysine inhibition is rather specific; histones and protamines could not be accurately tested since they caused the precipitation of the substrate phosphorylase *a*.

Salts at relatively high concentrations also inhibit the enzyme (40 to 96% inhibition at 0.1 to 0.3 M NaCl); inhibition follows the Hofmeister series decreasing in the order: $Mg^{2+} > Ba^{2+} > Na^+ > K^+$ and $SO_4^{2-} > Cl^- > CNS^- > I^-$.

The enzyme is maximally activated by SH compounds such as 50 mM 2-mercaptoethanol or 1 mM dithiothreitol or reducing agents (e.g., 1 mM ascorbic acid). By contrast, inhibition is rapid and quantitative with cystamine, 2-hydroxyethyl disulfide, or 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2), and somewhat weaker with oxidized glutathione, D-pantethine, or cystine. Titration with Nbs_2 for 15 min at room temperature leads to 90% inactivation which is irreversible unless an excess of SH groups is added within minutes; even then the enzyme is unstable and undergoes irreversible inactivation upon standing. Surprisingly, 50 mM iodoacetic acid or iodoacetamide caused only a 30% inhibition after 1 h at 30 °C.

Molecular Weight and Aggregation. The molecular weight of the phosphatase was difficult to determine by conventional procedures because of the tendency the enzyme has to aggregate. Aggregation could be demonstrated under many different experimental conditions and is strongly dependent upon protein concentration, ionic strength, and aging of the solutions. For example, during sedimentation velocity experiments, the asymmetric protein peak seen close to the meniscus often vanishes while heavy material accumulates at the bottom of the cell. No secondary peak remains indicating that no component in the 60 000–150 000 mol wt range can be present. The influence of protein concentration was exemplified, first in sedimentation equilibrium runs where ln c vs. r^2 plots (Yphantis, 1964) showed a pronounced curvature, and second, by sucrose density gradient centrifugation. In this latter case, at 20 mg/mL most of the enzyme appeared in a 4.6S peak (mol wt ca. 65 000) together with faster sedimenting species (Figure 3A). As the enzyme concentration was lowered (Figures 3B and 3C), the faster sedimenting species decreased and a more

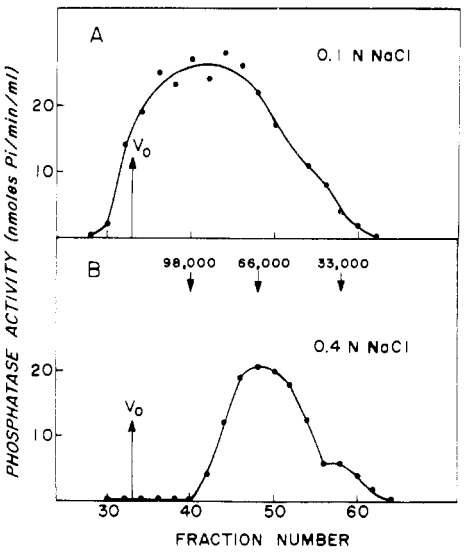

FIGURE 4: Effect of ionic strength on phosphatase elution pattern from Sephadex G-100. Sample from the Sepharose-polylysine column was chromatographed on a 1.5 × 90 cm Sephadex G-100 column equilibrated with buffer B containing 0.1 M (diagram A) or 0.4 M NaCl (diagram B). The column was precalibrated with marker proteins. Fractions (1.4 mL) were collected. Void volume and molecular weights are indicated by arrows.

uniform profile emerged. Complete dissociation was achieved in urea (Figure 3D) as will be discussed below.

The influence of ionic strength is illustrated in Figure 4: by increasing NaCl concentration from 0.1 to 0.4 M, the activity profile obtained by filtration through Sephadex G-100 (or G-200 and G-150; not illustrated) evolved from a typical aggregating system characterized by a broad peak covering the 30 000–90 000 mol wt range into a major symmetrical peak containing material of mol wt ca. 65 000 and a smaller percentage of component with mol wt ca. 33 000.

Finally, aging phosphatase solutions were shown to favor aggregation. Samples freshly eluted from Sephadex with an apparent mol wt of ca. 60 000 were completely excluded from the gel after concentration and storage at −20 °C for 3 days. This time-dependent aggregation could also be observed by high-speed sedimentation analysis resulting in an increase in the *s* value.

In any event, sizes of the aggregates appear to be multiples of ca. 30 000, and there is a direct correlation between the molecular weight of the samples (as determined on Sephadex) and the extent to which they migrate on polyacrylamide gels. Higher aggregates do not penetrate the gels. Nevertheless, all the results (extrapolation to zero concentration, sedimentation constants between 2.2 and 3 S obtained under a variety of conditions, high ionic strength, etc.) are in good agreement and yield a value of ca. 33 000 mol wt for the smallest active species. It should be emphasized that the monomeric species are active and that the polymeric species always display a lower specific activity suggesting that aggregation results in a loss of activity. Moreover, 6 M urea disaggregates the phosphatase as shown by sucrose density gradient centrifugation where the material now sediments as a single symmetrical 2.7S peak of ca. 33 000 mol wt (see Figure 3D). The denaturing agent can also convert the higher aggregates eluted from Sepharose-polylysine with 1 M NaCl to the lower 33 000 mol wt species.

Discussion

For a number of reasons, the purification of rabbit muscle phosphorylase phosphatase has presented a difficult problem. First, the enzyme is found in the muscle in far lesser amounts than both phosphorylase or phosphorylase kinase; whereas phosphorylase crystallizes after a ca. 25-fold enrichment, more than a 5000-fold purification is required for the phosphatase. As a result, very dilute solutions of the purified material are obtained that are difficult to concentrate; the enzyme is unstable and loses activity because of adsorption on glass surfaces. Second, since the enzyme has a strong tendency to aggregate and perhaps to bind to other proteins, variations are observed on Sephadex chromatography that ultimately yield material with varying degrees of purity.

The polylysine adsorption of the enzyme and its elution by urea used in the purification deserve some comments. The Sepharose-poly(L-lysine) step must be viewed as a sort of affinity chromatography in which the immobilized polypeptide mimics the enzyme substrate. The phosphorylated site in phosphorylase is highly basic. In an 88 amino acid, CNBr fragment of phosphorylase *a* that carries the phosphoseryl residue hydrolyzed by the phosphatase (Saari and Fischer, 1973), one out of every 5 residues is made up of either arginine or lysine resulting in an isoelectric point of ca. 10. Furthermore, an arginyl residue on the distal side of phosphoserine appears to play a crucial role in the specificity of the phosphatase (Graves et al., 1960). So, even though the enzyme is acidic and ionic bonds are to be expected, some more specific interactions, perhaps involving the active site of the enzyme, can be postulated. Indeed, elution by urea should be ascribed to a deformation of the enzyme that would interfere with this specific interaction since it is ineffective in eluting the phosphatase from other basic ion exchangers such as DEAE-Sephadex.

The purification procedure described herein offers several advantages: (a) it is rapid since it takes only 48 h to be carried out; (b) it yields ca. 6 mg of purified material per kg of muscle with a specific activity of 1 μmol of P_i released min^{-1} mg^{-1} of protein; (c) the purified preparation is essentially free of other enzymatic activities; and (d) it can be kept frozen for at least a month without loss of activity. There is no indication that the urea treatment has grossly affected the phosphatase because the purified enzyme has the same pH-activity profile, electrophoretic mobility, K_m, and resistance to proteolytic enzymes as the untreated material.

Many alternate procedures have been investigated, such as QAE or CM-Sephadex chromatography and gel filtration on various grades of Sephadex with or without sucrose, urea, or detergents such as Triton X-100 or deoxycholate: all led to unsatisfactory results. Sulfoethylcellulose and other ion exchangers that require acidic conditions could not be used due to the lability of the enzyme at low pH.

The inhomogeneity displayed by the purified enzyme on polyacrylamide gel electrophoresis is surprising in view of the high degree of purification obtained; in fact, there seems to be little relationship between the specific activity of the sample examined and the number of secondary bands observed (see, e.g., Figure 2). Unfortunately, it cannot be readily determined whether some of these are due to aggregated forms of the enzyme or to contaminants since both are inactive. No major alteration in gel patterns could be seen following treatment with sodium dodecyl sulfate, trichloroacetic acid, trypsin, or chymotrypsin (on the native enzyme) or high concentrations of SH compounds. Furthermore, since the phosphatase contains no extraneous material (carbohydrate, lipid, nucleic acid, cofactor, or bound phosphate, etc.), none of these could be used as markers to evaluate the number of active molecules present in a purified preparation.

Nevertheless, several preliminary observations tend to support the assumption that the purification procedure de-

scribed herein yields a rather pure material, perhaps a mixture of isoenzymes as suggested by the two active peaks seen in Figure 2B. For example, amino acid analysis carried out according to Houston (1971) on bands migrating in different positions gave similar compositions. Furthermore, a peptide map performed after performic acid oxidation and tryptic digestion of the purified material gave a simple pattern suggesting the presence of only one major protein component. These have not been reported herein because of uncertainties as to the homogeneity of the purified material obtained. Of course, the purification procedure could have been selected for a given type of molecules having properties in common, and more experiments will be needed to clarify the origin of the multiple bands appearing on polyacrylamide gels.

Attempts at using immobilized phosphorylase columns either to purify the phosphatase, or to determine the stoichiometry of the phosphorylase–phosphatase complex from which a specific activity for the pure enzyme could be evaluated, failed. The K_i of the phosphatase for phosphorylase *b* (150 μM, Gratecos et al., 1974) is too low to be of much use for affinity chromatography and phosphorylase *a*, despite its strong binding to the phosphatase (K_m = 5 μM) and the low turnover of the reaction, is too rapidly dephosphorylated. "Thiophosphorylase *a*" prepared by phosphorylase kinase and adenosine 5′-*O*-3-thiotriphosphate (ATPγS) is not attacked by the phosphatase and is a strong competitive inhibitor with a K_i = 3 μM (Gratecos and Fischer, 1974). This derivative possesses many of the features required for affinity chromatography.

The factors that govern the aggregation of the phosphatase are not clearly understood. The tertiary structure of the active enzyme is such that, at neutral pH, it is resistant to protease and to nonionic denaturing agents. Stability in detergents such as deoxycholate, various Tweens, Triton X-100, or in alcohol, etc., could imply that these reagents prevent the polymerization process. Aggregation might involve hydrophobic interactions and the same hydrophobic regions on the surface of the enzyme might also be implicated in the binding of phosphorylase phosphatase to the protein–glycogen complex.

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