Phosphorylase Phosphatase Complex from Skeletal Muscle. Activation of One of Two Catalytic Subunits by Manganese Ions†

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With the Technical Assistance of Curtis Diltz

ABSTRACT: Sarcoplasmic phosphorylase phosphatase extracted from ground skeletal muscle was recovered in a high molecular weight form (M_r = 250 000). This enzyme has been purified from extracts by anion-exchange and gel chromatography to yield a preparation with three major protein components of M_r 83 000, 72 000, and 32 000 by sodium dodecyl sulfate gel electrophoresis. The phosphorylase phosphatase activity of the complex form was activated more than 10-fold by Mn^{2+}, with a $K_{0.5}$ of 10^{-5} M, but not by Mg^{2+} or Ca^{2+}. Manganese activation occurred over a period of several minutes and resulted primarily in an increase in V_{max} of a phosphatase that was sensitive to trypsin. Activation persisted after gel filtration, and the active form of the enzyme did not contain bound manganese measured by using $^{54}Mn^{2+}$. A contaminating *p*-nitrophenylphosphatase was activated by either Mn^{2+} ($K_{0.5}$ of 10^{-4} M) or Mg^{2+} ($K_{0.5}$ of 10^{-3} M). Unlike the protein phosphatase this enzyme was inactive following removal of the metal ions by gel filtration. The phosphatase complex could be dissociated into its component subunits by precipitation with 50% acetone at 20 °C in the presence of an inert divalent cation, reducing agent, and bovine serum albumin. Two catalytic subunits were quantitatively recovered; one of M_r 83 000 was a trypsin-sensitive manganese-activated phosphatase and the second of M_r 32 000 was trypsin-stable and metal ion independent. Both enzymes were effective in catalyzing the dephosphorylation of either phosphorylase *a* or the regulatory subunit of adenosine cyclic 3′,5′-phosphate (cAMP) dependent protein kinase, but neither subunit possessed *p*-nitrophenylphosphatase activity.

Investigation of protein phosphatases was initiated with the studies by the Cori's of the phosphorylase "prosthetic group removing" (PR)[1] enzyme that converted the active form of glycogen phosphorylase to its inactive form (Cori & Cori, 1945). Only after it was found that the phosphorylase *a* to *b* conversion involved removal of a covalently bound phosphoryl group (Krebs & Fischer, 1956; Wosilait & Sutherland, 1956) was it realized that the PR enzyme was a phosphorylase *a* phosphatase (EC 3.1.3.17). Most of the extant information on the structure and function of protein phosphatases has been produced by continuing studies of enzymes that dephosphorylate phosphorylase *a* mainly because this enzyme is readily prepared in large amounts and contains a single phosphorylated site. Preparations of the protein phosphatases from several different tissue sources not only act on phosphorylase *a*, but also act on other enzymes involved in glycogen metabolism, in particular phosphorylase *b* kinase, glycogen synthase, and the regulatory subunit of cAMP-dependent protein kinase (Nakai & Thomas, 1973, 1974; Killilea et al., 1976; Khandelwal et al., 1976; Antoniw et al., 1977; Chou et al., 1977). Even though it has been proposed that the dephosphorylation of these proteins is carried out by a single "multifunctional" enzyme, there is evidence that "specific" protein phosphatases that preferentially use phosphorylase, phosphorylase kinase, glycogen synthase, or troponin I as substrate exist in different tissues such as liver and skeletal muscle [Ray & England, 1976; Antoniw & Cohen, 1976; Gratecos et al., 1977; Kikuchi et al., 1977; Tan & Nuttall, 1978; Laloux et al., 1978; also see the review by Krebs & Beavo (1979)]. The mechanistic or structural basis for this substrate specificity is not yet understood.

Purification of a homogeneous phosphatase from tissue extracts by combinations of chromatographic methods has been a frustrating problem over the last decade. Fractionation procedures produced such a complicated array of enzyme forms that the subunit composition of the native enzyme has yet to be unambiguously determined. Recent indications are that the phosphatase may contain two subunits, one of M_r 70 000 and another one of M_r 35 000 (Lee et al., 1977; Imaoka et al., 1980; Tamura et al., 1980).

Precipitation in 80% ethanol at room temperature was reported by Brandt et al. (1974, 1975) to cause a significant increase in the total phosphatase activity with concomitant reduction in molecular weight. From these precipitates a polypeptide of M_r 35 000 was identified as phosphorylase phosphatase and has been prepared in different laboratories by either Sepharose–histone chromatography (Khandelwal et al., 1976) or adsorption to columns of Sepharose–polylysine and elution with 6 M urea (Gratecos et al., 1977). More recently, it was reported that the M_r 35 000 phosphorylase phosphatase prepared by ethanol precipitation possessed significant *p*-nitrophenylphosphatase activity in the presence of divalent metal ions, probably due to contamination with an "alkaline-type" phosphatase of similar molecular weight (Li et al., 1979). It is not yet clear whether or not the multifunctional nature of the phosphorylase phosphatase of M_r 35 000 is due to this preparation being an admixture of different phosphatases.

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; pNPP, *p*-nitrophenyl phosphate; PMSF, phenylmethanesulfonyl fluoride; TPCK, tosylphenyl chloromethyl ketone; cAMP, adenosine cyclic 3′,5′-phosphate; PR, prosthetic group removing; ATP, adenosine 5′-triphosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DEAE, diethylaminoethyl.

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This paper presents the properties of a soluble form of phosphorylase phosphatase present in the cytoplasm of rabbit skeletal muscle. The enzyme was recovered as a high molecular weight species referred to as a "complex" since it could be dissociated into two catalytic subunits: a protein phosphatase of M_r 32 000 and a second enzyme of M_r 83 000 that is activated by Mn^{2+}. This reaction converts the enzyme to a stable, active form that is maintained even after removal of the metal ions. A preliminary report of this investigation has been presented (Brautigan & Fischer, 1980).

Materials and Methods

Crystalline rabbit muscle phosphorylase *b* was prepared according to Fischer & Krebs (1958) and converted to the *a* form by reaction with purified muscle phosphorylase kinase as described by Krebs et al. (1964). For radioactive phosphorylase *a* the [^{32}P]ATP (triethylammonium salt, 3000Ci/mmol) used was purchased from Amersham. Homogeneous, protease-free α-amylase was prepared from human parotid gland exudate as described by Kaufman et al. (1970) and Watanabe & Keller (1974).

Purified catalytic and regulatory subunits of bovine cardiac cAMP-dependent protein kinase were prepared according to Peters et al. (1977) and Dills et al. (1979), respectively.

The formulation of Anderson & McClure (1973) was modified for preparation of scintillation fluid: 100 g of Omnifluor (New England Nuclear) was dissolved in 4 gal of xylene, and 5 L of Triton X-100 was added as solubilizer. Typically, 10% (v/v) aqueous samples were counted in this scintillant with no significant quenching.

Polyacrylamide gel electrophoresis was performed with the system of Ornstein (1964) and Davis (1964) as described by Brewer et al. (1974) in Tris–glycine buffer, pH 8.3. Electrophoresis in the presence of 0.1% sodium dodecyl sulfate ($NaDodSO_4$)[1] was performed according to Weber & Osborne (1969) as modified by Kerrick et al. (1979).

Protein was determined by the Coomassie dye binding method of Bradford (1976) with bovine serum albumin as standard.

Phosphorylase phosphatase activity was measured by mixing 100 μL of 2 mg/mL [^{32}P]phosphorylase *a* (final concentration 10 μM, 2×10^4–5×10^4 cpm/nmol) and 100 μL of enzyme solution, both prepared in either 20 mM Tris- or imidazole–acetate buffer, pH 7.5, 0.1 mM DTT, and 5% glycerol at 30 °C in 5-mL polystyrene tubes. After 5 min, 100 μL of 25 mg/mL bovine serum albumin (Miles Laboratories fraction V) and 700 μL of ice cold 10% trichloroacetic acid were added; the tubes were chilled for 5 min and then centrifuged for 5 min at top speed in a clinical centrifuge. A 500-μL aliquot of the supernatant was mixed with 5.0 mL of scintillation fluid and counted in a Beckman LS 7000 liquid scintillation spectrometer. Total available radioactivity was determined by counting 50 μL of the substrate and the background (usually <1%) by substituting buffer for enzyme. This assay was linear with respect to time and enzyme concentration when <60% of the total $^{32}P_i$ was released. Units of activity are expressed as nanomoles of $^{32}P_i$ released per minute under these conditions.

Phosphorylase phosphatase activity was also measured by the phosphorylase *a* to *b* conversion using the same incubation mixture, but, instead of adding albumin and trichloroacetic acid, the reaction was diluted 10-fold with 0.1 M sodium maleate, pH 6.5, 50 mM 2-mercaptoethanol, and 1 mg/mL bovine serum albumin. Aliquots were further diluted, and the phosphorylase activity was determined according to Hedrick & Fischer (1965). A unit of phosphatase activity, defined as above, was calculated from the loss of phosphorylase *a* activity.

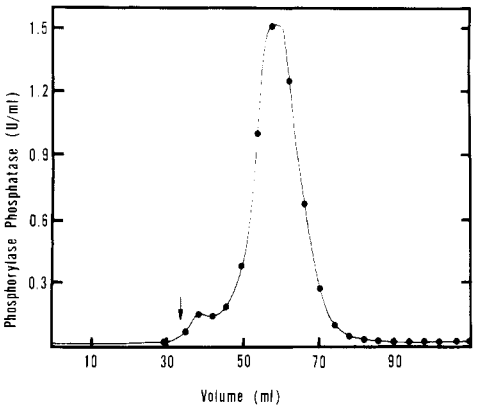

FIGURE 1: Chromatography of a skeletal muscle extract on Sepharose 6B-CL. Hypotonic extract was centrifuged at 20000*g* for 15 min. A 3.0-mL sample of the supernatant was eluted at 25 mL/h from a 2.5 × 23 cm column of Sepharose 6B-CL equilibrated with 10 mM imidazole–acetate, pH 7.5, and 5% glycerol at 4 °C. The phosphorylase phosphatase (●) appeared as a single peak eluting at the same volume as bovine liver catalase (M_r 250 000), and no activity was present in the fractions eluting after hemoglobin ($V_e \simeq 80$ mL, M_r 68 000). The void volume (V_0) is indicated by an arrow.

p-Nitrophenylphosphatase was measured in the Tris or imidazole buffer at pH 7.5, the optimal pH for this activity as determined in a series of Tris–cacodylate buffers (data not shown). The 200-μL reaction volume contained 50 mM disodium *p*-nitrophenyl phosphate and 1 mM manganese(II) acetate. After incubation at 30 °C the reaction was terminated by addition of 800 μL of 2 M Na_2CO_3; the absorbance at 410 nm was used to calculate the concentration of *p*-nitrophenolate by using a molar absorbtivity of 1.75×10^4 (pH 11). Units of activity are expressed as nanomoles of *p*-nitrophenol formed per minute under these conditions.

Results

Phosphorylase Phosphatase in a Sarcoplasmic Preparation. The initial steps of this preparation were designed to separate the sarcoplasm from skeletal muscle. In contrast to the generally used crude extracts prepared with high-speed blenders, this "sarcoplasmic preparation" is a neutral, EDTA-containing, hypotonic extract of coarsely ground muscle. Protein concentration in these extracts increased rapidly to a maximum of ~20 mg/mL within the first 5 min. The phosphorylase phosphatase activity was extracted more slowly than the majority of sarcoplasmic proteins, reaching a maximum yield after 15 min, and decreased by as much as 20% after that time. Identical results were obtained with hypotonic or isotonic buffers made with 250 mM sucrose. Addition of protease-free human salivary α-amylase had no significant effect on the extraction.

Phosphorylase phosphatase activity present in these extracts was inhibited ~50% by treatment with the cAMP-dependent protein kinase catalytic subunit; this treatment is expected to inhibit the enzyme by phosphorylating the heat-stable inhibitor (Huang & Glinsmann, 1975; Nimmo & Cohen, 1978a,b) or other proteins that would serve as competitive inhibitors. Addition of MgATP alone resulted in an insignificant increase in phosphatase activity, providing no evidence for the presence of an ATP-dependent form of the enzyme (Yang et al., 1980).

In these sarcoplasmic extracts, phosphorylase phosphatase is present predominantly as a single form of apparent molecular weight of 250 000 (Figure 1). Concentration by a variety of precipitation procedures including (a) isoelectric precipitation by addition of 1 M acetic acid to pH 6.0, (b)

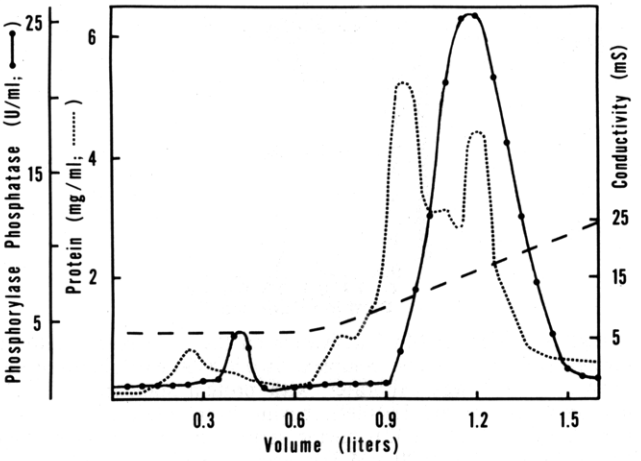

FIGURE 2: Elution of sarcoplasmic phosphorylase phosphatase from DEAE-Sephadex A50 as described under Results. Following elution, protein in milligrams per millilter (···), conductivity (---), and phosphorylase phosphatase activity (●) were determined. Samples were diluted 10-fold with salt-free buffer prior to assay.

treatment with −20 °C ethanol (25% v/v), and (c) treatment with polyethylene glycol (5% w/v) at pH 6.3 yielded only high molecular weight forms with no evidence for lower molecular weight species. These precipitation procedures yielded enzyme with variable polypeptide composition from preparation to preparation. This problem was alleviated by direct batch adsorbtion on DEAE-Sephadex A50 which provided consistent preparations.

Purification of Phosphorylase Phosphatase Complex. Female New Zealand white rabbits were sacrificed by cervical dislocation and exsanguinated. The back and hind leg muscles were removed (1025 g), put into plastic bags, and submerged in ice water. This initial procedure was typically completed within 3 min. After 10–15 min the tissue was passed through a chilled meat grinder fitted with a coarse cutting plate and immediately mixed with 2 volumes of ice-cold 5 mM EDTA, pH 7.0, to which was freshly added 1 mL/L of 2% (v/v) PMSF in 2-propanol. All subsequent operations were carried out at or below 4 °C. The suspension was vigorously stirred with a Teflon rod for 10 min, and ~10 g of granulated activated charcoal was added to adsorb nucleotides. Starting exactly 15 min after addition of the buffer, we centrifuged the suspension at 6000 g in a Sorval RC3 for 30 min. The supernatant (2100 mL; 5100 units at 0.13 unit/mg, measured with or without Mn^{2+}) was filtered through a glass wool plug, adjusted to pH 7.0 with 1 M NaOH, and stirred with 200 mL of DEAE-Sephadex A50 for 1 h. The anion exchanger was collected on a 15-cm Büchner funnel, washed by repeated suspension and suction filtration with 20 mM Tris–acetate, pH 7.5, 1 mM DTT, and 10% glycerol and with 0.5 L of this buffer containing 0.1 M NaCl. Routinely, more than 80% of the activity was adsorbed by this procedure. The gel was suspended and poured onto a 5 × 22 cm column of DEAE-Sephadex, forming a segment of 11 cm. This column was eluted with a linear gradient established between 2 volumes (0.75 L) buffer with 0.1 and 0.5 M NaCl at 75 mL/h as shown in Figure 2. The active fractions were pooled (9200 units total), concentrated by ultrafiltration with an Amicon XM-100 membrane, and chromatographed in two 40-mL portions on a 4 × 90 cm column of Sepharose 4B-CL. The single active peak was pooled, concentrated by ultrafiltration, dialyzed against Tris buffer containing 50% (v/v) glycerol, and stored at −20 °C (2750 units at 2 units/mg assayed without Mn^{2+}). The activity of the product in the presence of Mn^{2+} was consistently 10-fold higher, representing an ~150-fold purification. This enzyme has been stable to storage for over 1 year.

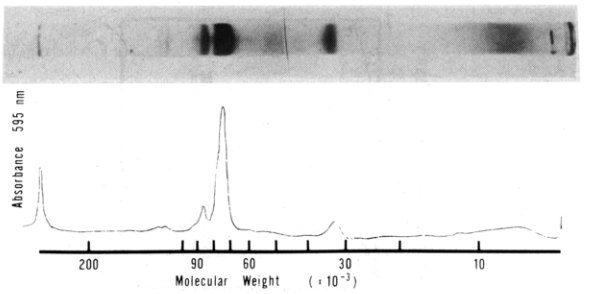

FIGURE 3: $NaDodSO_4$ gel electrophoresis of sarcoplasmic phosphorylase phosphatase complex of M_r 250 000 obtained after Sepharose 6B-CL chromatography (see Figure 5). Fractions with the highest activities were pooled, concentrated, and subjected to electrophoresis on a 8.75% polyacrylamide gel in the presence of reducing agent and 0.1% $NaDodSO_4$. Staining with Coomassie blue revealed three major bands of M_r 83 000, 72 000, and 32 000 in relative molar proportions of 1:6.4:1.

Polyacrylamide gel electrophoresis in the presence of 0.1% $NaDodSO_4$ revealed that the fractions with the highest phosphorylase phosphatase activity after elution from Sepharose contained three polypeptides of molecular weights 83 000, 72 000, and 32 000 with a stoichiometry of ca. 1:6:1, calculated on the basis of Coomassie blue staining (Figure 3). Gel electrophoresis of this preparation under nondenaturing conditions revealed two active bands of $R_f \simeq 0.4$ and 0.6, both stimulated by Mn^{2+}, in about a 3:1 proportion, respectively (data not shown). When these active bands were removed and subjected to $NaDodSO_4$ gel electrophoresis, a similar polypeptide pattern was observed, perhaps indicating partial dissociation of the enzyme under these conditions.

Mn^{2+} Stimulation of the Protein Phosphatase Complex. Divalent metal ions, especially Mn^{2+}, have long been known to stimulate protein phosphatase activity. In the absence of divalent metal ions the protein phosphatase *complex* catalyzed the dephosphorylation of either phosphorylase *a* or the phosphorylated form of the regulatory subunit (R_{II}) of cardiac cAMP-dependent protein kinase. However, it did not hydrolyze *p*-nitrophenyl phosphate. Addition of Mn^{2+} stimulated the protein phosphatase activity more than 10-fold, whereas Mg^{2+} and Ca^{2+} stimulated the activity less than three- and two-fold, respectively (Figure 4). Treatment of the complex with either Mn^{2+} or Mg^{2+} exposed a low level of *p*-nitrophenylphosphatase activity, a reaction not mimicked by Ca^{2+}, Co^{2+}, or Zn^{2+}. This activity had a pH optimum of 7.5 and was ascribed to the presence of a "neutral" rather than an "alkaline" phosphatase. It was not inhibited by millimolar concentrations of tetramisole, a potent inhibitor of mammalian alkaline phosphatases (Van Belle, 1972).

Half-maximum stimulation of phosphorylase phosphatase required 10^{-5} M Mn^{2+}, a concentration 1 order of magnitude lower than that required for the activation of *p*-nitrophenylphosphatase (Figure 4), suggesting that separate enzymes were involved. The Hill coefficient for the stimulation of phosphorylase phosphatase was 0.7 (correlation coefficient 0.99), suggesting that Mn^{2+} stimulation does not involve cooperative interactions between metal binding sites on the enzyme. The time course of the stimulation was relatively slow with a $t_{1/2}$ of ~2 min (data not shown). The complex exhibited a K_m value of 2 μM and a V_{max} of ~90 nmol/min prior to Mn^{2+} activation. Addition of the lowest concentrations of Mn^{2+} effective in increasing activity (see Figure 4) caused an abrupt shift in both K_m (from 2 to 10 μM) and V_{max} (from 90 to 770 nmol/min). Increasing the Mn^{2+} concentration from 1 μM

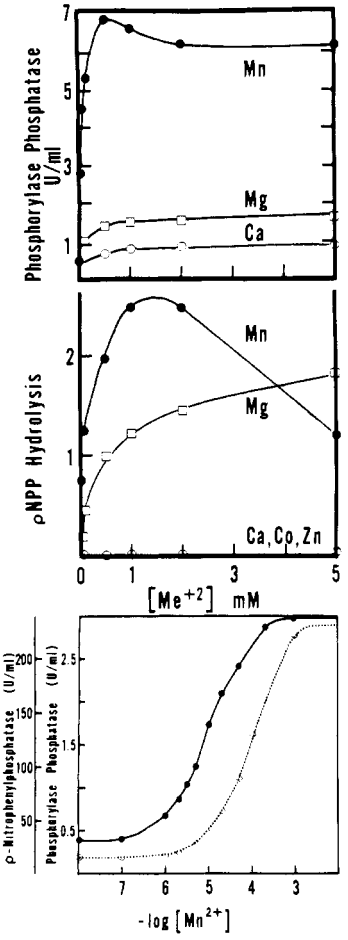

FIGURE 4: Stimulation of phosphorylase phosphatase (upper frame) and *p*-nitrophenolphosphatase (center frame) by divalent cations. The phosphatase complex was incubated with the indicated concentrations of Mn^{2+} (●), Mg^{2+} (□), or Ca^{2+} (○) (acetate or chloride salts) in 20 mM Tris–acetate, pH 7.5, 1 mM DTT, and 10% glycerol as described under Materials and Methods. The *p*-nitrophenylphosphatase activity expressed here as absorbance units at 410 nm per milliliter was not detected in the presence of Ca^{2+}, Co^{2+}, or Zn^{2+}. The bottom frame displays the stimulation of both sarcoplasmic phosphatase activities by Mn^{2+}. Samples of phosphatase complex were incubated for 15 min with various concentrations of manganese(II) acetate prior to assay for both *p*-nitrophenolphosphatase (○) and phosphorylase phosphatase (●) activities as described under Materials and Methods.

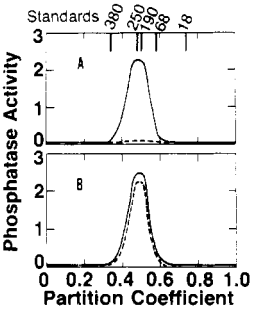

FIGURE 5: Effect of Mn^{2+} activation on the molecular weight of sarcoplasmic phosphatase complex. A 0.9 × 50 cm column of Sepharose 6B-CL was calibrated with protein standards: [^{32}P]-phosphorylase *a* (M_r 380000), bovine liver catalase (250000), phosphorylase *b* (190000), bovine serum albumin (68000), and troponin C (18000) in 20 mM Tris–acetate, pH 7.5, and 0.1% DTT containing 0.1 M NaCl. The partition coefficients are marked with solid bars and molecular weights in thousands at the top of the figure. Samples of phosphatase complex (500 μL) incubated for 30 min at 30 °C with either buffer alone (frame A) or buffer containing 1 mM Mn^{2+} (frame B) were chromatographed, and the activity of individual fractions was determined after a 20-min incubation in buffer alone (---) or with 1 mM Mn^{2+} (—).

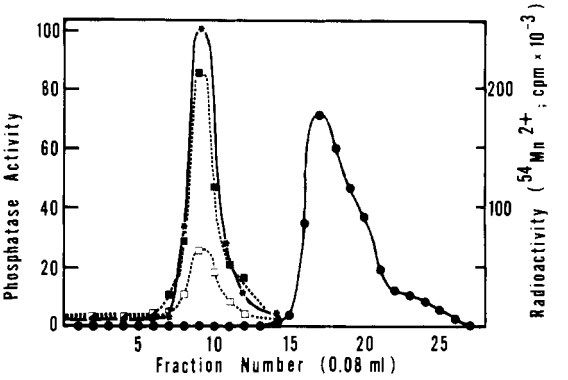

FIGURE 6: $^{54}Mn^{2+}$ binding to the activated phosphatase complex. Sarcoplasmic phosphatase complex (100 μL) was desalted on a 0.5 × 7 cm column of Sephadex G25 Fine in 20 mM imidazole–acetate, pH 7.5, 0.1% DTT, and 5% glycerol containing 0.1 M NaCl. Each fraction was diluted with 200 μL of salt-free buffer and the phosphorylase phosphatase activity measured after a 15-min incubation with (*) and without (□) 1 mM Mn^{2+}. A second sample of enzyme incubated 15 min at 30 °C with 1 mM $^{54}MnCl_2$ (Amersham Corp., evaporated to dryness and dissolved in buffer to 2.7×10^5 cpm/nmol) was desalted on the column, and fractions were diluted and assayed with (*) and without (■) 1 mM Mn^{2+}. Aliquots of 10 μL were counted for the $^{54}Mn^{2+}$ (●). Fraction 9 displayed fewer than 300 cpm over background.

up to 1 mM had no effect on the apparent K_m but further increased the V_{max} of the enzyme. Results illustrated in Figure 5A,B show that bound Mn^{2+} was not required to maintain the enzyme in the active form. Following chromatography on Sepharose 6B-CL, individual fractions were stimulated more than 10-fold when assayed in the presence of Mn^{2+} (Figure 5A). However, treatment of the enzyme with Mn^{2+} prior to the chromatography (a procedure expected to remove the metal ion) resulted in an enzyme retaining 80% of the activity of the fully stimulated form (Figure 5B). In contrast, the *p*-nitrophenylphosphatase was almost totally inactive after removal of Mn^{2+} by Sephadex G25 chromatography, and the 15-fold increase in activity with added Mn^{2+} indicated that the metal ion was required for activity (data not shown). The enzyme did not dissociate as a result of the activation process since all of the Mn^{2+}-activated phosphorylase phosphatase eluted as a single peak of identical molecular weight.

For confirmation that Mn^{2+} was not bound to the enzyme in the active state, the complex was incubated with radioactive $^{54}Mn^{2+}$, passed through a column of Sephadex G25 Fine, and assayed with and without Mn^{2+} (Figure 6). In this particular experiment the phosphatase exhibited about a five-fold activation with Mn^{2+}, and 85% of this activity persisted following gel filtration. The binding of $^{54}Mn^{2+}$ was equivalent to 14 pmol/mg of protein. If one assumes that the molecular weight of this complex obtained by gel filtration is accurate (M_r 250000), the amount of $^{54}Mn^{2+}$ present in the peak of enzyme activity would represent 3.5×10^{-3} g-atom of Mn^{2+}/mol of enzyme. Thus, even if this preparation were presumed to be only 1% pure, the enzyme would contain <1 equiv of Mn^{2+} in the active form. Furthermore, additions of the metal ion chelators EDTA or ATP did not inhibit the activated form of the enzyme significantly more than the nonactivated form (Table I). If the 10-fold increase in phosphatase activity had resulted from Mn^{2+} binding to the enzyme, the +/− activity ratio would be ~0.1.

Digestion with 5% (w/w) TPCK–trypsin for 30 min followed by addition of a 10-fold excess of soybean trypsin inhibitor and 5-fold dilution increased the activity of the phosphatase from 5.0 to 8.8 units/mL assayed in the absence

Table I: Effects of Mn^{2+} and Chelating Agents on Skeletal Muscle Phosphorylase Phosphatase[a]

phosphatase preparation	activity ratio +/− EDTA, 2.5 mM	+/− ATP, 0.5 mM	+/− Mn^{2+}, 1 mM
control	0.62	0.70	10
Mn^{2+} activated	0.59	0.58	1.5

[a] Samples of complex were assayed for phosphorylase phosphatase activity with and without the agents at the indicated concentration following a 10-min incubation at 30 °C. The enzyme was activated by incubation with 1 mM Mn^{2+} for 20 min and gel filtered on Sephadex G25 to remove the metal ion.

Table II: Effect of Subunit Dissociation on the Mn^{2+} Stimulation of Sarcoplasmic Phosphatase Complex[a]

	phosphorylase phosphatase complex (A)	acetone, 20 °C →	dissociated subunits (B)	
			B (units/mL)	
	A (units/mL)		$-Mn^{2+}$	$+Mn^{2+}$
control	0.6		1.2	6.9
Mn^{2+} activated	8.1		7.9	8.1

[a] Complex (3 volumes) was mixed with 25 mg/mL bovine serum albumin and 4 mM Ca^{2+} (1 volume) and precipitated by addition of 4 volumes of acetone (final concentration 50%) at 20 °C. The precipitate was collected by centrifugation at 8000*g* for 2 min and extracted with 4 volumes of imidazole–acetate buffer, pH 7.5, 0.1 mM DTT, and 5% glycerol at 4 °C. For the Mn^{2+}-activated enzyme, Mn^{2+} was substituted for Ca^{2+}. Samples were assayed before and after precipitation both with and without a 15-min incubation with 1 mM Mn^{2+}.

of Mn^{2+}, whereas in the presence of Mn^{2+} the control and trypsin-treated activities were 40.1 and 10.3, respectively, indicating that of the total activity ($+Mn^{2+}$) 75% was trypsin sensitive and 25% trypsin resistant.

Isolation of Two Catalytic Subunits from the Protein Phosphatase Complex. Investigations of phosphorylase phosphatase from 25 years ago (Keller & Cori, 1955) as well as those employing the purified M_r 35 000 form in this and other laboratories (Brandt et al., 1975; Gratecos et al., 1977; Li et al., 1978) have shown that divalent cations are either inhibitory or without effect. In contrast, a high molecular weight form of protein phosphatase, which presumably contains the M_r 35 000 subunit, was strongly stimulated by millimolar concentrations of Mn^{2+} (Kato & Bishop, 1972). Since the sarcoplasmic phosphatase complex exhibited Mn^{2+} stimulation, we hoped to find whether or not this occurred only in the complex. In other words, did one of the other subunits possess a Mn^{2+}-stimulated catalytic activity?

Attempts to dissociate the complex into a M_r 35 000 form by 80% ethanol precipitation at room temperature as reported by Brandt et al. (1974, 1975) yielded little or no activity. However, nearly quantitative yields could be obtained by precipitation with 50% acetone at 20 °C in the presence of bovine serum albumin, reducing agent, and divalent cations to facilitate the precipitation (Table II). Increasing the percentage of acetone decreased the yields of activity. As with tryptic digestion, solvent precipitation caused a ca. two-fold activation, presumably by disrupting some inhibitory protein–protein interactions. This form of the enzyme could be stimulated by Mn^{2+}, indicating that a second catalytic activity survived the procedure. If the complex were first reacted with Mn^{2+}, a treatment that activates the enzyme more than 10-fold, all activity could be recovered (Table II). This was not

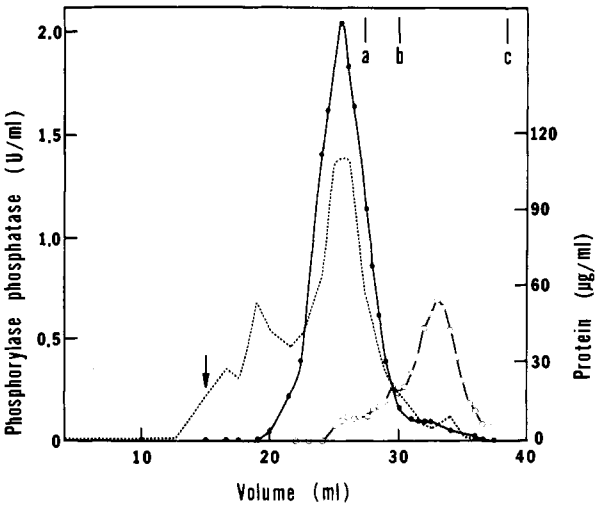

FIGURE 7: Recovery of two catalytic subunits following acetone precipitation of sarcoplasmic phosphatase complex. The complex was precipitated without bovine serum albumin by 50% acetone at 20 °C in the presence of 1 mM Ca^{2+}. After centrifugation for 2 min at 8500*g*, the pellet was extracted and 500 μL applied to a 1.5 × 30 cm column of Sephacryl S200 equilibrated in 15 mM (morpholino)propanesulfonic acid buffer, pH 7.5, 15 mM 2-mercaptoethanol, and 50 mM NaCl. Fractions of 0.5 mL were collected at 8 mL/h and analyzed for protein concentration (···) and phosphorylase phosphatase activity after a 15-min incubation with 1 mM Mn^{2+} (●). The major peak of activity (V_e = 25 mL) corresponded to a molecular weight of ~90 000 and was poorly active in the absence of Mn^{2+}. Virtually no M_r 35 000 enzyme was recovered due to the absence of albumin in the column buffer. In a separate experiment an identical sample of phosphatase complex was precipitated and chromatographed in the presence of 0.1 mg/mL bovine serum albumin. The phosphorylase phosphatase activity measured without Mn^{2+} (○) appeared at V_e = 33 mL corresponding to a M_r of 35 000. This activity was the same when measured with or without Mn^{2+}. Standards were (a) bovine serum albumin (M_r 68 000), (b) ovalbumin (45 000), and (c) ribonuclease (14 700); the void volume (V_0) is indicated by an arrow.

the case for the *p*-nitrophenylphosphatase activity which could not be detected after acetone treatment even in the presence of Mn^{2+}.

To separate the Mn^{2+}-sensitive activity from the M_r 32 000 phosphatase, we precipitated the complex with 50% acetone and chromatographed it on Sephacryl S200 as described in the legend to Figure 7. As anticipated, the phosphorylase phosphatase activity measured without Mn^{2+} was recovered in a peak corresponding to a M_r of 35 000 (32 000 by NaDodSO$_4$ gel electrophoresis, data not shown). It was necessary to include reducing agent and 0.1 mg/mL bovine serum albumin in the column buffer to recover this enzyme. Phosphatase activity was also found in Sephacryl S200 fractions corresponding to a M_r of ~90 000 but only when assayed in the presence of Mn^{2+}.

The characteristics of the Mn^{2+} activation for the M_r 90 000 phosphatase were the same as for the 250 000 molecular weight complex. Mn^{2+} activation persisted after gel filtration on Sephadex G25, and 80% of the total activity was recovered. Similarly, the Mn^{2+} stimulation of the complex was abolished by trypsin digestion, and, indeed, the phosphatase of M_r 90 000 lost more than 85% of its activity after 30 min with 5% (w/w) TPCK–trypsin.

The Sephacryl S200 fractions containing the trypsin-sensitive activity were pooled; gel electrophoresis under nondenaturing conditions revealed three protein bands, only one of which possessed phosphorylase phosphatase activity (Figure 8). The band containing the active protein was removed and subjected to NaDodSO$_4$ gel electrophoresis. This identified the Mn^{2+}-activated phosphatase as the protein with a M_r of

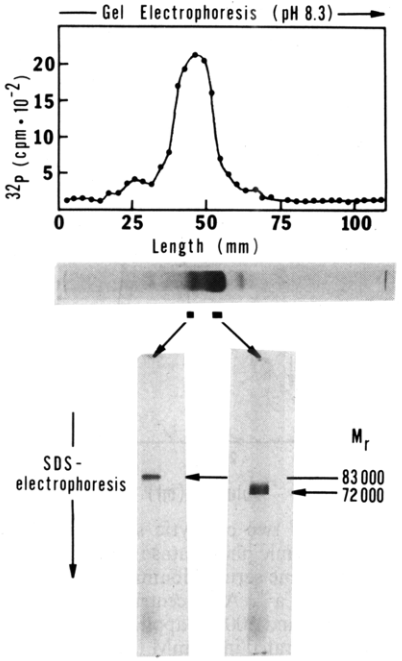

FIGURE 8: Gel electrophoresis of the Mn^{2+}-activated phosphatase from Sephacryl S200 chromatography (Figure 7). The fractions containing Mn^{2+}-activated phosphatase (M_r ~90 000) were pooled, concentrated, and subjected to gel electrophoresis at pH 8.3 as described under Materials and Methods. Several samples were run simultaneously; one gel was stained with Coomassie blue (displayed horizontally with migration from left to right); a second gel was cut into 2-mm slices and assayed for phosphorylase phosphatase after soaking for 15 h in imidazole buffer containing 1 mM Mn^{2+} [(●) top frame]. The single peak of activity coincided with the protein band of lowest mobility ($R_f \simeq 0.4$). From a third gel a 2-mm segment of this region and a 3-mm segment containing the major protein band were removed, minced, and applied to polyacrylamide gels containing 0.1% Na-DodSO$_4$ as shown. These two proteins corresponded to the two high molecular weight components of the complex form of the enzyme (Figure 4).

83 000 (90 000 by gel filtration). Together these experiments demonstrate that the properties of the sarcoplasmic complex can be accounted for by the behavior of the two individual phosphatase subunits.

The complex, as well as each of the individual active subunits, catalyzed the dephosphorylation of phosphorylase *a* and the phosphorylated form of the regulatory subunit of cAMP-dependent protein kinase (R_{II}). The specific activity of the M_r 250 000, 83 000, and 32 000 forms of the enzyme after incubation with manganese were ca. 20, 35, and 150 units/mg, respectively, when either phosphorylase *a* or R_{II} was used as substrate.

Discussion

Sarcoplasmic phosphorylase phosphatase extracted from coarsely ground muscle displays the same apparent molecular weight (250 000) as the protein phosphatase present in liver, heart, brown adipose tissue, and reticulocytes (Lee et al., 1977; Titanji & Pahlman, 1978; Killilea et al., 1979; Iamoka et al., 1978; Knight & Skala, 1979; Mumby & Traugh, 1979). This form of the enzyme is susceptible to proteolysis, and the appearance of multiple artifactual species has been attributed to the procedure used for tissue disruption (Mellgren et al., 1979; Killilea et al., 1979). In the present study, the rapid chilling of the excised tissue, the inclusion of metal chelators and protease inhibitors in the extract, and the relatively low protease content of muscle are all factors that minimize proteolysis. Yields of the enzyme were not increased by addition of α-amylase to the extracting buffer providing no evidence for the binding of sarcoplasmic phosphorylase phosphatase to glycogen particles. However, the lack of an amylase effect could also be due to some glycogen degradation during muscular activity following cervical dislocation. A second extraction would yield half again as much activity as obtained in the first extract; however, it is usually not carried out to expedite the operation. Some phosphatase activity is retained by the tissue; it may be a membrane-bound form that has not been studied.

Half the phosphorylase phosphatase activity can be inhibited by phosphorylation of the extract with cAMP-dependent protein kinase catalytic subunit, suggesting that some forms of the enzyme may be immune from the effects of the heat-stable inhibitor. Insensitivity to the phosphorylated inhibitor has been reported for some preparations of protein phosphatase (Laloux et al., 1978; Nimmo & Cohen, 1978b; Knight & Teal, 1980). The extent of inhibition here does not change with time, corroborating the conclusion that the enzyme is not subject to modification under these extraction conditions. Changes in the sensitivity of the phosphatase to heat-stable inhibitors occur with trypsin proteolysis of tissue extracts (Laloux & Hers, 1979).

The sarcoplasmic phosphatase has been partially purified by a procedure that avoids the use of any kind of precipitation. Such treatments have precluded to date the recovery of a preparation of consistent polypeptide composition. Advantage was taken of the acidic nature of the enzyme by adsorbing it directly to an anion-exchange gel. The purification of only 150-fold obtained here, as opposed to the more than 1000-fold reported in the literature, appears to be due in part to the difficulties encountered in correctly assessing phosphatase activity in the initial extract. The final specific activity of the complex after Mn^{2+} activation compares favorably with that of other preparations, including a recent purification involving 11 individual steps (Tamura et al., 1980). The enzyme appears to contain equal molar amounts of subunits with M_r of 83 000 and 32 000. The M_r 72 000 protein has yet to be assigned a function; it is not clear whether this material is an integral part of the complex in spite of the fact that it copurified with the enzyme even after acetone precipitation.

The initial study by Kato & Bishop (1972) on skeletal muscle protein phosphatase using glycogen synthase and histone as substrates revealed the specific effects of Mn^{2+} and has been in large part responsible for the routine inclusion of this metal ion in the preparation and assay of protein phosphatases. Phosphorylase phosphatase activity in the initial sarcoplasmic extract was not affected by Mn^{2+}, but the extent of its activation by this ion increased during the course of purification particularly following solvent precipitation. Manganese activation was a relatively slow reaction and brought about a stable modification of the enzyme. Radioactive $^{54}Mn^{2+}$ was not incorporated into the protein as part of the activation process, and incubation with metal chelators did not inhibit the activated form significantly more than the inactive form. Thus, there is no evidence to indicate that phosphorylase phosphatase is a "metallo-" or "manganese-dependent" enzyme. The precise mechanism of the manganese activation and the nature of the reverse reaction remain to be established.

Manganese activates both a protein (phosphorylase) phosphatase and a neutral *p*-nitrophenylphosphatase. Evidence that these are two separate enzymes includes the following: (a) activation of protein phosphatase by Mn^{2+} only, not by

Mg^{2+}, whereas *p*-nitrophenylphosphatase is activated by both ions; (b) the half-effective concentration for Mn^{2+} activation differs by 1 order of magnitude; (c) the fact that Mn^{2+} stimulation of phosphorylase phosphatase persists after removal of the metal ion in contrast to the *p*-nitrophenylphosphatase that is inactivated by this treatment.

Attempts to recover the Mn^{2+}-independent phosphatase led to the finding that two catalytic moieties could be derived from the complex form of the phosphatase. The former is the well-known "phosphorylase" phosphatase of M_r 32 000 that is exceptionally stable to tryptic attack (Gratecos et al., 1974, 1977; Killilea et al., 1979). This enzyme was recovered in good yield only when the solvent precipitation and subsequent steps were carried out in the presence of both bovine serum albumin and reducing agents. These results completely agree with earlier findings from this laboratory (Gratecos et al., 1977) that preparations of the M_r 32 000 enzyme require free sulfhydryl groups for activity and are difficult to recover and manipulate because of adsorption onto surfaces and a strong tendency to undergo aggregation. The other phosphatase is a trypsin-sensitive, Mn^{2+}-activated protein of M_r 83 000 that accounts for most of the activity present and is readily recovered following acetone precipitation.

Evidence indicates that one-fourth of the activity of the complex is due to the M_r 32 000 catalytic subunit and three-fourths is due to the Mn^{2+}-activated species. Gel electrophoresis of the complex under nondenaturing conditions yielded two peaks of activity in a 3:1 ratio, and the major active peak comigrated with that of the high molecular weight subunit ($R_f \simeq 0.4$, see Figure 8). The two subunits were also recovered with a 70:30 activity ratio after acetone precipitation of the complex and gel chromatography (see Figure 7). It is conceivable that the two active subunits are derived from two distinct high molecular weight forms of the phosphatase. But then, these separate enzymes would have to have the same molecular size, behave identically upon ion-exchange chromatography, and be recovered in identical amounts since the two active subunits are present in a stoichiometric ratio on $NaDodSO_4$ gels (see Figure 3). This possibility could not be resolved, for instance, by isoelectric focusing because the enzyme undergoes denaturation at its acidic isoelectric point.

Three substrates were used to monitor the phosphatase activity, namely, phosphorylase *a*, cAMP-dependent protein kinase regulatory subunit (R_{II}), and *p*-nitrophenyl phosphate. The two protein substrates were chosen because each contained a single site of phosphorylation with known amino acid sequence (Nolan et al., 1964; Huang et al., 1979). Furthermore, these two proteins are phosphorylated by distinctly different kinases, the regulatory subunit by cAMP-dependent kinase itself and phosphorylase by the calcium-dependent phosphorylase kinase. The two kinases display no cross-reactivity with regard to their substrates (Krebs & Beavo, 1979). This permitted an unambiguous examination of whether or not the same structural determinants recognized by the kinases would also be distinguished by any of the forms of the phosphatase. Our results indicate that the phosphatases do not exhibit the same substrate specificity as the kinases.

The presence of two active phosphatase subunits in a high M_r complex may clarify some previous observations. Since the larger, Mn^{2+}-activated subunit is extremely sensitive to trypsin, it is not surprising that from tissues that contain considerable proteolytic activity such as liver and heart, most of the phosphatase recovered following solvent precipitation is the trypsin-resistant subunit of M_r 35 000 (Brandt et al., 1975; Khandelwal et al., 1976; Chou et al., 1977; Hsiao et al., 1977). However, a Mn^{2+}-activated phosphatase of M_r 61 000 has been recovered from heart (Li et al., 1978), and reports of phosphatase preparations from adrenal cortex (Li, 1979) or brown adipose tissue (Knight & Skala, 1979) have shown two forms of the enzyme with M_r of ca. 75 000 and 35 000. Imaoka et al. (1980) separated these proteins from a M_r 240 000 form and demonstrated reassociation into a complex, reporting the larger protein as an inactive component; however, the activity was unfortunately not assayed with Mn^{2+}. Under other conditions, only the larger subunit has been recovered from adrenal cortex (Ullman & Perlman, 1975). This last result is similar to those obtained with reticulocyte lysates. The phosphatase in lysates is a M_r 270 000 species (Mumby & Traugh, 1979), and purification employing 80% ethanol precipitation has not produced an enzyme of M_r 35 000 but instead only a Mn^{2+}-activated form of M_r 78 000 (Grankowski et al., 1980). This enzyme may be the same or closely related to the M_r 83 000 enzyme examined here. In fact, the sarcoplasmic phosphatase complex in the presence of Mn^{2+} catalyzed the dephosphorylation of the smallest subunit of eukaryotic initiation factor 2, eIF2α.[2] The association of multiple catalytic subunits into high molecular weight phosphatase complexes may in part explain the "multifunctional" nature of these enzymes and presumably reflects a functionally significant physiological adaptation.

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