Subunit Structure and Activation of Inactive Phosphorylase Phosphatase†

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ABSTRACT: A phosphorylase phosphatase existing in a completely inactive form has been purified 4000-fold from rabbit skeletal muscle by a combination of acetone precipitation, gel filtration, ion-exchange chromatography, and affinity chromatography on polylysine–Affi-Gel 10. The nearly homogeneous enzyme was recovered with a yield of 22%; it has a specific activity of 7800 units/mg and accounts for 85% of the total phosphorylase phosphatase activity in crude extracts. The native enzyme displays a M_r of 70 000 by gel filtration and appears to consist of at least two subunits in an approximately 1:1 molar ratio: a M_r 38 000 catalytic subunit and a M_r 31 000 inhibitor probably identical with inhibitor 2. Activation of the phosphatase can occur in two ways. First, limited proteolysis in the presence of Mn^{2+} fully activates the enzyme in a biphasic reaction associated with rapid destruction of the inhibitor and slow degradation of the catalytic subunit to discrete species of M_r between 33 000 and 38 000. It is hypothesized that Mn^{2+} either causes the release of an inhibitory fragment still bound to the enzyme or induces a change in conformation in the catalytic subunit that exposes the active site. The trypsin/Mn^{2+} treatment also causes the simultaneous appearance of a *p*-nitrophenyl phosphatase activity. Second, the inactive enzyme can also be activated by a protein kinase termed F_A [Vandenheede, J. R., Yang, S.-D., Goris, J., & Merlevede, W. (1980) *J. Biol. Chem. 255*, 11768–11774] in a Mg^{2+}-ATP-dependent reaction resulting in phosphorylation of the inhibitory subunit. This phosphorylation is reversed when the kinase reaction is blocked by chelation of Mg^{2+}, with subsequent inactivation of the phosphatase.

It is well-known that many enzymes are regulated by reversible phosphorylation–dephosphorylation mechanisms, and for this reason, there has been much interest in the control of the kinases and phosphatases that catalyze these reactions [for reviews, see Cohen (1978), Krebs & Beavo (1979), Lee et al. (1980), and Li (1982)]. Divalent cations, particularly Mn^{2+} and Co^{2+}, activate a number of phosphorylase phosphatases (Cori & Cori, 1945; Brautigan et al., 1980, 1982a,b; Khandelwal & Kamani, 1980), and it has been suggested that the enzyme is a metalloprotein (Burchell & Cohen, 1978; Hsiao et al., 1978, Khatra & Soderling, 1978; Mackenzie et al., 1980). However, no radioactivity was detected in a partially purified phosphatase preparation following activation with $^{54}Mn^{2+}$ (Brautigan et al., 1980), and plasma atomic emission spectroscopy of the rabbit liver enzyme did not reveal significant amounts of Mn, Co, Mg, Ca, or other metals (Yan & Graves, 1982). Therefore, the role of divalent cations is not yet understood.

Also of current interest are two heat-stable protein inhibitors of phosphatase activity. Inhibitor 1 is active only after phosphorylation by cAMP-dependent protein kinase (Huang & Glinsmann, 1976a; Nimmo & Cohen, 1978), and its phosphorylation state appears to be under hormonal control (Foulkes & Cohen, 1979; Foulkes et al., 1980, 1982; Khatra et al., 1980). Inhibitor 2 was first observed in rabbit skeletal muscle (Huang & Glinsmann, 1976a,b) and has since been identified as a modulator protein that maintains a phosphatase referred to as F_C in an inactive state (Vandenheede et al., 1981a; Yang et al., 1981b). The F_C enzyme is completely inactive until it has been exposed to protein factor F_A and Mg^{2+}-ATP (Goris et al., 1979; Yang et al., 1980). Although F_A possesses kinase activity (Vandenheede et al., 1980) and is probably identical with glycogen synthase kinase 3 (Cohen et al., 1982), the activating reaction has not yet been shown to involve phosphorylation of the F_C protein (Yang et al., 1980, 1981a). However, it has recently been found that a Mg^{2+}-ATP-dependent phosphatase can be reconstituted by using purified phosphatase 1 catalytic subunit and inhibitor 2 and that activation with glycogen synthase kinase 3 results from phosphorylation of the inhibitor (Hemmings et al., 1982).

We previously reported the recovery of a Mn^{2+}-activated phosphatase following acetone precipitation of a M_r 250 000 form of the enzyme (Brautigan et al., 1980). The activation was specific for Mn^{2+} or Co^{2+} and was not reversed by removal of the metal ions. Sensitivity toward the cation increased progressively during purification, and this behavior was attributed to limit proteolysis (Brautigan et al., 1982a): addition of protease inhibitors throughout the preparation allowed the recovery of an inactive form of the enzyme that was poorly activated by Mn^{2+} alone but strongly activated by trypsin plus Mn^{2+}. This reaction decreased the size of the phosphatase as determined by gel filtration from M_r 70 000 to ca. 35 000. The preparations used were also shown to contain a heat-stable, trypsin-labile phosphatase inhibitor. It was therefore suggested that the native enzyme was made up either of a single polypeptide chain composed of catalytic, Mn^{2+}-binding, and inhibitory domains or of separate, very tightly bound subunits.

We report in this paper that the inactive M_r 70 000 phosphatase indeed consists of separate catalytic and inhibitory subunits. Activation of the enzyme occurs either through destruction of the inhibitor by trypsin and Mn^{2+} or through its phosphorylation by factor F_A in the presence of Mg^{2+}-ATP.

Materials and Methods

Rabbit muscle phosphorylase *b* was purified according to Fischer & Krebs (1958) as modified by DeLange et al. (1968), and phosphorylase kinase was purified according to Cohen (1973). The catalytic subunit of bovine cardiac cAMP-dependent protein kinase was isolated by the method of Peters et al. (1977). Phosphorylase *a* was prepared as described by

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Krebs et al. (1964); the [^{32}P]ATP (3000 Ci/mmol) used to make radioactive phosphorylase *a* was purchased from New England Nuclear. Tosylphenylalanyl chloromethyl ketone treated trypsin and lima bean trypsin inhibitor were purchased from Worthington; *p*-nitrophenyl phosphate (pNPP),[1] polylysine, PMSF, ATP, and benzamidine were from Sigma; BSA was obtained from Miles Labs; Brij-35 was purchased from Pierce, and Affi-Gel 10 was from Bio-Rad. Goat anti-rabbit albumin immunoglobulin G (IgG) and 4 times crystallized rabbit albumin were purchased from ICN.

Phosphorylase phosphatase was measured by release of ^{32}P from phosphorylase *a* as described by Brautigan et al. (1980) but with two modifications: the final substrate concentration was increased to 20 μM, and the assay buffer contained 20 mM imidazole, pH 7.5, 20 mM glucose, 5 mM theophylline, 1 mM DTT, and 1 mg/mL BSA. Full activation of the phosphatase was achieved by incubating the enzyme at 30 °C in assay buffer (except where otherwise noted) containing 1 mM Mn^{2+}, followed 2 min later by addition of 1 μg of trypsin. A 6-fold excess of lima bean trypsin inhibitor was added after 5 min, and the phosphatase was assayed either in a direct manner or after further dilution with the assay buffer to lower the Mn^{2+} concentration. Activation with F_A was carried out in the assay buffer, except where noted, containing 2–4 units/mL inactive phosphatase, 0.5 mM ATP, 1 mM Mg^{2+}, and F_A at concentrations indicated under Results. Phosphorylase *a* was added after a 15- or 30-min incubation. A unit of phosphatase activity is defined as 1 nmol of P_i released per min.

p-Nitrophenyl phosphatase activity was measured at 30 °C in 50 mM imidazole, 15 mM 2-ME, and 1 mg/mL BSA at pH 7.5. The 500-μL reaction mixture contained 10 mM pNPP and 10 mM Mn^{2+}, except where noted. The reaction was stopped with 500 μL of 2 M Na_2CO_3 containing 15 mM EDTA, and the absorbance at 410 nm was measured. A molar absorptivity of 1.75×10^4 M^{-1} cm^{-1} (pH 11) was used to calculate the *p*-nitrophenolate concentration. Units are expressed as nanomoles of *p*-nitrophenol formed per minute.

Phosphatase inhibitor was assayed by adding heat-treated material to 2–4 units/mL fully active phosphatase in the assay buffer. Phosphorylase *a* was added 5 min later.

Partially purified F_A was obtained from rabbit skeletal muscle as follows. Muscle extract (4 °C) was made 5% in poly(ethylene glycol) (M_r 20 000), and the pH was lowered to 5.9 by using 6 M acetic acid. Protein pellets were collected by centrifugation at 6000*g* for 30 min and were redissolved in 50 mM glycerophosphate buffer, pH 8.0, containing 3 mM Mg^{2+}, 3 mM EGTA, 0.002% benzamidine, 7 mM 2-ME, 10% glycerol, and 0.1% Brij-35. The solution was centrifuged at 80000*g* for 1 h to remove most of the glycogen; endogenous phosphorylase was activated by addition of AMP to 1 mM in order to digest some of the glycogen remaining in the supernatant. After 5 min at 30 °C, the temperature was lowered to 4 °C, and the solution was passed through a column of DEAE-Sepharose CL-6B. All of the F_A appeared in the breakthrough fraction and was subsequently adsorbed to and eluted from CM-Sephadex C-50 in a batchwise manner with 20 mM sodium phosphate, 0.1 mM EDTA, and 250 mM NaCl, pH 7.0. The protein was concentrated by ammonium sulfate fractionation (30–65% saturation) and was then chromatographed on Bio-Gel A-0.5m. The active fractions were pooled, dialyzed against 10 mM Na_2HPO_4, pH 7.0, 150 mM NaCl, and 40% glycerol, and stored at −20 °C. F_A was assayed by its ability to activate inactive phosphorylase phosphatase as indicated above.

Polyacrylamide gel electrophoresis was performed according to Ornstein (1964) and Davis (1964) as described by Brewer et al. (1974). Electrophoresis in the presence of $NaDodSO_4$ was carried out as per Laemmli (1970) and Studier (1973). Slab gels were stained by the silver method (Merrit et al., 1981a,b). Autoradiographs were made on Kodak X-Omat AR film. Densitometric analyses were performed on a double-beam recording MK IIIC microdensitometer (Joyce, Loebl and Co.) equipped with a peak integrator.

Polylysine (400 mg, average M_r of 25 000) was coupled to 25 mL of Affi-Gel 10 in 50 mL of 100 mM Mops, pH 7.5. The slurry was agitated at 4 °C overnight, and the gel was washed extensively with 1.0 M NaCl before use.

Protein was determined by the method of Bradford (1976).

Results

Enzyme Purification. (A) Crude Extract. Two female New Zealand white rabbits were sacrificed by cervical dislocation and exsanguination; the leg and back muscles were quickly removed and chilled. All further procedures were carried out at 4 °C. The tissue was minced in a meat grinder and extracted for 15 min with 3 volumes (v/w) of 5 mM EDTA, pH 7.0, containing 0.002% PMSF, 0.1 mM benzamidine, 15 mM 2-ME, and approximately 5 g of granular activated charcoal. The mixture was centrifuged at 6000*g* for 20 min; the pH of the supernatant was adjusted to 7.0 with 5 N NaOH, the conductivity was raised to 6 mS by addition of 5 M NaCl, and the extract was filtered through glass wool.

(B) Batch Adsorption to DEAE-Sepharose. The crude extract was poured slowly through 120 mL of DEAE-Sepharose CL-6B in a 10-cm Büchner funnel. The gel was washed with 250 mL of pH 7.5 buffer containing 10 mM imidazole, 0.1 mM EDTA, 15 mM 2-ME, 0.002% PMSF, 0.1 mM benzamidine (buffer A), and 0.1 M NaCl. The ion exchanger was removed from the funnel, stirred with 1 volume of buffer A containing 0.4 M NaCl, and returned to the funnel, where the eluate was collected by suction. This step was repeated twice, and the three eluate fractions were pooled.

(C) Acetone Precipitation. Magnesium acetate (1.0 M) was added to the protein solution to a final concentration of 1.1 mM Mg^{2+}, and 1 volume of room temperature acetone was added slowly with stirring. The milky solution was centrifuged for 5 min at 6000*g*, and the protein pellet was gently resuspended with a glass–Teflon homogenizer in 130 mL of buffer A containing 1.0 M NaCl. The mixture was dialyzed against buffer A until its conductivity reached 6.5 mS and centrifuged at 10000*g* for 20 min. The supernatant was retained.

(D) Gel Filtration Chromatography on Bio-Gel A-0.5m. Protein in the supernatant was concentrated by adding solid ammonium sulfate to 70% saturation. After 1 h at 0 °C, the suspension was centrifuged at 20000*g* for 20 min, and the pellet was redissolved in approximately 25 mL of buffer B (20 mM imidazole, 0.1 mM DTT, 0.1 mM benzamidine, 0.1 mM EDTA, 0.1 M NaCl, and 0.01% Brij-35, pH 7.5) containing 0.002% PMSF. The protein solution was centrifuged at

[1] Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; 2-ME, 2-mercaptoethanol; Mops, 4-morpholinepropanesulfonic acid; pNPP, *p*-nitrophenyl phosphate; pNPPase, *p*-nitrophenyl phosphatase; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; buffer A, 10 mM imidazole, 0.1 mM EDTA, 15 mM 2-ME, 0.002% PMSF, and 0.1 mM benzamidine, pH 7.5; buffer B, 20 mM imidazole, 0.1 mM DTT, 0.1 mM benzamidine, 0.1 mM EDTA, 0.1 M NaCl, and 0.01% Brij-35, pH 7.5; buffer C, 20 mM imidazole, 0.1 mM DTT, 5% glycerol, 0.1 mM EDTA, and 0.01% Brij-35, pH 7.5.

Table I: Purification of Inactive M_r 70 000 Phosphorylase Phosphatase

step	volume (mL)	protein (mg)	total units of activity: spontaneous[a]	total units of activity: after trypsin/Mn^{2+} treatment[b]	sp act.[c] (units/mg)	yield[d] (%)
crude extract	3150	30776	11340	72 450	2	100
batch DEAE-Sepharose	380	1279	15620	71 440	44	91
acetone pellet extract	142	242	10395	42 600	133	53
Bio-Gel A-0.5m peak I	150	65.4	75	21 150	322	34
DEAE-Sephadex A-50	182	21.1	0	15 505	735	25
polylysine-Affi-Gel 10[e]	11.3	1.7	3	13 300	7824	22

[a] Activity measured with no prior treatment. [b] Phosphatase activity measured after limited proteolysis as described under Materials and Methods. [c,d] Specific activity and yield of the trypsin/Mn^{2+}-activated enzyme only, calculated by subtracting the spontaneous activity from the total activity obtained after trypsin/Mn^{2+} treatment. [e] Activity and protein concentration were measured after dialysis against 50% glycerol.

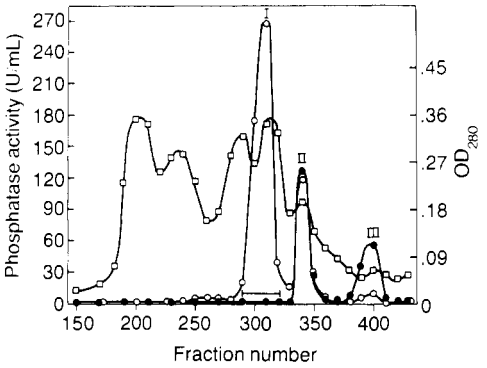

FIGURE 1: Gel filtration chromatography of the acetone pellet extract after concentration with 70% ammonium sulfate. The 27.5-mL sample was applied to a 5 × 127 cm (2490-mL) column of Bio-Gel A-0.5m equilibrated in buffer B. The flow rate was 100 mL/h, 4.5-mL fractions were collected. (○) Phosphatase activity after trypsin/Mn^{2+} treatment; (●) spontaneously active phosphatase; (□) OD_{280}. No phosphatase was detected in fractions 1–150. The bar indicates pooled fractions.

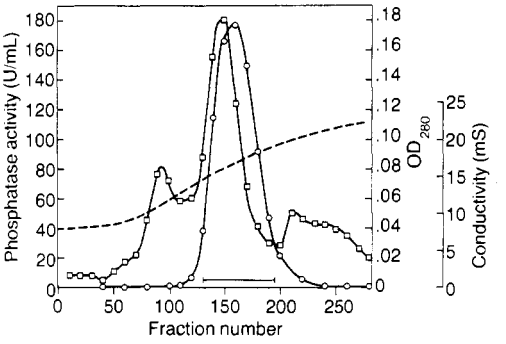

FIGURE 2: Ion-exchange chromatography of inactive peak I phosphatase. The NaCl concentration of the pooled peak I fractions was increased to 0.14 M before the solution was passed through a 2.5 × 17.5 cm (86-mL) column of DEAE-Sephadex A-50 equilibrated in buffer C containing 0.14 M NaCl. The column was washed with 450 mL of the same buffer at a flow rate of 30 mL/h. The enzyme was eluted with a linear salt gradient established between 425 mL each of buffer C containing 0.14 or 0.4 M NaCl. The flow rate was 55 mL/h, and 2.7-mL fractions were collected. (○) Phosphatase activity after trypsin/Mn^{2+} treatment; (□) OD_{280}; (---) conductivity. The bar indicates pooled fractions.

27000*g* for 20 min, filtered through glass wool, and applied to a sizing column of Bio-Gel A-0.5m equilibrated in buffer B. Three phosphatase species of M_r 70 000 (peak I), 46 000 (peak II), and 35 000 (peak III) were resolved (Figure 1). The enzyme in peak I was almost completely inactive prior to trypsin/Mn^{2+} treatment, whereas the two smaller phosphatase species were spontaneously active and partially inactivated by limited proteolysis. The peak I fractions, containing 75% of the total phosphatase activity recovered at this step, were pooled as indicated.

(*E*) *Ion-Exchange Chromatography.* The concentration of NaCl in the peak I fraction was increased to approximately 0.14 M before the solution was applied to a column of DEAE-Sephadex A-50 equilibrated in buffer C (20 mM imidazole, 0.1 mM DTT, 5% glycerol, 0.1 mM EDTA, and 0.01% Brij-35, pH 7.5) containing 0.14 M NaCl. The column was washed with 5 volumes of this buffer and then eluted with a linear gradient from 0.14 to 0.40 M NaCl (Figure 2). Fractions containing the inactive phosphatase were pooled as indicated. When this material was examined by NaDodSO$_4$–polyacrylamide gel electrophoresis, one major protein band of M_r 70 000 was visualized. This protein does not represent the phosphatase, but a contaminant due in part to rabbit serum albumin: the band comigrates with purified rabbit albumin during electrophoresis, and the enzyme preparation at this stage of purification reacts with anti-rabbit albumin IgG to give a precipitin line of identity in an Ouchterlony double-diffusion test (data not shown).

(*F*) *Polylysine–Affi-Gel 10 Chromatography.* The conductivity of the protein solution was decreased to 11.3 mS by addition of buffer C and was then applied to a 20-mL column of polylysine–Affi-Gel 10 prepared as described under Materials and Methods. The column was washed extensively with buffer C containing 0.23 M NaCl until no protein emerged; the phosphatase was then eluted stepwise with 0.40 M NaCl in buffer C. The active fractions were pooled and dialyzed against buffer C, and the protein was concentrated on a 2.5-mL column of DEAE-Sephadex A-50. The active fractions were dialyzed against 50 mM imidazole, 0.1 mM EDTA, 1 mM DTT, and 50% glycerol, pH 7.5, and stored at −20 °C.

(*G*) *Purity of the Enzyme Preparation.* The phosphatase was purified approximately 4000-fold by this procedure and had a specific activity of 7800 units/mg of protein (Table I). The enzyme was virtually inactive and only very slightly stimulated by 0.5 mM Mn^{2+} alone (15 vs. 13 300 units obtained after trypsin plus Mn^{2+}). Two major bands of M_r 38 000 and 31 000, accounting for ca. 90% of the protein, were seen on NaDodSO$_4$–polyacrylamide gels stained by the silver procedure (see below, Figure 5B, lane a). At light protein loads, the M_r 38 000 band was resolved into a closely spaced doublet. It is not yet known whether one of these bands might represent a contaminant or whether it might be due to proteolysis during the isolation procedure or to heterogeneity of the phosphatase. Densitometric scans of the silver-stained gels showed a ca. 1:1 stoichiometry of the 38- and 31-kdalton bands, assuming that the proteins stain in a linear fashion. Similar results were obtained with Coomassie blue stained gels.

Structure and Activation of the Phosphatase. (*A*) *Identification of Inhibitory and Catalytic Subunits.* As reported previously (Brautigan et al., 1982a), this enzyme preparation

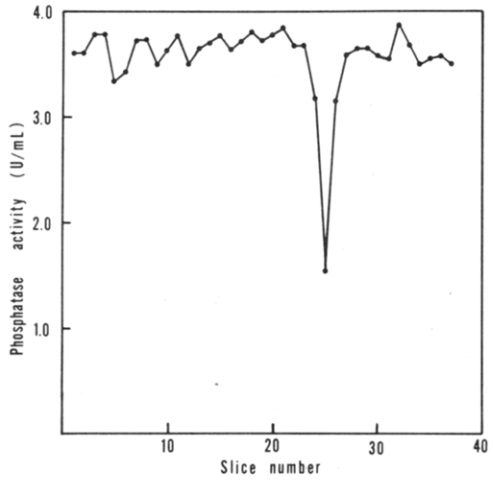

FIGURE 3: Identification of the inhibitory subunit following NaDodSO$_4$–polyacrylamide gel electrophoresis. Two 5-μg samples of phosphatase were subjected to electrophoresis in adjacent lanes on a 10% acrylamide slab gel with a 5% stacking gel. The gel was cut between the two lanes; one was stained for protein and the other sliced in 2-mm segments. The slices were soaked overnight at 4 °C in 150 μL of 100 mM imidazole and 15 mM 2-ME, pH 7.0, and assayed for phosphatase inhibitor as described under Materials and Methods. The inhibitory peak has an R_f of 0.68.

exhibits considerable phosphatase inhibitory activity after being boiled for 2–3 min. The relative homogeneity of the preparation allowed us to identify the inhibitory protein. Two phosphatase samples were subjected to electrophoresis side by side on a denaturing polyacrylamide slab gel; one lane was stained for protein, and the other was sliced and assayed for inhibitor. Figure 3 shows that all of the inhibitor was located in a sharp peak migrating with an R_f of 0.68. This corresponds exactly to the position of the M_r 31 000 protein band (see Figure 5B, lane a).

Positive identification of the phosphatase itself has been more difficult since we have not been able as yet to separate the inhibitory and catalytic moieties with retention of both activities. However, all data suggest that the catalytic subunit corresponds to the M_r 38 000 protein. When the inactive enzyme was subjected to electrophoresis under nondenaturing conditions, all the activity (measured after trypsin/Mn^{2+} treatment) was recovered in a few slices covering a narrow region of the gel (Figure 4A). Sodium dodecyl sulfate gel electrophoresis of these slices (19–29) showed that only two major protein bands of M_r 31 000 and 38 000 correlated with enzyme activity; these two bands presumably represent the inhibitory and catalytic subunits, respectively (Figure 4B).

(*B*) *Activation with Trypsin and* Mn^{2+}. We reported earlier (Brautigan et al., 1982a) that activation of the M_r 70 000 phosphatase by limited proteolysis and Mn^{2+} was accompanied by reduction in the size of the enzyme to M_r 35 000. The mechanism of tryptic activation is reexamined here by using a relatively high phosphatase:trypsin ratio. As shown in Figure 5A (closed circles), there appears to be two stages to this reaction: an initial rapid rise in activity from 0 to 5 min, followed by a more gradual increase up to 60 min. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of samples removed in the course of the reaction showed that the inhibitory subunit was rapidly destroyed so that it was no longer detectable after 5 min (Figure 5B). A low molecular weight protein of M_r 17 000, perhaps a fragment of the inhibitor, appeared transiently during this period. The catalytic moiety remained active throughout this experiment and was only slightly altered by the protease to yield several discrete protein bands in the range of M_r 38 000–33 000. The M_r 33 000

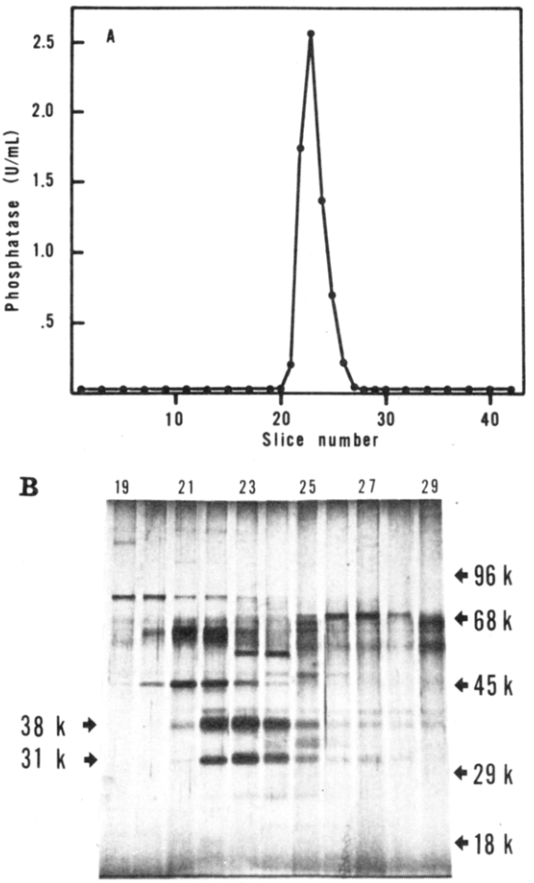

FIGURE 4: Identification of the phosphatase catalytic subunit. Phosphatase (10 μg) was subjected to electrophoresis on a nondenaturing gel, which was then cut into 2-mm segments. (A) Half of each slice was soaked overnight at 4 °C in 100 μL of 100 mM imidazole, pH 7.0, 5% glycerol, 15 mM 2-Me, and 0.01% Brij-35. The samples were assayed after trypsin/Mn^{2+} activation. (B) The other half of each slice was boiled for 2 min in 60 μL of NaDodSO$_4$ sample buffer and soaked overnight at 4 °C. The eluted proteins from slices 19–29 were analyzed by electrophoresis in the presence of NaDodSO$_4$. The gel was purposely overloaded in order to visualize contaminating proteins. The marker proteins include phosphorylase (96K), BSA (68K), actin (45K), carbonic anhydrase (29K), and troponin C (18K).

protein accumulated and was very stable to further proteolytic action.

Phosphorylase phosphatase fully activated by trypsin/Mn^{2+} does not require divalent metal ions for activity; in fact, Mn^{2+} is slightly inhibitory. However, the enzyme forms generated at early times during the activation reaction were stimulated by 1 mM Mn^{2+} (Figure 5A, compare open and closed circles). The effect was most prominent in the first 5 min, where increases of 70–120% were observed. Slight activations were observed at early times in the presence of 10 mM Mn^{2+}, but enzyme activity leveled off at a much lower value (ca. 6 units/mL). As indicated above, essentially no activity was observed if either trypsin or Mn^{2+} treatment was omitted.

There has been some controversy as to whether protein phosphatases possess activity toward low molecular weight phosphate esters such as *p*-nitrophenyl phosphate (pNPP). The majority of the pNPPase activity is due to a separate enzyme since it can be easily separated from the protein phosphatase early in the purification procedure. However, there exists a pNPPase activity that copurifies with the inactive phosphorylase phoshatase and that is most likely an intrinsic property of the same enzyme. It is essentially inactive prior to trypsin/Mn^{2+} treatment, and activation follows a somewhat similar time course (Figure 5A, squares). Furthermore, as

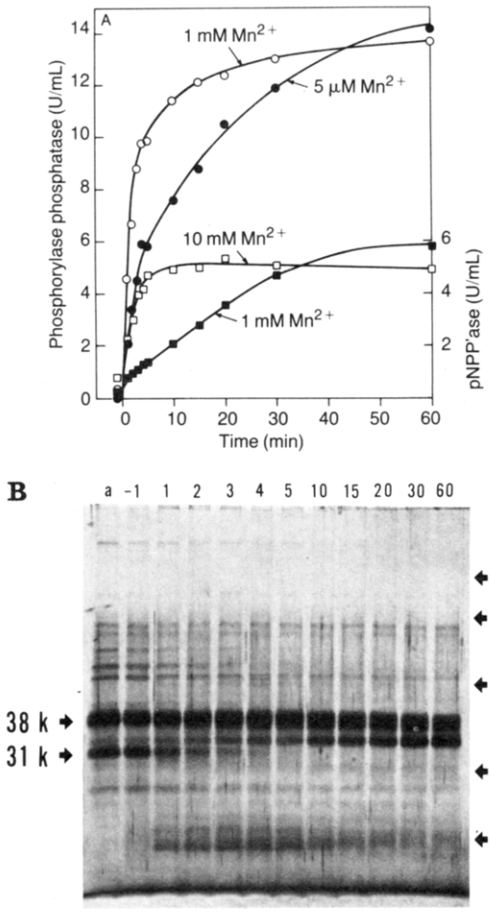

FIGURE 5: Activation of M_r 70000 phosphatase with trypsin/Mn^{2+}. Phosphatase (21.5 μg/mL) was incubated at 30 °C in 20 mM imidazole, pH 7.5, 0.1 mM DTT, 5% glycerol, and 1 mM Mn^{2+}; after 5 min (t = 0), trypsin was added to a final concentration of 0.24 μg/mL. At each time point, aliquots were removed and added to a 6-fold excess of lima bean trypsin inhibitor. (A) Samples were either diluted 100-fold and assayed for phosphorylase phosphatase in the presence of 5 μM (●) or 1 mM (○) Mn^{2+} or diluted 40-fold and assayed for pNPPase with 1 (■) or 10 mM (□) Mn^{2+}. (B) In an identical experiment, aliquots taken at each time point were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis; each lane contains 0.65 μg of phosphatase. Lane a contains enzyme that was exposed to neither Mn^{2+}, trypsin, nor lima bean trypsin inhibitor.

with the protein phosphatase, activation by Mn^{2+} was seen at the start of the incubation with protease but not after 1 h. There are, however, differences between the two activities: the pNPPase shows an absolute requirement for divalent cations ($Mg^{2+} > Mn^{2+} > Ca^{2+}$ at pH 7.5) and is less sensitive to high (e.g., 10 mM) Mn^{2+} concentrations. The K_m for pNPP is 3 mM and the specific activity ca. 2600 units/mg, i.e., 3-fold lower than that measured with phosphorylase *a* as substrate.

(*C*) *Activation with F_A and Mg^{2+}-ATP.* The inactive phosphatase, like the F_C enzyme described by Yang et al. (1980), can also be activated by the kinase F_A in a reaction requiring Mg^{2+}-ATP. The reaction was strongly dependent on F_A concentration (Figure 6); however, even when saturating amounts (2 μg) of F_A were added, the phosphatase exhibited only 40–45% of the maximum activity obtained after trypsin/Mn^{2+} treatment (10–15% when Mn^{2+}-ATP was used instead of Mg^{2+}-ATP). Addition of excess EDTA, which blocks the kinase reaction, brought about a 10% decrease in activity over a 10-min period. The time course of F_A activation was slow and again depended upon kinase concentration (Figure 6, inset). At relatively low F_A concentrations (0.8 μg/mL), phosphatase activity was still increasing after 30 min, whereas the reaction was complete after 20 min with 3 times more of

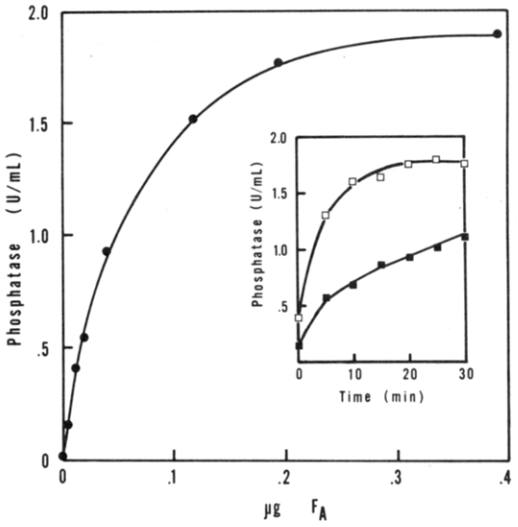

FIGURE 6: F_A activation of the M_r 70000 phosphatase. Phosphatase (0.030 μg) was incubated with increasing concentrations of F_A for 30 min in a total volume of 50 μL as described under Materials and Methods and assayed without further dilution. The *inset* shows the kinetics of activation using 0.039 (■) or 0.117 μg (□) of F_A. Each reaction mixture contained phosphatase and F_A at 30 °C; activation was initiated by addition of Mg^{2+}-ATP at the appropriate time.

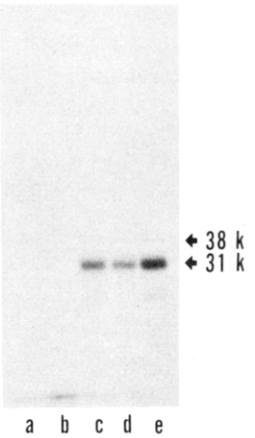

FIGURE 7: Phosphorylation of the phosphatase inhibitor by F_A and Mg^{2+}-ATP. Phosphatase (1.1 μg) and F_A (1.4 μg) were incubated for 30 min at 30 °C in 150 μL of 5 mM Mops, pH 7.0, containing 15 mM 2-ME, 1.0 mM Mg^{2+}, and 0.5 mM [γ-^{32}P]ATP (300 cpm/pmol). The protein was precipitated by addition of 6.2% trichloroacetic acid with 0.012% deoxycholate as a carrier prior to $NaDodSO_4$–polyacrylamide gel electrophoresis. An autoradiograph of the silver-stained gel is shown. (Lane a) F_A alone; (b) phosphatase alone; (c) F_A plus phosphatase; (d) identical with (c), except that 4 mM EDTA was added for an additional 10 min after the 30-min incubation; (e) identical with (c), except that 10 mM pyrophosphate was included in the reaction mixture.

the kinase. Purified catalytic subunit of cAMP-dependent protein kinase or phosphorylase kinase had no effect, even when present in large excess over the phosphatase.

Activation by F_A is associated with phosphorylation of the inhibitory subunit (Figure 7). Neither the F_A preparation nor the phosphatase alone contained detectable ^{32}P-labeled proteins after incubation with [γ-^{32}P]ATP (lanes a and b). However, after the two were mixed, a radioactive band corresponding to the inhibitory subunit was observed. Two other labeled proteins of M_r 38000 and M_r 25000 appeared very faintly on the autoradiograph. As described above for the phosphatase activity, EDTA decreased the extent of phosphorylation of the inhibitor by ca. 10% over 10 min. Pyrophosphate at 10 mM, which inhibits the phosphatase 75%, allowed a 2-fold increase in phosphorylation to occur.

Discussion

This study shows that the major phosphorylase phosphatase in muscle can be isolated as an inactive M_r 70 000 complex between at least two tightly bound proteins, a catalytic subunit (M_r 38 000) and an inhibitor (M_r 31 000). Two treatments—limited proteolysis in the presence of Mn^{2+} and phosphorylation of the inhibitor with F_A—have been found to activate the enzyme. In addition to the inhibited species, two spontaneously active phosphatases were also resolved (Figure 1). The M_r 46 000 enzyme (peak II) is similar to the inactive complex in that trypsin treatment generates a catalytic core of M_r 35 000; by contrast, Mn^{2+} is not required for the reaction, and there is only a slight increase in activity. The peak I and peak II phosphatases also show similar responses to inhibitors such as inhibitors 1 and 2 and various sulfhydryl-modifying reagents.[2] Although the interrelationship between these two forms is not yet known, the active peak II enzyme may consist of either free catalytic subunit derived from the inactive complex or a unique catalytic moiety. The second spontaneously active phosphatase (peak III, M_r 35 000) probably represents a distinct enzyme, since it is insensitive to inhibitor 2 (data not shown): the active fragments of the peak I and peak II phosphatases are always susceptible to inhibitor 2, even after prolonged tryptic digestion.

It was previously thought that a protein band of M_r 83 000 on $NaDodSO_4$–polyacrylamide gels represented the inactive phosphatase, in which the catalytic and inhibitory domains were covalently linked to one another, forming a single polypeptide chain (Brautigan et al., 1982a). Data presented here indicate that the M_r 83 000 protein is a contaminant of the inactive complex and that the two domains of the enzyme are distinct polypeptides. Though the possibility remains that the native enzyme actually exists as one protein and that endogenous proteases have generated the separate subunits, this is unlikely for several reasons: (1) a cocktail of protease inhibitors was present throughout the purification; (2) the native enzyme is insensitive to Mn^{2+} alone, whereas limited proteolysis produces a phosphatase species that is activated by Mn^{2+}; and (3) no phosphatase activity could be correlated with a protein band displaying a M_r of 70 000–83 000 on $NaDodSO_4$ gel electrophoresis.

The purified enzyme also dephosphorylates pNPP. Unlike the protein phosphatase reaction, activity toward the low molecular weight phosphate ester has an absolute requirement for divalent cations. The physiological significance of this reaction is unclear, although it has been suggested to reflect phosphotyrosine protein phosphatase activity (Li, 1982). So far, we have been unable to detect any activity toward phosphotyrosyl residues in proteins by using the fully active enzyme (data not shown). A M_r 35 000 protein phosphatase from canine cardiac muscle has also been found to use pNPP as a substrate (Li et al., 1979; Li & Chan, 1981); although these two activities had somewhat different properties, it was thought that they might be ascribed to two interconvertible forms of the same enzyme. The canine pNPPase is similar to the one described here in that it also requires divalent cations. Other M_r 30 000–35 000 phosphorylase phosphatases purified from rabbit muscle (Gratecos et al., 1977), rabbit liver (Khandelwal et al., 1976), and bovine cardiac muscle (Chou et al., 1977) have been reported to be devoid of pNPPase activity.

Both of the activating mechanisms discussed here are directed at least in part toward the inhibitory subunit. Our previous observations that trypsin/Mn^{2+} treatment fully activates the phosphatase and decreases its size to M_r 35 000 (Brautigan et al., 1982a) can now be explained in terms of degradation of the inhibitor. However, only 40–45% of the maximal activity is expressed at a time when the M_r 31 000 protein has completely disappeared. It can be assumed that the remaining activity is exposed by further proteolysis either of a more resistant fragment of the inhibitor or of inhibitory regions of the catalytic subunit. The M_r 38 000 protein appears to contain several trypsin-sensitive sites that give rise to a series of large active fragments, one of which (M_r 33 000) is particularly stable to further degradation. These forms may represent the multiple species of M_r 30 000–35 000 that have been isolated from various tissues. It is interesting to note that phosphatase activated with F_A and Mg^{2+}-ATP also displays only 40–45% of the maximal activity; this is not due to Mg^{2+}-ATP inhibition. This might suggest that proteolytic degradation of the inhibitory subunit and its phosphorylation are equivalent processes and that full activation with trypsin/Mn^{2+} probably requires modification of the catalytic subunit.

The strong dependence of phosphatase activation on F_A concentration illustrated in Figure 6 is undoubtedly due to the fact that the phosphatase dephosphorylates its associated inhibitor, thereby causing its own inactivation. In other words, with these two reactions competing with one another, phosphorylase phosphatase activity reaches a steady state whose level would vary with the relative proportion of the two enzymes. There is no evidence that the activation observed here in the presence of F_A is due to a contaminating protease: (a) there is an absolute requirement for Mg^{2+}-ATP; (b) after activation, no change in protein pattern is observed upon $NaDodSO_4$ gel electrophoresis; and (c) the phosphatase can undergo cycles of activation and inactivation which would not be observed in an irreversible proteolytic reaction. The low amount of radioactivity seen in the M_r 38 000 region on $NaDodSO_4$ gels may be due to direct phosphorylation of the catalytic subunit by F_A or to formation of a phosphoenzyme intermediate in the course of the dephosphorylation of the inhibitor.

The inhibitor associated with the peak I enzyme has many properties identical with those of inhibitor 2, including heat stability, molecular size, susceptibility to trypsin, and phosphorylation by F_A. Further evidence that the two proteins are the same comes from the fact that purified inhibitor 2 (Yang et al., 1981c) can substitute for the endogenous inhibitor in the system described herein.[2] Thus, this phosphatase complex resembles F_C, which also copurifies with inhibitor 2 (Yang et al., 1981b). Other similarities between the two phosphatases are the following: (1) they are both isolated as inactive M_r 70 000 species; (2) trypsin treatment causes irreversible activation and conversion to a size of M_r 32 000–38 000 (Vandenheede et al., 1981b); (3) both are activated by F_A and Mg^{2+}-ATP in a time- and concentration-dependent manner; and (4) Mn^{2+}-ATP is less effective than Mg^{2+}-ATP in the activation reaction (Yang et al., 1981a). On this basis, one might conclude that the two phosphatases are different forms of the same enzyme, if not identical. On the other hand, substantially different characteristics have been reported for F_C. Most importantly, the homogeneous enzyme was said to display one major protein band of M_r 70 000 on $NaDodSO_4$–polyacrylamide gels, and no ^{32}P incorporation was observed upon F_A activation. In fact, it was proposed that activation resulted from an F_A-mediated insertion of Mg^{2+} into the phosphatase (Vandenheede et al., 1981b). It is possible that the M_r 70 000 protein actually represents a native, un-

[2] Emma Villa Moruzzi, Lisa M. Ballou, and Edmond H. Fischer, unpublished results.

proteolyzed phosphatase; alternatively, this major protein could simply be a contaminant, for instance rabbit albumin. It is interesting to note that some F_C preparations were reported to have protein bands in the M_r 30000–40000 range on Na-DodSO$_4$ gels and some ^{32}P was indeed found in this area (Yang et al., 1980). A second difference between the two phosphatases is that treatment of F_C with trypsin/Mn^{2+} resulted only in a partial activation of the enzyme and that the same level of activity was obtained with Mn^{2+} alone (Yang et al., 1981a). This may be explained on the basis that the F_C preparation had sustained limited proteolysis that made it susceptible to Mn^{2+} activation and that the inactive peak I phosphatase described here is less susceptible to F_A activation. Finally, whereas the M_r 70000 complex described here was not affected by the isolated catalytic subunit of cAMP-dependent protein kinase, F_C was shown to be activated by this enzyme, and the effect was abolished by the heat-stable protein kinase inhibitor (Goris et al., 1980).

Of the two activating mechanisms presented here, the phosphorylation reaction is undoubtedly of more physiological significance. The speculation that the phosphorylation state of inhibitor 2 might be hormonally controlled raises intriguing possibilities as to the regulation of phosphorylase phosphatase in vivo.

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Registry No. Phosphorylase phosphatase, 9025-74-5; *p*-nitrophenyl phosphatase, 9073-68-1; *p*-nitrophenyl phosphate, 330-13-2; trypsin, 9002-07-7; manganese, 7439-96-5; protein kinase, 9026-43-1; magnesium ATP, 1476-84-2.

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