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Activation of Skeletal Muscle Phosphorylase Phosphatase. Effects of Proteolysis and Divalent Cations†

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ABSTRACT: Much of the phosphorylase phosphatase in skeletal muscle exists in an inactive state; this study explores various mechanisms for its activation. Although purified preparations of the phosphatase can be activated by Mn^{2+} up to 20-fold, in fresh muscle extracts the activity is inhibited by millimolar concentrations of Mn^{2+}. Limited proteolysis using trypsin or chymotrypsin exposes a phosphorylase phosphatase that is activated by Mn^{2+}. This proteolytic conversion of inactive phosphorylase phosphatase can be demonstrated either with a M_r 250 000 complex or with a lower molecular weight enzyme derived from it (M_r 83 000 by dodecyl sulfate–polyacrylamide gel electrophoresis or M_r 70 000 by gel filtration). The dependence on trypsin concentration and the time course for the appearance of Mn^{2+}-activated phosphatase decrease after a rapid initial rise, indicating that the enzyme exposed by limited proteolysis in the absence of metal ions is susceptible to further degradation. During prolonged tryptic digestion in the presence of divalent metal ions, this decrease is not observed because a stable form of the phosphatase is produced. Proteolysis of partially purified M_r 83 000 phosphatase without metal ions generates a trypsin-sensitive, Mn^{2+}-dependent species of M_r 53 000, whereas proteolysis in the presence of metal ions such as Mn^{2+}, Co^{2+}, and Mg^{2+} yields a trypsin-resistant fragment of M_r 35 000 whose activity is independent of divalent cations. The M_r 53 000 form can also be converted to a M_r 35 000 enzyme by the combined action of Mn^{2+} and trypsin. The enzyme preparations described here contain heat-stable phosphatase inhibitor activity which comigrates with the phosphatase activity during nondenaturing polyacrylamide gel electrophoresis and isoelectric focusing. On the basis of these results we propose that the native phosphatase catalytic subunit is a single polypeptide of M_r 83 000, composed of catalytic, metal-binding and inhibitory domains linked by trypsin-susceptible regions.

Divalent metal ions, in particular Mn^{2+}, have been known to affect phosphorylase phosphatase activity from the time of the first reported preparation (Cori & Cori, 1945). Ever since, Mn^{2+} has been used to increase and to stabilize the activity of phosphatases during their isolation. A number of protein phosphatases of different molecular sizes have been designated as Mn^{2+} dependent or Mn^{2+} activated (Kato & Bishop, 1972; Ullman & Perlman, 1975; Antoniw & Cohen, 1976; Li & Hsiao, 1977; Li et al., 1978; Knight & Skala, 1979; Brautigan et al., 1980; Grankowski et al., 1980), but the role of the metal ion is still not understood. Inhibition of some phosphatase preparations by fluoride, pyrophosphate, and EDTA[1] and subsequent reactivation by Mn^{2+} has led to the proposal that this metal ion is essential for activity (Hsiao et al., 1978; Khatra & Soderling, 1978; Mackenzie et al., 1980; Khandelwal & Kamani, 1980). However, no compelling evidence, such as stoichiometric Mn^{2+} binding to the enzyme, has been presented. Not all preparations of phosphorylase phosphatase are activated by Mn^{2+} (Keller & Cori, 1955; Nakai & Thomas, 1974; Brandt et al., 1975a; Gratecos et al., 1977; Khandelwal, 1977; Ingebritsen et al., 1980; Krakower & Kim, 1981), indicating that there may be more than one type of catalytic subunit, each with a different response to divalent metal ions. We previously reported the resolution of a M_r 250 000 form of phosphorylase phosphatase into a Mn^{2+}-activated enzyme of M_r 83 000 and a metal ion independent phosphatase of M_r 32 000 (Brautigan et al., 1980). The activation by Mn^{2+} was not mimicked by Mg^{2+} or Ca^{2+}; it was slow and was not significantly reversed by gel filtration or addition of chelating agents. No $^{54}Mn^{2+}$ was detected in the partially purified phosphatase following activation, suggesting that the metal ion might catalyze the interconversion of stable conformers.

During preparation of phosphorylase phosphatase from skeletal muscle extracts, a progressive increase in the degree

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; IDG buffer, 20 mM imidazole–acetate, pH 7.5, 0.1 mM dithiothreitol, and 5% glycerol; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)-aminomethane.

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of manganese sensitivity was observed. In fresh extracts the enzyme is unaffected or mildly inhibited by Mn^{2+}, whereas the purified material is activated severalfold by this cation. We show in this paper that an inactive phosphatase precursor, insensitive to Mn^{2+}, is converted by limited proteolysis into an inactive form that is greatly activated by the metal ion. Divalent cations alter the pattern of tryptic cleavage, yielding an active M_r 35000 fragment produced only in the presence of metal ions. A model is proposed delineating the relationship between the various phosphatase species.

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). The [^{32}P]ATP (triethylammonium salt, 3000 Ci/mmol) used to make radioactive phosphorylase *a* was purchased from Amersham.

Phosphorylase phosphatase activity was measured by release of ^{32}P from phosphorylase *a* [final concentration of 10 μM, (2–5) × 10^4 cpm/nmol]. Except where otherwise noted, both substrate and phosphatase were diluted with 20 mM imidazole–acetate, pH 7.5, 0.1 mM dithiothreitol, and 5% glycerol (IDG buffer)[1] containing 1 mg/mL bovine serum albumin and 1 mM theophylline (assay buffer). The assay and scintillation counting procedures have been described; units of activity are expressed as nanomoles of P_i released per minute under conditions where the substrate concentration is below K_m (Brautigan et al., 1980).

Nondenaturing polyacrylamide gel electrophoresis was performed according to Ornstein (1964) and Davis (1964) as described by Brewer et al. (1974) in Tris–glycine buffer, pH 8.3. Tosylphenyl chloromethyl ketone treated trypsin, lima bean trypsin inhibitor, and α-chymotrypsin were purchased from Worthington, PMSF was from Sigma, and bovine serum albumin fraction V was from Miles Labs.

Preparation of Muscle Extract. Back muscles of a New Zealand white female rabbit were excised following cervical dislocation and exsanguination. The tissue was placed in plastic bags, submerged in ice water until chilled, and minced with an electric meat grinder. The ground muscle was extracted for 15 min with 1 volume (v/w) of 5 mM EDTA, pH 7.0, containing 1 mL/L 2% PMSF in 2-propanol and then centrifuged at 6000*g* for 20 min. The supernatant was adjusted to pH 7.0 with 6 N ammonia, filtered through glass wool and fluted paper, and concentrated 2-fold by ultrafiltration under 30 psi of N_2 using an Amicon XM-30 membrane. A 12-mL portion of the concentrated extract was freed of nucleotides and metabolites by gel filtration on a column of Sephadex G-25 fine (4 × 20 cm) in 20 mM imidazole–acetate, pH 7.5, and 10% glycerol. Fractions with the highest protein concentrations were pooled, usually amounting to 50 mL at 10 mg/mL protein. This is the muscle extract used in the experiments described below.

Results

Effect of Trypsin on Inactive Phosphatase. Although preparations of skeletal muscle phosphorylase phosphatase obtained according to our purification procedure were activated from 2-fold to 20-fold by addition of Mn^{2+} (9.9 ± 5.8, n = 8), the phosphatase activity in fresh extracts was unaffected, if not slightly inhibited, by addition of 1 mM Mn^{2+}. Activation by manganese ions was a property of the phosphatase acquired during purification. Precipitation of skeletal muscle extracts with ammonium sulfate or organic solvents such as alcohol or acetone reproducibly resulted in a significant but variable

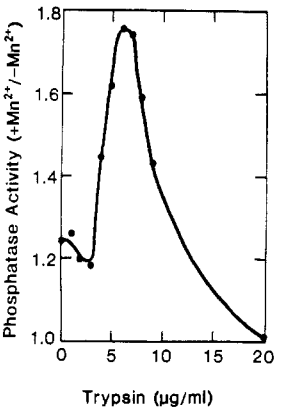

FIGURE 1: Desalted muscle extract, prepared as described under Materials and Methods, was incubated at 30 °C with the indicated concentrations of trypsin. After 15 min, lima bean trypsin inhibitor was added to each tube to a final concentration of 120 μg/mL. Aliquots were diluted 5-fold with 20 mM imidazole–acetate, pH 7.5, 10% glycerol, and 1 mM theophylline and assayed for phosphatase activity following a 15-min incubation with or without 1 mM Mn^{2+}.

increase in the sensitivity of the phosphatase to Mn^{2+} activation. It was suspected that the Mn^{2+}-sensitive phosphatase might be present in the extracts in a form that was unresponsive to the metal ion and that during purification this enzyme became exposed by either limited proteolysis or removal of a presumed protein inhibitor.

Following gel filtration of muscle extracts on Sephadex G-25 fine, incubation with low concentrations of trypsin unmasked a form of phosphorylase phosphatase that is activated by Mn^{2+}. A plot of the ratio of enzyme activity measured with and without Mn^{2+} as a function of trypsin concentration is biphasic (Figure 1); at low concentrations of protease the phosphatase becomes increasingly susceptible to Mn^{2+} activation, while at higher concentrations of the protease the enzyme is degraded. It was previously shown that a Mn^{2+}-activated phosphatase of M_r 83000 was inactivated by trypsin (Brautigan et al., 1980) while a M_r 35000 form of the enzyme was remarkably resistant (Brandt et al., 1974, 1975a; Gratecos et al., 1977; Killilea et al., 1979). Incubation of the extracts with chymotrypsin gave similar results except that more protease was required for optimum activation and the sensitivity to Mn^{2+} was not increased to the same extent. These results indicate that a protease-sensitive block prevents Mn^{2+} activation of a phosphatase that itself is susceptible to proteolysis.

Activation of Phosphatase by Trypsin and Mn^{2+}. Two preparations of skeletal muscle phosphorylase phosphatase were used to further examine the relationship between proteolysis and Mn^{2+} activation. The first was a native form of the enzyme (M_r 250000) prepared according to Brautigan et al. (1980) by adsorption of the tissue extract onto DEAE-cellulose, followed by gel chromatography on Bio-Gel A 0.5 m. The second was a phosphatase (M_r 83000 by dodecyl sulfate–polyacrylamide gel electrophoresis and M_r 70000 by gel filtration on Bio-Gel A 0.5 m) derived from the M_r 250000 complex by precipitation with 50% acetone at room temperature in the presence of divalent cations, followed by resolution of the two active species extracted from the precipitate as previously described (Brautigan et al., 1980). Various preparations of the M_r 250000 form are activated by Mn^{2+} to different extents, while the M_r 83000 species always has been recovered in an inactive state that requires treatment with Mn^{2+} for activity.

A closer examination of the effects of limited proteolysis on phosphatase activity revealed that two distinct reactions were taking place, depending on whether or not Mn^{2+} was

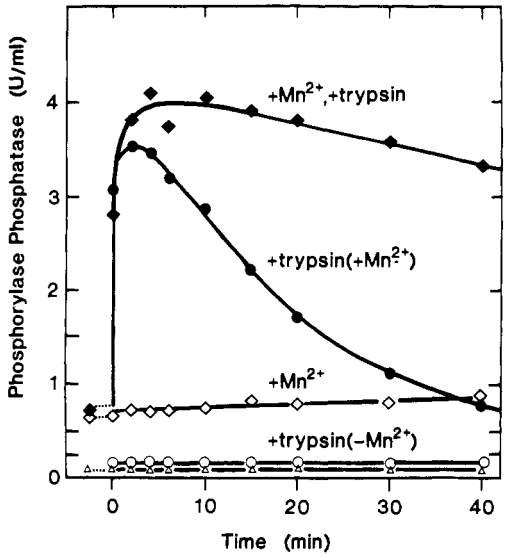

FIGURE 2: Effects of Mn^{2+}, trypsin, and Mn^{2+} plus trypsin on phosphorylase phosphatase. Partially purified M_r 250 000 phosphorylase phosphatase (final concentration 5.3 mg of protein/mL) was preincubated at 30 °C in IDG buffer containing 1 mM Mn^{2+} alone. After 14 min (at minus 2 min) aliquots were diluted 10-fold with assay buffer containing 30 μg/mL lima bean trypsin inhibitor and assayed. At time = 0, trypsin [final concentration 50 μg/mL (♦)] or IDG buffer (◇) was added. At the indicated times, aliquots were removed, diluted 10-fold, and assayed. In a second experiment samples were preincubated without Mn^{2+} in IDG buffer. At time = 0, trypsin was added to a concentration of 50 μg/mL, and at each time point, samples were diluted and assayed after a 15-min incubation with (●) or without (○) 1 mM Mn^{2+}. The control reaction (△) was incubated without Mn^{2+}, and IDG buffer was added instead of trypsin.

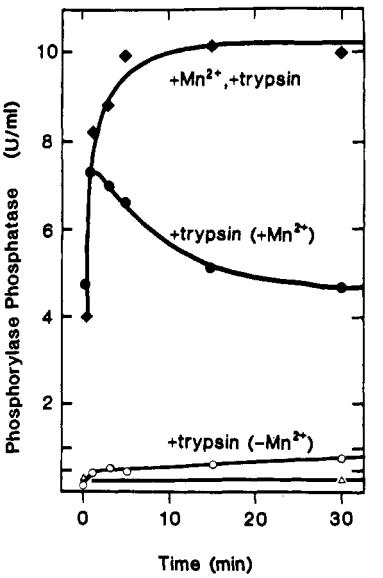

FIGURE 3: Effects of Mn^{2+}, trypsin, and Mn^{2+} plus trypsin on M_r 83 000 phosphorylase phosphatase. Partially purified M_r 83 000 phosphatase (final concentration 0.75 mg of protein/mL) was preincubated at 30 °C in IDG buffer containing 1 mM Mn^{2+}. After 15 min (time = 0) an aliquot was diluted 20-fold and assayed. All dilutions were carried out with IDG buffer containing 25 μg/mL lima bean trypsin inhibitor. One minute later, trypsin (♦) was added to a final concentration of 20 μg/mL. At the indicated times, aliquots were diluted and assayed after a 15-min incubation in buffer containing 0.05 mM EDTA. In a second experiment, samples were preincubated in IDG buffer without Mn^{2+}. After 16 min, trypsin was added to a final concentration of 20 μg/mL. At each time point, samples were diluted with either 1 mM Mn^{2+} (●) or 0.05 mM EDTA (○) and assayed after a 15-min incubation. The control (△) was incubated in IDG buffer without Mn^{2+} or trypsin.

present. Incubation of the M_r 250 000 form with trypsin in the absence of divalent metal ions slightly increased the phosphatase activity; the low level of activity observed with trypsin alone indicates that the lima bean trypsin inhibitor effectively blocked protease activity during the phosphatase assay (Figure 2). The phosphatase was activated by Mn^{2+} alone approximately 7-fold, within the range of values typically found for this type of preparation. The effects of trypsin or Mn^{2+} alone did not vary appreciably with time up to 1 h. However, a combination of these two treatments induced a completely different pattern of time-dependent changes in activity. Following trypsin digestion and then addition of excess lima bean trypsin inhibitor, samples assayed with 1 mM Mn^{2+} displayed phosphatase activities more than 20 times higher than those measured without the metal ion. The amount of Mn^{2+}-activated phosphatase decreased as incubation continued, indicating once more that this form of the enzyme was susceptible to further tryptic degradation. When proteolysis was carried out in the presence of Mn^{2+}, a similar increase in activity was observed, but under these conditions the phosphatase was resistant to further tryptic attack, retaining more than 80% of its peak activity after 40 min of incubation.

The M_r 83 000 phosphatase exhibited a similar response to trypsin and Mn^{2+} (Figure 3). As before, trypsin alone rapidly converted the enzyme into a form that was highly susceptible to Mn^{2+} activation; this activity was lost upon prolonged exposure to protease. Trypsin added together with Mn^{2+} had a synergistic effect, resulting in a remarkable 50-fold overall increase in activity that resisted further tryptic attack for up to 1 h.

Thus, it appears that two distinct forms of the phosphatase are produced by limited proteolysis: first, a Mn^{2+}-activated and trypsin-labile species generated by tryptic attack without divalent cations and, second, a Mn^{2+}-independent, trypsin-resistant species resulting from proteolysis in the presence of Mn^{2+}.

Changes in the Molecular Size of the Phosphatase upon Trypsin/Mn^{2+} Treatment. We wondered whether activation of the M_r 83 000 enzyme by trypsin was accompanied by a change in the size of the protein. This phosphatase, which eluted from Bio-Gel A 0.5 m just ahead of bovine serum albumin at a volume corresponding to M_r 70 000, required Mn^{2+} for activity and was further stimulated by treatment with trypsin plus Mn^{2+} (Figure 4A). In the absence of divalent cations, proteolytic cleavage for as little as 1 min completely converted the enzyme into a M_r 53 000 form that was totally dependent on Mn^{2+} for activity (Figure 4B). In contrast, trypsin treatment in the presence of Mn^{2+} generated a M_r 35 000 species which was no longer stimulated by the divalent cation (Figure 4C); in fact, addition of 1 mM Mn^{2+} to the assay inhibited its activity approximately 50%. Both of these trypsin-generated phosphatases were rather unstable, with a $t_{1/2}$ of about 12 h.

Even after prolonged treatment with trypsin in the absence of Mn^{2+}, no phosphatase activity was detected at M_r 35 000; the major activity peak remained at M_r 53 000. However, in the presence of metal ions, this M_r 53 000 species can be converted to the M_r 35 000 form. When Mn^{2+} was added just prior to the trypsin inhibitor in the experiment described in the legend of Figure 4B, all the activity was recovered at M_r 35 000 rather than at 53 000 (data not shown).

Other divalent cations could substitute for Mn^{2+} in effecting the fragmentation and activation of the enzyme. In fact, Co^{2+} and trypsin resulted in activation 50% greater than that observed with Mn^{2+} and trypsin, and all of the activity appeared at M_r 35 000 (see Table I and Figure 4). Proteolysis in the

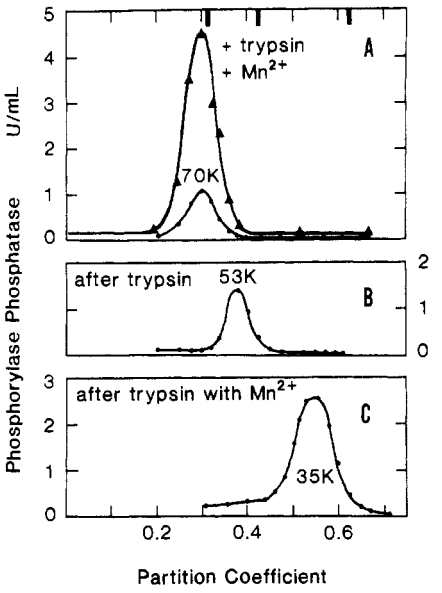

FIGURE 4: Proteolytic conversion of M_r 83 000 phosphatase to species of lower molecular weight. Samples of partially purified enzyme were treated as described below and applied to a 1 × 44 cm column of Bio-Gel A 0.5 m in IDG buffer containing 0.1 M NaCl and 0.01% Brij 35. Five-drop fractions (107 μL) were collected at a flow rate of 4 mL/h. (A) Phosphatase (185 μL of 230 μg of protein/mL) was eluted from the column and assayed plus 1 mM Mn^{2+} (●) or after treatment with trypsin plus Mn^{2+} (▲) as follows: Column fractions were diluted with assay buffer containing 1 mM Mn^{2+}, and after 5 min at 30 °C, trypsin was added to a final concentration of 20 μg/mL. A 6-fold excess of lima bean trypsin inhibitor was added 5 min later, and the samples were assayed for phosphatase activity. (B) Phosphatase (200 μg of protein/mL) was incubated with 10 μg/mL trypsin in assay buffer containing 1 mg/mL bovine serum albumin. Excess trypsin inhibitor was added 1 min later, and a 185-μL sample was applied to the column. Fractions were assayed plus 1 mM Mn^{2+}. (C) Phosphatase (200 μg protein/mL) was incubated for 5 min in assay buffer with 1 mM Mn^{2+} before addition of trypsin (20 μg/mL final concentration). Excess trypsin inhibitor was added 5 min later, and a 185-μL sample was applied to the column; the fractions were assayed without Mn^{2+}. The column was calibrated by using bovine serum albumin (M_r 68 000), ovalbumin (M_r 45 000), and carbonic anhydrase (M_r 29 000) as standards, indicated by vertical bars at the top of frame A.

Table I: Effects of Trypsin and Divalent Cations on the Activity and Molecular Size of Phosphoprotein Phosphatase

	phosphorylase phosphatase act. (units/mL)		
	before trypsin[a]	after trypsin[b]	M_r[c]
control	0.18	7	53 000
Mn^{2+}	2.76	790	35 000
Co^{2+}	3.12	1200	35 000
Mg^{2+}	1.10	275	35 000
Ca^{2+}	0.20	4	35 000

[a] Activity of M_r 83 000 phosphatase was determined after a 5-min preincubation in 1 mM of the indicated cations. [b] Phosphatase was treated with trypsin and cations as described in the legend to Figure 4C and assayed after a 100-fold dilution. [c] Relative size determined by chromatography on a column of Bio-Gel A 0.5 m as described in the legend to Figure 4C.

presence of Mg^{2+} had the same effect on the size of the enzyme, but the extent of activation was significantly less. Almost no activity was recovered after tryptic attack in the presence of Ca^{2+}; what could be detected eluted at a volume corresponding to M_r 35 000 material.

Irreversibility of Trypsin/Mn^{2+} Activation. After activation of phosphorylase phosphatase by protease and Mn^{2+} treatment,

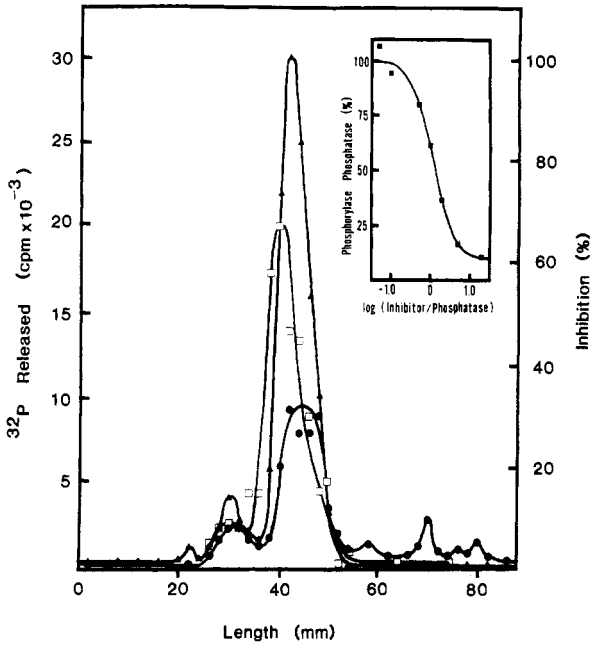

FIGURE 5: Comigration of heat-stable phosphatase inhibitor and phosphatase on nondenaturing polyacrylamide gels. Phosphatase of M_r 83 000 was subjected to electrophoresis at pH 8.3 as described under Materials and Methods. The gels were sliced in 2-mm segments and soaked overnight in 125 μL of 150 mM imidazole, pH 7.5, containing 1 mM dithiothreitol. Slices from one gel were assayed after incubation in 1 mM Mn^{2+} for 5 min and addition of 10 μg/mL trypsin, followed by an excess of trypsin inhibitor 15 min later (▲). Slices from a second gel were assayed after a 20-min incubation in 1 mM Mn^{2+} (●), and those from a third gel were assayed for inhibitory activity as follows: They were heated at 90 °C for 15 min and then incubated for 20 min in 1 mM Mn^{2+} and Mn^{2+}-stimulated M_r 83 000 phosphatase before the addition of substrate (□). All assays were started by mixing 50 μL of 3 mg/mL [^{32}P]phosphorylase *a* with 150 μL of solution containing the gel slice. The inset shows a titration curve of the M_r 35 000 phosphatase, prepared as described in the legend of Figure 4C, with heat-stable inhibitor. The inhibitor was generated by diluting M_r 83 000 enzyme 10-fold in H_2O and boiling for 3 min. The ratio of inhibitor to phosphatase was determined on a w/w basis of the original phosphatase sample.

the enzyme was not inactivated by procedures expected to remove the metal ion. For instance, 100% of the activity was recovered following gel chromatography of the M_r 35 000 fragment (see Figure 4C). This yield takes into account the $t_{1/2}$ of 12 h which is observed when the phosphatase is stored in buffer containing Mn^{2+}. In addition, chelating agents such as EDTA or ATP did not reverse the activation, even after a 45-h incubation with 4 mM reagents. Pyrophosphate (4 mM) did cause 75% inhibition when compared to controls after 5 h at 4 °C, but this inactivation was not reversed by Mn^{2+}. Thus, inhibition by pyrophosphate cannot be attributed to the removal of a metal ion bound to the phosphatase.

Phosphatase Inhibitor Associated with the Enzyme. All enzyme preparations used in this study contain some heat-stable phosphatase inhibitor. Samples of the phosphatase placed in boiling water for 3–5 min inhibited both the Mn^{2+}-activated M_r 83 000 species and its M_r 35 000 fragment; the lower molecular weight enzyme was inhibited 50% at approximately a 1:1 (w/w) ratio (Figure 5, inset). The inhibitor was destroyed by trypsin and, unlike phosphatase inhibitor 1, did not require prior phosphorylation by cAMP-dependent protein kinase for activity (Huang & Glinsmann, 1975; Nimmo & Cohen, 1978). There was no evidence that the inhibitor was present as a contaminant. On the contrary, all data appeared to indicate that it was part of the phosphatase molecule itself: the activities could not be separated in the

course of purification and comigrated on nondenaturing polyacrylamide gels (Figure 5) as well as during isoelectric focusing.

Discussion

This study shows that the phosphorylase phosphatase in muscle extracts exists as an inactive high molecular weight complex that is insensitive to activation by Mn^{2+}. Limited tryptic or chymotryptic attack converts the enzyme to a form that subsequently can be activated 20-fold or more by Mn^{2+}. In the course of its purification a progressive increase in the $+Mn^{2+}/-Mn^{2+}$ activity ratio has been observed (Cohen, 1978; Brautigan et al., 1980). Various preparations were activated to different extents; in several instances the Mn^{2+} sensitivity increased after the enzyme had been concentrated, e.g., by salt precipitation. Since Mn^{2+} activation requires prior proteolysis, it is possible that the precipitation steps favor this reaction by rendering the enzyme more susceptible to contaminating proteases. It has been reported that certain Ca^{2+}-dependent proteases copurify with the phosphatase, even during ion-exchange chromatography (Mellgren et al., 1979). These would prevent isolation of the enzyme in its native, inactive form. Even though tissue extracts are generally treated with protease inhibitors, these have usually not been included in later purification steps. Addition of fresh PMSF throughout the purification has resulted in the recovery of an enzyme that is poorly activated by Mn^{2+} alone but strongly activated by the combined action of Mn^{2+} and trypsin.

We show that an inactive catalytic subunit of M_r 83 000 derived from a M_r 250 000 complex can be cleaved into either of two fragments of M_r 53 000 (inactive in the absence of Mn^{2+}) or 35 000 (active). The M_r 35 000 enzyme can be generated from the M_r 53 000 or from the M_r 83 000 protein, but only if metal ions such as Mn^{2+}, Co^{2+}, or Mg^{2+} are present. Thus, these ions appear to direct the proteolytic attack, perhaps by binding to the phosphatase and inducing a conformational change which exposes a trypsin-sensitive region.

In the past, trypsin, organic solvents, and 6 M urea have been used to disrupt the high molecular weight forms of the phosphatase (Brandt et al., 1974, 1975b; Gratecos et al., 1977; Laloux et al., 1978; Killilea et al., 1979); in each case, an active species of M_r 30 000–35 000 was recovered. Although these enzymes have been purified from several tissues and studied extensively, their interrelationship is still unclear [see review by Lee et al. (1980)]. A M_r 35 000 phosphatase purified from canine cardiac muscle was rapidly inactivated by ATP and pyrophosphate and could be reactivated with Mn^{2+} or Co^{2+} (Hsiao et al., 1978). This is unlike the active fragment described here; it is unaffected by ATP, and its inhibition by pyrophosphate is not reversed by divalent cations. In addition, we have examined the effects of several chemical and protein inhibitors on the M_r 32 000 phosphatase recovered after acetone precipitation (Brautigan et al., 1980) and the M_r 35 000 fragment derived from the M_r 83 000 phosphatase. Preliminary evidence suggests that these two proteins have significantly different properties with respect to their reactivities toward urea, 5,5′-dithiobis(2-nitrobenzoate), and the heat-stable inhibitor associated with the enzyme (data not shown). Thus, it is probable that there are different protein phosphatases, each with a similar trypsin-resistant catalytic core in the M_r 30 000–35 000 range.

A question remains to be answered: namely, what is the structure of the native catalytic subunit? The increase in phosphatase activity accompanying denaturing treatment has generally been attributed to the removal of an inhibitor or a regulatory protein thought to be associated with a M_r 35 000 catalytic subunit. Since the phosphatase described here does indeed contain some heat-stable inhibitor activity, it is possible that it exists as a noncovalent complex between catalytic and inhibitory components. If this were true the complex would have to be an unusually stable one, since these two activities copurify through several chromatographic steps, acetone precipitation at room temperature, high salt (1 M NaCl) treatment, and isoelectric focusing. In addition, when the band containing Mn^{2+}-activated phosphatase was excised from a nondenaturing polyacrylamide gel and the material therein rerun in the presence of dodecyl sulfate, a single band of M_r 83 000 was visualized by Coomassie blue staining (Brautigan et al., 1980). It is, of course, always possible that the 83 000-dalton material merely represents a contaminant and that the enzyme and the inhibitor stain so poorly that they remain undetected.

Phosphatase inhibitor 2 has been implicated in maintaining a skeletal muscle phosphorylase phosphatase referred to as F_C in an inactive state (Yang et al., 1981a). This enzyme is activated by protein factor F_A in the presence of MgATP (Yang et al., 1980; Vandenheed et al., 1980). Manganese ions in the absence of F_A and ATP are able to partially activate F_C (Vandenheede et al., 1981; Yang et al., 1981b). Therefore, one might wonder whether or not the M_r 83 000 phosphatase described here and F_C are the same. Both are sensitive to trypsin and are activated by Mn^{2+} and Co^{2+} but poorly by Mg^{2+}; both react with several phosphoprotein substrates and after activation are inhibited by phosphatase inhibitor 1[2] (Stewart et al., 1981). However, attack of F_C by trypsin in the presence of Mn^{2+} did not increase its activity (Yang et al., 1981b), in contrast to the large activation described above (Figure 3). In addition, activated F_C undergoes rapid and spontaneous inactivation (Yang et al., 1981b), whereas the M_r 83 000 phosphatase, after activation by Mn^{2+} and removal of the metal ion, remains active for days. Recent evidence indicates that F_C copurifies with a modulator protein (phosphatase inhibitor 2) to which it is bound. Removal of the modulator protein produces an enzyme that can be activated by Mn^{2+} (Yang et al., 1981a). Preparations contain different amounts of this modulator, thus accounting for the variable extents of MgATP or of Mn^{2+} activation. According to this interpretation the M_r 83 000 and 53 000 enzymes described here might represent the F_C–modulator complex and free F_C, respectively.

On the other hand, our results appear to be more consistent with a model in which the native phosphatase catalytic subunit is a single polypeptide chain. We propose that the protein would be composed of three structural domains linked by trypsin-sensitive regions: a trypsin-resistant catalytic domain of M_r 35 000, a divalent metal ion binding domain of M_r 18 000, and an inhibitory domain of M_r 17 000. This would account for the overall size of the molecule (as obtained by gel filtration) (Figure 6). Cleavage of the enzyme by brief trypsin treatment without divalent metal ions would eliminate the inhibitory domain, yielding a Mn^{2+}-dependent fragment of M_r 53 000 composed of the catalytic and metal binding regions. Addition of divalent metal ions to either the native subunit or the M_r 53 000 fragment exposes another site to proteolysis, producing an active, metal-independent enzyme of M_r 35 000 representing the catalytic core. This model might explain the variable sensitivity of the M_r 83 000 phosphatase to activation by Mn^{2+} if one assumes that the polypeptide chain

[2] D. L. Brautigan, B. Khatra, T. Soderling, and E. H. Fischer, unpublished results.

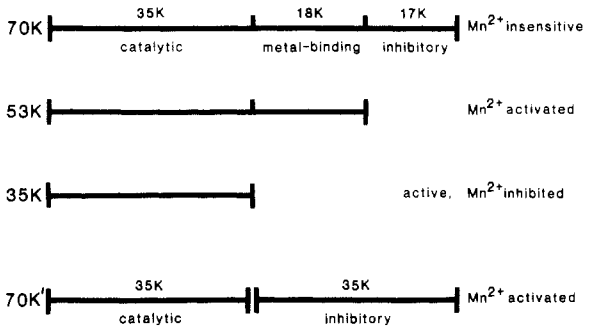

FIGURE 6: Proposed model for the structure of the Mn^{2+}-dependent phosphatase. Relative molecular sizes were determined by gel filtration chromatography.

can be clipped near the middle by endogenous proteases but that the two halves remain attached to one another (represented as 70K′ in Figure 6). This nicked enzyme would be activated by Mn^{2+} or Co^{2+} without requiring dissociation. The proposed metal binding domain would be of approximately the same size as the well-known Ca^{2+}-binding proteins such as troponin C, parvalbumin, and calmodulin [for reviews see Cheung (1980), Klee et al. (1980), and Fuchs (1974)] and would presumably be part of the inhibitory half of the molecule. Inhibitor 2 could originate from this fragment, and one might wonder whether various preparations of the inhibitor might bind divalent metal ions such as Mn^{2+} or Co^{2+}.

It is not known to what extent proteolysis of the phosphatase and the conformational transitions induced by metal ions described here contribute to the activation of the enzyme in vivo. The mechanisms involved in the physiological regulation of phosphatase activity remain an intriguing problem.

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