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EVIDENCE FOR A LATENT FORM OF PROTEIN PHOSPHATASE 1 ASSOCIATED WITH CARDIAC MYOFIBRILS

Keith K. Schlender, Wei Wang, and Susan E. Wilson

Department of Pharmacology
Medical College of Ohio
Toledo, OH 43699

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SUMMARY: Detergent-purified myofibrils from bovine heart contained very little spontaneously active protein phosphatase 1 activity. Phosphatase 1, extracted from the myofibrils by freeze-thawing in the presence of 500 mM KCl, was markedly activated by cobalt/trypsin treatment. Myofibril phosphatase 1 was separated from phosphatase 2A by chromatography on heparin-Sepharose. The phosphatase 1 was isolated in a latent form. Pretreatment with trypsin released free catalytic subunit and increased activity about 25-fold. Addition of cobalt with the trypsin increased activity another 2-fold. The latent myofibril phosphatase 1 did not appear to be the same as previously characterized forms of protein phosphatase 1. We suggest that cardiac myofibril phosphatase 1 contains a unique inhibitory subunit which directs the enzyme to the myofibril and regulates the dephosphorylation of myofibril phosphoproteins.

Reversible phosphorylation of several myofibril proteins including myosin light chain, troponin I, and C-protein is important in the regulation of the contractile activity of cardiac muscle (reviewed in Ref. 1). Although a number of kinases, including cAMP-dependent protein kinase and myosin light chain kinase, have been identified for the phosphorylation of these myofibril proteins (1), much less is known about the phosphatases involved. Mumby, et al. (2) reported that there was very little myosin light chain phosphatase or troponin I phosphatase activity associated with the 18,000xg myofibril-particulate fraction of bovine cardiac muscle. However, they purified two forms of protein phosphatase 2A (nomenclature reviewed in Ref. 3,4) from the soluble fraction which readily dephosphorylated myosin light chain and troponin I. We have reported that the catalytic subunits of either phosphatase 1 or phosphatase 2A can dephosphorylate C-protein phosphorylated by cAMP-dependent protein kinase (5).

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Recently, Chisholm and Cohen (6) established that over half of the phosphatase 1 activity of rabbit skeletal muscle homogenates was associated with purified myofibrils and they suggested that the phosphatase associated with the myofibril was a unique form of phosphatase 1. A smaller but significant fraction of the phosphatase 2A activity was also associated with the skeletal muscle myofibrils. They also reported that a spontaneously active phosphatase 1 associated with rabbit skeletal muscle or bovine cardiac myofibrils could dephosphorylate native myosin (7). These findings prompted us to further examine the protein phosphatases associated with cardiac myofibrils. In this paper, we report that bovine cardiac myofibrils contain substantial amounts of a latent form of protein phosphatase 1 which can be activated by treatment with trypsin.

MATERIALS AND METHODS

Materials: Heat-stable protein phosphatase inhibitor 2 (8), glycogen synthase kinase 3 (9), phosphatase 1_I [ATP-Mg-dependent protein phosphatase] (10), and the catalytic subunit of protein phosphatase 1 (11), were purified from rabbit skeletal muscle. [^{32}P]Phosphorylase *a* (12) and histone H1 (13) were prepared as previously described. [^{32}P]Histone phosphorylated on serine 103 by protein kinase C was prepared as in (14). Chicken heart [^{32}P]C-protein was phosphorylated by cAMP-dependent protein kinase to 2.2 mol ^{32}P/mol protein as already described (5). Trypsin, treated with L-(tosylamido 2-phenyl)ethyl chloromethylketone, and soybean trypsin inhibitor were purchased from Worthington Biochemical Corp.

Preparation of cardiac myofibrils: Detergent-purified myofibrils were prepared from the left ventricle of fresh bovine heart by the method of Solaro, et al. (15). The purified myofibrils were suspended in 20 mM Tris-HCl (pH 7.1 at 22°), 2 mM EDTA, 14 mM β-mercaptoethanol, 650 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 μg pepstatin A/ml and stored at -70°.

Protein phosphatase assays: Protein phosphatase was assayed by measuring the release of [^{32}P]Pi from [^{32}P]phosphorylase *a* as in (16). If there was significant phosphatase 2A activity present, inhibitor-sensitive phosphatase 1 activity was calculated by subtracting activity obtained in the presence of 0.3 units inhibitor 2/ml from activity obtained in the absence of inhibitor 2. The phosphatase 2A assay contained 8 μg histone H1/ml. If there was significant phosphatase 1 activity present, 0.3 units inhibitor 2/ml was included. One unit of protein phosphatase activity removes one μmol ^{32}P/min. Inhibitor 2 was assayed by measuring the inhibition of rabbit skeletal muscle phosphatase 1 catalytic subunit (6). One unit of inhibitor 2 activity was the amount required to inhibit one milliunit phosphatase 1/ml by 50%.

Cobalt/trypsin pretreatment: Latent protein phosphatase activity was activated by cobalt/trypsin pretreatment (17). Aliquots of enzyme samples were preincubated at 30° for 5 min with 0.2 mM $CoCl_2$, then incubated for 10 min with $CoCl_2$ and 20 μg trypsin/ml.

The reaction was stopped with excess soybean trypsin inhibitor. Samples were then diluted and assayed for phosphatase 1 activity.

RESULTS AND DISCUSSION

Approximately 90% of the phosphorylase phosphatase activity of bovine heart homogenates was due to phosphatase 2A (40 milliunits/gram tissue). About 25% of the total phosphatase 2A activity was associated with highly purified detergent-washed myofibrils. There was very little spontaneously active phosphatase 1 in bovine heart extracts or in purified myofibrils. Most of the phosphatase activity of the purified myofibrils could be extracted by freeze-thawing the myofibrils in the presence of 500 mM KCl. When the extracted myofibril protein phosphatases were pretreated with cobalt/trypsin, there was little change in the phosphatase 2A activity but there was a marked increase in the phosphatase 1 activity suggesting that most of the phosphatase 1 was present in a latent form. In a number of myofibril preparations, the latent phosphatase averaged about 5 milliunits activity from myofibrils obtained from one gram of heart muscle.

To further characterize the latent form of myofibril phosphatase 1, we used a heparin-Sepharose column to separate phosphatase 1 from phosphatase 2A (18). Virtually all of the phosphatase 2A activity was in the flow-through fraction of the column (Figure 1). Phosphatase 1 bound to the column and was eluted by buffer containing 500 mM KCl. Nearly all of the phosphatase 1 was in an inactive form which was activated by preincubation of the fractions with cobalt/trypsin (Figure 1). As shown in Figure 2, preincubation with cobalt alone had no effect upon phosphatase activity. Preincubation with trypsin rapidly activated the enzyme and revealed about half of the activity obtained by pretreatment with both cobalt and trypsin (Figure 2). The phosphatase activity of the cobalt/trypsin treated myofibril enzyme had virtually the same sensitivity to inhibition by inhibitor 2 as purified rabbit skeletal muscle protein phosphatase catalytic subunit (Figure 3). When cardiac myofibril C-protein phosphorylated by cAMP-dependent protein kinase was used as the substrate, the activity of the myofibril phosphatase 1 was also latent until pretreated with cobalt/trypsin (Figure 4). We previously reported that the catalytic subunit of rabbit skeletal muscle phosphatase 1 dephosphorylated only a phosphothreonine residue of [^{32}P]C-protein which was on the tryptic phosphopeptide designated T3 (5). Tryptic phosphopeptide maps showed that cobalt/trypsin activated myofibril

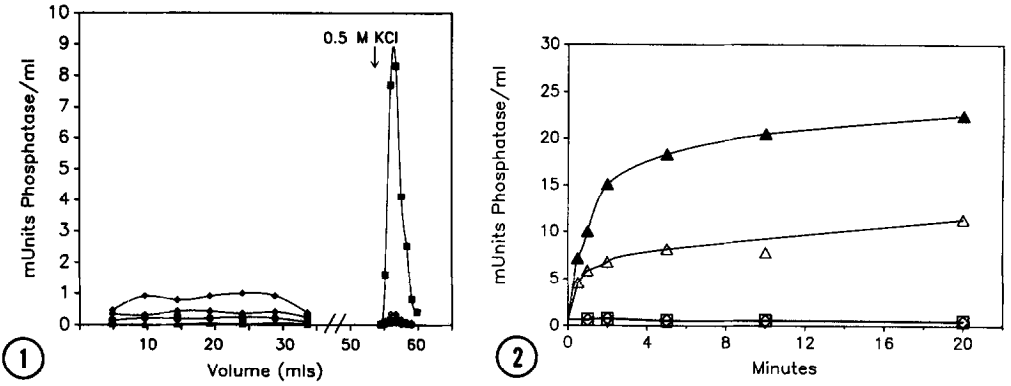

Figure 1. Separation of bovine heart myofibril protein phosphatase 1 and phosphatase 2A by heparin Sepharose chromatography. A freeze-thaw extract of myofibrils from 6 grams of cardiac muscle, prepared as described in Materials and Methods, was dialyzed at 4° against 20 mM Tris (pH 7.1 @ 22°), 0.1 mM EDTA, 5% glycerol, and 1 mM dithiothreitol (buffer A). After clarification by centrifugation, the supernatant was applied to a heparin Sepharose column (0.9 x 2.4 cm) that was equilibrated in buffer A containing 100 mM KCl. The column was washed with equilibration buffer and then eluted with buffer A containing 500 mM KCl. Fractions were assayed for phosphorylase phosphatase activity [(inhibitor sensitive (▲), inhibitor insensitive (●), histone H1 stimulated (◆), and activity after cobalt/trypsin pretreatment (■)]. Fractions were also assayed for histone H1 phosphatase activity (◇) as previously described (22).

Figure 2. Activation of bovine heart myofibril protein phosphatase 1 . The myofibril protein phosphatase was prepared through the 500 mM fraction described in Figure 1. The enzyme was preincubated for 5 min without cobalt and then incubated with no additions (□) or with 20 μg trypsin/ml (△). A second sample of enzyme was preincubated with 0.2 mM cobalt and then incubated with no further additions (◇) or with the addition of 20 μg trypsin/ml (▲). At the indicated times samples were removed and stopped with soybean trypsin inhibitor, and phosphatase activity was determined as described in Materials and Methods.

phosphatase also selectively dephosphorylated the phosphothreonine of peptide T3 (data not shown).

There are two well characterized forms of protein phosphatase 1. Phosphatase 1_I (also referred to as the ATPMg-dependent protein phosphatase) is a latent form which is composed of a 37 kDa catalytic subunit and a 23 kDa inhibitor 2 subunit in a 1:1 molar ratio (4). Phosphatase 1_I has an apparent molecular weight on gel filtration of approx. 250,000 (4). The holoenzyme can be activated by a manganese/trypsin treatment or by a cobalt/trypsin treatment similar to that described in this paper. Trypsin degrades the inhibitor 2 subunit and the divalent cation activates the resulting free catalytic subunit (4). Phosphatase 1_I can also be activated by phosphorylation with glycogen synthase kinase 3 on a threonine residue in the inhibitor 2 subunit (4). Phosphatase 1_G is a

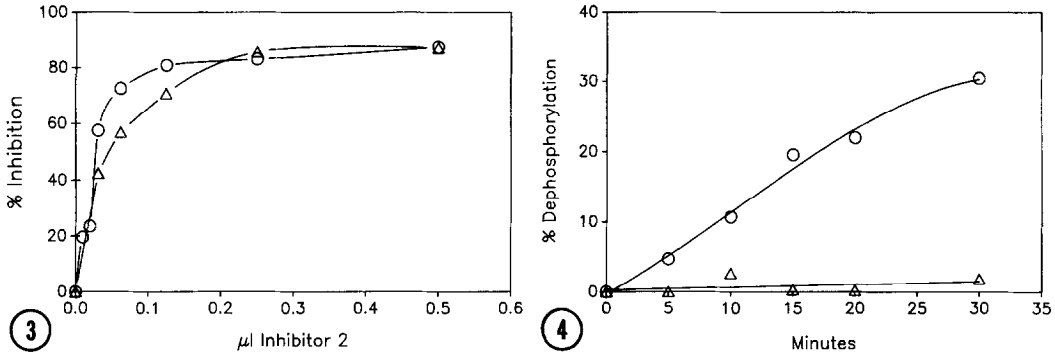

Figure 3. Effect of inhibitor 2 on the activity of cobalt/trypsin activated myofibril protein phosphatase 1 and the activity of rabbit skeletal muscle protein phosphatase 1 catalytic subunit. The myofibril protein phosphatase 1 was prepared through the 500 mM fraction described in Figure 1 and the skeletal muscle catalytic subunit was prepared as noted in Materials and Methods. The myofibril phosphatase was activated by cobalt/trypsin pretreatment. The phosphatase activity of the pretreated myofibril enzyme (O) and the skeletal muscle catalytic subunit (Δ) was determined as described in Materials and Methods with increasing amounts of inhibitor 2 added.

Figure 4. Dephosphorylation of [^{32}P]C-protein by myofibril protein phosphatase 1. The myofibril protein phosphatase was prepared through the 500 mM fraction described in Figure 1. The enzyme was preincubated without (Δ) or with cobalt/trypsin (O) and then assayed for C-protein phosphatase activity as previously described (5).

spontaneously active form which is associated with the glycogen particle (19,20). It is composed of a 37 kDa catalytic subunit and 161 kDa glycogen-binding subunit (1:1 molar ratio). The enzyme has an apparent molecular weight on gel filtration of >250,000 (19). Several properties of the myofibril protein phosphatase 1 purified through the heparin-Sepharose column were examined to determine if they were similar to phosphatase 1_I or phosphatase 1_G. The activity of myofibril phosphatase 1 was not changed when the enzyme was incubated as previously described (21) with glycogen synthase kinase 3 and ATP-Mg (data not shown). Under identical conditions, the activity of skeletal muscle phosphatase 1_I was increased about 4-fold. The preparation was also assayed for heat-stable inhibitor activity. The preparation contained only a small amount of phosphatase inhibitor activity (data not shown). Even if it were assumed that all of the inhibitory activity in the preparation was due to inhibitor 2, there was much less than stoichiometric amounts of inhibitor 2. A sample of the myofibril phosphatase 1 was applied to a Superose 12 HPLC column and the eluted fractions were

assayed for phosphatase activity with and without pretreatment with cobalt/trypsin. Phosphatase 1 activity was dependent upon cobalt/trypsin treatment and the major activity peak eluted with an apparent Mr of about 70,000 (data not shown), a value much smaller than that reported for either phosphatase 1_I or 1_G (4, 19). When the phosphatase was treated with cobalt/trypsin before gel filtration, the major phosphatase activity eluted from the column with an apparent Mr of approx. 34,000. Thus, our evidence is consistent with the recent suggestion that phosphatase 1 associated with myofibrils is not identical to either phosphatase 1_I or 1_G (6,7). However, unlike Chisholm and Cohen (6,7) we found nearly all of the phosphatase 1 to be in a latent form.

Mumby et al. (2) reported that nearly all of the phosphatase activity of bovine heart muscle was due to phosphatase 2A. However, Chisholm and Cohen (7) reported that both skeletal muscle and cardiac muscle myofibrils contained phosphatase 1 activity. Our results indicate that there are substantial amounts of protein phosphatase 1 associated with bovine cardiac myofibrils, but most of it is in a latent form. Myofibril phosphatase 1 activity was markedly increased by preincubation with trypsin and the activity was only increased another two-fold when cobalt ion was included in the preincubation. This would suggest that the holoenzyme form of cardiac myofibril phosphatase 1 contains an inhibitory subunit which was destroyed by trypsin treatment. Since there was only a small further increase in activity when cobalt was added, it would appear that, unlike phosphatase 1_I (4), the liberated catalytic subunit did not require the metal ion to convert it into an active conformation. We suggest that the latent myofibril protein phosphatase 1 is composed of a catalytic subunit and a specific inhibitory subunit. The myofibril inhibitory subunit may direct the enzyme to the myofibril and confer upon it properties which are important for the regulation of the dephosphorylation of myofibril proteins. Further studies on purified myofibril phosphatase 1 will be required to determine the properties of the myofibril-specific regulatory subunit and to establish how the phosphatase is activated *in vivo*.

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