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HISTONE H1-STIMULATED PHOSPHORYLASE PHOSPHATASE FROM RABBIT SKELETAL MUSCLE

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A major rabbit skeletal muscle phosphorylase phosphatase activity which is markedly stimulated by histone H1 has been resolved from inhibitor-sensitive phosphorylase phosphatase (type-1 phosphatase), glycogen synthase kinase 3-activated phosphatase, phosphatase heat-stable inhibitor proteins, and alkaline phosphatase activity by various purification techniques. Evidence is presented that this phosphatase is a high-molecular weight form of a type-2 phosphatase. Our data suggest that this phosphatase may be regulated by histone H1, protamine or analogous polycationic compounds.

Cohen and his colleagues have classified the catalytic subunits of the major phosphorylase phosphatases in various mammalian tissues as type-1 and type-2 phosphatases (1). Type-1 phosphatases can be inhibited by heat-stable inhibitor proteins (2,3) and are relatively insensitive to inhibition by ATP. Type-2 phosphatases are sensitive to inhibition by 0.1 mM ATP but are not inhibited by the heat-stable inhibitor proteins. We recently reported that a rabbit renal cortical phosphorylase phosphatase preparation was activated by a heat-stable protein from the same source (4). Subsequently, we found that the activator was histone H1 (5). In this communication, we report that a major phosphorylase phosphatase activity in rabbit skeletal muscle extracts is markedly activated by histone H1. This phosphatase appears to be a high molecular weight form of type-2 phosphatase.

MATERIALS AND METHODS

Materials: DEAE-Cellulose 52 (Whatman), DEAE-Sepharose CL-6B (Pharmacia), bovine liver catalase, cadaverine dihydrochloride, putrescine dihydrochloride, spermine tetrahydrochloride, spermidine trihydrochloride, protamine base, and disodium ATP were obtained from Sigma. Bio-Gel A-1.5m was purchased from Bio Rad. Mixed histones were obtained from Aldrich. Calf thymus histones H2b and H4, and horse spleen ferritin were obtained from Boehringer Mannheim.
Buffers: Column chromatography was routinely performed utilizing buffer A: 50 mM imidazole-HCl, 0.2 mM EGTA, 2 mM dithiothreitol, pH 7.4. Buffer B was the same as buffer A, except that it contained 5 mM EGTA.

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Enzymes and other proteins: Phosphorylase a was prepared from crystallized phosphorylase b and γ-^{32}P-ATP (6). Rabbit muscle protein phosphatase inhibitor-1 was prepared to apparent homogeneity by a modification of the procedure in (7). Core histones were prepared from mixed histone as previously described (5). Histone H1 was prepared from calf thymus by the method of Sanders (8). Rabbit muscle protein phosphatase-1 catalytic subunit (type I phosphatase) was purified to near-homogeneity by a procedure which will be described in detail elsewhere (R. Mellgren, manuscript in preparation). The purification involved batch chromatography on DEAE-cellulose (0.1-0.4 M NaCl fraction) followed by Bio-Gel A-1.5m, Phenyl-Sepharose and Sephadex G-200 chromatography steps. The catalytic subunit had a molecular weight of 31,000. It could be inhibited >90% by inhibitor-1, but not by 0.1 mM ATP. H1-stimulated phosphatase was obtained as a side-product in this protocol. In contrast to phosphatase-1 catalytic subunit, which was eluted from the Phenyl-Sepharose column by a combined decreasing ammonium sulfate (1 M to 0 M) and increasing dioxane (0 to 10%) gradient, H1-stimulated phosphatase remained on the column and was eluted with 4 M urea. This urea fraction was extensively dialyzed and stored at -20°C in the presence of buffer A containing 50% glycerol, 10 mM benzamidine, 1 µg/ml pepstatin A and 10 µg/ml leupeptin. Glycogen synthase kinase 3 was a generous gift from Dr. T. Soderling of Vanderbilt University.

Analytical methods: Phosphorylase phosphatase was assayed as previously described (6). When histone H1 was added, it was present at a concentration of 7.2 µg/ml unless otherwise stated. A unit of phosphorylase phosphatase produces one nMole of phosphorylase b dimer per minute. Heat-stable phosphatase inhibitor was assayed as previously described (9). A unit of inhibitor activity produces a 10% inhibition of 1 milliunit of phosphatase-1 catalytic subunit.

RESULTS

Preparation of a histone H1-stimulated phosphatase: Chromatography of a 10,000 x g supernatant from rabbit muscle on DEAE-cellulose revealed three major regions of phosphorylase phosphatase activity eluting at 120 mM, 170 mM, and 260 mM chloride (Fig. 1A). These activity peaks will be referred to as peaks I, II, and III respectively. When histone H1 was included in the phosphorylase phosphatase assay, peak I phosphatase was fully inhibited while peak III phosphatase was activated several-fold (Fig. 1A). The H1-stimulated phosphatase activity was well separated from the heat-stable phosphatase inhibitory activity. The peak III phosphatase had a Stokes radius of 5.5 nm (apparent Mr 260,000) on Bio-Gel A-1.5m chromatography (not shown).

The H1-stimulated phosphatase could be partially resolved from H1-inhibited phosphatase activity by Phenyl-Sepharose chromatography (see Methods). The concentrated urea fraction contained a major H1-stimulated phosphatase activity which chromatographed as a 5.5 nm form on Bio-Gel A-1.5m (Fig. 1B) and displayed approximately the same degree of stimulation by histone H1 as the peak III phosphatase from DEAE-cellulose chromatography. The urea

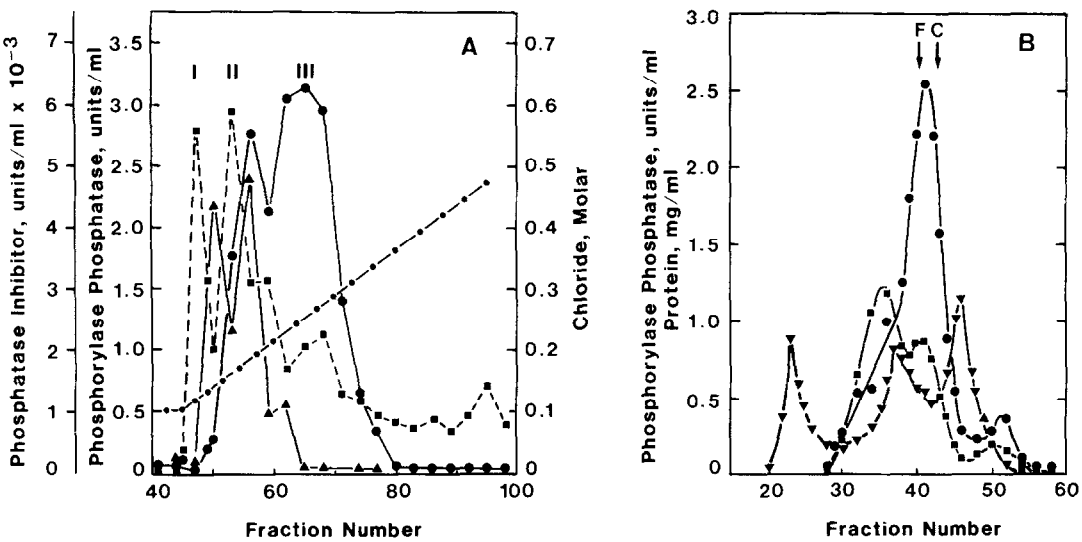

Figure 1. A. DEAE-Cellulose chromatography of a rabbit muscle low-speed supernatant. Ten grams of fresh rabbit skeletal muscle was homogenized in 20 ml of ice-cold buffer B containing 250 mM sucrose and centrifuged for 20 min at 10,000 x g at 4°C. A 15 ml sample was applied to a 1.6 x 10 cm column of DEAE-cellulose equilibrated in buffer B and washed successively with one column volume of buffer B and three column volumes of buffer B containing 0.10 M NaCl. A linear gradient of 0.10-0.60 M NaCl in 200 ml of buffer B was applied and 2 ml fractions were collected. The fractions were assayed for phosphorylase phosphatase activity in the absence (■) or presence (●) of 7.2 µg/ml of histone H1. Aliquots of the fractions were heated at 100°C for 5 min, centrifuged to remove denatured protein, and assayed for phosphatase inhibitor activity (▲). B. Bio-Gel A-1.5m chromatography of Phenyl-Sepharose purified H1-stimulated phosphatase. A 0.5 ml sample of the concentrated urea fraction from Phenyl-Sepharose chromatography of a rabbit muscle phosphatase preparation (see Methods) was applied to a Sephadex G-25 column in buffer A containing 0.10 M NaCl to remove the glycerol. The sample in a volume of ca. 0.7 ml was applied to a 1.0 x 45 cm column of Bio-Gel A-1.5m equilibrated with buffer A containing 0.10 M NaCl. Fractions of 0.64 ml were collected and assayed for phosphorylase phosphatase activity in the absence (■) or presence (●) of 7.2 µg/ml of histone H1. Protein concentrations (▼) were determined by the method of Bradford (17). The marker proteins ferritin (F, 5.85 nm Stokes radius) and catalase (C, 5.15 nm Stokes radius) were chromatographed separately.

fraction contained no detectable heat-stable phosphatase inhibitor activity. The most active H1-stimulated phosphatase fractions from Bio-Gel chromatography of the urea fraction were pooled. Except where noted this pooled fraction was used for the studies described below.

The H1-stimulated phosphatase activity was relatively stable during storage at 5°C, losing less than 10% of its original activity after 14 days. However, activity measured in the absence of histone H1 decreased by 81% over the same time. Thus, with ageing the preparation became more dependent on histone H1 for activity . Ageing of a phosphatase preparation while bound to

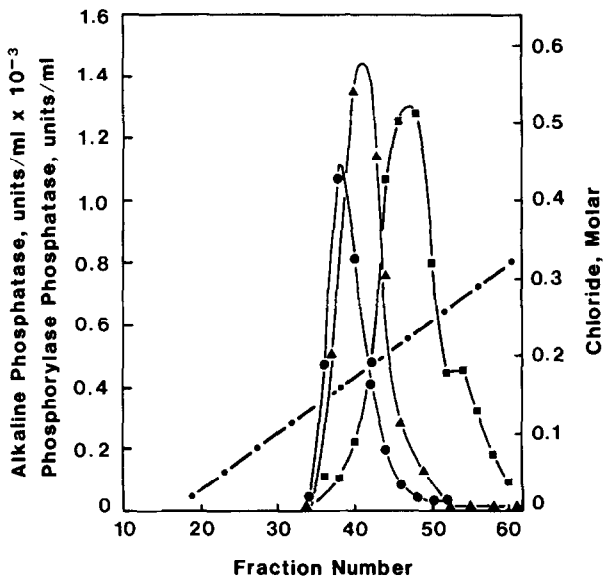

Figure 2. DEAE-Sepharose chromatography of an aged preparation of rabbit muscle phosphatase. A rabbit skeletal muscle extract, prepared as described in Figure 1, was applied to a DEAE-cellulose column and washed with 3 to 4 bed volumes of Buffer A containing 0.1 M NaCl. The column was left at 2-5°C for 7 days, and then eluted with Buffer A containing 0.4 M NaCl. The pooled protein peak was concentrated by dialysis against a 20% solution of polyethylene glycol (Mr 20,000) in buffer A and chromatographed on a Bio-Gel A-1.5m column. The tubes eluting at ca. 5.5 nm Stokes radius were pooled, dialyzed against buffer A containing 50% glycerol, and stored at -20°C. A 60 ml sample was dialyzed against buffer A for several hours to remove glycerol. The dialyzed sample containing 34 units of H1-stimulatable phosphatase was applied to a 2.6 x 10 cm column of DEAE-Sepharose equilibrated in buffer A. Phosphatase activities were eluted with a linear gradient of 0 to 0.50 M NaCl in 500 ml of buffer A. Fractions of 5.5 ml were collected and assayed for phosphorylase phosphatase activity in the presence of histone H1 (■). Samples of the fractions were incubated with 1 mM Mg-GTP and glycogen synthase kinase 3 at 30°C for 30 min. These samples were then assayed for phosphorylase phosphatase in the absence of added histone H1 (▲). The fractions were also assayed for alkaline phosphatase activity (●) by the method of Li et al. (18). There was no detectable phosphorylase phosphatase activity in any of the fractions nor in the sample applied to the column, when assayed without preincubation with GTP and synthase kinase, or in the absence of histone H1.

DEAE-cellulose resulted in a totally H1-dependent phosphatase. Chromatography of this preparation on DEAE-Sepharose resolved the H1-dependent activity from glycogen synthase kinase 3-modulated phosphatase (10,11), and alkaline phosphatase activities (Fig. 2). This aged phosphatase preparation retained its high molecular weight on gel filtration (not shown).

Characterization of the histone H1-stimulated phosphatase: The H1-stimulated phosphatase had maximum activity at 4-8 µg histone H1/ml (Fig. 3). The enzyme sample used in this experiment had aged for several days.

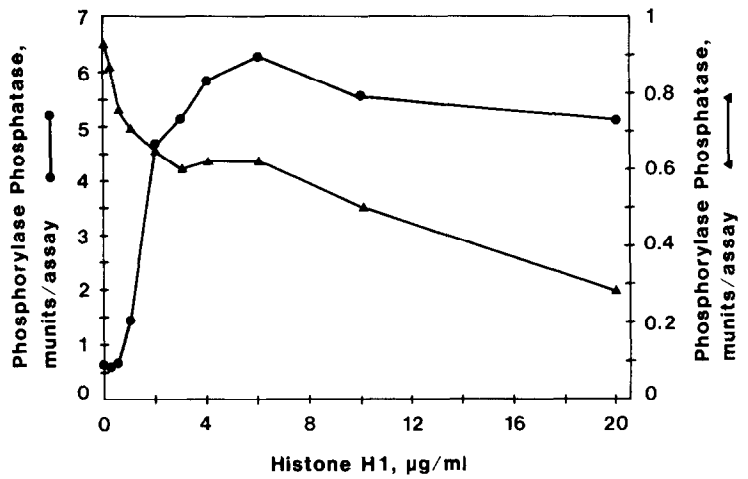

Figure 3. The effect of histone H1 concentration on the activities of rabbit muscle H1-stimulated phosphatase (●) and the purified catalytic subunit of rabbit muscle phosphatase-1 (▲).

Thus, the extent of activation by histone H1 was greater than observed in the experiments presented in Fig. 1. The H1-stimulated phosphatase was not inhibited by 100 units of purified rabbit muscle inhibitor-1/ml. However, it could be completely inhibited by 0.1 mM ATP. The same results were obtained whether the phosphatase was assayed alone or in the presence of 4.0 µg/ml histone H1. In the absence of histone H1 the phosphatase was activated 30% to 50% by 1 to 5 mM Mn^{2+}. Activation was not observed with Ca^{2+} or Mg^{2+}. The same concentrations of Mn^{2+} effectively reversed histone H1 activation of the phosphatase. Freezing and thawing the phosphatase in the presence of 0.2 M mercaptoethanol converted the phosphatase to a 2.3 nm form as determined by Bio-Gel A-1.5m chromatography. This low molecular weight form of the phosphatase was activated by histone H1, inhibited by ATP, and it was not inhibited by inhibitor-1. The H1-stimulated phosphatase could be activated by protamine as well as histone H1 (Table I). Other histones and the polyamines were either inhibitory or had no significant effect. Inhibition of phosphorylase phosphatase activities by polyamines has been reported elsewhere (12,13). In contrast, purified rabbit muscle phosphatase-1 was inhibited by very low concentrations of histone H1 (Fig. 3). Histone H1 did not influence the ability of inhibitor-1 to inhibit this phosphatase.

Table I: The Effect of Various Histones, Protamine, and Polyamines on the H1-stimulated Phosphorylase Phosphatase.

Addition	Concentration µg/ml	Relative Phosphatase Activity
None	-	(100)
Histone H1	4	670
Histone H2b	4	98
	40	107
Histone H4	4	93
	40	25
Core Histones	4	103
	40	65
Mixed Histone	4	163
	40	86
Protamine	4	222
	50	30
Spermine	4	65
	101 (0.5 mM)	51
Spermidine	4	86
	72 (0.5 mM)	59
Cadaverine	4	103
	51 (0.5 mM)	86
Putrescine	4	94
	44 (0.5 mM)	84

DISCUSSION

The rabbit renal phosphatase employed in earlier studies from our laboratories (4,5) was a relatively impure preparation which may have contained several different phosphatase activities. Therefore, the present studies were designed to characterize the major phosphorylase phosphatase activity in rabbit muscle which is stimulated by histone H1. The histone H1-stimulated phosphatase described here was activated slightly by Mn^{2+}, inhibited by low concentrations of ATP, and not inhibited by high concentrations of inhibitor-1. The phosphatase did not contain heat-stable inhibitor activity. Thus, the activation by histone H1 did not appear to be the result of reversal of a heat-stable inhibitor. This was confirmed by the observation that histone H1 does not reverse inhibition of purified phosphatase-1 catalytic subunit by inhibitor-1, and by the resolution of the H1-stimulated phosphatase from

glycogen synthase kinase 3-activated phosphatase (a type-1 phosphatase : inhibitor-2 complex - ref. 11). These results suggest that the H1-stimulated phosphatase is a type-2 phosphatase in the classification scheme of Ingebritsen, et al. (1). The H1-stimulated phosphatase was larger than other type-2 phosphatases (14). It had the same Stokes radius as the classic phosphatase H holoenzyme of Lee et al. (15). The latter may be a mixture of type-1 and type-2 phosphatases (1). Thus, the exact classification of the H1-stimulated phosphatase remains to be established, and it may represent a new form of type-2 protein phosphatase. At any rate, in the presence of histone H1, a substantial amount of latent phosphorylase phosphatase activity present in rabbit muscle extracts is revealed (Fig. 1A). In our earlier studies, histone H1 appeared to activate an inhibitor-sensitive rabbit renal cortex phosphatase (4). We have since reinvestigated this preparation and find that histone H1 appears to dramatically activate a small amount of type-2 phosphatase in the preparation. Histone H1 was a potent inhibitor of purified phosphatase-1 catalytic subunit (Fig. 3).

In a simultaneous but independent study, DiSalvo et al. (16) have characterized an H1-stimulated phosphatase from bovine vascular smooth muscle. The latter enzyme appears to be very similar in properties to the rabbit skeletal muscle enzyme. Thus, in two different muscle tissues latent phosphatases exist which may be regulated by polycationic macromolecules.

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