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PURIFICATION OF A LATENT PROTEIN PHOSPHATASE FROM RABBIT SKELETAL MUSCLE

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A high molecular weight protein phosphatase (M_r=260K) has been isolated from rabbit skeletal muscle. The enzyme has a very low activity towards phosphorylase *a* isolated from the same tissue, but its activity towards this substrate is stimulated several fold after dissociation by 2-mercaptoethanol treatment. The purified phosphatase shows one major protein staining band on non denaturing polyacrylamide gel electrophoresis, and contains four subunits with molecular weights of 95K, 75K, 65K and 38K. The catalytic activity resides in the M_r=38K subunit and is not sensitive to inhibition by the heat stable protein phosphatase inhibitor-1 or modulator protein. Polyamines stimulate the holoenzyme in a dose dependent, biphasic manner, but inhibit the activity of the dissociated M_r=38K catalytic subunit.

Rabbit skeletal muscle contains several types of protein phosphatases which dephosphorylate phosphorylase *a* at a considerable rate (1,2). Several reports (3-7) have mentioned the existence of inactive or latent protein phosphatase enzymes whose activity towards phosphorylase *a* as a substrate can be greatly enhanced by specific treatments such as freeze-thawing in the presence of 2-mercaptoethanol, limited proteolysis, or addition of stimulatory factors such as lysine rich histones or polylysine. None of these enzymes has however been purified to near homogeneity. Although their physiological role as phosphorylase phosphatases is not at all certain, their potential activity towards this substrate is quite remarkable. The present report describes the purification and the subunit structure of a high molecular weight protein phosphatase (M_r=260K) whose catalytic subunit (M_r=38K) has a phosphorylase phosphatase activity of 2400 units per mg protein.

MATERIALS AND METHODS

Most materials and procedures, including the preparation of ^{32}P-labeled phosphorylase *a*, polylysine-Sepharose 4B

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and phosphatase modulator have been described in previous reports (8,9).

The potential activity of the latent protein phosphatase was measured either by the addition of 50 µg/ml of histone H1 (Sigma type V-S) into the phosphorylase phosphatase assay, or after freeze-thawing of the enzyme in the presence of 0.2 M 2-mercaptoethanol, as indicated in the text. The phosphatase activity unit is defined as the amount of enzyme which releases 1 nmol of (^{32}P) phosphate/min at 30°C from ^{32}P-labeled phosphorylase *a* (2 mg/ml). The assay time is 5 min.

Sucrose density gradient centrifugations were performed at 4°C for 15 hrs at 40,000 rpm in a Beckman SW 50.1 rotor. ^{14}C-ovalbumin (M_r=43K) was used as internal marker. 5-20% sucrose gradients were used made in 20 mM Tris-HCl, 1 mM dithiothreitol pH 7.0 (buffer A).

RESULTS AND DISCUSSION

An extract was prepared from 1.5 kg of rabbit skeletal muscle as in (8) and directly absorbed batchwise onto one liter of DEAE Sephadex A-50 equilibrated in buffer A. After extensive washing with 0.2 M NaCl in buffer A, the resin was packed into a (5x40 cm) column and further washed with this buffer until the absorbance at 280 nm was below 0.1. The absorbed proteins were then eluted with a linear salt gradient (2x500 ml) from 0.2 M to 0.5 M NaCl in buffer A, and assayed for phosphorylase phosphatase activity in the presence or absence of 50 µg/ml of histone H1 (Fig. 1A). A histone H1-stimulated phosphatase activity eluted late in the gradient and was pooled as indicated, concentrated by precipitation with solid ammonium sulfate (30-50% saturation) and applied to an Ultrogel ACA-34 column (2.5x90 cm). The latent phosphatase eluted just in front of the marker protein catalase, which is indicative of a molecular weight of about 260K (Fig. 1B). The histone H1-stimulated fractions were directly absorbed onto a (0.9x10 cm) column of polylysine-Sepharose 4B, which was extensively washed with buffer A containing 0.2 M NaCl, and the phosphatase was eluted with a linear salt gradient (2x50 ml) from 0.2 M to 0.6 M NaCl in buffer A (Fig. 1C). The latent phosphatase eluted in a sharp symmetrical peak, was concentrated by dialysis against 10% polyethylene glycol (M_r=10,000) and applied to sucrose density gradients. The enzyme migrated with an apparent molecular weight of about 120K and was again concentrated by dialysis against polyethylene glycol (Table I).

Electrophoresis on 6% non-denaturing polyacrylamide gels showed one major protein staining band containing all of the

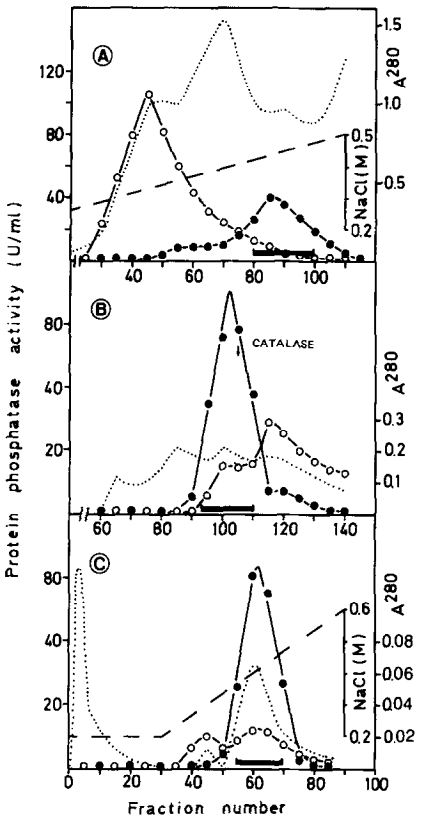

Fig. 1. *Purification of rabbit skeletal muscle latent protein phosphatase.*
(A) DEAE Sephadex A-50 gradient elution profile; (B) Ultrogel ACA 34 elution with marker protein catalase (250K); (C) Polylysine-Sepharose 4B gradient elution. Assays for phosphorylase phosphatase activity were performed in the presence (●) or absence (○) of histone H1 as outlined in the text. The dotted line indicates the absorbance at 280 nm.

Table I

Purification of a latent protein phosphatase from rabbit skeletal muscle

Step	Total protein	Total activity[1]	Specific activity
	mg	units	units/mg
1. DEAE Sephadex A-50	140	4400	32
2. Ultrogel ACA 34	6	1800	300
3. Polylysine Sepharose 4B	1.3	900	700
4. Preparative sucrose gradient centrifugation	1.0	800	800
5. 2-mercaptoethanol treatment	0.25	600	2400

[1] Phosphorylase phosphatase activity measured after maximal stimulation by histone H1 except for step 5 enzyme.

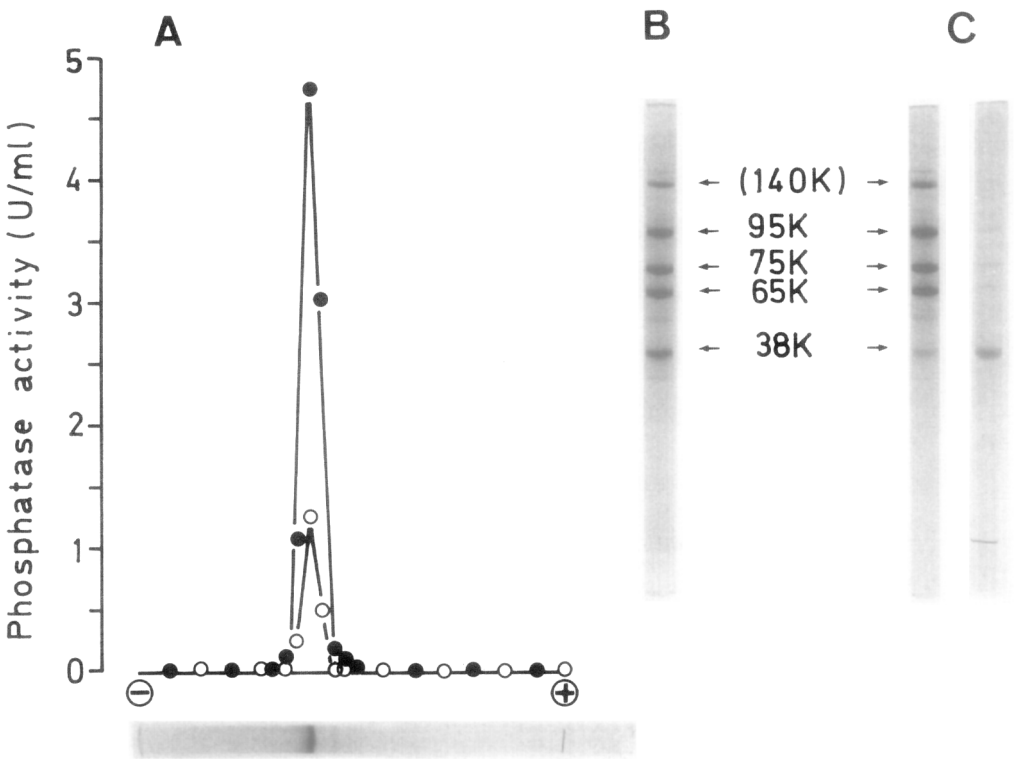

Fig. 2. *Polyacrylamide gel electrophoresis of the purified "native" and "2-mercaptoethanol treated" latent phosphatase.*
(A) 6% non-denaturing polyacrylamide gels: one gel was stained for proteins and a second gel sliced, the soluble proteins extracted in buffer A, and the phosphatase activity measured in the presence (●) or absence (○) of histone H1.
(B) and (C) 7.5% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate: (B), native enzyme; (C) 2-mercaptoethanol treated enzyme: precipitated and supernatant proteins respectively. Molecular weight marker proteins were as in (8).

latent phosphatase activity and also a minor protein contaminant without any activity (Fig. 2A). Electrophoresis in the presence of sodium dodecyl sulfate consistently showed four equally dense protein stained bands with respective molecular weights of 95K, 75K, 65K and 38K, and a M_r=140K band which varied in intensity with each preparation, and therefore does not seem to be related to the enzyme activity (Fig. 2B).

The inactive enzyme had a specific activity of about 160 U/mg when measured as phosphorylase phosphatase without the addition of histone H1. Maximal stimulation by histone H1 produced a 5 fold increase in the phosphorylase phospha-

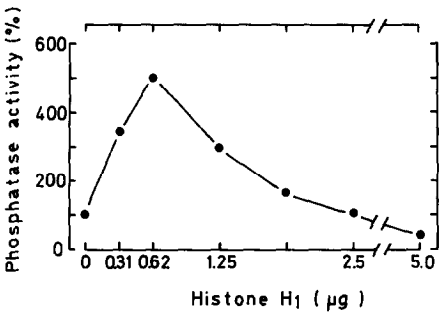

Fig. 3. *Histone H1-mediated stimulation of the latent protein phosphatase.*
The amount of histone H1 used is expressed as μg per assay; the 100% phosphatase activity represent the activity of 50 ng of latent protein phosphatase measured in the absence of histone H1.

tase activity. The stimulation by histone H1 was dose dependent and biphasic (Fig. 3). Freezing and thawing of the preparation in the presence of 0.2 M 2-mercaptoethanol produced a similar activity increase. About 75% of the protein was precipitated by this treatment and could be removed from the soluble protein which exhibited high enzymatic activity in the absence of histone H1: this enzyme was either not affected or inhibited by all concentrations of histone H1 tested. The precipitated protein was readily soluble in buffer A, and contained some residual enzyme activity (20%). Readdition of this fraction to the high specific activity enzyme (i.e. to the 2400 U/mg soluble fraction) did not inhibit the activity. Electrophoresis in the presence of sodium dodecyl sulfate revealed that the soluble, active enzyme fraction contained the M_r=38K subunit as major protein, while the precipitated proteins also contained the other subunits (Fig. 2C). The dissociated enzyme migrated in sucrose density gradients as a M_r=40K protein (not shown).

The latent phosphorylase phosphatase enzyme, before or after stimulation by histone H1 or 2-mercaptoethanol treatment was completely insensitive to inhibition by the phosphatase modulator or phosphorylated inhibitor-1 (not shown). The enzyme did not contain any measurable modulator activity. It was noted that 2-mercaptoethanol treatment performed in the early stages of purification gave poor recoveries of the (stimulated) enzyme activity and was therefore not used to localize the latent enzyme throughout the isolation procedure. The isolated enzyme was very susceptible to limited

proteolysis by trypsin, which irreversibly destroyed the catalytic activity. The histone H1 proteins only stimulated the latent protein phosphatase and strongly inhibited the rabbit skeletal muscle multisubstrate protein phosphatase described in (10).

The physiological significance of this protein phosphatase, as a potential phosphorylase phosphatase and the regulatory role of lysine rich histones or other naturally occuring polyamines in the activation process remains to be established. The amount of phosphorylase phosphatase activity formed in this way is less than 10% of the total phosphorylase phosphatase present in rabbit skeletal muscle.

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REFERENCES

1. Li, H.C. (1982) Curr. Top. Cell. Regul. *21*, 129-174.
2. Merlevede, W., Vandenheede, J.R., Goris, J. and Yang, S.-D. (1984) Curr. Top. Cell. Regul. *23*, 177-215.
3. Laloux, M. and Hers, H.G. (1979) FEBS Lett. *105*, 239-243.
4. Killilea, S.D., Mellgren, R.L., Aylward, J.H., Metieh, M.E. and Lee, E.Y.C. (1979) Biochim. Biophys. Acta *193*, 130-139.
5. Ingebritsen, T.S., Foulkes, J.G. and Cohen, P. (1983) Eur. J. Biochem. *132*, 263-274.
6. Di Salvo, J., Waelkens, E., Gifford, D., Goris, J. and Merlevede, W. (1983) Biochem. Biophys. Res. Commun., in press.
7. Wilson, S.E., Mellgren, R.L. and Schlender, K.K. (1983) Biochem. Biophys. Res. Commun. *116*, 581-586.
8. Yang, S.-D., Vandenheede, J.R., Goris, J. and Merlevede, W. (1980) J. Biol. Chem. *255*, 11759-11767.
9. Yang, S.-D., Vandenheede, J.R. and Merlevede, W. (1981) FEBS Lett. *132*, 293-295.
10. Vandenheede, J.R., Yang, S.-D. and Merlevede, W. (1981) J. Biol. Chem. *256*, 5894-5900.