Volume 271, number 1,2, 76–78 FEBS 08895 October 1990

A low-molecular weight acid phosphatase present in crystalline preparations of rabbit skeletal muscle glycogen phosphorylase *b*

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Received 24 July 1990

Crystalline preparations of glycogen phosphorylase *b* contain traces of acid phosphatase activity. Non-denaturing gel electrophoresis of phosphorylase *b* reveals a single band of 1-naphthyl phosphate phosphohydrolase activity which co-migrates with phosphorylase. The two enzymes can be separated by Sephadex G-200 column chromatography, where the phosphatase exhibits an apparent M_r of 17000. The contaminant enzyme hydrolyzes effectively the phenolic ester of monoorthophosphate with optimal activity for *p*-nitrophenyl phosphate and L-phosphotyrosine between pH 5.5 and 6.0. The phosphatase is insensitive to inhibition by L(+)-tartrate but strongly inhibited by μM vanadate and Zn^{2+}.

Phosphorylase *b*; Acid phosphatase

1. INTRODUCTION

Glycogen phosphorylase (EC 2.4.1.1) plays the key role in glycogenolysis and it is one of the best studied enzymes which is regulated by covalent phosphorylation and by substrates and effectors [1]. In skeletal muscle, a substantial proportion of glycogen phosphorylase, together with a number of other enzymes related to the biochemical machinery of glycogen metabolism is associated with glycogen particles [2].

Acid phosphatases (orthophosphoric-monoester phosphohydrolases (acid optimum) EC 3.1.3.2) are ubiquitous in nature and possess widely diverse properties [3]. Although the large M_r forms are of lysosomal origin, the low M_r forms appear to be soluble cytoplasmic enzymes [4], while there is evidence that some of the nonlysosomal acid phosphatases might function as phosphotyrosyl protein phosphatases [5].

Several years ago Johnson et al. [6] showed that AMP aminohydrolase contaminates rabbit muscle phosphorylase *b* crystals. In this communication we report that another contaminant enzyme activity, that of acid phosphatase, is constantly present in crystalline preparations of phosphorylase *b*. A preliminary characterization of this phosphatase is also presented.

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Abbreviations: pNPP, *p*-nitrophenyl phosphate; PAGE, polyacrylamide gel electrophoresis; IC_{50}, inhibitor concentration causing 50% inhibition; Mes, 4-morpholineethanesulfonic acid

2. MATERIALS AND METHODS

Rabbit skeletal muscle phosphorylase *b* was prepared as in [7]. The enzyme was recrystallized four times, passed through a Sephadex G-25 column and stored in 25 mM β-glycerophosphate buffer (pH 6.8), containing 25 mM 2-mercaptoethanol, 0.5 mM EDTA and 50% (v/v) glycerol, at −20°C.

Unless otherwise specified, acid phosphatase activities present in phosphorylase *b* were assayed at 30°C in 50 mM triethanolamine buffer (pH 6.8) containing 5 mM pNPP as substrate, 0.2–0.3 mU/ml of the phosphatase (0.5 mg/ml of phosphorylase *b*) and 0.1 mM 2-mercaptoethanol. After 20–60 min, the reaction was stopped with NaOH (final concentration 0.1 M) and the absorbance was read at 405 nm ($\epsilon_{405} = 1.9 \times 10^4\ M^{-1} \cdot cm^{-1}$). Units of phosphatase activity are expressed as μM of *p*-nitrophenol released per min. Specific activity is expressed as mU/mg of protein determined according to Lowry [8] or from absorbance measurements at 280 nm [9].

The substrate specificity studies and the pH dependence studies for tyrosine phosphate hydrolysis were carried out at 30°C, by determining the release of inorganic phosphate using a malachite green procedure [10] after deproteinization with perchloric acid (3% v/v). In this case, phosphorylase *b* used as a source of acid phosphatase activity was dialysed against 50 mM Tris-HCl buffer (pH 6.8), containing 10 mM 2-mercaptoethanol and 1 mM EDTA.

Non-denaturing PAGE (7.5% acrylamide) was essentially performed as in [11]. Rod gels (6 × 75 mm) were run at 4 mA/tube for about 90 min. Protein was stained with Coomassie blue. Acid phosphatase activity in gels was detected in 0.2 M Tris/maleate buffer (pH 6.0), with naphthylphosphate and fast blue [11]. All chemicals used were of the highest grade commercially available.

3. RESULTS AND DISCUSSION

We found that crystalline preparations of phosphorylase *b* from rabbit skeletal muscle are constantly contaminated by traces of a pNPP phosphatase activity. After four successive crystallizations of phosphorylase *b* the contaminant phosphohydrolase

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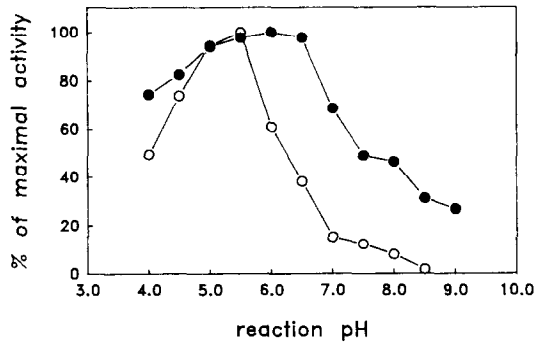

Fig. 1. Relative pH optima for the pNPP and phosphotyrosine dephosphorylation reactions. pNPP (5 mM) and phosphotyrosine (0.5 mM) were used to assay pNPP phosphatase (●) and phosphotyrosine phosphatase (○) activities present in phosphorylase *b* (0.5 mg/ml). The buffers, sodium acetate (50 mM), Mes (50 mM) and Tris-HCl (50 mM) were used for reactions with pH 4 to 5.5, 6 to 7 and 7.5 to 9, respectively. Other conditions as described in section 2.

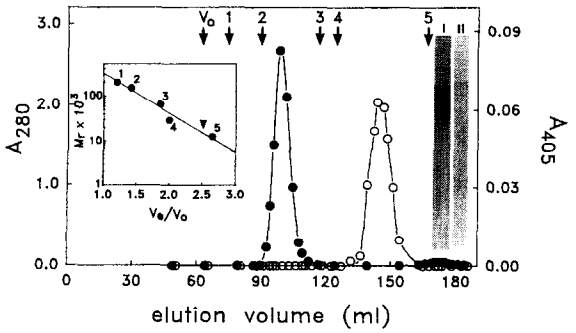

Fig. 2. Gel filtration and non-denaturing PAGE of acid phosphatase present in phosphorylase *b*. 20 mg of phosphorylase *b* (1 ml) was applied to a Sephadex G-200 column (1.5 × 78 cm) equilibrated in 20 mM Tris-HCl (pH 7.0), 0.1 mM EDTA, 10 mM 2-mercaptoethanol. The flow rate was 6 ml/h. Aliquots of each fraction were assayed for protein at 280 nm (●) and for pNPP phosphatase activity (○) as described in section 2. The arrows and numbers indicate the elution positions of the M_r markers (Sigma): V_o, void volume; 1, cytochrome *c* (12.4 kDa); 2, carbonic anhydrase (29 kDa); 3, albumin (66 kDa); 4, alcohol dehydrogenase (150 kDa); 5, β-amylase (200 kDa). V_e, elution volume. Left inset: calibration curve of the Sephadex G-200 column. The position of the phosphatase is shown by the arrowhead. Right inset: PAGE. 75 μg of phosphorylase *b* was applied on each gel. I, protein staining; II, acid phosphatase activity staining. Other conditions as in section 2.

Table I

Substrate specificity of acid phosphatase present in phosphorylase *b*

Substrate	Concentration (mM)	% Activity
pNPP	0.5	100
o-Phospho-L-tyrosine	0.5	44
Glucose-6-phosphate	0.5	15
Pyridoxal 5-phosphate	0.5	7
β-Glycerophosphate	0.5	0
AMP	0.5	0
pNPP	0.1	40
1-Naphthyl phosphate[a]	0.1	29
ATP[a]	0.1	28
Prednisolone 21-phosphate[a]	0.1	2

[a] Assayed at lower concentration because of high blank values

All assays were done in 50 mM triethanolamine buffer (pH 6.8) with 0.5 mg/ml of phosphorylase *b*. Other conditions as described in section 2.

Table II

Effect of various compounds on the activity of acid phosphatase present in phosphorylase *b*

Compound	Concentration (mM)	Relative activity
None	–	100
L-(+) Tartrate	100	99
Vanadate	0.0015	15
Zn^{2+}	0.05	6
NaF	50	66
KCl	50	99
Orthophosphate	10	65
Pyrophosphate	5	60
Molybdate	0.1	53
ATP	3	7
AMP	10	99

Phosphatase activity with pNPP was measured under standard conditions as described in section 2.

activity decreased more than 3-fold to a value of 0.5 ± 0.1 mU · mg^{-1} (mean ± SD for six preparations) at pH 6.8, although some phosphatase activity persisted at a constant level even after five recrystallizations. The enzyme activity was maximal at pH 6.0 with pNPP and at pH 5.5 with phosphotyrosine (Fig. 1).

Non-denaturing PAGE of phosphorylase *b* preparations revealed only one acid phosphatase activity band, which shows similar mobility with that of phosphorylase (Fig. 2). However, the contaminant phosphatase activity can be effectively separated from phosphorylase *b* by gel filtration on a Sephadex G-200 column, where it shows one symmetric activity peak with an apparent M_r of 17000 (Fig. 2).

The activity of acid phosphatase, present in phosphorylase *b* preparations, towards a number of phosphate esters is shown in Table I. At pH 6.8, pNPP gives the highest reaction rate, while the natural substrates ATP and *o*-phosphotyrosine were also efficiently hydrolyzed.

Various known inhibitors of acid phosphatases and protein phosphatases were tested for their effect on the pNPP phosphatase activity (Table II). The enzyme is a tartrate-resistant acid phosphatase, strongly inhibited by vanadate, Zn^{2+} and molybdate, known inhibitors of several phosphotyrosine protein phosphatases [5,12]; vanadate the most potent inhibitor, exhibited an IC_{50} value of 200 nM. ATP, which is a substrate of the phosphatase (Table I) and an allosteric inhibitor of phosphorylase [1] effectively inhibited the phosphatase

activity when assayed in presence of pNPP, with an IC_{50} value of 0.2 mM.

In conclusion, our results suggest that a low M_r acid phosphatase might be a contaminating enzyme normally present in phosphorylase *b* preparations. This enzyme is possibly related to an acid phosphatase activity which accompanies a fraction of protein–glycogen complex purified from rabbit skeletal muscle [2]. The purification and characterization of the above acid phosphatase(s) will offer the opportunity to examine their relationship (if any) with the control of glycogen metabolism.

Acknowledgements: We are grateful to Drs A.E. Evangelopoulos and N.G. Oikonomakos for helpful discussions.

REFERENCES

[1] Graves, D.J. and Wang, J.H. (1972) in: The Enzymes, vol. 7 (Boyer, P.D. ed.) pp. 435–482, Academic Press, New York.
[2] Meyer, F., Heilmeyer, Jr., L.M.G., Haschke, R.H. and Fischer, E.H. (1970) J. Biol. Chem. 245, 6642–6648.
[3] Hollander, P.V. (1971) in: The Enzymes, vol. 4 (Boyer, P.D. ed.) pp. 449–498, Academic Press, New York.
[4] Baxter, J.H. and Suelter, C.H. (1985) Arch. Biochem. Biophys. 239, 29–37.
[5] Lau, K.-H.W., Farley, J.R. and Baylink, D.J. (1989) Biochem. J. 257, 23–36.
[6] Johnson, L.N., Madsen, N.B., Mosley, J. and Wilson, K.S. (1974) J. Mol. Biol. 90, 703–717.
[7] Fischer, E.H. and Krebs, E.G. (1962) Methods Enzymol. 5, 369–373.
[8] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[9] Kastenschmidt, L.L., Kastenschmidt, J. and Helmreich, E. (1968) Biochemistry 7, 3590–3608.
[10] Baykov, A.A., Evtushenko, O.A. and Avaeva, S.M. (1968) Anal. Biochem. 171, 266–270.
[11] Schweingruber, A.-M., Schoenholzer, F., Keller, L., Schwaninger, R., Trachsel, H. and Schweingruber, M.E. (1986) Eur. J. Biochem. 158, 133 140.
[12] Tonks, N.K., Diltz, C.D. and Fischer, E.H. (1988) J. Biol. Chem. 263, 6731–6737.