NATIVE AND LATENT FORMS OF SKELETAL MUSCLE PHOSPHORYLASE PHOSPHATASE

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1. Introduction

A protein phosphatase active on several substrates including phosphorylase *a*, glycogen synthase *b*, phosphorylase kinase, histone, the R subunit of cyclic AMP-dependent protein kinase, casein and troponin I, has been extracted from liver [1–4], muscle [4–7], heart [8,9], kidney and brain [4]. This so-called multifunctional protein phosphatase can be inhibited by two trypsin-sensitive polypeptides extracted from boiled tissue homogenates and called inhibitor 1 and inhibitor 2 [10–16]. Inhibitor 1 is active only when it has been phosphorylated by cyclic AMP-dependent protein kinase [11]. Cohen et al. [17,18] proposed to use the property to be inhibited by inhibitor 2 for the specific recognition of the multifunctional phosphatase, presently called 'protein phosphatase I' [18,19] and previously referred to as protein phosphatase III [17]. On this basis, these authors reported that 90% of the phosphorylase phosphatase and glycogen synthase phosphatase activity measured in a fresh muscle extract are catalyzed by this multifunctional protein phosphatase [17].

In contrast with these findings, we have observed that in a fresh liver extract, phosphorylase phosphatase is insensitive to the heat-stable inhibitors. If, however, the extract was not kept strictly at 0°C or if it was incubated in the presence of trypsin, an enzyme which was highly sensitive to inhibitor 2 was formed [20]. The present work was therefore undertaken in order to check if a similar situation exists in muscle. We report that the phosphorylase phosphatase present in a fresh muscle extract was not affected by concentrations of inhibitors 1 or 2 which inhibited by > 90% the same preparation when treated with trypsin. A partial activation of the inhibitor-sensitive protein phosphatase occurred during the prolonged centrifugation which is the first step of several [4–7,21] purification procedures.

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2. Materials and methods

Male Wistar rats were killed by decapitation, male rabbits by cervical dislocation. Muscle of the hind limb was excised and placed in ice. The muscle was cut into small pieces and homogenized with an Ultra-Turrax (Janke und Kunkel, Staufen im Breisgau) for 30 s (speed 7–8), in 3 vol. of an ice-cold solution containing 50 mM imidazol and 0.5 mM dithiothreitol (pH 7.4). The homogenate was centrifuged in the cold for 5 min at 12 000 rev./min (extract final temp. 0°C) or 45 min at 6000 rev./min (final temp. 5–6°C) in a Sorvall centrifuge. It was verified that the supernatant protein concentration was the same in both procedures. The supernatant was passed through a Sephadex G-25 column equilibrated with the homogenization buffer. In some experiments, the muscle was homogenized in 3 vol. of a solution containing 4 mM EDTA and 15 mM β-mercaptoethanol (pH 7), and the extract filtered on Sephadex equilibrated in 50 mM Tris–HCl, 1 mM EDTA, 15 mM β-mercaptoethanol as in [17]; these experi-

ments yielded results undistinguishable from the others. The Sephadex filtrate was either used as such or diluted 3-fold with the buffer used for the filtration and mixed with trypsin added at final conc. 1.3 mg trypsin/ml and the mixture incubated for 15 min at room temperature. The reaction was stopped by addition of a 4-fold excess of soybean trypsin inhibitor.

Phosphorylase phosphatase was measured as in [20]. One unit of phosphorylase phosphatase is the amount of enzyme which inactivates one unit of phosphorylase *a* per minute under our assay conditions. In most assays, 20–40 mU of enzyme were present in 0.1 ml total vol. Inhibitor 1 was prepared by the method in [22], with some modifications. The rabbit muscle was minced, homogenized in 3 vol. of a solution containing 4 mM EDTA and 2% trichloroacetic acid, and centrifuged for 5 min at 12 000 rev./min. The pellet, containing inhibitor 2 [22], was discarded; the supernatant was incubated for 15 min in a boiling water bath and centrifuged for 5 min at 12 000 rev./min. The supernatant was filtered on Sephadex G-25, equilibrated with 50 mM glycylglycine (pH 7.4). This filtrate was then incubated for 2 h at 30°C with cyclic AMP-dependent protein kinase, 0.01 mM cyclic AMP, 0.2 mM ATP, 2 mM Mg acetate [13]. It was verified that a shorter incubation period yielded the same amount of inhibitor, indicating that the phosphorylation was complete. To stop the reaction the mixture was immersed in a boiling water bath for 15 min and filtered on Sephadex G-25 in 50 mM glycylglycine (pH 7.4). This preparation was stored at −20°C. As a control, the boiled extract was incubated 2 h at 30°C, without any addition, boiled and filtered. No inhibition of trypsin-activated phosphorylase phosphatase was observed with the latter preparation, indicating that inhibitor 2 was absent. Cyclic AMP-dependent protein kinase (a gift of Dr G. van den Berghe) was purified from beef muscle [23]. Inhibitor 2 was partially purified by the method in [17], up to and including the DEAE-cellulose step, with the following modifications. The DEAE cellulose eluate was concentrated by Amicon filtration (PM 30), filtered on Sephadex G-25 equilibrated in 50 mM glycylglycine (pH 7.4) and stored at −20°C. One unit of inhibitor 1 and 2 is the amount which causes a 50% inhibition of the trypsin-activated phosphatase under our assay conditions.

3. Results

Figure 1 and fig.2 show the sensitivity of various types of skeletal muscle phosphorylase phosphatase to inhibitor 1 and inhibitor 2, respectively. The phosphatase present in the trypsin-treated extract was the most sensitive and could be inhibited 100% by relatively low concentrations of both inhibitors. In contrast, the enzyme present in a fresh muscle extract, obtained by a 5 min centrifugation of the homogenate, was minimally affected. Indeed no inhibition was detected at concentrations of the inhibitors that brought about a 90% inhibition of the trypsin-treated enzyme. A complete inhibition was, however, observed at very high concentrations of inhibitor 2, to which the fresh enzyme was ~70–100-fold less sensitive than the trypsin-treated extract. The phosphatase present in an extract obtained by a more prolonged centrifugation displayed intermediary properties. The percent of inhibition of this type of enzyme was however variable from one preparation to another. We have collected in table 1 the results obtained in 5 different preparations in which the effect of both

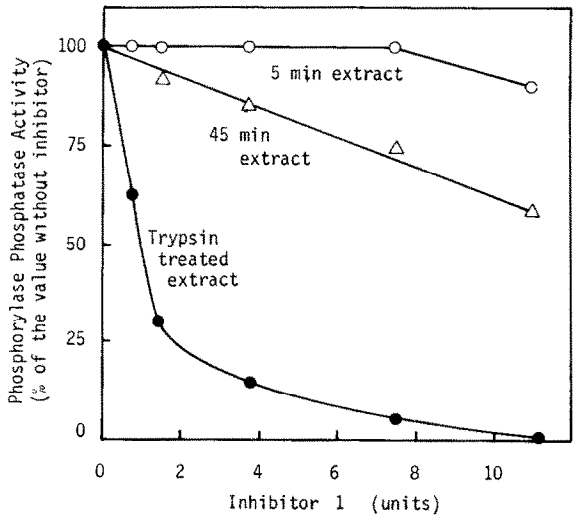

Fig.1. Effect of inhibitor 1 on the activity of phosphorylase phosphatase in a rat muscle extract. The extract was obtained as in section 2 by a centrifugation of 5 min (5 min extract) or of 45 min (45 min extract). A portion of the 5 min extract was further diluted 3-fold and treated with trypsin. 10 μl of each preparation containing about 25 mU of phosphorylase phosphatase was used for the assay.

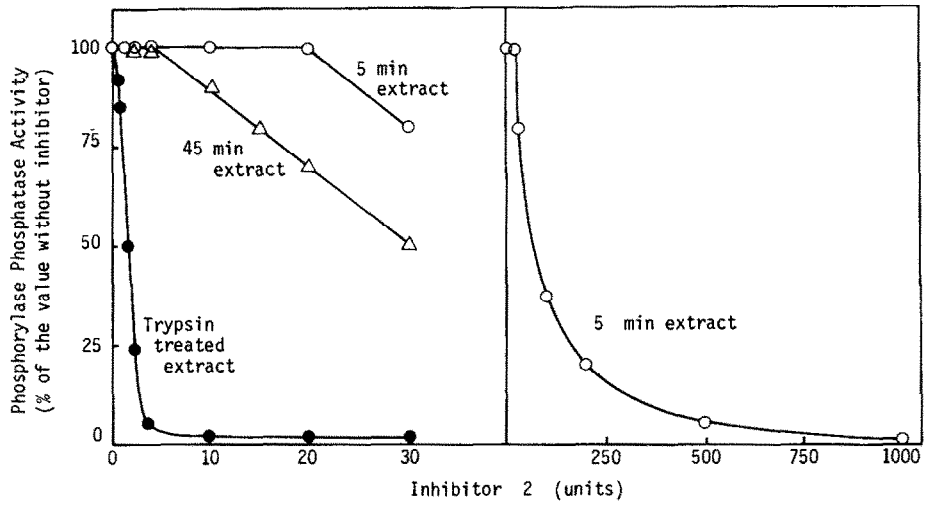

Fig.2. Effect of inhibitor 2 on the activity of phosphorylase phosphatase in a rat muscle extract. Same procedure as in fig.1.

inhibitors has been studied in parallel. It has also been observed on several occasions that the addition of 5 mM EGTA to the homogenization medium decreased the sensitivity of the extract to inhibitor 2 (not shown). Erratic results were obtained with extracts prepared from frozen rabbit muscle. In some of these preparations, no phosphorylase phosphatase activity was detectable (not shown).

Table 2 shows that a fresh muscle extract has little if any inhibitory property on the phosphorylase phosphatase present in a trypsin treated extract and that the inhibitory property is revealed by heating the extract in a boiling water bath.

4. Discussion

From these results we conclude that the multifunctional, inhibitor-sensitive, protein phosphatase purified from various tissues (see section 1) is not present in a fresh muscle extract. This enzyme is generated, presumably by proteolysis, during the

Table 1
Effect of inhibitors 1 and 2 on the activity of phosphorylase phosphatase present in a muscle extract

	Phosphorylase phosphatase (mU phosphorylase inactivated/min)					
	5 min extract			45 min extract		
Addition:	None	Inhibitor 1 (4.5 U)	Inhibitor 2 (3 U)	None	Inhibitor 1 (4.5 U)	Inhibitor 2 (3 U)
Rat	28	27	28	33	23	24
Rat	19	18	18	17	10	10
Rat	22	21	22	21	11	15
Rabbit	24	25	25	42	28	25
Rabbit	44	43	45	47	31	33

General procedure as in fig.1. In each assay 10 μl extract was used in 0.1 ml total vol. [20]. The amount of inhibitor added caused an ~90% inhibition of the trypsin-activated enzyme (see fig.1,2)

Table 2
Inhibition of the trypsin-activated phosphorylase phosphatase by boiled and unboiled muscle extracts

Muscle source	Addition	Phosphorylase phosphatase act. (% of theoretical)
Rat (n = 4)	5 min extract	95 ± 4.1
	Boiled extract	36 ± 3.5
Rabbit (n = 3)	5 min extract	94 ± 2.3
	Boiled extract	49 ± 4.8

A trypsin-activated extract (10 μl) was incubated with an equivalent amount of the same extract not treated with trypsin and which has or has not been immersed for 15 min in a boiling water bath. The activities are expressed in percent of the theoretical value obtained by additioning the activity of the two components of the mixture, measured separately. Values shown are means ± SEM

prolonged centrifugation which is an early step in several published purification procedures [4–7,21]. The situation in muscle resembles that described for the liver [20]. In the latter tissue, the native phosphorylase phosphatase is not inhibited by heat-stable inhibitors. It is distinct from synthase phosphatase and is entirely bound to particulate glycogen. There is little or no phosphorylase phosphatase activity in a freshly prepared high-speed supernatant from liver, although a large activity can be generated from the same preparation upon trypsin treatment. This activity is highly sensitive to heat-stable inhibitors and obviously originates from a previously completely inactive, latent, proenzyme, which is distinct from the native enzyme. Furthermore, the activation by trypsin was not entirely explained by the proteolytic destruction of the heat-stable inhibitors. The inhibitor-sensitive enzyme can also be formed by treatment with ethanol at 20°C, a procedure initially introduced [1,2] for the purification of the liver enzyme and later on used to purify the heart enzyme [8,9]. Trypsin digestion has an effect similar to that of warm ethanol [20,24] and may be the simplest procedure to obtain from any tissue a protein phosphatase which is highly sensitive to heat-stable inhibitors. From these data we conclude that in the muscle, like in the liver, there is at present no proof that the multifunctional protein phosphatase plays a role in glycogen metabolism. Other roles have been recently proposed for this enzyme [18].

The physiological role of the heat-stable inhibitors also appears very problematic since not only do they have little activity on the native phosphorylase phosphatase, but also their efficiency is greatly increased by boiling the extract. The inhibition reported in table 2 is presumably attributable to both inhibitors, since inhibitor 1 is more abundant in muscle than inhibitor 2 [19] and is normally ~30% in the phosphorylated form [22]. Figure 1 indicates that inhibitor 1 is apparently not rapidly dephosphorylated in the presence of a crude muscle extract since its activity was clearly apparent on unpurified preparations.

Our data are also illustrative of the danger of drawing conclusions from the properties of purified enzymes which might be artefactually modified or generated during the purification procedure.

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