

Activation of the PCS_M-protein phosphatase by a Ca²⁺-dependent protease

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The inhibitor-1 phosphatase but not the phosphorylase phosphatase activity of a newly discovered 250 kDa polycation-stimulated (PCS_M) protein phosphatase in rabbit skeletal muscle is increased up to 10-fold by a Ca²⁺-dependent protease. The enzyme-directed protease effect to which the PCS_H and PCS_L phosphatases are insensitive was progressively lost during purification of the enzyme. This could be explained by either a slow conversion of the enzyme to an active form of the enzyme with a change in specificity, or the loss of a protease-sensitive inhibitor of the inhibitor-1 phosphatase activity, resulting in a PCS phosphatase characterized by its high inhibitor-1/phosphorylase *a* activity ratio. The Ca²⁺-dependent protease is completely inhibited by EGTA or leupeptin.

Polycation-stimulated protein phosphatase Phosphorylase a Inhibitor-1 Ca²⁺-dependent protease
Modulator protein (inhibitor-2)

1. INTRODUCTION

In 1982 Wilson et al. [1] reported that an acid- and heat-stable protein isolated from porcine kidney markedly stimulated phosphorylase phosphatase activity. Less than a year later, the same authors [2] identified the stimulatory protein as histone H₁ and polycation-stimulated protein phosphatases were described in bovine aortic smooth muscle [3] and rabbit skeletal muscle [4], using highly basic polypeptides such as histone H₁, protamine and polylysine. Based on the stimulation of phosphorylase phosphatase activity by polycations as well as on inhibitor-1 phosphatase activity, we [5,6] have purified from rabbit skeletal muscle 3 polycation-stimulated (PCS) protein phosphatases, denoted according to their molecular mass in the early purification stages: PCS_H (390 kDa), PCS_M (250 kDa) and PCS_L (200 kDa) phosphatase [7]. These enzymes are all insensitive to phosphorylated inhibitor-1 as well as to the modulator protein and display a relatively high specificity for the α -subunit over the β -subunit of phosphorylase kinase. The PCS_H and PCS_L phos-

phatases are probably identical to phosphatases 2A₁ and 2A₂ respectively described by Cohen and co-workers [8], while a novel PCS_M phosphatase elutes between these 2 enzymes during early DEAE-Sephacel chromatography. Its identification, as reported here, is based on its sensitivity to a Ca²⁺-dependent protease, resulting in a 3–10-fold increase in inhibitor-1 phosphatase activity without affecting its activity towards phosphorylase *a*.

2. MATERIALS AND METHODS

The PCS protein phosphatases were purified from rabbit skeletal muscle [5,6] using both phosphorylase *a* in the presence of 10 μ g per ml protamine (Sigma) and inhibitor-1 as substrates. The specific activity of PCS_H, PCS_M and PCS_L phosphatase was 400, 600 and 3000 U per mg in the absence and 1600, 4500 and 5500 U per mg in the presence of 10 μ g per ml protamine, using phosphorylase *a* as a substrate. In the inhibitor-1 assay the specific activity of the enzymes was 40, 130 and 200, respectively. The PCS_M phosphatase, which elutes at $\pm$ 0.37 M NaCl in DEAE-Sephacel (Phar-

macia) chromatography, represents $\pm 50\%$ of the total inhibitor-1 phosphatase activity of the PCS phosphatases in the presence of 3 mM Ca^{2+} .

Rabbit muscle phosphorylase *b* [9], protein phosphatase inhibitor-1 [10], deinhibitor protein [5,11], and calmodulin [12] were purified according to published methods. Phosphorylase kinase [13] and the catalytic subunit of cyclic AMP-dependent protein kinase [14] were gifts from D.A. Walsh (Davis, USA). ^{32}P -labeled phosphorylase *a* [3] and inhibitor-1 [15] were prepared and phosphorylase phosphatase [3] and inhibitor-1 phosphatase [16] activity measured as described. Phenylmethylsulfonyl fluoride (PMSF), *p*-tosyl-L-lysine chloromethyl ketone (TLCK), benzamidine, leupeptin and dithiothreitol were purchased from Sigma.

Azocoll (Calbiochem-Behring) or deinhibitor protein [11] were used as substrates for the detection of Ca^{2+} -dependent proteolytic activity. The sample ($100 \mu\text{l}$) was added to 1 ml containing 10 mg per ml Azocoll suspended in 20 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol and 3 mM Ca^{2+} and incubated at 37°C for up to 2 h . The digestive action of the protease on Azocoll was terminated by filtering the incubation mixture and the absorption was read at 520 nm . In the deinhibitor-proteolysis assay method samples were incubated with an appropriate amount of the deinhibitor for 10 min in the presence or absence of Ca^{2+} . After the boiling step the remaining deinhibitor was measured as in [11].

3. RESULTS AND DISCUSSION

3.1. Elution of the Ca^{2+} -stimulated PCS phosphatase and Ca^{2+} -dependent protease during DEAE-Sephacel chromatography

Rabbit skeletal muscle (starting material $\sim 1.3 \text{ kg}$) was homogenized in 2 vols ice-cold 50 mM Tris-HCl buffer, pH 8.0, containing 0.5 mM dithiothreitol, 0.5 mM benzamidine, 1 mM EGTA, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM TLCK and 0.25 M sucrose. The homogenate was centrifuged 60 min at $6000 \times g$ and the supernatant applied batch-wise to 500 ml DEAE-Sephacel equilibrated in 20 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol, 0.5 mM benzamidine, 1 mM EGTA and 1 mM EDTA (buffer A). The DEAE-Sephacel was washed extensively with 3 l buffer A containing

200 mM NaCl to remove calcineurin and the different forms of ATP, Mg-protein phosphatase, and the slurry packed into a $5 \times 36 \text{ cm}$ column. The proteins eluting from the column with 1500 ml of a $0.2\text{--}0.5 \text{ M}$ NaCl linear gradient in buffer A were collected in 12-ml fractions and assayed for phosphorylase phosphatase activity in the presence or absence of $10 \mu\text{g}$ per ml protamine or for inhibitor-1 phosphatase activity in the presence or absence of 3 mM Ca^{2+} . As shown in fig.1 there are

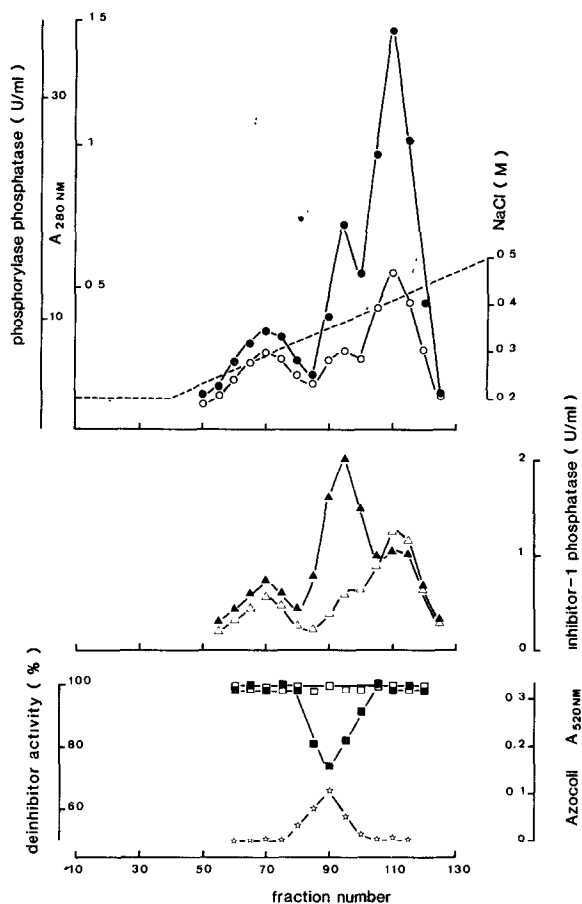


Fig.1. DEAE-Sephacel elution profile of the polycation-stimulated protein phosphatases and the Ca^{2+} -dependent protease activity. Fractions were assayed for phosphorylase phosphatase activity in the presence (●) or (○) of protamine and for inhibitor-1 phosphatase activity in the presence (▲) or absence (Δ) of 3 mM Ca^{2+} . Protease activity, using Azocoll, was measured after 2 h incubation in the presence of Ca^{2+} (☆) and using the deinhibitor protein in the presence (■) or absence (□) of 3 mM Ca^{2+} . Absorbance at 280 nm . Procedures are as described in section 2 (...).

3 distinct peaks of protein phosphatase activity, corresponding to the PCS_H, PCS_M and PCS_L phosphatases [7], with a clear stimulation of inhibitor-1 phosphatase activity in the presence of 3 mM Ca²⁺ (usually 3–10-fold) of the PCS_M protein phosphatase.

Assaying for protease activity using Azocoll or the deinhibitor protein (see section 2) revealed a strictly Ca²⁺-dependent protease activity, slightly preceding the PCS_M protein phosphatase peak (fig.1). According to the manufacturer's instructions, Azocoll should produce substantial absorption readings after a 15 min preincubation at 37°C with 30 µg pancreatin, 0.11 units pronase or 1 NF unit trypsin. Since the color yield was quite low even after 2 h, the degradation of the purified deinhibitor protein, which was shown to be sensitive to pronase or trypsin [11], was adopted as far more sensitive and reliable in this Ca²⁺-dependent protease activity assay.

3.2. Effect of the Ca²⁺-dependent protease on the PCS_H, PCS_M and PCS_L protein phosphatases

Combination of the peak fractions of the PCS protein phosphatases with a fraction containing a high protease/phosphatase activity ratio shows that only the inhibitor-1 phosphatase activity of the PCS_M enzyme is clearly increased (fig.2). No stimulation was observed with the PCS_H and PCS_L phosphatases. During further isolation of the

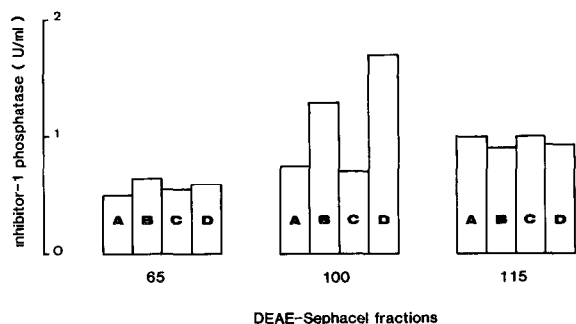


Fig.2. Ca²⁺-dependent protease stimulated inhibitor-1 phosphatase activity of the polycation-stimulated enzyme fractions obtained by DEAE-Sephacel chromatography. Inhibitor-1 phosphatase activity was measured as in section 2 under basal conditions (A), after 20 min preincubation at 30°C in the presence of 3 mM Ca²⁺ (B), fraction 85 from fig.1 as source of protease activity (C) or in the presence of both (D).

PCS_M phosphatase there is a gradual increase of the inhibitor-1 phosphatase/phosphorylase phosphatase activity ratio [6] and the protease effect was progressively lost during purification.

Stimulation of the inhibitor-1 phosphatase activity of the PCS enzyme by the protease is completely Ca²⁺-dependent. Half-maximal stimulation was observed with 1 mM Ca²⁺. Protease activity in the Azocoll as well deinhibitor-proteolysis assay was equally Ca²⁺-dependent, and this effect was completely abolished by 3 mM EGTA or 20 µg per ml leupeptin. Attempts to purify the protease were unsuccessful, probably because of the lack of stability of the enzyme.

The protease activity was enzyme- and not substrate-directed since all radioactivity released by the phosphatase preparations using ³²P-labelled phosphorylase *a* as well as inhibitor-1 as substrates, under conditions where the protease was clearly stimulated, was recovered as free ³²P according to the method of Shacter [17]. This is remarkable feature as to the effect of the protease because of the selectivity at both the substrate and the phosphatase level. Neither the substrates nor the PCS_H and PCS_L phosphatases are affected, except the PCS_M protein phosphatase and then specifically in its activity towards inhibitor-1.

The physiological mechanism responsible for the regulation of the polycation-stimulated protein phosphatases in vivo is still a matter of conjecture. The role of the naturally occurring polycations such as spermine, spermidine, putrescine and histone H₁ in the regulation of these protein phosphatases is questionable. Even if proteolysis cannot be considered a likely procedure for regulation of enzyme activity, the protease effect described here can point towards the understanding of the regulation of the PCS_M phosphatase. The high inhibitor-1/phosphorylase activity ratio which characterizes the PCS_M phosphatase could be explained by a slow conversion of the enzyme involving an activation and/or a change in specificity of the enzyme, or by the loss of a specificity inducing subunit which is particularly sensitive to this Ca²⁺-dependent protease.

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