# Activation of the $PCS_M$-protein phosphatase by a $Ca^{2+}$-dependent protease

Etienne Waelkens, Jozef Goris and Wilfried Merlevede

*Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium*

Received 10 October 1985

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 $Ca^{2+}$-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 $Ca^{2+}$-dependent protease is completely inhibited by EGTA or leupeptin.

*Polycation-stimulated protein phosphatase* *Phosphorylase* a *Inhibitor-1* *$Ca^{2+}$-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 $H_1$ 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 $H_1$, 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 $\alpha$-subunit over the $\beta$-subunit of phosphorylase kinase. The $PCS_H$ and $PCS_L$ phosphatases are probably identical to phosphatases $2A_1$ and $2A_2$ 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 $Ca^{2+}$-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 $\mu$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 $\mu$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-

*Published by Elsevier Science Publishers B.V. (Biomedical Division)*
00145793/85/$3.30 

macia) chromatography, represents ± 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 $\mu$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 ~1.3 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 × *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 × 36 cm column. The proteins eluting from the column with 1500 ml of a 0.2–0.5 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 $\mu$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

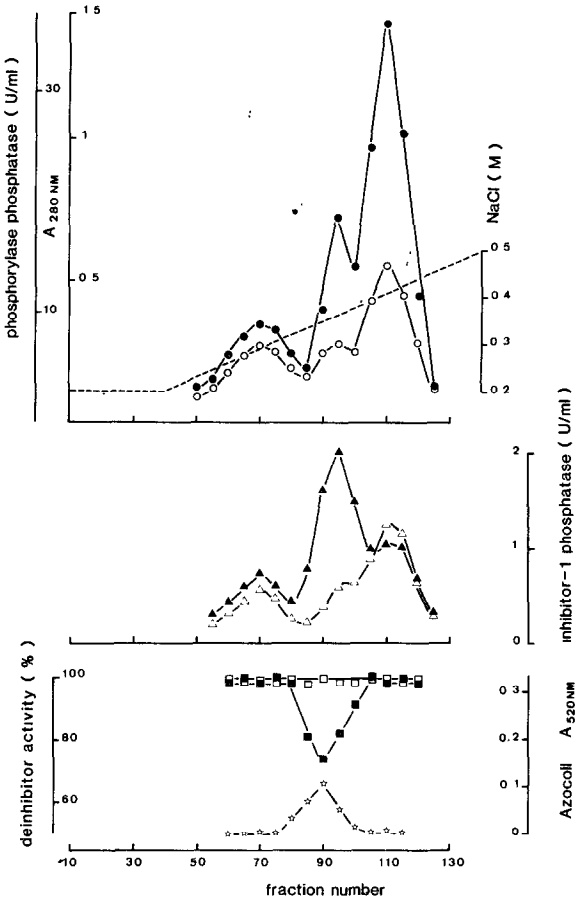


Fig.1. DEAE-Sephacel elution profile of the polycation-stimulated protein phosphatases and the $Ca^{2+}$-dependent protease activity. Fractions were assayed for phosphorylase phosphatase activity in the presence (●) or (○) of protamine and for inhibitor-1 phosphatase activity in the presence (▲) or absence (△) 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 $Ca^{2+}$ (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 $Ca^{2+}$-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 $\mu$g 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 $Ca^{2+}$-dependent protease activity assay.

### 3.2. *Effect of the $Ca^{2+}$-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 $PCS_M$ 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.

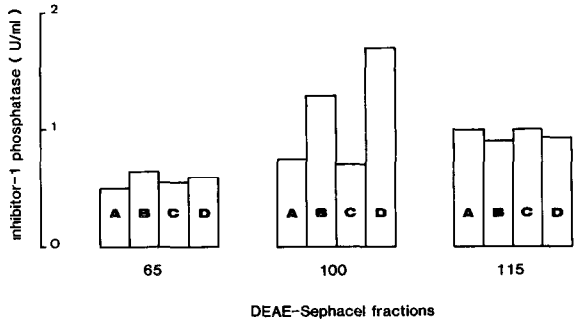


Fig.2. $Ca^{2+}$-dependent protease stimulated inhibitor-1 phosphatase activity of the polycation-stimulated enzyme fractions obtained by DEAE-Sephacel chromatography. Inhibitor-1 phosphatase activity was measured as in section 2 under basal conditions (A), after 20 min preincubation at 30°C in the presence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

Stimulation of the inhibitor-1 phosphatase activity of the PCS enzyme by the protease is completely $Ca^{2+}$-dependent. Half-maximal stimulation was observed with 1 mM $Ca^{2+}$. Protease activity in the Azocoll as well deinhibitor-proteolysis assay was equally $Ca^{2+}$-dependent, and this effect was completely abolished by 3 mM EGTA or 20 $\mu$g 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 $^{32}P$-labelled phosphorylase *a* as well as inhibitor-1 as substrates, under conditions where the protease was clearly stimulated, was recovered as free $^{32}P$ 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 $H_1$ 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 $Ca^{2+}$-dependent protease.

## ACKNOWLEDGEMENTS

These studies were supported by the Fonds voor Wetenschappelijk Geneeskundig Onderzoek. The

authors are grateful to Ms R. Verbiest and Ms R. Bollen for expert technical assistance.

## REFERENCES

[1] Wilson, S.E., Mellgren, R.L. and Schlender, K.K. (1982) FEBS Lett. 146, 331-334.
[2] Wilson, S.E., Mellgren, R.L. and Schlender, K.K. (1983) Biochem. Biophys. Res. Commun. 116, 581-586.
[3] Di Salvo, J., Waelkens, E., Gifford, D., Goris, J. and Merlevede, W. (1983) Biochem. Biophys. Res. Commun. 117, 493-500.
[4] Mellgren, R.L. and Schlender, K.K. (1983) Biochem. Biophys. Res. Commun. 117, 501-508.
[5] Goris, J., Waelkens, E. and Merlevede, W. (1985) FEBS Lett. 188. 262-266.
[6] Waelkens, E., Goris, J. and Merlevede, W. (1985) in preparation.
[7] Merlevede, W. (1985) Adv. Protein Phosphatases 1, 1-18.
[8] Tung, H.Y.L., Alemany, S. and Cohen, P. (1985) Eur. J. Biochem. 148, 253-263.
[9] Fischer, E.H. and Krebs, E.G. (1958) J. Biol. Chem. 231, 65-71.
[10] Aitken, A., Bilham, T. and Cohen, P. (1982) Eur. J. Biochem. 126, 235-246.
[11] Goris, J., Waelkens, E., Camps, T. and Merlevede, W. (1984) Adv. Enzyme Regul. 22, 467-484.
[12] Gopalakrishna, R. and Anderson, W.B. (1982) Biochem. Biophys. Res. Commun. 104, 830-836.
[13] Pickett-Gies, C.A. and Walsh, D.A. (1985) J. Biol. Chem. 260, 2046-2056.
[14] Cooper, R.H., Sul, H.S., McCullough, T.E. and Walsh, D.A. (1980) J. Biol. Chem. 255, 11794-11801.
[15] Goris, J., Camps, T., Defreyn, G. and Merlevede, W. (1981) FEBS Lett. 134, 189-193.
[16] Waelkens, E., Goris, J., Di Salvo, J. and Merlevede, W. (1984) Biochem. Biophys. Res. Commun. 120, 397-404.
[17] Shacter, E. (1984) Anal. Biochem. 138, 416-420.