Identification of the phosphatase deinhibitor protein phosphatases in rabbit skeletal muscle

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In rabbit skeletal muscle the polycation-stimulated (PCS) protein phosphatases [Merlevede (1985) *Adv. Protein Phosphatases* 1, 1–18] are the only phosphatases displaying significant activity toward the deinhibitor protein. Among them, the PCS_H protein phosphatase represents more than 80% of the measurable deinhibitor phosphatase activity associated with the PCS phosphatases. The deinhibitor phosphatase activity co-purifies with the PCS_H phosphatase to apparent homogeneity. In the last purification step two forms of PCS_H phosphatase were separated (PCS_{H1}, containing 62, 55 and 34 kDa subunits, and PCS_{H2}, containing 62 and 35 kDa subunits), both showing the same deinhibitor/phosphorylase phosphatase activity ratio. The activity of the PCS_H phosphatase toward the deinhibitor is not stimulated by polycations such as protamine, histone H1 or polylysine, unlike the stimulation observed with phosphorylase as the substrate. The phosphorylase phosphatase activity of PCS_H phosphatase is inhibited by ATP, PP_i and P_i, whereas the deinhibitor phosphatase activity of the enzyme is much less sensitive to these agents.

INTRODUCTION

The deinhibitor is a small thermostable protein, first described in the dog liver glycogen pellet (Defreyne *et al.*, 1977) associated with a particular protein phosphatase representing an active form of the ATP,Mg-dependent protein phosphatase (Stewart *et al.*, 1981; Vandenheede *et al.*, 1981a,b). This enzyme can be regulated *in vitro*, in two ways. The intrinsic activity state of the catalytic subunit F_C can be increased through a mechanism involving a transient phosphorylation (Hemmings *et al.*, 1982; Jurgensen *et al.*, 1984) of the modulator (M) subunit (Yang *et al.*, 1981b) by kinase F_A. It is also regulated by the interaction with inhibitor-1, extra bound modulator and deinhibitor protein (Merlevede *et al.*, 1984a,b).

The deinhibitor protein could be of crucial importance in the conversion of the inactive form into the spontaneously active form of the phosphatase *in vivo*. Not only does it antagonize the inhibitory effect of inhibitor-1 and modulator protein, but it also stabilizes the ATP,Mg-dependent phosphatase in its activated state, and thus prevents the time-dependent inactivation of the enzyme by the modulator protein (Goris *et al.*, 1984b).

The deinhibitor protein is regulated through phosphorylation–dephosphorylation (Goris *et al.*, 1984a). Because of this, the activity of the ATP,Mg-dependent protein phosphatase *in vivo* may be regulated by the state of phosphorylation of the deinhibitor protein, which itself depends on the relative activities of the cyclic AMP-dependent protein kinase and the protein phosphatases that act on the deinhibitor. Among the protein phosphatases tested to date, only the PCS_H phosphatase has shown substantial deinhibitor phosphatase activity. The other polycation-stimulated enzyme forms, their catalytic subunit, the ATP,Mg-dependent phosphatase,

and calcineurin are each relatively inefficient in dephosphorylating the deinhibitor protein (Goris *et al.*, 1985a,b).

We now report that, in rabbit skeletal muscle, more than 80% of the soluble deinhibitor phosphatase activity is associated with the PCS_H phosphatase and co-purifies with this enzyme to apparent homogeneity. The phosphorylase phosphatase and deinhibitor phosphatase activities of this purified preparation are affected differentially by effectors such as polycations, P_i, PP_i and ATP.

EXPERIMENTAL

Materials

Rabbit skeletal-muscle phosphorylase *b* (Fischer & Krebs, 1958), the inactive ATP,Mg-dependent protein phosphatase (Yang *et al.*, 1980) and kinase F_A (Vandenheede *et al.*, 1980), protein phosphatase inhibitor-1 (Aitken *et al.*, 1982) and modulator protein (Yang *et al.*, 1981a) were purified by the published methods. Rabbit skeletal-muscle phosphorylase *b* kinase (Pickett-Gies & Walsh, 1985) and bovine heart cyclic AMP-dependent protein kinase (Beavo *et al.*, 1974) were generously given by Dr. D. A. Walsh (Davis, CA, U.S.A.). Dog liver deinhibitor protein was purified as described by Goris *et al.* (1984b, 1985a) and phosphorylated as described by Goris *et al.* (1985a). ³²P-labelled phosphorylase *a* and inhibitor-1 were prepared as described by Krebs *et al.* (1958) and Goris *et al.* (1981) respectively. Phenylmethanesulphonyl fluoride, tosyl-l-tyrosylchloromethane ('TLCK'), protamine hydrochloride and dithiothreitol were obtained from Sigma; Ultrogel AcA-34 was from LKB; DEAE-Sephacel, aminohexyl-Sepharose, Blue Dextran, ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa) were from Pharmacia.

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Assays

Phosphorylase phosphatase (Yang *et al.*, 1980) and inhibitor-1 phosphatase (Waelkens *et al.*, 1984) activities were measured with 10 μ M and 2 μ M substrate respectively. The ATP,Mg-dependent protein phosphatase was measured after activation in the presence of 0.2 mM-ATP and 1 mM-magnesium acetate and the minimal amount of kinase F_A needed to obtain full activation. The PCS phosphatases were assayed in the presence or absence of 10 μ g of protamine/ml. One unit of enzyme is the amount that releases 1 nmol of [³²P]P_i/min at 30 °C. The assay time was 5 min unless indicated otherwise. Deinh inhibitor phosphatase activity was measured in a 5 min assay based on the activity change (Goris *et al.*, 1985a) with 10–20 μ g of deinh inhibitor protein (specific activity 100–200 units/mg)/ml. Modulator was measured in an inhibition assay with 0.03 unit of the deinh inhibitor-free phosphatase (Goris *et al.*, 1984b) in a total volume of 30 μ l. In the crude fractions the effect of the modulator, which can interfere in a deinh inhibitor phosphatase assay, was negligible, considering the assay dilutions used; only in the crude extract and in the pH 5.9 supernatant the deinh inhibitor phosphatase activity could be slightly underestimated.

Preparation of the crude cellular fractions

Fresh skeletal muscle (1.6 kg from four rabbits) was homogenized in 4 litres of buffer containing 50 mM-Tris/HCl, pH 8, 0.5 mM-dithiothreitol, 1 mM-EDTA, 1 mM-EGTA, 0.1 mM-tosyl-lysylchloromethane, 0.1 mM-phenylmethanesulphonyl fluoride, 0.5 mM-benzamidine and 250 mM-sucrose, with a Robot Minute SEB blender. This, and all consecutive steps, were carried out at 4 °C. The homogenate was then centrifuged for 60 min at 6000 g and the supernatant filtered through glass wool. This crude muscle extract was then acidified to pH 5.9 with 1 M-acetic acid, left for 10 min on ice, and then centrifuged for 20 min at 8000 g to separate the microsomal and particulate glycogen fraction from the cytosol. The pH of the supernatant was adjusted to 7.5 with 1 M-NaOH. The pellet was redissolved in the homogenization buffer without sucrose, with a Dounce hand homogenizer (400 ml total vol.), and centrifuged at 30000 rev./min for 90 min in the Beckman Ti 35 rotor. The pellet thus obtained was redissolved in the same way.

RESULTS AND DISCUSSION

Identification of the major soluble deinh inhibitor phosphatase in rabbit skeletal muscle

In previous studies (Merlevede, 1985; Goris *et al.*, 1985a,b), purified preparations of three different forms of polycation-stimulated (PCS) protein phosphatases (PCS_H, PCS_M and PCS_L) and their catalytic subunit (PCS_C), as well as the ATP,Mg-dependent protein phosphatase in its spontaneously active or activated form, and calcineurin were tested for their ability to re-activate the deinh inhibitor protein. On the basis of their phosphorylase/deinh inhibitor phosphatase activity ratio, only the PCS_H protein phosphatase could be considered as an effective deinh inhibitor phosphatase *in vitro*. However, this observation did not exclude other, possibly more important, protein phosphatases that could play a role in the re-activation of the deinh inhibitor protein. To investigate this question, the cytosol was first separated from the microsomal and particulate glycogen fraction by a mild acidification step. This method is known to precipitate also most of the active form of the ATP,Mg-dependent phosphatase (Cohen, 1978), which has a very low deinh inhibitor phosphatase activity (Goris *et al.*, 1985a). As shown in Table 1, about 80% of the deinh inhibitor phosphatase activity found in the extract was recovered in the pH 5.9 supernatant. The precipitate contained less than 8% of the deinh inhibitor phosphatase activity. Most of the phosphorylase phosphatase activity present in this pellet is the active form of the ATP,Mg-dependent phosphatase, since it is inhibited by the phospho-form of inhibitor-1 and can be converted into the inactive form by the modulator protein (Vandenhede *et al.*, 1983; Merlevede *et al.*, 1984b; results not shown). The phosphorylase phosphatase activity of the resuspended pH 5.9 pellet was recovered in the 30000 rev./min pellet as an active form of the ATP,Mg-dependent phosphatase, as expected (Cohen, 1978). The low deinh inhibitor phosphatase activity in the pellet (4%) or in the supernatant (2.6%) was due either to an active form of the ATP,Mg-dependent phosphatase or, more probably, to a contaminating PCS phosphatase.

The pH 5.9 supernatant was then chromatographed on DEAE-Sephacel and screened for different protein phosphatase activities, with phosphorylase α , inhibitor-1

Table 1. Distribution of deinh inhibitor and phosphorylase phosphatase activity observed during the crude cellular fractionation

For experimental details see the text. The deinh inhibitor phosphatase recovered in the different preparations is expressed as a percentage of the phosphatase activity present in the crude extract.

Fraction	Vol. (ml)	Deinh inhibitor phosphatase (a)			Phosphorylase phosphatase (b)		Ratio a/b
		(units/ml)	(total units)	(%)	(units/ml)	(total units)	
Crude extract	5000	30.3	151 500	100	65.7	328 500	0.46
pH 5.9 precipitation							
Supernatant	4800	25.2	121 000	79.8	6.1	29 280	4.13
Pellet	400	29.3	11 720	7.7	315.3	126 120	0.09
30000 rev./min resuspended pH 5.9 pellet							
Supernatant	3000	13.0	3900	2.6	21.8	6 540	0.59
Pellet	400	15.0	6000	4.0	215.3	86 120	0.07

Fig. 1. Protein phosphatase activity toward different substrates during DEAE-Sephacel chromatography of the pH 5.9 supernatant.

Details of the protein phosphatase fraction used and the development of the column are described in the text; 5.6 ml fractions were collected. Phosphorylase phosphatase activity measured as such and after activation by kinase F_A in the presence of ATP and Mg^{2+} (not shown, but the peak fraction is indicated by arrow A), in the presence of 330 units of phospho-inhibitor-1/ml in the absence ($\blacktriangle$) or presence ($\triangle$) of $10 \mu g$ of protamine/ml, and the deinhibitor phosphatase activity ($\circ$) are shown in panel (a). Inhibitor-1 phosphatase activity was measured in the absence ($\square$) or in the presence ($\blacksquare$) of 3 mM- $CaCl_2$ as shown in panel (b). All these phosphatase activities were measured after a 40-fold dilution in 20 mM-Tris/HCl (pH 7.5)/0.5 mM-dithiothreitol containing 1 mg of bovine serum albumin/ml (buffer B).

and deinhibitor protein as substrates (Fig. 1). Some 800 ml of the pH 5.9 supernatant, adjusted to pH 7.5 with 1 M-NaOH, was mixed with 150 ml (settled volume) of DEAE-Sephacel equilibrated in the homogenization buffer without sucrose. After 15 min on ice with intermittent mixing the slurry was poured into a Buchner funnel, and the eluate was re-applied on to the ion exchanger and subsequently washed with 4×150 ml of the same buffer. The slurry was then packed in a 2.5 cm-diam. column and washed further until the A_{280} was below 0.1. The proteins were eluted from the column with 1000 ml of a 0–0.5 M-NaCl linear gradient in buffer A (20 mM-Tris/HCl, pH 7.5, 0.5 mM-dithiothreitol, 1 mM-EDTA, 1 mM-EGTA, 0.5 mM-benzamidine) as

Fig. 2. Co-purification of the PCS_H protein phosphatase and the deinhibitor phosphatase activity on Ultrogel AcA-34 molecular sieving.

The dialysed 60% -satd.-(NH_4) $_2$ SO $_4$ precipitate of the PCS_H phosphatase pool after DEAE-Sephacel (11 ml from 1.2 kg of rabbit skeletal muscle) was applied on to a 2.5 cm $\times$ 95 cm Ultrogel AcA-34 column equilibrated in buffer A; 2.6 ml fractions were collected and diluted 20-fold in buffer B for the phosphorylase phosphatase assay in the presence ($\triangle$) or absence ($\blacktriangle$) of $10 \mu g$ of protamine/ml and for deinhibitor phosphatase ($\circ$) activity. The M_r markers are Blue Dextran (V_0), ferritin (1), catalase (2) and aldolase (3). Fractions were pooled as indicated by the horizontal bar.

shown in Fig. 1. The phosphorylase phosphatase activity was measured in the presence or absence of protamine to localize the polycation-stimulated protein phosphatase(s), and with and without the phospho-form of inhibitor-1 to localize the spontaneously active form of the ATP, Mg-dependent protein phosphatase. The inactive form of this enzyme was determined by an incubation with kinase F_A and ATP, Mg. The PCS_M phosphatase was localized by its specific characteristic of being activated by a Ca^{2+} -dependent proteinase when inhibitor-1 was used as substrate (Waelkens *et al.*, 1985).

The ATP, Mg-dependent phosphatase is inhibited by polycations (Merlevede, 1985), and, since stimulation of phosphorylase phosphatase activity of the PCS enzyme could be hidden if both types of phosphatase were present in the same fractions, the PCS phosphatase activity was measured in the presence of the phospho-form of inhibitor-1 in order to suppress the ATP, Mg-dependent phosphatase. It was concluded that the phosphatase eluted at position A in Fig. 1 corresponds to the ATP, Mg-dependent protein phosphatase enzyme forms (Merlevede *et al.*, 1984b), for the following reasons. (i) The phosphorylase phosphatase is inhibited by over 66% by the phospho-form of inhibitor-1 (330 units/ml). This residual activity is rather resistant to further inhibition,

Fig. 3. Co-purification of the PCS_H protein phosphatase and the deinhibitor phosphatase activity during aminohexyl-Sepharose 4B affinity chromatography

The PCS_H phosphatase from the previous step (55 ml) was pooled and, after addition of glycerol (final concn. 2%), was applied to an aminohexyl-Sepharose 4B column (0.9 cm × 5 cm) equilibrated in buffer A containing 2% glycerol. The column was washed with 40 ml of 0.4 M-NaCl in the same solution and subsequently developed with 160 ml of a linear gradient to 0.8 M-NaCl in the same buffer. Fractions of 2.6 ml (wash) and 1.8 ml (gradient) were collected and assayed (same symbols) as in Fig. 2 after a 10-fold dilution. Fractions were pooled as indicated by the horizontal bar.

possibly because of the presence of the deinhibitor protein, which is difficult to detect in the presence of modulator protein (see below). It could be further decreased by an additional 40% of the remaining activity by inhibitor-1 (1200 units/ml). (ii) The phosphorylase phosphatase activity is increased by incubation with kinase F_A in the presence of ATP, Mg. (iii) The modulator protein, a subunit of this phosphatase, is eluted in the same position as the phosphorylase phosphatase (results not shown).

As shown in Fig. 1, the ATP, Mg-dependent phosphatase was eluted in the gradient well separated from three different polycation-stimulated protein phosphatase activity peaks, that correspond to the PCS_H, PCS_M and PCS_L protein phosphatases respectively (Waelkens *et al.*, 1985). The major portion (about 80%) of deinhibitor phosphatase activity was located in the peak corresponding to the PCS_H protein phosphatase and was eluted to a minor extent at the place of the PCS_L phosphatase. The asymmetry at the leading edge of the PCS_H, inhibitor-1 and deinhibitor phosphatase peak is probably due to the active form of the ATP, Mg-dependent phosphatase, or possibly to a still unidentified protein phosphatase. Quantitatively this represented only a minor fraction of the deinhibitor phosphatase

Fig. 4. Co-purification of the PCS_H protein phosphatase and the deinhibitor phosphatase activity during Mono Q fast protein liquid chromatography

The pooled PCS_H phosphatase from the previous step (41 ml), concentrated by dialysis against 20% poly(ethylene glycol) in buffer A and then against 60% glycerol in the same buffer, was applied on to a Mono Q column equilibrated in buffer A containing 2% glycerol and 0.25 M-NaCl. After washing with 5 ml of the same solution, the phosphatase was eluted with 30 ml of a linear gradient to 0.45 M-NaCl in the same buffer. Fractions (1 ml) were collected and assayed (same symbols) as in Fig. 2 after a 20-fold dilution.

activity. No deinhibitor activity could be detected in any other elution fraction. At this stage recovery of the main peak of deinhibitor phosphatase activity with reference to the crude extract, as can be gathered from Table 1 and Fig. 1, was only 15–20%. This loss of deinhibitor phosphatase activity cannot be explained at present, but might be due to the removal of a stimulatory or stabilizing factor.

Co-purification of the deinhibitor phosphatase and the PCS_H protein phosphatase

To determine whether the major deinhibitor phosphatase activity that co-eluted with the PCS_H protein phosphatase could be ascribed to the same enzyme, the PCS_H phosphatase was purified to apparent homogeneity and the deinhibitor phosphatase activity was monitored during this procedure. The crude extract was applied to DEAE-Sephacel (with a procedure similar to that described above) and the different forms of ATP, Mg-dependent phosphatase were removed by extensive washing with 0.2 M-NaCl in buffer A. After development of the gradient (as in Fig. 1, but with a 0.2–0.5 M-NaCl linear gradient in buffer A), a 30–60% -satd.-(NH₄)₂SO₄ fraction of the pooled PCS_H phosphatase was made and the enzyme was further purified by molecular sieving in Ultrogel AcA-34 (Fig. 2), aminohexyl-Sepharose 4B affinity chromatography (Fig. 3) and Mono Q fast protein liquid chromatography (Fig. 4). In these successive purification steps most of the deinhibitor phosphatase activity co-eluted perfectly with the polycation-stimulated phosphorylase phosphatase activity of the PCS_H enzyme. The lower-*M_r* deinhibitor phosphatase

Table 2. Purification of rabbit muscle phosphatase deinhibitor protein phosphatase

Details of the experimental procedures are described in the text. The activity of the DEAE-Sephacel eluate was taken as 100%.

Purification step	Activity (units/ml)	Volume (ml)	Total activity (units)	Yield (%)
DEAE-Sephacel PCS _H pool	13	250	3250	100
30–60%-satd.-(NH ₄) ₂ SO ₄ fraction	259	11	2850	87.6
Ultrogel AcA-34 eluate	25	55	1375	42.3
Aminohexyl-Sepharose 4B eluate	7	41	287	8.8
Mono Q eluate				
PCS _{H1}	22	5	110	
PCS _{H2}	13	5	75	
Total PCS _{H1+2}			185	5.6

peak found in the Ultrogel AcA-34 gel filtration (Fig. 2) was clearly separated from the PCS_H phosphatase and co-migrated with a mixture representing some spontaneously active ATP, Mg-dependent protein phosphatase as well as PCS_L phosphatase (E. Waelkens, unpublished work). This mixture was removed in the aminohexyl-Sepharose 4B chromatography step (Fig. 3). The Mono Q fast protein liquid chromatography (Fig. 4) separated two forms of PCS_H phosphatase, both showing a deinhibitor/phosphorylase phosphatase activity ratio of about 4:1: PCS_{H1}, characterized by a 62, 55 and 35 kDa subunit structure, and PCS_{H2}, containing only 62 and 35 kDa subunits (results not shown). The latter enzyme form appears indistinguishable in its subunit structure from the PCS_L phosphatase (E. Waelkens, unpublished work). Nevertheless PCS_L phosphatase displays a distinct deinhibitor/phosphorylase phosphatase activity ratio which is lower by two orders of magnitude than that of the PCS_{H2} phosphatase. This clearly demonstrates that the subunit structure is an insufficient criterion to distinguish between PCS phosphatases.

A summary of the purification is shown in Table 2. In calculating the yields of deinhibitor phosphatase, the activity of the enzyme at the DEAE-Sephacel chromatography step was taken as 100%.

Effects of P_i, PP_i, ATP and polycations on the phosphatase activity of PCS_H enzyme with deinhibitor protein and phosphorylase α as substrates

As shown in Fig. 5, the phosphorylase phosphatase activity of the PCS_{H1} phosphatase (and also of the PCS_{H2} phosphatase; results not shown) is completely abolished by PP_i, and the same is true for P_i and ATP (results not shown). However, when both the deinhibitor protein and phosphorylase were used as substrates for the PCS_H phosphatase, it was observed that the deinhibitor phosphatase activity is comparatively less sensitive to P_i, PP_i and ATP (Fig. 5, Table 3). In addition, the phosphatase activity of the PCS_H enzyme toward the deinhibitor is not stimulated by polycations such as protamine, histone H1 or polylysine, in contrast with the clear stimulation of dephosphorylation of the phosphorylase observed with the same enzyme. It therefore appears that these effectors induce a change in the efficiency of the phosphatase towards phosphorylase, whereas the dephosphorylation of the deinhibitor protein is less affected.

The basal phosphorylase phosphatase activity of the PCS_H phosphatase *in vivo* is presumably low, on the basis

Fig. 5. Effect of PP_i on phosphorylase phosphatase and deinhibitor phosphatase activities of the PCS_H enzyme

The purified PCS_{H1} phosphatase was preincubated with the indicated concentrations of PP_i during 10 min at 30 °C. Subsequently the deinhibitor (□) and the phosphorylase phosphatase activity were measured in the presence (Δ) or absence (○) of 10 μg of protamine/ml. The activities are expressed as % of the control value without treatment.

Table 3. Effects of P_i, PP_i and ATP on the deinhibitor and phosphorylase phosphatase activity of the PCS_{H1} phosphatase

The PCS_{H1} phosphatase was preincubated for 10 min at 30 °C with the different effectors. Phosphorylase and deinhibitor phosphatase activities were then assayed as described in the Experimental section after addition of an equal volume containing the substrate. The concentrations of the effectors indicated I₅₀ were those in the preincubation that gave 50% inactivation of the enzymes

Effector	I ₅₀ (mM)	
	Phosphorylase	Deinhibitor
P _i	3	6
PP _i	0.005	0.11
ATP	0.4	No inhibition*

* With concentrations up to 6 mM.

of its cellular concentration and specific activity of the enzyme, and is probably still further suppressed by the prevailing concentrations of the effectors (ATP, PP_i and P_i) in the cell. PCS_H phosphatase could be active *in vivo* as a deinhibitor phosphatase, since it is much less sensitive to these effectors. One should bear in mind, however, that, although the polycation stimulation is a useful tool for classification of protein phosphatases (Merlevede, 1985), it may represent a completely artificial way to stimulate these enzymes.

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