Identification of the phosphatase deinhibitor protein phosphatases in rabbit skeletal muscle

Jozef GORIS,* Etienne WAELKENS and Wilfried MERLEVEDE
Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit Leuven, Campus Gasthuisberg, Leuven B-3000, Belgium

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, PPi, and P1, whereas the deinhibitor phosphatase activity of the enzyme is much less sensitive to these agents.

INTRODUCTION

The deinhibitor is a small thermolabile protein, first described in the dog liver glycogen pellet (Defreyn 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; Jurgenensen 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., 1984a).

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, Pi, PPi 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, tosyllysylchloromethane ("TLCK"), protamine hydrochloride and dithiothreitol were obtained from Sigma; Ultrogel AAc-34 was from LKB; DEAE-Sephalac, aminoethyl-Sepharose, Blue Dextran, ferritin (440 kDa), catalase (232 kDa) and aldolase (158 kDa) were from Pharmacia.

* To whom reprint requests should be addressed.

Vol. 239
Assays

Phosphorylase phosphatase (Yang et al., 1980) and inhibitor-I phosphatase (Waelkens et al., 1984) activities were measured with 10 μM and 2 μM 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, needed to obtain full activation. The PCS phosphatases were assayed in the presence or absence of 10 μg of protamine/ml. One unit of enzyme is the amount that releases 1 nmol of [32P]P/μmol at 30 °C. The assay time was 5 min unless indicated otherwise. Deinhibitor phosphatase activity was measured in a 5 min assay based on the activity change (Goris et al., 1985a) with 10–20 μg of deinhibitor protein (specific activity 100–200 units/mg)/ml. Modulator was measured in an inhibition assay with 0.03 unit of the deinhibitor-free phosphatase (Goris et al., 1984b) in a total volume of 30 μl. In the crude fractions the effect of the modulator, which can interfere in a deinfibrator phosphatase assay, was negligible, considering the assay dilutions used; only in the crude extract and in the pH 5.9 supernatant the deinfibrator 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 deinhibitor 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, PCS and PCS) and their catalytic subunit (PCS), 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 deinhibitor protein. On the basis of their phosphorylase/deinhibitor phosphatase activity ratio, only the PCS protein phosphatase could be considered as an effective deinfibrator 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 deinfibrator 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 deinfibrator phosphatase activity (Goris et al., 1985a). As shown in Table 1, about 80% of the deinfibrator phosphatase activity found in the extract was recovered in the pH 5.9 supernatant. The precipitate contained less than 8% of the deinfibrator 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-I and can be converted into the inactive form by the modulator protein (Vandenheede 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 deinfibrator 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 a, inhibitor-I

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

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol. (ml)</th>
<th>Deinhibitor phosphatase (a)</th>
<th>Phosphorylase phosphatase (b)</th>
<th>Ratio a/b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(units/ml)</td>
<td>(total units)</td>
<td>(units/ml)</td>
</tr>
<tr>
<td>Crude extract</td>
<td>5000</td>
<td>30.3</td>
<td>151500</td>
<td>100</td>
</tr>
<tr>
<td>pH 5.9 precipitation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>4800</td>
<td>25.2</td>
<td>121000</td>
<td>79.8</td>
</tr>
<tr>
<td>Pellet</td>
<td>400</td>
<td>29.3</td>
<td>11720</td>
<td>7.7</td>
</tr>
<tr>
<td>30000 rev./min resuspended pH 5.9 pellet</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant</td>
<td>3000</td>
<td>13.0</td>
<td>3900</td>
<td>2.6</td>
</tr>
<tr>
<td>Pellet</td>
<td>400</td>
<td>15.0</td>
<td>6000</td>
<td>4.0</td>
</tr>
</tbody>
</table>

For experimental details see the text. The deinfibrator phosphatase recovered in the different preparations is expressed as a percentage of the phosphatase activity present in the crude extract.
Deinhibitor phosphatases in skeletal muscle

Fig. 1. Protein phosphatase activity toward different substrates during DEAE-Sephael 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 $F_\Lambda$ 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 (▲) or presence (▲) of 10 μg of protamine/ml, and the deinhibitor phosphatase activity (○) are shown in panel (a). Inhibitor-1 phosphatase activity was measured in the absence (□) or in the presence (■) 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).

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

The dialysed 60% saturated $(NH_4)_2SO_4$ precipitate of the PCS$_H$ phosphatase pool after DEAE-Sephael (11 ml from 1.2 kg of rabbit skeletal muscle) was applied on to a 2.5 cm × 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 (▲) or absence (▲) of 10 μg of protamine/ml and for deinhibitor phosphatase (○) 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 $F_\Lambda$ and ATP,Mg. The PCS$_H$ 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., 1984), for the following reasons. (i) The phosphorylase phosphatase is inhibited by over 60% by the phospho-form of inhibitor-1 (330 units/ml). This residual activity is rather resistant to further inhibition,
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-I (1200 units/ml). (ii) The phosphorylase phosphatase activity is increased by incubation with kinase F, 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 PCSH, PCSM and PCSL protein phosphatases respectively (Waelkens et al., 1985). The major portion (about 80%) of deinhibitor phosphatase activity was located in the peak corresponding to the PCSH protein phosphatase and was eluted to a minor extent at the place of the PCSL phosphatase. The asymmetry at the leading edge of the PCSH, inhibitor-I 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 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 I 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 PCSH protein phosphatase

To determine whether the major deinhibitor phosphatase activity that co-eluted with the PCSH protein phosphatase could be ascribed to the same enzyme, the PCSH phosphatase was purified to apparent homogeneity and the deinhibitor phosphatase activity was monitored during this procedure. The crude extract was applied to DEAE-Sepharose (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% saturation of (NH4)2SO4 fraction of the pooled PCSH 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 PCSH enzyme. The lower-M, deinhibitor phosphatase
peak found in the Ultrogel AcA-34 gel filtration (Fig. 2) was clearly separated from the PCS\textsubscript{H} phosphatase and co-migrated with a mixture representing some spontaneously active ATP,Mg-dependent protein phosphatase as well as PCS\textsubscript{H}, 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\textsubscript{H} phosphatase, both showing a deinhibitor/phosphorylase phosphatase activity ratio of about 4:1: PCS\textsubscript{H1}, characterized by a 62, 55 and 35 kDa subunit structure, and PCS\textsubscript{H2}, containing only 62 and 35 kDa subunits (results not shown). The latter enzyme form appears indistinguishable in its subunit structure from the PCS\textsubscript{H}, phosphatase (E. Waelkens, unpublished work). Nevertheless PCS\textsubscript{H} phosphatase displays a distinct deinhibitor/phosphorylase phosphatase activity ratio which is lower by two orders of magnitude than that of the PCS\textsubscript{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-Sepahcel chromatography step was taken as 100%.

Effects of P\textsubscript{i}, PP\textsubscript{i}, ATP and polycations on the phosphatase activity of PCS\textsubscript{H1} enzyme with deinhibitor protein and phosphorylase \textit{a} as substrates

As shown in Fig. 5, the phosphorylase phosphatase activity of the PCS\textsubscript{H1} phosphatase (and also of the PCS\textsubscript{H2} phosphatase; results not shown) is completely abolished by PP\textsubscript{i}, and the same is true for P\textsubscript{i} and ATP (results not shown). However, when both the deinhibitor protein and phosphorylase were used as substrates for the PCS\textsubscript{H1} phosphatase, it was observed that the deinhibitor phosphatase activity is comparatively less sensitive to P\textsubscript{i}, PP\textsubscript{i} and ATP (Fig. 5, Table 3). In addition, the phosphatase activity of the PCS\textsubscript{H2} 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\textsubscript{H} phosphatase in vivo is presumably low, on the basis

---

**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-Sepahcel eluate was taken as 100%.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Activity (units/ml)</th>
<th>Volume (ml)</th>
<th>Total activity (units)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE-Sepahcel PCS\textsubscript{H} Pool</td>
<td>13</td>
<td>250</td>
<td>3250</td>
<td>100</td>
</tr>
<tr>
<td>30-60% satd.-{(NH_4)}\textsubscript{2}SO\textsubscript{4} fraction</td>
<td>259</td>
<td>11</td>
<td>2850</td>
<td>87.6</td>
</tr>
<tr>
<td>Ultrogel AcA-34 eluate</td>
<td>25</td>
<td>55</td>
<td>1375</td>
<td>42.3</td>
</tr>
<tr>
<td>Aminohexyl-Sepharose 4B eluate</td>
<td>7</td>
<td>41</td>
<td>287</td>
<td>8.8</td>
</tr>
<tr>
<td>Mono Q eluate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCS\textsubscript{H1}</td>
<td>22</td>
<td>5</td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>PCS\textsubscript{H2}</td>
<td>13</td>
<td>5</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>Total PCS\textsubscript{H1+2}</td>
<td></td>
<td></td>
<td>185</td>
<td>5.6</td>
</tr>
</tbody>
</table>

---

**Table 3. Effects of P\textsubscript{i}, PP\textsubscript{i}, and ATP on the deinhibitor and phosphorylase phosphatase activity of the PCS\textsubscript{H1} phosphatase**

The PCS\textsubscript{H1} phosphatase was preincubated with the indicated concentrations of P\textsubscript{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>
<thead>
<tr>
<th>Effector</th>
<th>Phosphorylase</th>
<th>Deinhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>P\textsubscript{i}</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>PP\textsubscript{i}</td>
<td>0.005</td>
<td>0.11</td>
</tr>
<tr>
<td>ATP</td>
<td>0.4</td>
<td>No inhibition*</td>
</tr>
</tbody>
</table>

* 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\textsubscript{i} and P\textsubscript{i}) in the cell. PCS\textsubscript{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.

These investigations were supported by the ‘Fonds voor Geneeskundig Wetenschappelijk Onderzoek’ and by the ‘Onderzoeksfonds K. U. Leuven’. We are grateful to Ms. R. Bollen, Ms. R. Verbiest and Ms. T. Crabbé for expert technical assistance.

REFERENCES


Received 3 February 1986/14 April 1986; accepted 10 June 1986