Dephosphorylation of the deinhibitor protein by the PCS_H protein phosphatase

Jozef Goris, Etienne Waelkens and Wilfried Merlevede

Afdeling Biochemie, Faculteit Geneeskunde, Campus Gasthuisberg, Katholieke Universiteit te Leuven, B-3000 Leuven, Belgium

Received 20 June 1985

The deinhibitor protein, responsible for the decreased sensitivity of the ATP,Mg-dependent protein phosphatase to inhibitor-1 and the modulator protein, is inactivated by cyclic AMP-dependent protein kinase and reactivated by dephosphorylation. The specificity of this reaction was tested with the ATP,Mg-dependent phosphatase in its activated or spontaneously active form, four different forms of polycation-stimulated phosphatases (PCS_H, PCS_M, PCS_L and PCS_C) and calcineurin. Only the high -M_r polycation-stimulated protein phosphatase (PCS_H), but not its catalytic subunit (PCS_C), shows a high degree of specificity for the deinhibitor protein. Deinhibitor phosphatase activity of PCS_H is affected neither by polycations nor by Mn ions.

Deinhibitor protein (activation) *ATP,Mg-dependent phosphatase* *Inhibitor-1*
Modulator protein (inhibitor-2) *Polycation-stimulated phosphatase*

1. INTRODUCTION

The deinhibitor, one of the heat-stable regulatory proteins of the ATP, Mg-dependent phosphatase [1], was discovered in 1977 [2] as a factor neutralizing the effect of inhibitor-1 and modulator protein (then still called inhibitor-2) on dog liver phosphorylase phosphatase. The deinhibitor protein not only protects the ATP, Mg-dependent phosphatase in its activated or spontaneously active form from inhibition by inhibitor-1 and the modulator protein, but also prevents the inactivation of the enzyme brought about by the modulator protein and stabilizes the phosphatase in its active conformation after activation by protein kinase F_A [3–5]. Furthermore the dephosphorylation and inactivation of inhibitor-1 by a protein phosphatase isolated from the glycogen pellet are controlled by the deinhibitor protein [6].

Recently, we have shown [7] that the deinhibitor protein can be phosphorylated and inactivated by cyclic AMP-dependent protein kinase. Dephosphorylation and reactivation were observed with a protein phosphatase isolated from vascular smooth muscle [7]. The same phosphatase appeared to be the major inhibitor-1 phosphatase in this tissue and was stimulated several-fold by polycations using phosphorylase *a* as a substrate [8]. Based on the stimulation of phosphorylase phosphatase activity by polycations as well as on inhibitor-1 phosphatase activity, we have purified [9,10] to homogeneity 3 (PolyCation-Stimulated) protein phosphatases, called according to their apparent M_r in gel filtration: PCS_H (390000), PCS_M (250000) and PCS_L (200000). We now report that only PCS_H protein phosphatase is a highly specific deinhibitor phosphatase in contrast to the other polycation-stimulated enzyme forms, their catalytic subunit (PCS_C), the ATP, Mg-dependent phosphatase and calcineurin.

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

2. MATERIALS AND METHODS

Rabbit muscle phosphorylase *b* [11], the inactive ATP, Mg-dependent protein phosphatase [12] and activating factor protein kinase F_A [13], protein phosphatase inhibitor-1 [14], modulator protein [15] and brain calmodulin [16] were purified according to published methods. Bovine brain calcineurin [17] was a gift from J.H. Wang (Calgary, Canada) phosphorylase *b* kinase [18] from D.A. Walsh (Davis, USA) and bovine heart cyclic AMP-dependent protein kinase [19] from P.J. Parker (London, England). Dog liver deinhibitor protein was purified according to [5], but the last two purification steps (second DEAE-cellulose and Blue Sepharose chromatography) were performed in 20 mM ammonium bicarbonate and the deinhibitor protein eluted with 0.4 M ammonium bicarbonate. The lyophilisation was followed by Sephadex G-15 gel filtration. The different polycation-stimulated protein phosphatases were isolated [9,10] from rabbit skeletal muscle, the purification steps including DEAE-Sephacel ion exchange, Ultrogel AcA 34 (LKB) gel filtration, aminohexyl-Sepharose (Pharmacia) for the PCS_H protein phosphatase and polylysine-Sepharose 4B [20] for the PCS_M and PCS_L protein phosphatases, followed by Mono-Q-FPLC (Pharmacia) for all 3 enzymes. The PCS_H (390 kDa), PCS_M (250 kDa) and PCS_L (200 kDa) protein phosphatases had a specific activity of 400, 600 and 3000 U/mg, respectively, in the absence of polycations and 1600, 4500 and 5500 U/mg in the presence of 0.01 mg/ml protamine (Sigma) using phosphorylase *a* (1 mg/ml) as the substrate. With inhibitor-1 as the substrate (2 μM) the specific activity of the phosphatases was 40, 130 and 200 U/mg, respectively. The catalytic subunits of both the ATP, Mg-dependent and polycation-stimulated protein phosphatases from rabbit skeletal muscle were purified essentially as in [21]. Both catalytic subunits were subsequently separated without cross-contamination by polylysine-Sepharose 4B chromatography, resulting in specific activities of 9200 U/mg for the PCS_C phosphatase and 6800 U/mg for the catalytic subunit of the ATP, Mg-dependent phosphatase, using phosphorylase *a* as a substrate. The specific activity of calcineurin was 19.2 U/mg in the presence of 50 μM Ca^{2+} and 0.4 μM calmodulin, using 4 μM inhibitor-1 as a substrate. The deinhibitor-free spontaneously active form (40 000 U/mg) of the ATP, Mg-dependent protein phosphatase was isolated from the glycogen pellet according to [5]. All units are expressed as nmol ^{32}P released/min.

^{32}P-labeled phosphorylase *a* [22] and ^{32}P-labeled inhibitor-1 [6] were prepared as described. The deinhibitor protein (0.1 mg/ml) was phosphorylated and completely inactivated with 5 U/ml of the catalytic subunit of cyclic AMP-dependent protein kinase in a mixture containing 0.2 mM ATP, 1 mM $MgCl_2$ and 20 mM Mes buffer, pH 6. After 60 min

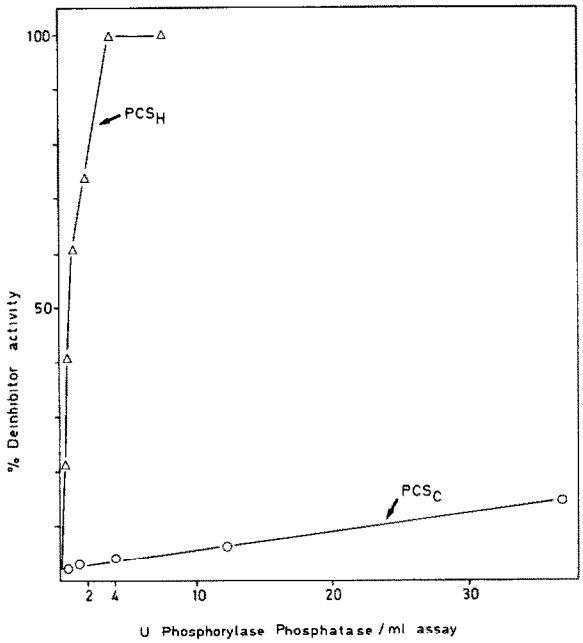

Fig.1. Reactivation of the phosphoform of the deinhibitor protein by PCS_H and PCS_C protein phosphatase. PCS_H- and PCS_C protein phosphatase, both enzymes expressed in concentrations of phosphorylase phosphatase activity measured in the absence of polycations, were incubated in a 10 μl assay containing 6 μg/ml of the deinhibitor protein phosphorylated by cyclic AMP-dependent protein kinase. After 5 min the dephosphorylation reaction was stopped by addition of 20 μl of a mixture containing 20 mM Tris, pH 7.5, 0.05 mM dithiothreitol and 0.1% Triton X-100 and boiling for 5 min. The resulting deinhibitor activity was then measured as in [5]. The 0 and 100% values represent controls of the fully phosphorylated and dephosphorylated deinhibitor preparation in the same assay conditions.

incubation, 20% Triton X-100 was added to obtain a final concentration of 0.1% and the mixture boiled for 5 min to destroy the protein kinase. Phosphorylase phosphatase [23] and inhibitor-1 phosphatase [8] activity was measured as described.

3. RESULTS AND DISCUSSION

To quantitate deinhibitor phosphatase activity, an assay method was developed based on activity changes of the deinhibitor protein, taking advantage of its heat stability. To establish the linearity of the deinhibitor phosphatase assay, the activity was measured incubating the protein phosphatase with the phosphoform of deinhibitor protein (30 μg/ml) in the absence or presence of 0.01 mg/ml protamine or 1 mM Mn^{2+}. At the appropriate time intervals 5-μl samples were diluted in 75 μl of a mixture containing 20 mM Tris, pH 7.5, 0.5 mM dithiothreitol and 0.1% Triton X-100, and boiled for 5 min. The resulting deinhibitor activity was then measured as in [5] using 20–30 mU activated ATP, Mg-dependent protein phosphatase, inhibited about 50% by inhibitor-1 in a 30 μl assay. One unit of deinhibitor phosphatase increases the deinhibitor activity by 1 unit, the assay being linear up to 0.3 U/ml.

Fig.1 shows the reactivation of the phosphorylated deinhibitor (6 μg/ml) by PCS_H and PCS_C. In table 1 deinhibitor phosphatase activity of different homogeneously purified protein phosphatases was measured, using 30 μg deinhibitor protein/ml, followed by a 5-fold higher dilution in the subsequent boiling step, to stay within the linear assay conditions for the determination of deinhibitor activity. In the same table the results are expressed in the last two columns as activity ratios when deinhibitor and phosphorylase (column 5) or deinhibitor and inhibitor-1 (column 6) are used as substrates to make comparison between the different protein phosphatases easier. As can be seen in fig.1 as well as in table 1, only the high M_r polycation-stimulated protein phosphatase (PCS_H) shows substantial deinhibitor phosphatase activity.

Table 1

Reactivation of the phosphoform of the deinhibitor protein by different protein phosphatases

Protein phosphatase	Phosphorylase phosphatase		Inhibitor-1 phosphatase (U/ml) (C)	Deinhibitor phosphatase (U/ml) (D)	Ratio D/A	Ratio D/C
	− Polycations (U/ml) (A)	+ Polycations (U/ml) (B)				
PCS_H	0.28	2.22	0.025	0.89	3.17	35.60
PCS_M	3.79	24.70	0.094	0.59	0.15	6.28
PCS_L	11.29	30.90	0.112	0.35	0.03	3.12
PCS_C	7.0	8.6	–	0.05	0.007	–
ATP,Mg-dependent						
-activated	5.00	inhibited	n.d.	0.03	0.006	–
-active (muscle)	7.00	inhibited	n.d.	0.02	0.003	–
-active (liver)	4.00	inhibited	n.d.	0.02	0.005	–
Calcineurin						
+ calmodulin	–	–	0.18	n.d.	–	–
− calmodulin	–	–	0.07	n.d.	–	–

The different protein phosphatases were assayed with 10 μM phosphorylase *a* in the absence or presence of 10 μg/ml protamine, 2 μM inhibitor-1 or 30 μg/ml deinhibitor protein. Deinhibitor phosphatase activity was measured in a 5 min assay as described in section 3. The concentrations of the phosphatases were adapted in an attempt to obtain comparable or measurable deinhibitor phosphatase activity. The ATP, Mg-dependent protein phosphatase was activated by kinase F_A in the presence of ATP and Mg^{2+} as described [12]. Non-detectable effects are indicated as n.d.

Taking phosphorylase phosphatase activity as a reference (table 1), much higher concentrations of protein phosphatase are necessary to obtain reactivation of the deinhibitor protein in the case of PCS_M, PCS_L and PCS_C. The low deinhibitor phosphatase activity observed with the activated form as well as with the spontaneously active forms of the ATP, Mg-dependent protein phosphatase obtained from skeletal muscle and liver is negligible. No reactivation of the deinhibitor protein could be observed with calcineurin even in the presence of calmodulin and Ca^{2+}. The inhibitor-1 phosphatase activity in the calcineurin preparation was blocked completely by trifluoperazine [24]. Polycations such as protamine or lysine-rich histone H_1, which increase the phosphorylase phosphatase activity of PCS_H, PCS_M and PCS_L several-fold, have no effect on the reactivation of the deinhibitor protein by the same enzymes. Mn ions have no effect either on the deinhibitor phosphatase activity of the polycation stimulated protein phosphatases (not shown).

From these results it appears that the reactivation of the deinhibitor protein by PCS_H is highly specific. A high degree of structural organization of the protein phosphatase is apparently required since the deinhibitor phosphatase activity observed with the catalytic subunit - still displaying a substantial phosphorylase phosphatase activity - is negligible. Whereas the phosphorylase phosphatase activity of PCS_H seems to be suppressed and can be stimulated by polycations, this is not the case when the deinhibitor protein is used as a substrate. These observations could be important in the study of the physiological events leading to the regulation of the ATP, Mg-dependent protein phosphatase. Indeed, the deinhibtior protein could be a key regulatory protein [1] determining the activity of the ATP, Mg-dependent phosphatase activity in vivo, not only by neutralizing the inhibitory effects of inhibitor-1 and modulator protein, but also by locking the phosphatase in its active conformation. The control of inhibitor-1 and the deinhibitor protein as well could be coordinated through the action of cyclic AMP-dependent protein kinase and PCS_H protein phosphatase, but calcineurin controls only the dephosphorylation of inhibitor-1. For the reactivation of the deinhibitor protein nature seems to have developed a very specific system, which is currently being investigated further in this laboratory.

ACKNOWLEDGEMENTS

These studies were supported by the Nationaal Fonds voor Geneeskundig Wetenschappelijk Onderzoek. The authors are grateful to Ms R. Bollen and Ms R. Verbiest for expert technical assistance.

REFERENCES

[1] Merlevede, W., Goris, J., Vandenheede, J.R., Waelkens, E. and Yang, S.-D, (1984) Proc. Soc. Exp. Biol. Med. 177, 3–11.
[2] Defreyn, G., Goris, J. and Merlevede, W. (1977) FEBS Lett. 79, 125–128.
[3] Goris, J., Waelkens, E. and Merlevede, W. (1983) Biochem. Biophys. Res. Commun. 116, 349–354.
[4] Merlevede, W., Vandenheede, J.R., Goris, J. and Yang, S.-D. (1984) Curr. Top. Cell. Regul. 23, 177–215.
[5] Goris, J., Waelkens, E., Camps, T. and Merlevede, W. (1984) Adv. Enzyme Regul. 22, 467–484.
[6] Goris, J., Camps, T., Defreyn, G. and Merlevede, W. (1981) FEBS Lett. 134, 189–193.
[7] Goris, J., Parker, P.J., Waelkens, E. and Merlevede, W. (1984) Biochem. Biophys. Res. Commun. 120, 405–410.
[8] Waelkens, E., Goris, J., Di Salvo, J. and Merlevede, W. (1984) Biochem. Biophys. Res. Commun. 120, 397–404.
[9] Waelkens, E., Goris, J. and Merlevede, W. (1985) Biochem. Soc. Trans., in press.
[10] Waelkens, E., Goris, J. and Merlevede, W. (1985) in preparation.
[11] Fischer, E.H. and Krebs, E.G. (1958) J. Biol. Chem. 231, 65–71.
[12] Yang, S.-D., Vandenheede, J.R., Goris, J. and Merlevede, W. (1980) J. Biol. Chem. 255, 11759–11767.
[13] Vandenheede, J.R., Yang, S.-D., Goris, J. and Merlevede, W. (1980) J. Biol. Chem. 255, 11768–11774.
[14] Aitken, A., Bilham, T. and Cohen, P. (1982) Eur. J. Biochem. 126, 235–246.
[15] Yang, S.-D., Vandenheede, J.R. and Merlevede, W. (1981) FEBS Lett. 132, 293–295.
[16] Gopalakrishna, R. and Anderson, W.B. (1982) Biochem. Biophys. Res. Commun. 104, 830–836.
[17] Sharma, R.K., Taylor, W.A. and Wang, J.A. (1983) Methods Enzymol. 102, 210–219.
[18] Pickett-Gies, C.A. and Walsh, D.A. (1985) J. Biol. Chem. 260, 2046–2056.
[19] Beavo, J. Bechtel, P. and Krebs, E.G. (1974) Methods Enzymol. 38, 299–308.

[20] Nevaldine, B. and Kassel, B. (1971) Biochim. Biophys. Acta 230, 207–209.
[21] Brandt, H., Capulong, Z.L. and Lee, E.Y.C. (1975) J. Biol. Chem. 250, 8038–8044.
[22] Krebs, E.G., Kent, A.B. and Fisher, E.H. (1958) J. Biol. Chem. 231, 73–83.
[23] Di Salvo, J., Waelkens, E., Gifford, D., Goris, J. and Merlevede, W. (1983) Biochem. Biophys. Res. Commun. 117, 493–500.
[24] Stewart, A.A., Ingebritsen, T.S. and Cohen, P. (1983) Eur. J. Biochem. 132, 289–295.