Isolation of a heat-stable protein activator of phosphorylase phosphatase

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1. INTRODUCTION

Phosphorylase phosphatase activity of protein phosphatase-1 [1] is believed to be regulated by:

(i) Specific interaction of ligands with phosphorylase *a*; i.e., by substrate directed-effects;
(ii) Competition between phosphorylase and various other phosphoprotein substrates;
(iii) Interaction of the phosphatase with heat-stable inhibitors [2].

Two heat-stable inhibitors have been isolated from skeletal muscle, inhibitor-1 and inhibitor-2 [3–5]. Inhibitor-1 exists in an active phosphorylated form and an inactive dephosphorylated form [3,4]. The degree of phosphorylation of inhibitor-1 is under hormonal control [1].

While studying the heat-stable protein inhibitors of phosphorylase phosphatase from kidney, we have found an activator of phosphorylase phosphatase. To our knowledge such an activator has not been described. We have purified the heat-stable protein activator from swine renal cortex ~300-fold and have studied some of its properties. The activator stimulates phosphorylase phosphatase activity primarily by decreasing the K_m of the enzyme for phosphorylase *a*.

2. MATERIALS AND METHODS

2.1. *Preparation of enzymes*

An enzyme, termed protein phosphatase C, was prepared from rabbit renal cortex as in [6]. The phosphatase preparation was >90% inhibited by renal heat-stable inhibitors and thus appears to be the putative catalytic subunit or active fragment of protein phosphatase-1 [1]. Rabbit muscle phosphatase C was purified through the first DEAE–Sephadex step [7] and further purified by chromatofocusing on a Pharmacia PBE 94 column. The phosphatase eluted as a single peak at pH 4.7. A high-M_r and presumably native form of bovine heart phosphorylase phosphatase was partially purified from a 10 000 × *g* extract by DEAE–Sepharose chromatography followed by Bio-Gel A-1.5m chromatography. [^{32}P]Phosphorylase *a* was prepared as in [7]. TPCK-treated crystalline trypsin and soybean trypsin inhibitor were purchased from Worthington. Catalytic subunit of cyclic AMP-dependent protein kinase [8] was a gift from Dr E. Reimann. Swine kidneys were obtained from a local slaughterhouse and transported to the laboratory on ice. The cortex was removed and stored at −70°C.

2.2. *Phosphorylase phosphatase activator assay*

Phosphorylase phosphatase was assayed by measuring the release of ^{32}P from [^{32}P]phosphorylase *a* [7]. A unit of phosphorylase phosphatase converts 1 nmol phosphorylase *a* dimer to phosphorylase *b*/min. Phosphorylase phosphatase activator was assayed in a 100 μl incubation mixture containing 10 munits/ml phosphatase C and 1.03 μM [^{32}P]phosphorylase *a* (all phosphorylase concentrations assume a dimer). A unit of activator doubles the phosphatase activity under standard reaction conditions. All activator assays and dilutions were carried out in glass test tubes treated with Prosil-28 (an organosilane surface-treating agent).

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3. RESULTS

3.1. *Isolation and purification of a heat-stable activator of phosphorylase phosphatase*

An extract of rabbit renal cortex [6] was heated to 95°C for 5 min, the denatured protein removed by centrifugation, and the supernatant assayed for heat-stable inhibitor. An aliquot of this preparation could completely inhibit phosphorylase phosphatase activity of phosphatase C (not shown). In an attempt to separate inhibitor-1 and inhibitor-2 we fractionated rabbit renal cortical extracts by precipitation between 0–2% and 2–15% (w/v) trichloroacetic acid [9]. The 0–2% trichloroacetic acid fraction which was expected to contain inhibitor-2 [9], did have a large amount of inhibitory activity. However, the 2–15% trichloroacetic acid fraction which was expected to contain inhibitor-1 actually stimulated phosphorylase phosphatase activity by >2-fold (not shown). Even after treatment with protein kinase to phosphorylate inhibitor-1 [4], the fraction still activated phosphorylase phosphatase activity.

A preliminary study of the 2–15% trichloroacetic acid fraction from swine renal cortex revealed a heat-stable, non-dialyzable and trypsin-labile phosphorylase phosphatase activator. For purification of the activator, the 2–15% trichloroacetic acid precipitate was prepared from 390 g cortical tissue as in [9]. The precipitate was suspended in 45 ml of 0.5 M potassium phosphate, 1.0 mM EDTA (pH 7.1). The pH of the suspension was adjusted to 7.1 by the addition of 6 N NH_4OH and extensively dialyzed against 10 mM potassium phosphate, 1 mM EDTA, pH 7.1 (PE buffer). The insoluble material was removed by centrifugation at 20 000 × *g* for 10 min and the supernatant (53 ml) was placed in a flask and heated in a boiling water bath. After the temperature reached 90°C (~5 min) heating was continued for 5 min. The suspension was chilled and centrifuged at 8000 × *g* for 10 min to remove the denatured protein. The heat-treated supernatant was mixed with 3 ml phosphocellulose equilibrated with PE buffer. After gently stirring for 45 min, the gel was poured into a column (0.9 cm) and washed with PE buffer. Phosphatase inhibiting activity was present in the effluent. The column was washed with four bed volumes of PE buffer containing 0.5 M NaCl and then the activator was eluted with PE buffer containing 1.0 M NaCl. The fractions were dialyzed against PE buffer to remove salt before assay.

The active fractions which contained 187 000 units of activator were pooled (2 ml) and could be

Table 1

Properties of heat-stable activator of phosphorylase phosphatase

Trypsin-labile[a]	+
Stokes' radius (r_S)	4.0 nm
App. M_r (gel filtration)	90 000
$s_{20,w}$	1,5
Frictional ratio	2.0
M_r (calc. [11])	26 000

[a]Activator was incubated with trypsin (activator/trypsin ratio, 40:1) in 50 mM imidazole, 5 mM EGTA, 2 mM DTT, pH 7.4 for 30 min at 30°C. The reaction was terminated with soybean trypsin inhibitor. The trypsin–trypsin inhibitor complex did not affect phosphatase activity

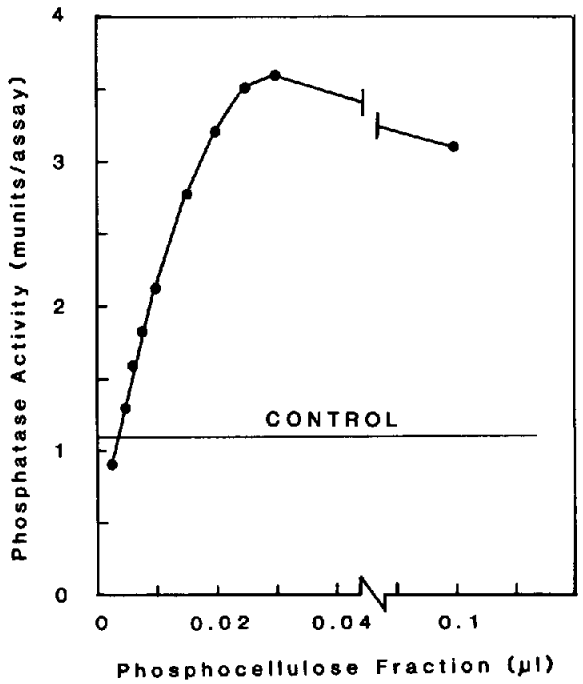

Fig.1. Effect of the activator on the activity of phosphatase C. The assay was performed as described in the text. The protein concentration of the activator was 2 µg/µl. Activity in the absence of activator (control) is shown as a solid horizontal line.

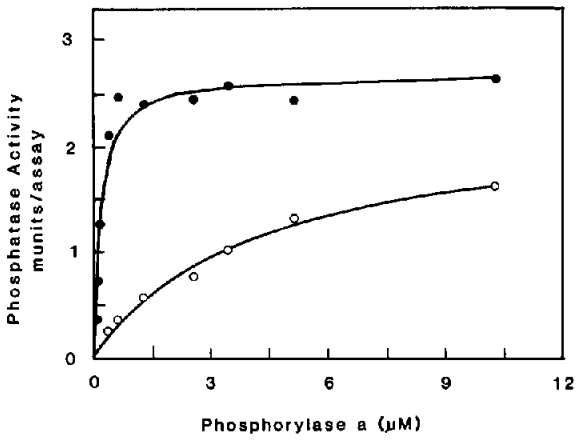

Fig.2. Kinetics of the activation of phosphatase C by the activator. The assay conditions were the same as described in the text except that the phosphatase was included at 5 munits/ml and the [^{32}P]phosphorylase concentration was varied. Assays were performed in the absence (0) and in the presence (•) of 0.045 μg activator preparation. Kinetics constants were determined by fitting the data to the Michaelis–Menten equation by nonlinear regression analysis [18]. V_{max}-values were: without activator, 2.28 munits/assay; with activator, 2.71 munits/assay. K_m-values were: without activator, 4.13 μM; with activator, 0.19 μM.

stored for several months at 4°C without loss of activity. Making the assumption that the assay of the 2–15% trichloroacetic acid fraction (the first step which could be assayed) measures all of the activator present in the extract, the activator was purified 320-fold by the procedure outlined above. On SDSgel electrophoresis [10], the preparation had 2 major protein bands (M_r 30 000–34 000) and a minor protein band (M_r ~28 000). Activator activity was destroyed by SDS treatment and could not be detected after elution of the gels. A small amount of inhibitory activity was stable during SDS electrophoresis and was eluted from the region of the gel containing the minor protein band. Activator purified through the phosphocellulose step was used for the studies reported in this paper.

3.2. *Properties of the phosphorylase phosphatase activator*

Some of the physical properties of the activator are summarized in table 1. Incubation of activator with trypsin completely abolished stimulation of phosphatase activity indicating that the activator is probably a polypeptide. When the activator was chromatographed on Sephadex G-200 both the activator and the protein emerged as a single peak with r_S 4.0 nm and app. M_r 90 000. The $s_{20,w}$ determined by sucrose density gradient centrifugation was 1.5. The activator appeared to be highly asymmetric with a frictional ratio of 2.0. The calculated M_r [11] was 26 000.

3.3. *Effect of activator on phosphorylase phosphatase activity*

Phosphorylase phosphatase activity of renal phosphatase C in the presence of activator was 3–4-times greater than in the absence of activator, under standard assay conditions (fig.1). Phosphatase C purified from rabbit skeletal muscle and a high M_r phosphorylase phosphatase isolated from bovine heart were stimulated to a similar extent by the activator (not shown). At high concentrations of activator, activition was decreased (fig.1). This may have been due to the slight contamination of the activator with heat-stable inhibitor. The activator itself had no phosphorylase phosphatase activity.

When the concentration of [^{32}P]phosphorylase *a* was varied, the activator increased the V_{max} by only ~20% (fig.2). In the absence of activator, the K_m for phosphorylase *a* was 4.13 μM. This was comparable to the value of 2.4 μM reported in [12] for skeletal muscle phosphatase. In the presence of activator, there was a marked decrease in the K_m for phosphorylase *a* to 0.19 μM.

4. DISCUSSION

To our knowledge, this is the first description of a heat-stable protein activator of phosphorylase phosphatase. Khandelwal and Zinman [13] reported on a heat-stable protein isolated from rabbit liver which stimulated the dephosphorylation of [^{32}P]histone catalyzed by a rabbit liver phosphoprotein phosphatase. However, their protein factor had no effect on phosphatase activity when [^{32}P]phosphorylase *a* was the substrate [13]. The properties of the purified phosphorylase phosphatase activator were similar to other heat-stable protein modulators which are believed to have little ordered structure [4,5,14]. It is possible that these proteins are active only after harsh treat-

ment. However, there is evidence that several are active in their native state [4,14].

The activator described here may represent a new mechanism for the regulation of phosphoprotein phosphatase activity. The activator increases phosphatase activity primarily by decreasing the K_m for phosphorylase *a* by ~20-fold. The activator may be of particular importance in tissues such as the kidney where the phosphorylase concentration is very low. The activity of phosphorylase in swine renal cortex is < 1% of the activity in rabbit skeletal muscle [15,16]. If one assumes that the specific activity of renal phosphorylase is similar to muscle phosphorylase [17], it can be calculated that the phosphorylase concentration of swine renal cortex is ~0.1 μM. Thus, the activator could have a dramatic effect on phosphorylase phosphatase activity in vivo. The brain is another tissue in which the phosphorylase concentration is low [15]. A similar heat-stable activator has been found in a 2–15% trichloroacetic acid precipitate of rat brain (unpublished).

It will also be of interest to see if other putative substrates of protein phosphatase-1 are affected by the activator. One of the major questions relating to a multifunctional phosphoprotein phosphatase is how the activity of an enzyme with several substrates is regulated [1,2,12]. An activator which can decrease the K_m for one substrate provides a mechanism by which relative specificity, and thus control, can be conferred on a multisubstrate enzyme.

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REFERENCES

[1] Cohen, P., Foulkes, J.G., Goris, J., Hemmings, B.A., Ingebritsen, T.S., Stewart, A.A. and Strada, S.T. (1981) in: Metabolic Interconversion of Enzymes 1980 (Holzer, H. ed) pp. 28–43, Springer-Verlag, Berlin, Heidelberg, New York.
[2] Krebs, E.G. and Beavo, J.A. (1979) Annu. Rev. Biochem. 48, 923–959.
[3] Huang, F.L. and Glinsmann, W.H. (1976) Eur. J. Biochem. 70, 419–426.
[4] Nimmo, G.A. and Cohen, P. (1978) Eur. J. Biochem. 87, 341–351.
[5] Foulkes, J.G. and Cohen, P. (1980) Eur. J. Biochem. 105, 195–203.
[6] Mellgren, R.L. and Schlender, K.K. (1982) J. Cyclic Nucl. Res. in press.
[7] Killilea, S.D., Aylward, J.H., Mellgren, R.L. and Lee, E.Y.C. (1978) Arch. Biochem. Biophys. 191, 638–646.
[8] Reimann, E.M. and Beham, R.A. (1982) Methods Enzymol. in press.
[9] Foulkes, J.G., Jefferson, L.S. and Cohen, P. (1979) FEBS Lett. 112, 21–24.
[10] Laemmli, U.K. (1970) Nature 227, 680–685.
[11] Siegel, L.M. and Monty, K.J. (1966) Biochim. Biophys. Acta 112, 346–362.
[12] Lee, E.Y.C., Silberman, S.R., Ganapathi, M.K., Petrovic, S. and Paris, H. (1980) Adv. Cyclic Nucl. Res. 13, 95–131.
[13] Khandelwal, R.L. and Zinman, S.M. (1978) Biochem. Biophys. Res. Commun. 82, 1340–1345.
[14] Weber, H. and Rosen, O.M. (1977) J. Cyclic Nucl. Res. 3, 415–424.
[15] Villar-Palasi, C. and Larner, J. (1960) Arch. Biochem. Biophys. 86, 270–273.
[16] Villar-Palasi, C. and Gazquez-Martinez, I. (1968) Biochim. Biophys. Acta 159, 479–489.
[17] Mendicino, J., Abou-Issa, H., Medicus, R. and Kratowick, N. (1975) Methods Enzymol. 42, 375–397.
[18] Cleland, W.W. (1967) Adv. Enzymol. 29, 1–32.