# A SECOND HEAT-STABLE PROTEIN INHIBITOR OF PHOSPHORYLASE PHOSPHATASE FROM RABBIT MUSCLE

Freesia L. HUANG and Walter GLINSMANN

*Section on Physiological Controls, Laboratory of Biomedical Sciences, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20014, USA*

Received 15 December 1975

## 1. Introduction

Glycogen phosphorylase phosphatase (EC 3.1.3.17) may be a hormonally-regulated enzyme since its activity in tissue extracts is controlled by a cyclic AMP-mediated reaction [1,2]. However, attempts to characterize the regulation of this enzyme have been difficult since the enzyme can be isolated in multiple molecular forms [3,4], appears to have broad substrate specificity [5,6], and loses the ability to be regulated by cyclic AMP and the cyclic AMP-dependent protein kinase when it is purified [7,8].

One possibility for regulation is that the enzyme contains a catalytic subunit which can dephosphorylate many proteins and that regulatory subunit(s) restrict catalytic attack and are also the site of cyclic AMP-stimulated phosphorylations. With both liver and muscle, relatively heat-stable phosphatase inhibitor proteins can be isolated from larger molecular weight proteins containing phosphatase activity [9,10]. Inactivation of the rabbit muscle enzyme by cyclic AMP-dependent protein kinase is associated with phosphorylation of one of these proteins [10].

The phosphorylated inhibitor is separable from the phosphatase by sucrose gradient centrifugation or gel permeation chromatography. However, following such separation, heat-stable inhibitor activity remains associated with the phosphatase. We now provide evidence that this residual activity is due to a second protein inhibitor of the phosphatase which does not require phosphorylation for activity.

## 2. Methods

Phosphorylase phosphatase from rabbit muscle was prepared as previously described [10]. Cyclic AMP-dependent protein kinase was prepared from rabbit muscle by the method of Wastila et al. [11] and was further purified by hydroxyapatite column chromatography [12].

Phosphorylase phosphatase activity was assayed by measuring the release of [$^{32}$P]phosphate from [$^{32}$P] phosphorylase *a* [10]. A unit of phosphatase is defined as that amount of enzyme which releases one $\mu$mol of [$^{32}$P]phosphate per min at 30°C. The inhibitor activity was determined in the assay system [10] to which 7 $\mu$U of phosphorylase phosphatase was added.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was done by the method of Shapiro et al. [13] using 100 mM Na-phosphate buffer (pH 7.1) containing 0.1% SDS. Protein was denatured in buffer containing 1% SDS and 1% 2-mercaptoethanol. Bovine serum albumin (BSA, 68 000), ovalbumin (OA, 45 000), chymotrypsinogen-A (CA, 25 000), and ribonulease-A (13 700) were used to calibrate gel columns and SDS-polyacrylamide gels.

## 3. Results and discussion

Phosphorylation of partially purified preparations of rabbit muscle phosphorylase phosphatase by cyclic

*North-Holland Publishing Company – Amsterdam*

AMP-dependent protein kinase inactivates the phosphatase by generating an inhibitory phosphoprotein that is stable to heating at 100°C for 5 min, very labile in the presence of trypsin (10–40 μg/ml, 30°C), and readily separable from the phosphatase [10]. Since we found that catalytic activity of the phosphatase is unaffected by incubation with trypsin, one of the ways we tested for relative inhibitor concentrations in phosphatase preparations was by examining the degree of activation which occurred when the enzyme was incubated with trypsin. Following maximal phosphorylation of the phosphatase by cyclic AMP-dependent kinase and separation of the phosphoinhibitor by gel permeation chromatography, we still observed tryptic activation of the phosphatase, and heat-stable inhibitor activity in the phosphatase preparation. Therefore, we were interested in whether this residual activity might be due to a second heat-stable phosphatase inhibitor.

When rabbit muscle phosphorylase phosphatase was prepared through a DEAE-cellulose chromatography step [10], and then chromatographed on columns of Sephadex (G150, G200) or Biogel (A-0.5m), a single heat-stable phosphatase inhibitor was observed; however, if the phosphatase was first treated with cyclic AMP-dependent kinase, two inhibitors were seen (fig.1). The one eluting with the smaller mol. wt. proteins was the one we described previously on the basis of its activity dependence on phosphorylation, its subunit mol. wt. (26 000), and its loss of inhibitory activity when incubated with a $Mn^{++}$-stimulated phosphatase [10]. The inhibitor activity eluting immediately after the phosphatase did not depend on prior phosphorylation and actually was not separated from phosphatase activity since treatment of these inhibitor-containing fractions with trypsin unmasked inactive phosphatase (fig.1).

The inhibitor fractions migrating with inactive phosphatase were pooled and heated (100°C, 5 min). Denatured protein was removed by centrifugation, and the supernatant fluid was chromatographed on a Biogel P-60 column. A single major peak of inhibitory activity eluted, as did the phospho-inhibitor [10], with globular proteins of 42 000 mol. wt. Fractions from Biogel P-60 containing inhibitor activity were pooled and concentrated by ultrafiltration (Amicon UM 2 membrane).

This inhibitor preparation did not lose inhibitory

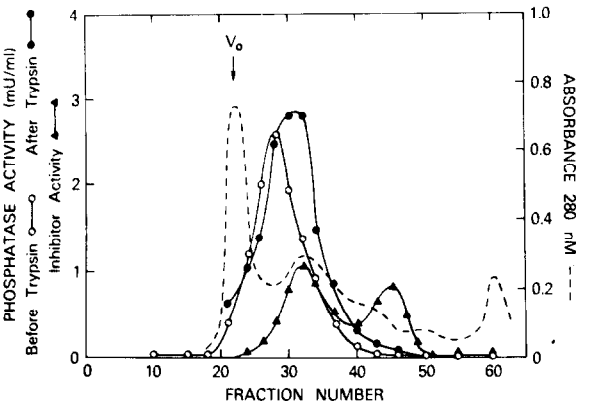



Fig.1. Column chromatography of phosphorylase phosphatase and its inhibitors on Bio-gel 0.5 m agarose. Phosphorylase phosphatase (0.1 unit) from a DEAE-cellulose step [10] was treated at 30°C with 50 μM ATP, 5 μM cyclic AMP, 5 mM $MgOAc_2$ and 0.15 unit of protein kinase for 30 min in 25 mM β-glycerophosphate buffer, pH 7.0 containing 1 mM DTT, 0.5 mM EDTA and 0.3 mM EGTA. At the end of incubation the reaction mixture was applied to a column (1.5 × 74 cm) which was developed with 20 mM Tris-Cl pH 7.4, containing 1 mM DTT, 1 mM EDTA and 0.1 M NaCl. Fractions of 2 ml were collected and were assayed for phosphorylase phosphatase before (○) and after trypsin treatment (●). Conditions for trypsin treatment were 1 μg trypsin/30 μl for 10 min at 30°C followed by addition of a 5-fold excess by weight of soybean trypsin inhibitor. Aliquots of fractions were also heated at 100°C for 5 min and 5 μl of heated supernatant fluid were tested for inhibitory activity (▲).

activity when it was incubated with a $Mn^{++}$-stimulated phosphatase that destroyed the activity of the phospho-inhibitor (fig.2A). When incubated with cyclic AMP-dependent kinase (fig.2B), the activity of the inhibitor did not change. Maximal incorporation of $^{32}P$ from γ-labelled ATP into protein in this fraction was less than 10% of that which occurred when the phosphorylatable inhibitor was used as substrate. Therefore we could find no evidence that conditions which altered phosphorylation and activity of the previously described inhibitor and any demonstrable effect on this second inhibitor.

Electrophoresis of the second inhibitor on SDS-containing gels demonstrated that it had a subunit mol. wt. of 33 000 which was distinguishable from that of the phospho-inhibitor (fig.3). The inhibitor was markedly effective at inhibiting phosphatase activity (fig.4), and it was labile when incubated with trypsin (fig.5).

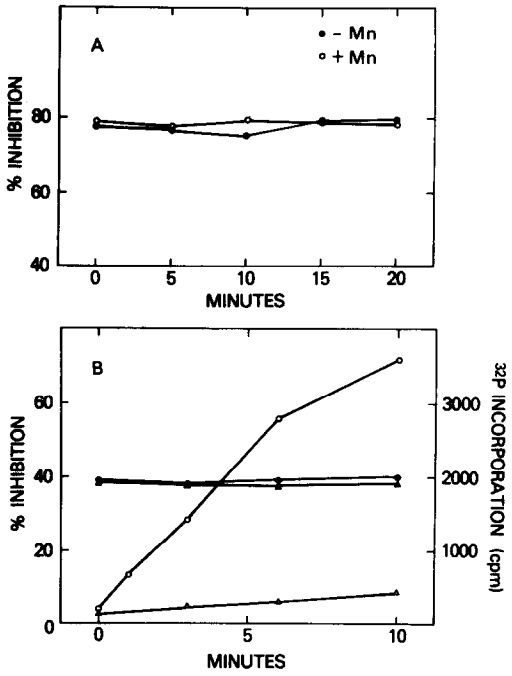



Fig.2. Lack of effect of phosphorylation and dephosphorylation on phosphatase inhibition activity. (A) Inhibitor preparation (50 μg protein) was incubated with 60 μU of $Mn^{++}$-stimulated phosphoprotein phosphatase prepared according to Kato and Bishop [3] in the presence (○) and absence (•) of 5 mM $MnCl_2$ in 125 μl buffer of 20 mM Tris-Cl, pH 7.4, containing 1 mM DTT and 1 mM EDTA. At indicated times, portions of these reactions were removed and heated for 5 min at 100°C. After centrifugation, 5 μl of supernatant fluid were determined for inhibitor activity. (B) Inhibitor (30 μg protein) was incubated with 50 μM[$\gamma$-$^{32}$P]ATP, 5 μM cyclic AMP, 5 mM $MgOAc_2$ and 5 mU protein kinase in 50 μl buffer of 25 mM β-glycerophosphate, pH 7.0, containing 1 mM DTT, 0.5 mM EDTA and 0.3 mM EGTA. At indicated times, 5 μl samples were removed for determination of $^{32}$P incorporation into trichloroacetic acid precipitable material (○). Control with protein kinase alone was checked for endogeneous phosphorylation (△). Inhibitor activity was determined from parallel reactions in which cold ATP was used, with (•) and without (▲) protein kinase; a 5 μl sample of these reactions was diluted 25-fold before assay.

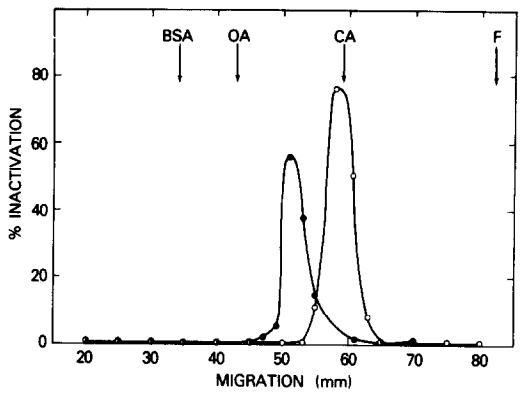



Fig.3. Electrophoresis of phosphatase inhibitors on SDS-polyacrylamide gel. Inhibitors prepared on Bio-gel P-60 columns were denatured and electrophoresed in SDS-containing polyacrylamide gels. After electrophoresis 1 mm fractions were eluted with buffer and were tested for inhibitory activity (•). The phosphorylated inhibitor (○) prepared previously [10] was run simultaneously. F = front; other abbreviations are as described in Methods.

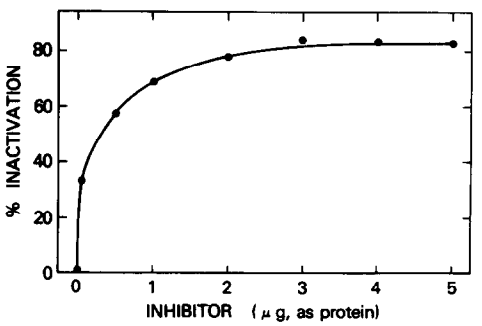
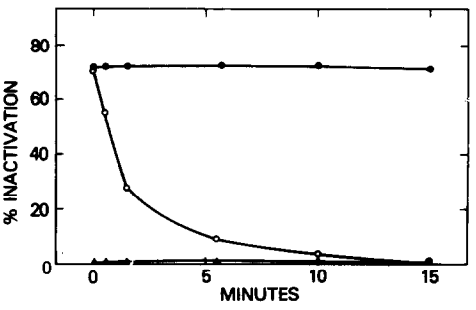



Fig.4. Concentration dependence of the inactivation of phosphorylase phosphatase by the heat-stable protein inhibitor. Inhibitor in a volume of 5 μl was added to 50 μl phosphatase assay system for inhibitor activity assay.

Fig.5. Effect of trypsin on the inhibitor of phosphorylase phosphatase. Inhibitor preparation (1 mg/ml) was incubated with bovine pancreatic trypsin (○) at a ratio of 25:1 by weight. At indicated times, tryptic digestion was terminated by the addition of a 5-fold excess by weight of soybean trypsin inhibitor. The inhibitor activity was then determined. Control reactions, to which trypsin inhibitor was added before the addition of trypsin (•), or omitting inhibitor (▲), were run simultaneously.



The results of this study provide evidence for the presence in muscle of a second heat-stable protein inhibitor of phosphorylase phosphatase. Another study from this laboratory describing an apparently non-phosphorylatable inhibitor of phosphorylase phosphatase in preparations of cyclic AMP-dependent protein kinase [14] lends further support for the existence in muscle of more than one phosphatase inhibitor.

The possible physiological function of the presently described inhibitor is unknown; however, its association with the substantially purified phosphatase suggests to us that it may be a regulatory subunit of a phosphatase analogous to the inhibitor of rabbit liver phosphatase described by Brandt et al. [9].

## References

[1] Merlevede, W. and Riley, G. A. (1966) J. Biol. Chem. 241, 3517–3524.
[2] Chelala, C. A. and Torres, H. N. (1970) Biochim. Biophys. Acta 198, 504–513.
[3] Kato, K. and Bishop, J. S. (1972) J. Biol. Chem. 247, 7420–7429.
[4] Kato, K. and Sato, S. (1974) Biochim. Biophys. Acta 358, 299–307.
[5] Nakai, C. and Thomas, J. A. (1974) J. Biol. Chem. 249, 6459–6467.
[6] Lee, E. Y. C., Capulong, Z. L. and Martin, S. (1973) Fed. Proc. 32, 554.
[7] Haschke, R. H., Heilmeyer, L. M. G., Meyer, F. and Fischer, E. H. (1970) J. Biol. Chem. 245, 6657–6663.
[8] Huang, F. L. and Glinsmann, W. H., in preparation.
[9] Brandt, H., Lee, E. Y. C. and Killilea, S. D. (1975) Biochem. Biophys. Res. Commun. 63, 950–956.
[10] Huang, F. L. and Glinsmann, W. H. (1975) Proc. Nat. Acad. Sci. (USA) 72, 3004–3008.
[11] Wastila, W. B., Stull, J. T., Meyer, S. E. and Walsh, D. A. (1971) J. Biol. Chem. 246, 1996–2003.
[12] Yamamura, H., Kumon, A. and Nishizuka, Y. (1971) J. Biol. Chem. 246, 1544–1546.
[13] Shapiro, A. L., Viñuela, E. and Maizel, J. V. (1967) Biochem. Biophys. Res. Comm. 28, 815–820.
[14] Nakai, C. and Glinsmann, W., in preparation.