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THE CONTROL OF PHOSPHORYLASE KINASE PHOSPHATASE ACTIVITY BY POLYCATIONS AND THE DEINHIBITOR PROTEIN

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The dephosphorylation of phosphorylase *b* kinase by the activated ATP,Mg-dependent protein phosphatase, which is highly specific for the β-subunit, is stimulated by the deinhibitor protein which neutralizes the effect of inhibitor-1 and the modulator protein on the phosphatase. The specific dephosphorylation of the α-subunit of phosphorylase *b* kinase by a "latent" protein phosphatase isolated from vascular smooth muscle is stimulated by histone H_1 but not affected by the deinhibitor protein. These observations show that there is no strict correlation between the insensitivity of a protein phosphatase to inhibitor-1 or modulator protein and the dephosphorylation of the α-subunit of phosphorylase *b* kinase. © 1984 Academic Press, Inc.

Phosphorylase *b* kinase is composed of four types of subunit α, β, γ, δ and has the structure $(\alpha,\beta,\gamma,\delta)_4$ (1,2). The δ-subunit is the Ca^{2+}-binding component identical to calmodulin (3). The α- and β-subunits are regulatory in nature and both are subject to phosphorylation. As discussed in (4) activation appears to be better correlated with phosphorylation of both α- and β-subunits of the enzyme than with the phosphorylation of either one alone. Dephosphorylation of the α- or β-subunit of phosphorylase *b* kinase is rather specific and this property has been used, together with the sensitivity to inhibitor-1 and modulator protein (5) as a criterion for the classification of protein phosphatases.

A high molecular weight (260K in gel filtration) "latent" protein phosphatase has been isolated from vascular smooth muscle. The enzyme activity towards phosphorylase *a* could be stimulated severalfold by polyamines (6) but the enzyme is

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spontaneously active towards phosphorylated inhibitor-1 (7,8) and deinhibitor protein (8,9). The deinhibitor protein enhances the activation of the ATP,Mg-dependent protein phosphatase (10) by kinase F_A and protects this phosphatase against inhibition by inhibitor-1 and modulator protein (10,11). We have shown previously that the ATP,Mg-dependent protein phosphatase dephosphorylates the β-subunit of phosphorylase *b* kinase preferentially (12) and the α-subunit rather than the β-subunit is dephosphorylated by the histone H_1-stimulated protein phosphatase isolated from rabbit skeletal muscle (13).

The present report describes the effect of the deinhibitor protein on the ATP,Mg-dependent phosphatase as well as the effect of lysine-rich histone H_1 on the "latent" protein phosphatase, utilizing as substrate phosphorylase *b* kinase phosphorylated on the α- and β-subunits to less than 1 phosphate per subunit.

MATERIALS AND METHODS

Rabbit muscle phosphorylase *b* (14), phosphorylase *b* kinase (4), the ATP,Mg-dependent phosphatase (15) and its activating protein factor kinase F_A (16), protein phosphatase inhibitor-1 (17), modulator protein, previously called inhibitor-2 (18), dog liver deinhibitor protein (11) and bovine heart cyclic AMP-dependent protein kinase catalytic subunit (19) were purified to homogeneity according to published methods. The histone H_1 and polylysine stimulated phosphatase was purified from bovine aortic smooth muscle according to (7) and had a specific activity of 660 U/mg in the presence of histone H_1 using phosphorylase *a* as a substrate or 15 U/mg using inhibitor-1 as a substrate. Phosphorylase and inhibitor-1 were ^{32}P-labeled up to one mole per mole per mole by phosphorylase *b* kinase and the catalytic unit of the cyclic AMP-dependent protein kinase respectively as in (20) and (21). Phosphorylase *b* kinase was ^{32}P-labeled by the catalytic unit of the cyclic AMP-dependent protein kinase. Autophosphorylation was minimized by working at a low Mg^{2+} concentration and stopping the reaction before the level of one mole P per mole α- and β-subunit was attained. Phosphorylation conditions were as follows: 1 mg/ml phosphorylase *b* kinase (6.8/8.2 activity ratio ∿0.08, pH 8.2, specific activity 1.35 U/mg), 3 mM $MgCl_2$, 4 U/ml protein kinase, 0.19 mM γ-^{32}P ATP (∿1000 CPM/pmole), 50 mM β-glycerophosphate pH 6.8, 2 mM EDTA, 25% sucrose, 2 mM benzamidine, 0.1 mM tosylphenylchloromethylketone were incubated for 10 min at 30°C. Samples were taken to determine protein bound phosphate stoichiometry by the filter paper method (22). To determine the extent of ^{32}P in each subunit, 30 λ samples were quenched with 30 λ sample buffer, boiled for 5 min and sodium dodecylsulfate polyacrylamide gel electrophoresis was performed in 7.5% gel as in (23). Histone H_1 (type V-S) was obtained from Sigma.

RESULTS AND DISCUSSION

The dephosphorylation of phosphorylase *b* kinase by the activated ATP,Mg-dependent protein phosphatase is stimulated by the deinhibitor protein (Fig. 1A). As previously observed with a partially purified dog liver and rabbit skeletal muscle enzyme (12) the purified ATP,Mg-dependent protein phosphatase from skeletal muscle is highly specific for the dephosphorylation of the β-subunit. Furthermore the deinhibitor protein even stimulates the dephosphorylation of the β-subunit by the ATP, Mg-dependent protein phosphatase, while the slow ^{32}P release

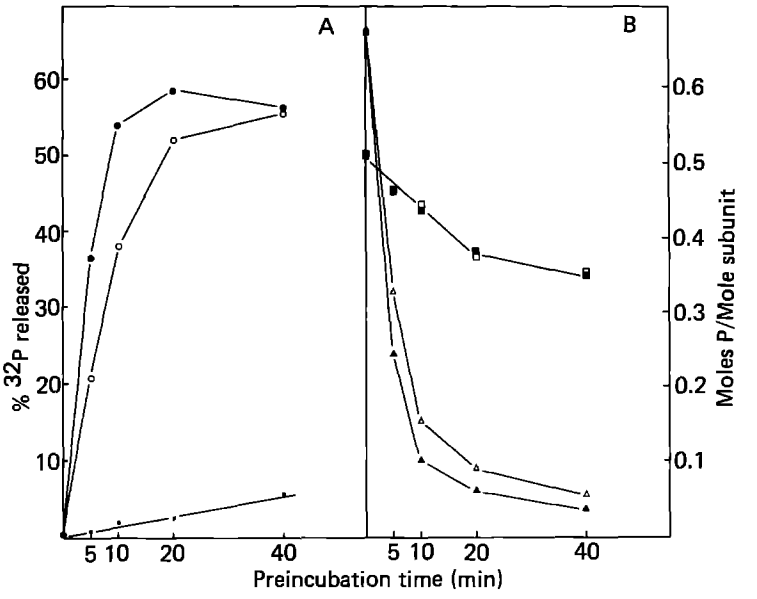

Fig. 1. *Effect of the deinhibitor protein on the dephosphorylation of phosphorylase b kinase by the ATP,Mg-dependent protein phosphatase.*
The ATP,Mg-dependent phosphatase was activated by the activating protein factor F_A, in the presence of 0.2 mM ATP, 1 mM $MgCl_2$, 1 mg per ml bovine serum albumin, 0.5 mM dithiothreitol and 20 mM Tris, pH 7.4 in the presence (closed symbols) or absence (open symbols) of deinhibitor protein (1 µg per ml) during 15 min at 30°C in a total vol of 75 µl, resulting respectively in 2.4 and 1.3 U of phosphorylase phosphatase activity per ml. After this preincubation 150 µl of 1.2 µM labeled phosphorylase *b* kinase (see methods) was added and at different time intervals 10 and 30 µl were taken to determine respectively the ^{32}P release, measured as 10% trichloroacetic acid soluble ^{32}P (panel A), and the ^{32}P remaining in each subunit, by adding 30 µl sodium dodecyl sulfate sample buffer, followed by boiling during 5 min and separation of the α- and β-subunits in 7.5% Laemmli gels (23). After electrophoresis the gels were stained with Coomassie blue, destained and the α- (squares) and β- (triangles) subunits excised and counted (panel B). Phosphate release from phosphorylase *b* kinase in the absence of phosphatase as indicated (.—.).

from the α-subunit is not affected at all (Fig. 1B). The stimulation of the β-subunit dephosphorylation by the deinhibitor protein occurs even in the absence of added inhibitor-1 or modulator protein, in accordance with the observed stimulation of phosphorylase *a* dephosphorylation by the ATP,Mg-dependent protein phosphatase (11).

The "latent" protein phosphatase isolated from vascular smooth muscle can also dephosphorylate phosphorylase *b* kinase and this reaction is stimulated by histone H_1 (Fig. 2A). The deinhibitor protein did not affect this reaction neither in the absence nor in the presence of histone H_1 (not shown). The dephosphorylation of the α-subunit is stimulated by histone H_1 while the slow ^{32}P release from the β-subunit is unaffected (Fig. 2B). The dephosphorylation of the α-subunit as well as of the β-subunit is unaffected by the deinhibitor protein in the presence as well as in the absence of histone H_1.

From these observations it appears that the effect of the deinhibitor protein is specific for the ATP,Mg-dependent protein phosphatase and for the dephosphorylation of the β-subunit of phosphorylase *b* kinase. The deinhibitor protein does not influence the "latent" protein phosphatase whether phosphorylase

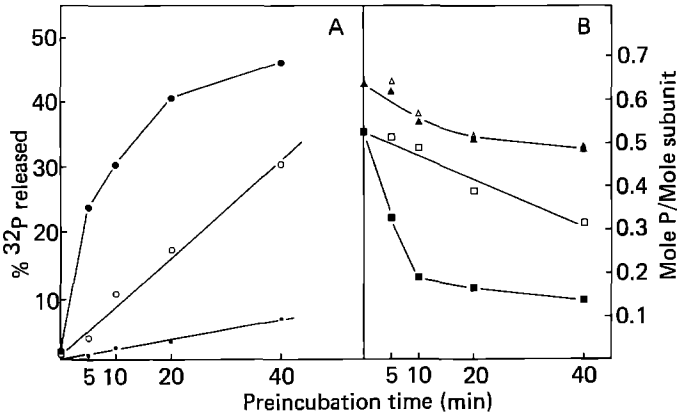

Fig. 2. *Effect of histone H_1 on the dephosphorylation of phosphorylase b kinase by the histone H_1 stimulated protein phosphatase.*
The histone H_1 stimulated phosphatase was preincubated with (closed symbols) and without (open symbols) 125 µg per ml histone H_1, 1 mg per ml bovine serum albumin, 0.5 mM dithiothreitol and 20 mM Tris, pH 7.4 during 5 min at 30°C in a total volume of 75 µl, resulting in respectively 1 and 0.2 U phosphorylase phosphatase activity per ml. After this preincubation labeled phosphorylase *b* kinase was added and the samples processed as in figure 1.

b kinase or phosphorylase *a* (not shown) is used as a substrate. Nevertheless, since the deinhibitor can stimulate some spontaneously active protein phosphatase, insensitive to inhibitor-1 and modulator protein (11) more information is necessary to know if an effect of the deinhibitor protein can be used to differentiate between different types of protein phosphatases. Vice versa the criterion of sensitivity of protein phosphatases to inhibitor-1 or the modulator protein for a classification of protein phosphatases (5) should be used carefully, since the presence of the deinhibitor protein could be responsible for a lack of inhibition of the phosphatase (11). Using phosphorylase *b* kinase, phosphorylated to less than one phosphate per α-subunit and up to one phosphate per β-subunit the deinhibitor stimulates the β-subunit dephosphorylation by the ATP,Mg-dependent protein phosphatase. Since under these conditions the phosphatase becomes insensitive to inhibition by inhibitor-1 and the modulator protein, there is no strict correlation between insensitivity to this type of inhibition and the lack of specificity towards the β-subunit of phosphorylase *b* kinase. Indeed Khatra and Soderling partially purified a phosphoprotein phosphatase with a high specificity towards dephosphorylation of the β-subunit of phosphorylase *b* kinase but insensitive to inhibition by the phosphorylated form of inhibitor-1 (24).

Histone H_1 has been shown to stimulate the activity of a "latent" protein phosphatase isolated from vascular smooth muscle towards phosphorylase *a* (6) and the α-subunit of phosphorylase *b* kinase (this paper) but the polycation has no clear stimulatory effect on the dephosphorylation of inhibitor-1 (7), the deinhibitor protein or casein phosphorylated by cyclic AMP-dependent protein kinase (unpublished observations from this laboratory). These complex observations neither confirm nor disprove the proposal (13) that the stimulation of the phosphatase activity by histone H_1 is only an enzyme-directed effect.

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