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THE DEINHIBITOR PROTEIN: REGULATION BY PHOSPHORYLATION-DEPHOSPHORYLATION

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The deinhibitor protein, which protects the multisubstrate protein phosphatase from inhibition by inhibitor-1 and the modulator protein, stabilizes the enzyme in its active conformation preventing its conversion to the ATP,Mg-dependent enzyme form and controls the dephosphorylation of inhibitor-1, was shown to exist under active and inactive forms. It can be inactivated by the catalytic unit of the cyclic AMP-dependent protein kinase and reactivated by an inhibitor-1 phosphatase, also described as histone-H_1 ("latent") stimulated protein phosphatase.

The protein phosphatase deinhibitor was discovered as a small molecular weight, heat stable regulatory protein associated with a glycogen bound protein phosphatase in dog liver (1). This multisubstrate protein phosphatase which exists as a spontaneously active and an inactive ATP,Mg-dependent enzyme form (2) could be a key target enzyme in hormonal and metabolic regulation of cellular function. The deinhibitor protein protects the ATP,Mg-dependent protein phosphatase from inhibition by the phospho-form of inhibitor-1 and the modulator protein (1,3). It has been purified to apparent homogeneity (3), and shown to control the dephosphorylation and inactivation of inhibitor-1 by a protein phosphatase isolated from the liver glycogen pellet (4). The deinhibitor also plays an important role in the interconversion of the ATP,Mg-dependent protein phosphatase, since it prevents the

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conversion of the active to the ATP,Mg-dependent enzyme form brought about by the modulator protein and enhances the activation of the inactive enzyme by kinase F_A, probably by stabilizing the protein phosphatase in its active conformation (3,5). We now present evidence that the deinhibitor itself can be phosphorylated and inactivated by the catalytic unit of the cyclic AMP-dependent protein kinase and that a major inhibitor-1 phosphatase isolated from vascular smooth muscle and previously described as the histone stimulated phosphorylase phosphatase (6), can dephosphorylate and reactivate the deinhibitor protein.

MATERIALS AND METHODS

The materials used and the experimental procedures were essentially as described in (3,6). The specific activity of the deinhibitor protein preparations used varied between 100 to 800 U per mg. The catalytic subunit of cyclic AMP-dependent protein kinase was prepared from bovine heart according to (7). The deinhibitor protein was assayed by its ability to relieve the inhibition of the ATP,Mg-dependent phosphatase by the modulator protein or by inhibitor-1 under non-dephosphorylating conditions (3).

To avoid diffusion of the deinhibitor protein out of the gel after sodium dodecyl sulfate polyacrylamide gel electrophoresis in phosphate buffer according to (8) proteins were cross-linked with 5 percent formaldehyde in 25 percent ethanol (9) before the Coomassie blue staining.

RESULTS AND DISCUSSION

The deinhibitor protein could be inactivated by the catalytic subunit of cyclic AMP-dependent protein kinase in a time- and concentration-dependent manner (Figure 1). This inactivation can be ascribed to a phosphorylation of the deinhibitor protein since reactivation was observed with a purified protein phosphatase (Figure 2). The enzyme used to obtain dephosphorylation has been shown to be a major inhibitor-1 phosphatase in vascular smooth muscle (6) previously described as a "latent" histone-H_1 and polylysine stimulated phosphorylase *a* phosphatase (10). The phosphate incorporated by the catalytic subunit of cyclic AMP-dependent protein kinase was removed by the protein phosphatase treatment as could be demonstrated by autoradiography (not shown). ^{32}P-labeled protein and deinhibitor activity comigrated in sodium

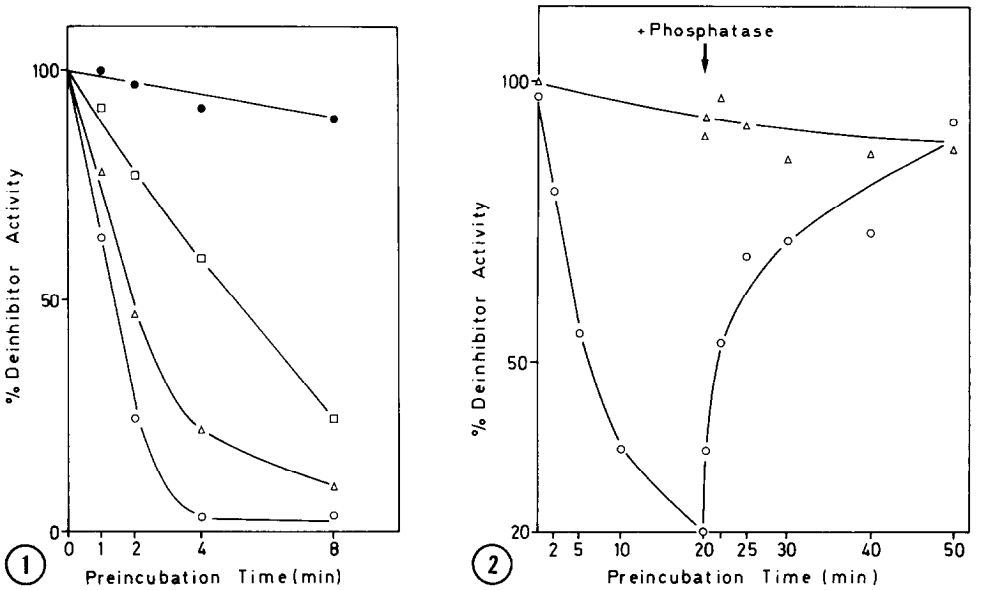
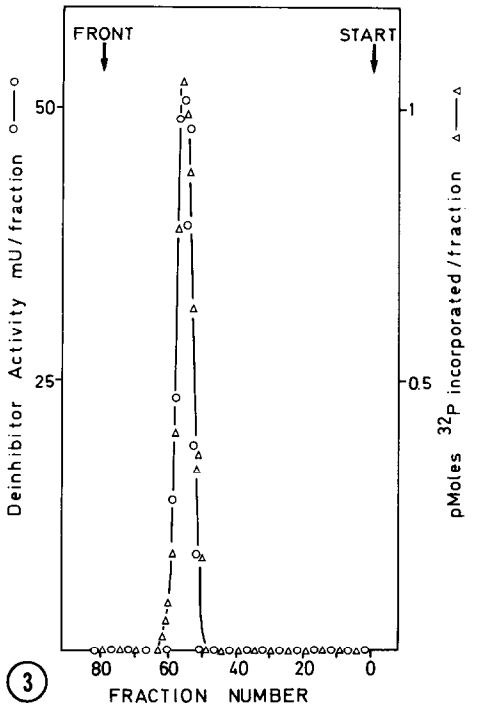


Fig. 1. *Inactivation of the deinhibitor protein by different concentrations of the catalytic subunit of cyclic AMP-dependent protein kinase.*
The deinhibitor protein (0.1 mg per ml) was preincubated at 30°C in a solution consisting of 20 mM Tris-HCl buffer (pH 7.4), 0.5 mM dithiothreitol, 1 mg per ml bovine serum albumin, 0.1 mM ATP and 0.5 mM $MgCl_2$ in the absence (●—●) or in the presence of 10 U per ml (o—o), 5 U per ml (Δ—Δ) or 2.5 U per ml (□—□) of the catalytic subunit of protein kinase. After preincubation 5 µl aliquots were added to 35 µl of a buffer at 100°C consisting of 20 mM Tris-HCl buffer (pH 7.4), 0.5 mM dithiothreitol and 0.1% Triton X-100 and the solution was kept boiling for 5 min. After cooling deinhibitor activity was measured as described in "Materials and Methods" and expressed as a percentage of the original activity.

Fig. 2. *Reactivation of the inactivated deinhibitor protein by the inhibitor-1 phosphatase.*
The deinhibitor protein (0.1 mg per ml) was preincubated with (o—o) or without (Δ—Δ) 5 U per ml of the catalytic unit of cyclic AMP-dependent protein kinase as in Fig. 1. After 20 min both preincubation mixtures were boiled, cooled and 2 µl containing 2.8 mU of inhibitor-1 phosphatase from smooth muscle were added to 70 µl of the solution. The incubation was continued at 30°C. Deinhibitor activity was measured on 5 µl aliquots as in Fig. 1.

dodecyl sulfate polyacrylamide gel electrophoresis (Figure 3) and the time course of incorporation of ^{32}P label into trichloroacetic acid precipitable protein correlated with the decrease in activity of the deinhibitor protein (Figure 4). The stoichiometry of the ^{32}P incorporation calculated on the basis of protein concentration was about 0.02 mol per mol. This observation might indicate that most of the deinhibitor protein is modified and inactive so that reactivation of this



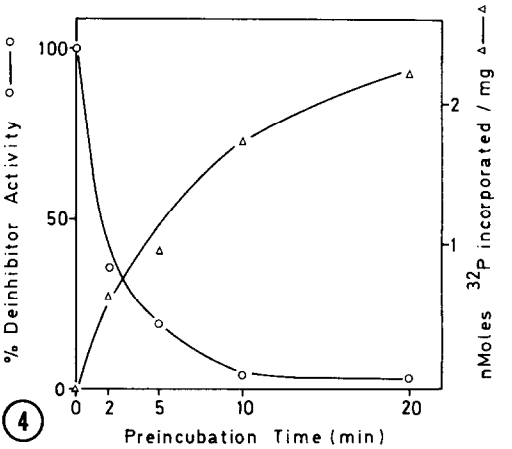


Fig. 3. *SDS polyacrylamide gel electrophoresis of the ^{32}P-labeled-inactive and active deinhibitor protein.*
5 µg deinhibitor was preincubated for 15 min at 30°C with a solution consisting of 0.1 mM [γ-^{32}P]ATP, 1 mM $MgCl_2$, 0.25 mM dithiothreitol and 10 mM Tris-HCl buffer (pH 7.4), in the presence or absence of 10 U per ml of the catalytic unit of cyclic AMP-dependent protein kinase. The preincubation was stopped by adding the same volume (12 µl) of a solution consisting of 20 mM phosphate buffer (pH 7.2), 2% SDS, 20 mM dithiothreitol, 20% glycerol and 0.002% bromophenol blue. After boiling for 5 min, 20 µl were applied to 15% polyacrylamide gels. The gel with the non-phosphorylated deinhibitor was sliced in 1 mm slices, extracted overnight in 100 µl containing 20 mM Tris pH 7.4, 0.5 mM dithiothreitol, 1 mg per ml bovine serum albumin and assayed for deinhibitor activity. The other gel was cross-linked with formaldehyde as described in "Materials and Methods", Coomassie blue stained and destained, sliced in 1 mm slices and radioactivity counted in the scintillation fluid.

Fig. 4. *Inactivation and phosphorylation of the deinhibitor protein by the catalytic unit of cyclic AMP-dependent protein kinase.*
The deinhibitor protein (0.1 mg per ml) was preincubated with 5 U per ml of the catalytic unit of cyclic AMP-dependent protein kinase and assayed as in Fig. 1. In a parallel incubation ATP was replaced by [γ-^{32}P]ATP (1,300 counts per min per picomol). At the indicated time periods 5 µl aliquots were spotted on Whatman filter paper (1x2 cm) and dropped in 20 percent trichloroacetic acid. The filter papers were washed 3 times with the trichloroacetic acid solution and the radioactivity counted.

form by the inhibitor-1 phosphatase is impossible. Another explanation which suggests itself would be that the deinhibitor protein is not pure. However the stoichiometry of the

interaction between deinhibitor protein and the phosphatase makes this explanation rather doubtful: half maximal effects are obtained when both the deinhibitor and the protein phosphatase are used in the nanomolar range (3).

The results presented clearly show that the deinhibitor protein can be inactivated by the cyclic AMP-dependent protein kinase and that it can be reactivated by a protein phosphatase which has been shown to be a major inhibitor-1 phosphatase in vascular smooth muscle. The concerted activation of inhibitor-1 and inactivation of the deinhibitor protein by cyclic AMP-dependent protein kinase and the reversal of the process by the same protein phosphatase, provide for a very sensitive modulation of the major cellular protein phosphatase by cyclic AMP. At high levels of cyclic AMP both inhibitor-1 and the deinhibitor protein should be phosphorylated. The neutralizing effect of the deinhibitor protein would thus disappear under conditions where the concentration of the phospho-form of inhibitor-1 increases as a consequence of a rising concentration of cyclic AMP. The reverse process would occur when the level of cyclic AMP decreases.

It is obvious that in this system the regulation of the multisubstrate protein phosphatase through the concerted modulation of inhibitor-1 and the deinhibitor protein is possible at the level of either the cyclic AMP-dependent protein kinase or the protein phosphatase. So far no regulation of inhibitor-1- or deinhibitor-phosphatase activity is known. However the same phosphatase is stimulated by cationic effectors such as histone-H_1 or polylysine when phosphorylase *a* is used as a substrate. Furthermore there remains the interesting possibility that the state of phosphorylation of inhibitor-1 and the deinhibitor protein could be regulated independently. The specific effect of the deinhibitor protein on the activation of the ATP,Mg-dependent phosphatase in the absence of inhibitor-1 would favour such a hypothesis. This is currently under investigation.

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