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ROLE OF THE DEINHIBITOR PROTEIN IN THE INTERCONVERSION OF THE ATP,Mg-DEPENDENT PROTEIN PHOSPHATASE

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The small molecular weight (± 9,000) heat stable deinhibitor protein, isolated from dog liver, not only protects the multisubstrate protein phosphatase from inhibition by inhibitor-1 and the modulator protein. It prevents the conversion of the active enzyme to the ATP,Mg-dependent enzyme form brought about by the modulator protein, and also affects the activation of the ATP,Mg-dependent protein phosphatase, probably by stabilizing the enzyme in its active conformation during the reversible activation by protein kinase F_A. Therefore the deinhibitor protein could be an important factor in the process of glycogen synthesis, which requires glycogen synthase and phosphorylase as dephosphorylated enzymes.

Mammalian tissues contain active and inactive forms of a multisubstrate protein phosphatase. The interconversion between the active and F_A-ATP,Mg-dependent enzyme forms has been studied in detail in rabbit skeletal muscle (1-4). The inactive enzyme represents a complex between the catalytic subunit (F_C) and a heat stable modulator protein (M), which can be activated in the presence of ATP,Mg by an activating factor (protein kinase F_A). The modulator protein, previously termed "inhibitor-2" (5), is strictly required for the reversible activation-inactivation of the ATP,Mg-dependent phosphatase. A transient phosphorylation of M by F_A results probably in a reversible intramolecular change in the catalytic subunit F_C of the protein phosphatase (1). In unboiled rabbit skeletal muscle preparations all the modulator activity appears to be associated with a heat labile protein component which co-

Abbreviations: F_A: protein kinase F_A, the activating factor of the ATP,Mg-dependent protein phosphatase also displaying synthase kinase activity; F_C: catalytic entity of the ATP,Mg-dependent protein phosphatase; M: modulator protein of the ATP,Mg-dependent protein phosphatase, previously called inhibitor-2.

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migrates in sucrose density gradient centrifugation (6). This complexed modulator appears to be involved in the reversible F_A-ATP,Mg- and time-dependent activation-inactivation of the phosphatase, while free modulator brings about an instantaneous inhibition of the phosphatase which cannot be reversed by a subsequent F_A-ATP,Mg-dependent activation step, which suggests another distinct binding mechanism of the modulator to the phosphatase (6). The inhibitory effect of protein phosphatase inhibitor-1, activated by cyclic AMP-dependent protein kinase (5), cannot be reversed by a subsequent incubation with kinase F_A in the presence of ATP,Mg (1).

The lack of sensitivity of the glycogen bound protein phosphatase from dog liver towards inhibitor-1 as well as to the modulator protein, led to the discovery of the deinhibitor protein neutralizing the effect of both inhibitors on phosphorylase phosphatase (7). Subsequently (8) we have also shown that the dephosphorylation and inactivation of inhibitor-1 by a protein phosphatase isolated from the liver glycogen pellet is controlled by the deinhibitor protein. The deinhibitor protein has been purified from dog liver (9). It was shown to be thermostable, resistant to ethanol- and trichloroacetic acid precipitation, but non-dialysable and destroyed by pronase or trypsin. The protein with a mol. wt. of about 9,000 has probably little ordered structure. We now report that the deinhibitor protein also affects the activation-inactivation process of the multisubstrate ATP,Mg-dependent protein phosphatase.

MATERIALS AND METHODS

The materials used and the experimental procedures are essentially as described in previous reports (10-12). Deinhibitor-free spontaneously active protein phosphatase (17,000 U/mg) was isolated from the glycogen pellet of dog liver according to (9). The phosphatase activity unit is defined as the amount of enzyme which releases 1 nmol of (^{32}P) phosphate/min at 30°C from ^{32}P-labeled phosphorylase a (1 mg/ml). Deinhibitor protein, with a specific activit of about 800 U/mg when assayed by its ability to relieve the inhibition of the ATP,Mg-dependent phosphatase by inhibitor-1 under non-dephosphorylating conditions, was prepared according to (9).

RESULTS AND DISCUSSION

The inactive ATP,Mg-dependent protein phosphatase represents a complex between the catalytic subunit (F_C) and the heat stable modulator protein (M) and can be activated by pro-

tein kinase F_A in the presence of ATP,Mg (1). When the activation is allowed to proceed in the presence of excess modulator, inhibition occurs. (F_CM) appears to be the correct substrate for the F_A-ATP,Mg-dependent activation, and extra modulator is probably bound at an inhibitory site (13). Since the deinhibitor protein neutralizes the effect of inhibitor-1 and the modulator protein on the spontaneously active as well as on the ATP,Mg-dependent phosphatase (7), the question arose if the deinhibitor also affects the interaction of the modulator with the catalytic subunit of the phosphatase or with kinase F_A in the activation process. Early experiments showed clearly that the deinhibitor protein could not substitute for the modulator in the F_A-ATP,Mg-dependent activation process (not shown).

To investigate further a possible interaction of the deinhibitor protein in the activation process, the F_A-ATP,Mg-dependent activation of the (F_CM)-complex was examined in the presence of suboptimal concentrations of kinase F_A (Fig. 1). Apparently the rate of activation, which is dependent upon the F_A concentration, is not influenced by the presence of the deinhibitor protein. However, instead of leveling off as in the absence of the deinhibitor, the activation proceeds further in a time-dependent manner in the presence of the deinhibitor protein, reaching eventually a new plateau, as shown with the low concentration of kinase F_A. This further increase in activity is not due to a stabilizing effect of the deinhibitor on one of the factors involved in the activation, since when the deinhibitor is added after the plateau is reached a further time-dependent activation is observed (not shown). From these results it appears that due to the action of the deinhibitor protein either more inactive phosphatase is made available for the activation process or, more likely, that the equilibrium between activation and inactivation of the phosphatase is shifted towards the active conformation.

Before the role of the modulator protein in the activation process became clear (14) evidence was provided that the protein had the capacity to inactivate in a time-dependent way the trypsin treated activated F_C-enzyme as well as a partially purified preparation of glycogen-bound phosphorylase phosphatase isolated from dog liver (15). Figure 2 shows the modulator-dependent inactivation (left) of the deinhibitor-free protein phosphatase isolated from the glycogen pellet of dog liver.

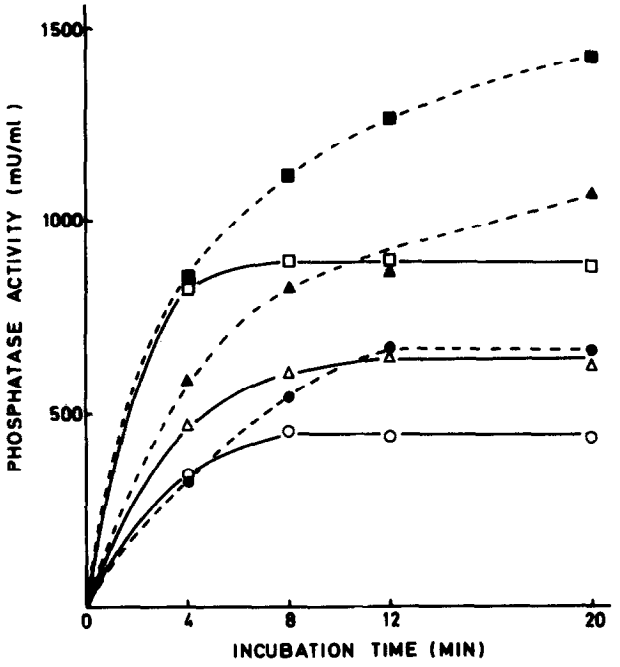

Fig. 1 *Effect of the deinhibitor protein on the activation of the ATP,Mg-dependent protein phosphatase by rate limiting concentrations of kinase* F_A.
(F_CM)-enzyme (6.6 nM) from rabbit skeletal muscle was incubated with 0.4 U (o,●), 0.8 U (△,▲) and 1.6 U (□,■) of protein kinase F_A per ml, 0.2 mM ATP, 1 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol and 1 mg per ml bovine serum albumin at 30°C in the presence (---) or absence (——) of 200 nM deinhibitor protein. For the phosphatase assay 10 µl samples were incubated with 10 µl of phosphorylase *a* (2 mg per ml) during 2 min.

As shown in figure 2 on the right, after the inactivation of the phosphatase by the modulator protein, saturating amounts of deinhibitor protein have no effect on the enzyme activity. When the phosphatase was reactivated with kinase F_A in the presence of ATP and Mg, the partial reactivation obtained with kinase F_A becomes complete only when performed in the presence of the deinhibitor protein. Since no effect of the deinhibitor protein is seen without a preincubation with F_A and ATP,Mg under the present experimental conditions, its effect cannot be explained by a simple "deinhibition". This conclusion becomes even more obvious from the observation that the activation by kinase F_A reaches a plateau after 3 min, but proceeds further in a time-dependent way in the presence of added deinhibitor protein. Most likely in the (re)activation of the enzyme the deinhibitor protein acts by its stabilizing effect on the active form(s) of phosphatase produced during the F_A-ATP,Mg-dependent activation.

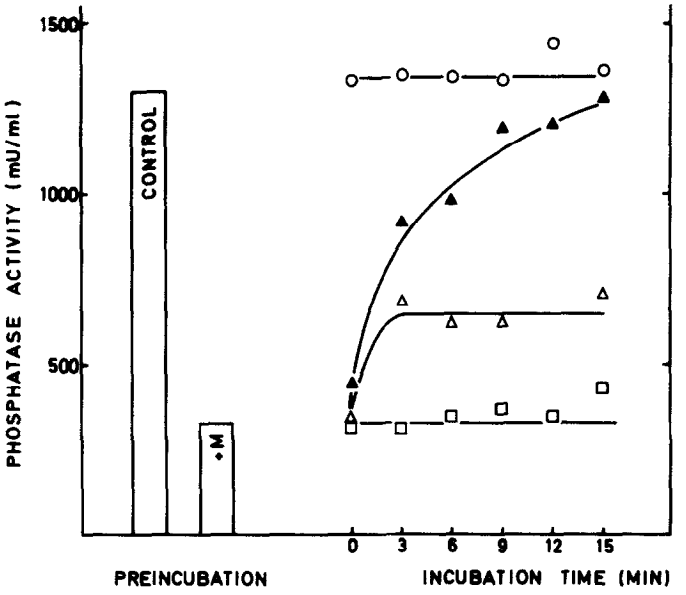

Fig. 2 *Effect of the deinhibitor protein on the reactivation of the modulator-inactivated deinhibitor-free protein phosphatase.* The spontaneously active protein phosphatase from dog liver was preincubated with (+ M) and without (control) 5 nM modulator protein and 20 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol and 1 mg per ml bovine serum albumin during 15 min at 30°C. Next aliquots of the preincubation mixture containing the inactivated phosphatase were incubated in the presence of 20 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol, 1 mg per ml bovine serum albumin and either 500 nM deinhibitor protein (□—□) or 1.8 U per ml kinase F_A, 0.1 mM ATP and 0.5 mM $MgCl_2$ in the presence (▲—▲) or in the absence (Δ—Δ) of 500 nM deinhibitor protein. The control sample was incubated in the presence of 1.8 U per ml kinase F_A, 0.1 mM ATP and 0.5 mM $MgCl_2$ and 500 mM deinhibitor protein (o—o). The phosphatase assay using phosphorylase *a* was carried out as in figure 1.

From these and previous (7,8) observations it appears that the deinhibitor protein affects the multisubstrate protein phosphatase activity in several ways. It neutralizes the effect of inhibitor-1 and modulator protein on the spontaneously active phosphatase (7), at high concentrations it controls the dephosphorylation and inactivation of inhibitor-1 (8) and now we have provided evidence for a stabilizing effect during the activation of the ATP,Mg-dependent protein phosphatase resulting in an increased enzyme activity. The deinhibitor, which is a relatively small protein with a mol. wt. of about 9,000, thus seems to have pronounced effects on the activation-inactivation process of the ATP,Mg-dependent protein phosphatase and on the interaction of the active phosphatase and inhibitor-1. Glycogen synthesis requires both phosphorylase and glycogen synthase

as dephosphorylated enzymes. Therefore the deinhibitor could be a factor -maybe some type of "second messenger"- regulating glycogen storage, since by keeping the multisubstrate protein phosphatase in its active conformation, the deinhibitor protein would be expected to facilitate amongst others the conversion of glycogen synthase to its active *a* form and phosphorylase to its inactive *b* form, resulting in glycogen synthesis according to the metabolic requirements.

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