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Control of the ATP,Mg-Dependent Protein Phosphatase by Heat-Stable Regulatory Proteins (41904)

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Protein Phosphatases Involved in Glycogen Metabolism. Covalent modifications are involved in the control of almost every type of cellular regulation, and the phosphorylation–dephosphorylation cycle appears to be the most prevalent among them. This type of regulation has assumed increasing importance not only in exploring enzyme regulation but also in the study of the signal transfer by receptors, muscular contraction, membrane transport systems, and by the observation that gene products of certain oncogenic viruses display protein kinase activity. The mode of action and regulation of a substantial number of protein kinases is well documented while development in the field of protein phosphatases, which has been reviewed in detail (1–4), is much more recent. This is probably so, because their activity and specificity is basically the result of the interaction of several regulatory proteins and because of the problems involved in the use of phosphoproteins in screening studies and early purification procedures.

Glycogen metabolism has always been an outstanding area in metabolic research resulting in remarkable contributions to the development of enzymology. Protein phosphatases have also been investigated most thoroughly in the regulation of the pathways of glycogen synthesis and glycogenolysis. Dephosphorylation by the ATP,Mg-dependent protein phosphatase of all the phosphoproteins known to be relevant in the regulation of glycogen metabolism (Fig. 1) and the activation of this phosphatase by kinase F_A have been reviewed recently (3, 4). A Ca^{2+}-calmodulin-dependent protein phosphatase that appears to be identical with calcineurin (5, 6) is present at high concentrations in brain and skeletal muscle but its concentration in liver (7) and vascular smooth muscle (E. Waelkens, J. Goris, and W. Merlevede, unpublished observations) is low. The Ca^{2+}-calmodulin-dependent phosphatase could promote glycogen synthesis through dephosphorylation of inhibitor-1 although this proposal is not easy to reconcile with the role of Ca^{2+} in muscular contraction. More recently a "latent" histone-H_1-stimulated phosphorylase phosphatase isolated from vascular smooth muscle (8) was shown to be an inhibitor-1 (9) and deinhibitor (10) phosphatase (see further). The enzyme has been purified from skeletal muscle and characterized as a 260,000-Da protein phosphatase containing four subunits of 95,000, 75,000, 65,000, and 38,000, the latter displaying the catalytic activity (11).

The ATP,Mg-dependent protein phosphatase dephosphorylates among other substrates (12) phosphorylase *a*, glycogen synthase *b*, and phosphorylase kinase *a* (β subunit) and is today's most thoroughly investigated protein phosphatase involved in glycogen metabolism (4). The enzyme is activated by protein kinase F_A, which also displays synthase kinase activity. In the regulation of the ATP,Mg-dependent protein phosphatase activity three known heat-stable regulatory proteins are involved. The modulator protein is the subunit through which the activation–inactivation process of the phosphatase catalytic subunit takes place; this process involves a reversible covalent modification of the modulator and probably a structural configuration change of the catalytic subunit (F_C). Inhibitor-1 is responsible for the cyclic AMP-dependent protein kinase induced inhibition of the ATP,Mg-dependent protein phosphatase, through phosphorylation of the heat-stable protein. Finally interaction of the deinhibitor protein with the protein phosphatase results in a decreased sensitivity of the enzyme to inhibition by the phosphorylated inhibitor-1 or the modulator protein and in

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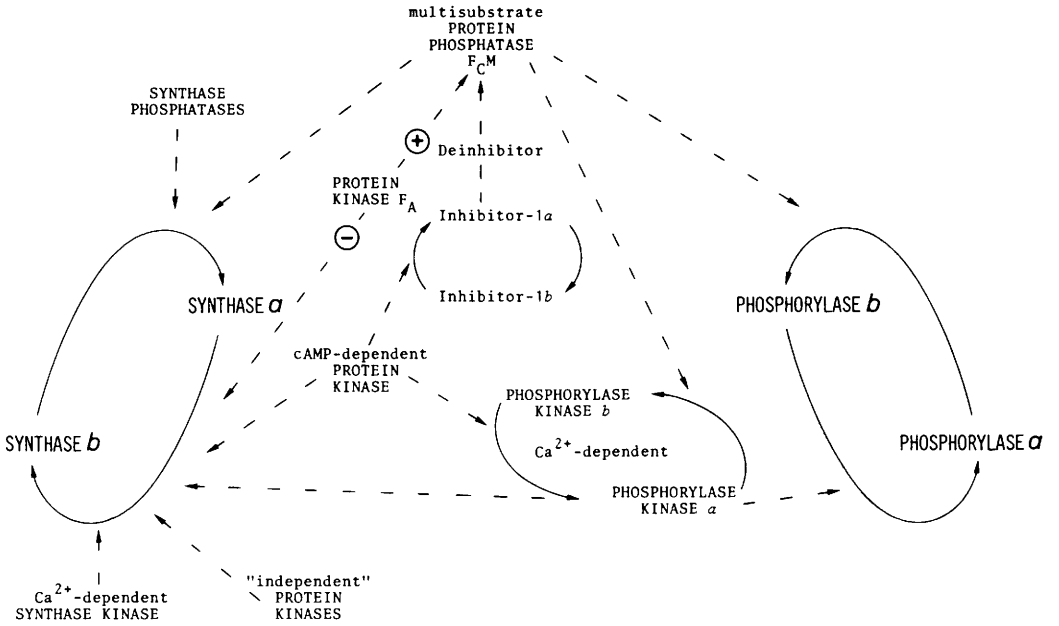

FIG. 1. The control of glycogen synthesis and glycogenolysis by reversible phosphorylation.

an increased resistance to the conversion of the enzyme to the inactive F_A–ATP,Mg-dependent form.

The Modulator Protein. Inhibitor-2 is now called modulator protein for the obvious reason that this regulatory protein not only brings about a time-dependent and stable inactivation of the phosphatase but also because it is an essential protein for the F_A–ATP,Mg-mediated activation (4). Strictly speaking the modulator protein was discovered in 1974 by Lee and co-workers (13) who were the first to present evidence for the existence of the protein phosphatase as an "enzyme–inhibitor complex." Since then the protein has been purified to apparent homogeneity from rabbit skeletal muscle (14, 15) and from liver (16). The amino acid composition of the modulator protein is distinctly different from that of inhibitor-1 (14), suggesting that they are unrelated proteins. The heat stability and the contrasting behavior in gel filtration (55,000 Da), gradient centrifugation (17,000 Da), and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (32,000 Da) suggest very little ordered structure. Reports about noncompetitive (16, 17) and mixed noncompetitive (18) inhibition should be reevaluated because of the dual function of the modulator protein in the ATP,Mg-dependent protein phosphatase interconversions.

Following the observation of the time dependency of the inhibitor 2-mediated inhibition of a partially purified phosphatase preparation from dog liver, the molecular role of the modulator protein became clear using two different approaches in the study of the interconversion between active and inactive protein phosphatase forms. The first studies involved the trypsin resistance of the activated ATP,Mg-dependent phosphatase and the second set of observations were the paradoxical results obtained during the purification of the protein phosphatase.

The activation of the ATP,Mg-dependent enzyme is very reversible: following removal of the kinase F_A, ATP, and Mg^{2+} a time-dependent reversal of the activated enzyme is observed. After treatment of the activated enzyme with trypsin, which proteolyzes quite selectively the modulator protein (17), the enzyme remained completely active even when incubated at 30°C. However, addition of the modulator protein reversed the activation in a time- and concentration-depen-

dent manner (19). Since the ATP,Mg-dependent protein phosphatase preparations contained modulator protein, it was assumed that this protein was responsible for the reversal of the activation in the absence of kinase F_A, ATP, and Mg^{2+}. On the other hand the final specific activity, after F_A–ATP,Mg-mediated activation, of the purified inactive phosphatase was found to vary from preparation to preparation: on some occasions, specific activities as low as 1000 units/mg were obtained for preparations that appeared like a pure protein upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Surprisingly these low activity preparations could be stimulated more than 10-fold when a titrated amount of modulator protein was included during the F_A and ATP,Mg-mediated activation. On the contrary an enzyme preparation with a specific activity of 20,000 units/mg, which always contained inhibitor 2 measurable in an additional protein staining band after sodium dodecyl sulfate–polyacrylamide gel electrophoresis, always decreased in activity upon addition of modulator protein. Henceforth it was clear that inhibitor 2, originally described as an inhibitory protein, was a true modulator essential for the reversible F_A–ATP,Mg-mediated activation of the protein phosphatase (20).

In unboiled rabbit skeletal muscle preparations the modulator activity (a 17,000-Da heat-stable protein when free), is present in a higher molecular weight complex which has ATP,Mg-dependent protein phosphatase activity: the modulator and the ATP,Mg-dependent phosphatase copurify completely in a constant activity ratio through several purification steps (21). This heat-labile (modulator–phosphatase) complex has an apparent molecular weight of 120,000 in gel filtration and 70,000 in sucrose density gradient centrifugation, which may reflect either an asymmetrical character of the complex or a dissociation by the centrifugal force into monomeric units (21). The major phosphorylase phosphatase present in rabbit skeletal muscle can be isolated from the glycogen pellet as a fully active enzyme, devoid of ATP,Mg-dependent phosphatase activity, which contains about 1 unit modulator bound per unit of phosphatase activity (22). Addition of extra modulator protein causes a time- and concentration-dependent conversion of the enzyme to an inactive F_A–ATP,Mg-dependent form. The modulator protein is used stoichiometrically in the activation of the free inactive catalytic subunit (F_C) of the ATP,Mg-dependent protein phosphatase (22), and this observation is in functional agreement with the presence of 1 unit of modulator per unit of native spontaneously active phosphatase.

Since the activation of the ATP,Mg-dependent protein phosphatase (F_CM) requires a kinase (kinase F_A) and ATP,Mg, a phosphorylation is expected to be involved in the mechanism of activation. Cohen and co-workers (23, 24), working with a "reconstituted" ATP,Mg-dependent phosphatase enzyme (a recombination of modulator protein with a catalytic subunit, isolated (25) according to a modified ethanol-precipitation procedure), reported a partial and transient phosphorylation of the modulator protein (up to 0.25 mole [^{32}P]phosphate per mole of modulator protein) followed by a complete dissociation of the inactive complex, yielding the "free" originally active catalytic phosphatase subunit. Fischer and co-workers (26) isolated a "Mn^{2+} and trypsin"-dependent protein phosphatase, using a purification protocol involving a room temperature acetone precipitation step. The enzyme was reported to be a complex consisting of a 38,000-Da catalytic subunit and the 31,000-Da modulator protein. This enzyme preparation could also be activated by kinase F_A and ATP,Mg, and phosphorylation of the modulator protein could be demonstrated. Discussion of the "native" catalytic subunit of the phosphatase is beyond the scope of this minireview but the reader should be reminded of the possibility that the 35,000-Da phosphatase unit which is usually obtained by the use of disruptive procedures on relatively crude extracts or enzyme preparations could be the combined result of protein unfolding and subsequent proteolysis by endogenous proteases (27).

We have also shown (28, 29) that phosphorylation of the modulator occurs during the activation of the ATP,Mg-dependent protein phosphatase by kinase F_A. In addition a net ATPase activity was observed during activation of the phosphatase, consistent with

a cyclic phosphorylation–dephosphorylation reaction. ^{32}P incorporation does not appear to directly parallel the activation of the phosphatase, an observation which was also made by Cohen and co-workers (23) with the reconstituted ATP,Mg-dependent protein phosphatase. These authors confirmed our earlier observation (30) that thiophosphorylation prevented the activation of the ATP,Mg-dependent phosphatase. The inactive ATP,Mg-dependent protein phosphatase (F_CM complex) migrates in sucrose density gradient centrifugation with an apparent molecular weight of 70,000. F_A–ATP,Mg-activated F_CM preparations spontaneously revert to their inactive form during the centrifugation when ATP,Mg is not included in the gradient. With ATP,Mg present in the gradient the activated phosphatase migrates also with an apparent molecular weight of 70,000; the enzyme still has all the modulator activity associated with it, is fully active, and cannot be activated further by kinase F_A (29). These results confirm earlier observations with enzyme preparations from liver and adrenal cortex, that the sedimentation coefficients of the phosphatase remained unchanged after activation (31). These findings demonstrate that the F_CM complex displays enzyme activity after activation and that dissociation of the modulator protein is not required for expression of activity. This observation, together with the fact that the spontaneously active protein phosphatase isolated from the glycogen complex contains modulator in a 1:1 activity ratio (22), clearly shows that the modulator is not an inhibitor which must be dissociated from the phosphatase for the enzyme to exhibit its activity. The dissociation observed by Cohen and co-workers with the "reconstituted" ATP,Mg-dependent phosphatase could be explained by the observation that the modulator is more tightly bound in the "native" than in the "reconstituted" enzyme (29).

From our results and those reported in the literature it appears that the ATP,Mg-dependent phosphatase exists as an inactive complex between the catalytic subunit F_C and the tightly bound modulator protein (M). During activation the modulator protein is first phosphorylated by the kinase F_A. This phosphorylation triggers a conformational change in the F_C catalytic subunit to activate the enzyme. The activated phosphatase is able to dephosphorylate the modulator protein without dissociation of the complex. After thiophosphorylation of the modulator protein either the conformational change of the catalytic unit does not happen or the expression of phosphatase activity is impossible because the modulator is irreversibly phosphorylated. Reversal of the activation is due to a slow protein isomerization by which the activated phosphatase catalytic subunit returns to its inactive form. It therefore seems that the phosphorylation of the modulator protein is transitory, but essential to induce the conformational change of the catalytic moiety into the active state.

Protein Phosphatase Inhibitor 1. Since the discovery of inhibitor 1 in rabbit skeletal muscle by Huang and Glinsmann (32) this phosphatase inhibitor has been identified in several tissues (9, 33–35). Inhibitor 1 has been purified and its phosphorylation investigated by Nimmo and Cohen (36, 37). The molecular weight measured by amino acid analysis is 20,800. Based on its behavior in gel filtration and sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and its acid and heat stability, it was suggested that inhibitor 1 possesses little ordered structure. Activation by cyclic AMP-dependent protein kinase correlated with the incorporation of 1 mole of phosphate per mole of protein on the threonine-35 residue. The complete primary structure of inhibitor 1 has been elucidated and during this study another "silent" phosphate residue whose function is unknown was revealed on serine-67 (38).

It was originally proposed by Cohen (39) that the multisubstrate protein phosphatase itself could be responsible for the dephosphorylation and inactivation of inhibitor 1. Nevertheless nonphysiological concentrations of Mn^{2+} are required for the reaction to take place at any respectable rate. However the phosphatase–deinhibitor complex as well as the deinhibitor-free protein phosphatase isolated from the liver glycogen complex incubated with the deinhibitor protein (see further) could dephosphorylate inhibitor 1 in the absence of Mn^{2+} (40). In the absence of deinhibitor the deinhibitor-free phosphatase could only inactivate inhibitor 1 in the pres-

ence of high concentrations of Mn^{2+}. Inhibitor 1 can also be dephosphorylated by a Ca^{2+}- and calmodulin-dependent protein phosphatase (5, 6) identical to CaM-BP_{80} (41), modulator binding protein (42, 43), or calcineurin (44). Significantly, the Ca^{2+} and calmodulin requirement of the enzyme can be mimicked by Mn^{2+} alone (45).

In skeletal muscle the extent of phosphorylation of inhibitor 1 is increased by epinephrine (46, 47) and is decreased by insulin which antagonizes the effects of low concentrations of β-adrenergic agonists (48). This antagonism apparently results from the ability of insulin to suppress the small rise in cyclic AMP produced by low concentrations of β agonists. However no *in vivo* correlation between the phosphorylated state of inhibitor 1 and the activity of the multisubstrate protein phosphatase has ever been put forward. It should be clear from the complexity of the regulation of the ATP,Mg-dependent protein phosphatase, that the assay of the enzyme in homogenates or crude extracts as a true reflection of its activity *in vivo* is hardly realistic yet.

The Deinhibitor Protein. The lack of sensitivity of the glycogen-bound protein phosphatase from dog liver toward inhibitor 1 as well as to the modulator protein (then called inhibitor 2) (49) was found to be intriguing, especially since most of the phosphorylase phosphatase in liver is proposed to be bound to particulate glycogen (50). This observation led to the discovery in 1977 of a novel factor neutralizing the effect of both inhibitory proteins on phosphorylase phosphatase: the name deinhibitor protein was proposed (49). The deinhibitor has now been purified extensively from dog liver (51). It was shown to be thermostable and resistant to ethanol or trichloroacetic acid precipitation and boiling in sodium dodecyl sulfate, but nondialyzable and destroyed by pronase or trypsin. The apparent molecular weight was estimated at about 17,500 by gel filtration, 8300 by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and 5500 by sucrose density gradient centrifugation—findings which are consistent with an asymmetrical protein with little ordered structure.

When the activation of F_C (catalytic subunit of the ATP,Mg-dependent protein phosphatase) by kinase F_A in the presence of ATP and Mg^{2+} is allowed to proceed in the presence of increasing concentrations of modulator protein, a gradual transition from stimulation to inhibition is observed (20). However, in the presence of deinhibitor protein the phosphatase activity reaches a higher level when using suboptimal levels of modulator and at higher concentrations of modulator the "inhibitory" effect of the protein is counteracted. When the activation of the ATP,Mg-dependent phosphatase was examined in the presence of suboptimal concentrations of kinase F_A, the rate of activation which depends upon the kinase 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 (51, 52). It therefore appears that due to the action of the deinhibitor protein either more inactive phosphatase is made available for the activation by kinase F_A or, more likely, that the equilibrium between activation and inactivation of the phosphatase is shifted toward the active conformation.

When the spontaneously active protein phosphatase is preincubated with the deinhibitor protein the phosphatase is clearly stabilized and much less sensitive to the effect of modulator protein at concentrations that otherwise completely inactivate the enzyme. Deinhibitor alone cannot reactivate the modulator-induced inactivation reaction, and the inactivated enzyme can only be partially reactivated by an incubation with kinase F_A in the presence of ATP and Mg^{2+}. Complete reactivation, however, can be obtained when the inactivated phosphatase is incubated with kinase F_A and ATP,Mg in the presence of the deinhibitor protein (51, 52).

The deinhibitor protein can completely neutralize the effect of inhibitor 1 on the spontaneously active phosphatase without adding kinase F_A and ATP,Mg, which also shows that the effect of inhibitor 1 on the phosphatase is truly an inhibition and not an inactivation. However, while the effect of inhibitor 1 on the phosphatase is non-time dependent, the effect of the deinhibitor re-

quires a preincubation period (53). Since under the experimental conditions no inactivation of inhibitor 1 is observed it is assumed that the deinhibition is obtained through a time-dependent conformational change of the protein phosphatase induced by the association of the deinhibitor with the enzyme or by a modification of the deinhibitor protein.

Protein kinase inhibitor and protein phosphatase deinhibitor have similar physical characteristics but unlike the insulin generated factor (54) which inhibits the cyclic AMP-dependent protein kinase and stimulates glycogen synthase phosphatase activity, neither the protein kinase inhibitor nor the protein phosphatase deinhibitor shares both activities (55). The deinhibitor shares also a number of physical characteristics with the reticulocyte phosphatase activator I described by Hardesty and co-workers (56). The latter regulatory protein stimulates a Mn^{2+}-stimulated phosphatase about twofold when phosphorylated reticulocyte 40 S ribosomal subunits or phosphohistones are used as a substrate. However, in a collaborative study (57) we have shown that the deinhibitor and the activator I, which both stimulate phosphatase activity through interaction with the enzyme and not with the substrate, display their effect through an interaction with a specific phosphatase.

Glycogen synthesis requires both phosphorylase and glycogen synthase as dephosphorylated enzymes. The interaction of the deinhibitor protein with the multisubstrate protein phosphatase results in several effects on the enzyme which when considered together could all facilitate the dephosphorylation of glycogen synthase and phosphorylase. Recently we have shown (10) that the deinhibitor protein is also subject to regulation by phosphorylation–dephosphorylation and exists under active and inactive forms. The deinhibitor can be inactivated by cyclic AMP-dependent protein kinase and reactivated by the histone H1-stimulated ("latent") phosphorylase phosphatase (10) which is also an inhibitor 1 phosphatase (9). 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

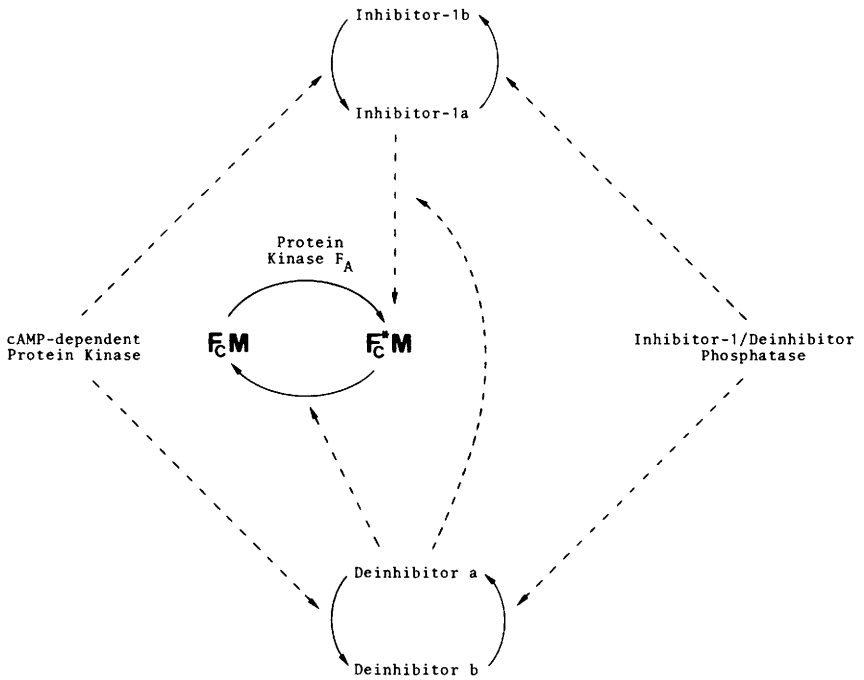

FIG. 2. Phosphorylation–dephosphorylation of inhibitor 1 and deinhibitor protein in the regulation of the ATP,Mg-dependent protein phosphatase.

(Fig. 2) provide a very sensitive modulation of the major cellular phosphatase by cyclic AMP and possibly a still unknown "second messenger" acting on the protein phosphatase. Furthermore, there remains the interesting possibility that the state of phosphorylation of inhibitor 1 and the deinhibitor could be regulated independently. The specific effect of the deinhibitor on the activation of the ATP,Mg-dependent phosphatase in the absence of inhibitor 1 and the fact that inhibitor 1 is also dephosphorylated by a Ca^{2+}-calmodulin-dependent protein phosphatase, would favor such an hypothesis.

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