The activation of the ATP,Mg-dependent protein phosphatase [Fₐ-M] has been shown to involve a transient phosphorylation of the modulator subunit (M) and consequent isomerization of the catalytic subunit (Fₐ) into its active conformation (Jurgensen, S., Shacter, E., Huang, C. Y., Cheek, P. B., Yang, S.-D., Vandenheede, J. R., and Merlevede, W. (1984) J. Biol. Chem. 259, 5864-5870). The modulator subunit constitutes the inactivating force for the enzyme, but the slow intramolecular inactivation of the phosphatase can be prevented or blocked by the addition of either the phosphorylated inhibitor-1 or Mg²⁺ ions. Autodephosphorylation of the modulator subunit is not prevented by the phosphorynhibitor-1, suggesting that the ATP,Mg-dependent phosphatase binds the phosphomodulator subunit in a very specific manner, different from the way it binds exogenous phosphoprotein substrates. Alternatively, the autodephosphorylation of the modulator subunit is catalyzed at a separate active site on the enzyme, which is not influenced by the binding of phosphorynhibitor-1. The phosphorynhibitor-1 does not prevent the activation of the enzyme by kinase Fₐ when added at concentrations that totally inhibit the potential phosphorylase phosphatase activity. These results, together with other already published information, suggest separate autonomic controls of the ATP,Mg-dependent phosphatase activity by inhibitor-1 and the modulator protein through the presence of specific regulatory subunits on the enzyme.

The ATP,Mg-dependent protein phosphatase constitutes a major regulatory enzyme in the control of glycogen metabolism in rabbit skeletal muscle (1). Its regulation has been unraveled down to the molecular level (2-8). The enzyme is usually isolated in the inactive form (2-11) and is activated by a transient phosphorylation of the modulator subunit by protein kinase Fₐ (2-8, 12) which induces the isomerization of the phosphatase catalytic subunit into the active conformation (4, 6, 7). Proteolysis in the presence of Mn²⁺ ions can mimic the kinase Fₐ-mediated activation of the inactive phosphatase (2, 3, 12), and, although this process may not be of any physiological relevance, it suggests a possible implication of metal ions in the phosphatase activity (10). Mg²⁺ ions were shown to be necessary for the autocatalytic dephosphorylation of the activated enzyme (6, 7), and it has recently been suggested that the isomerization of the phosphatase catalytic subunit involves the incorporation of Mg²⁺ during the kinase Fₐ-mediated phosphorylation reaction (13). The observation that the reversal of the phosphatase activation takes place after removal of ATP,Mg (23) contributed to the idea of a metal activation of the [Fₐ-M] complex (10). We now report that Mg²⁺ ions stabilize the activated ATP,Mg-dependent phosphatase in the absence of kinase Fₐ and ATP.

One has recently isolated and characterized (14) a glycogen-bound protein phosphatase which contains the same 38-kDa catalytic subunit as the ATP,Mg-dependent enzyme and a 103-kDa glycogen binding protein. This active enzyme does not contain a modulator subunit, but can be converted to an inactive kinase Fₐ-dependent form by addition of "free modulator." Its exact structural relationship to the [Fₐ-M] complex remains undefined.

The activity of the ATP,Mg-dependent phosphatase can be impaired in two different and probably unrelated ways (15). One process involves the reversal of the kinase Fₐ-mediated activation which is controlled by the modulator subunit of the phosphatase (16, 17) and can thus be considered as an intramolecular process. This time-dependent phosphatase inactivation has also been studied in recombination experiments using the isolated (active) catalytic subunit and low concentrations of modulator (15, 17). A different way to block the phosphatase activity is the addition of either phosphorylated inhibitor-1 (18, 19) or high concentrations of free modulator (11, 15); this causes an instantaneous inhibition of the enzyme. Inactivation and inhibition of the phosphatase are thought of as independent phenomena, controlled by two separate sites on the catalytic subunit (8, 11, 15, 29, 31). Binding of modulator to a high affinity binding site (site m) regulates the reversible activation-inactivation of the catalytic subunit and does not have an inhibitory effect on the phosphatase activity. A second molecule of modulator can attach itself at a low affinity binding site (site i) causing an instantaneous inhibition of the enzyme activity. Site i may also be the place where inhibitor-1 exerts its inhibitory action on the catalytic subunit.

Inhibitor-1 has been implicated in the hormonal control of the phosphatase activity (20-22), but, up until now, there has been no information published about the possible involvement of inhibitor-1 in the activation-inactivation process of the ATP,Mg-dependent phosphatase.

The present report shows that phosphoinhibitor-1 can prevent the reversal of the kinase Fₐ-mediated phosphatase activation, as well as the inactivation of the catalytic subunit by exogenously added modulator. This would suggest that, at least in vitro, inhibitor-1 and modulator protein can control
EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

Reversal of the Kinase F_A-mediated Activation of the ATP,Mg-dependent Phosphatase—The ATP,Mg-dependent phosphatase activity is only linear at very low enzyme concentrations (9). We recently confirmed that at low enzyme dilution, the expression of the phosphorylase phosphatase of the activated [F_c-M] complex is partially inhibited; however, the full activity could be revealed by proteolytic destruction of the inhibitory protein (8). This suggests that the kinase F_A-mediated activation of the inactive enzyme goes to completion at low as well as at high enzyme dilutions since it is known that only the activated conformation of the catalytic subunit is resistant to proteolysis (8, 20). These results enabled us to study the reversal of the kinase F_A-mediated activation by using concentrated [F_c-M] preparations in the activation step and subsequently diluting out the ATP,Mg to initiate the inactivation reaction.

When the phosphatase was fully activated by kinase F_A and subsequently diluted so that the concentration of ATP is lowered to micromolar levels, the activated enzyme slowly reverted to its inactive conformation (Fig. 1A). The amount of active phosphatase at each time point was measured after a trypsin treatment, which allows for the full expression of the activated catalytic subunit and destroys the potential activity of the inactive enzyme produced in the preincubation. Diluting the activated enzyme in 5 mM Mg²⁺ considerably slowed down this inactivation process, which would support the hypothesis of a metal-induced [F_c-M] activation through the kinase F_A-mediated phosphorylation of the modulator subunit (10, 13, 29). Surprisingly enough, addition of phosphorylated inhibitor-1 at concentrations which totally inhibited the potential phosphorylase phosphatase activity of the [F_c-M] preparation (Fig. 1B) blocked this inactivating process as well. It should be noted that the added inhibitor-1 is also destroyed by trypsin so that its inhibitory effect on the phosphatase is relieved before the phosphorylase phosphatase assay. This is illustrated in Fig. 1B, which also shows that a combined kinase F_A and trypsin treatment produces more phosphorylase phosphatase activity than kinase F_A alone, as already discussed in Ref. 8. Identical results were obtained whether the phosphoinhibitor-1 was added before or after the activation of the enzyme, which suggested that the inhibitor-1 did not prevent the kinase F_A-mediated phosphorylation or activation of the [F_c-M] complex. This is also illustrated in Fig. 1C, which shows that the kinase F_A-mediated activation of "free F_c" in the presence of 10 ng of modulator is not at all influenced by the addition of 100 ng of phosphoinhibitor-1. For reasons mentioned before, the phosphorylase phosphatase activities were measured after a trypsin treatment. The rate or extent of activation was the same if inhibitor-1 was added to the free F_c before or after the recombination of F_c with M to reconstitute the [F_c-M] complex. The phosphoinhibitor-1

1 Portions of this paper (including "Experimental Procedures," part of "Results," and Fig. 2) are presented in miniprint at the end of this paper. The abbreviation used is: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-2589, cite the authors, and include a check or money order for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

did not prevent the autodephosphorylation of the activated ATP,Mg-dependent phosphatase as described and illustrated in Fig. 2.

A common element in these two stabilization mechanisms is not evident unless one assumes that the presence of the phosphorylated inhibitor-1 at the inhibitory site could secure the active conformation of the catalytic subunit by stabilizing the necessary Mg²⁺ ion at the active site. Addition of millimolar concentrations of EDTA did not affect the stabilizing properties of the phosphoinhibitor-1 (not shown). It is possible that the phosphate moiety on inhibitor-1 is in the proximity of the active site of the enzyme where it could stabilize the active conformation as a substrate analogue. Inhibitor-1 is not being dephosphorylated by the ATP,Mg-dependent protein phosphatase in the absence of Mn²⁺ ions, which are not included in our assay mixtures. The dephosphoform of inhibitor-1 did not stabilize the phosphatase activity, and p-nitrophenyl phosphate or phosphoproteins, like phosvitin or...
casein and lysine-rich histones (both phosphorylated by the cyclic AMP-dependent protein kinase), could not substitute for the phosphoinhibitor (at nanomolar to micromolar concentrations). Less specific inhibitors of the phosphatase activity such as protease or synthetic polypeptide were also ineffective as stabilizing factors, and the substrate phosphoprotein a only retarded the inactivation of the phosphatase by about 30% at a concentration of 20 mg/ml (not shown).

**Inactivation of the Active Catalytic Subunit**—Addition of purified modulator to the active catalytic subunit of the ATP,Mg-dependent phosphatase converts the enzyme to its inactive form in a time-dependent way (Fig. 3). As shown for the reversal of the kinase Fα-mediated activation of the [Fc·M] complex, the phosphoinhibitor-1 could prevent this inactivation. Addition of phosphoinhibitor-1 at the 50% inactivation time point (arrow) stopped the process instantaneously (dotted line). This shows that the active catalytic subunit which is isolated with an ethanol precipitation step still exhibits the same regulatory properties and has similar modulator- and inhibitor-1 binding sites as the [Fc·M] complex or free Fc. Mg2+ ions were less efficient in blocking the inactivation of the catalytic subunit by added modulator than reported for the native [Fc·M] complex: the rate of inactivation was reduced by about 40% after addition of 5 mM Mg2+ (not shown).

The dog liver deinhibitor protein has also been reported to prevent the inactivation of the active catalytic subunit by the modulator (1, 35), but this heat-stable effector has not been demonstrated in rabbit skeletal muscle. The glycogen binding component of the rabbit muscle protein phosphatase (14) does not prevent the inactivation of the enzyme by added modulator protein (14, 15). Myelin basic protein, which is a potent inhibitor as well as a substrate for the ATP,Mg-dependent phosphatase activity in brain (32), is also able to block the inactivation of the catalytic subunit (33) so that different tissues may contain specific stabilizing proteins for the ATP,Mg-dependent protein phosphatase enzyme. It was noticed however that, like the deinhibitor protein, myelin basic protein did not stabilize the activation of the isolated [Fc·M] complex as described for inhibitor-1 in the previous paragraph, but only interfered in the inactivation of the (active) catalytic subunit by exogenously added modulator.

**CONCLUSIONS**

Rabbit skeletal muscle, like all tissues examined so far, contains a multisubstrate protein phosphatase whose catalytic subunit interconverts between an active and an inactive ATP,Mg-dependent conformation. This enzyme is isolated from different tissues or cellular localizations in various forms, which all contain the same 38-kDa catalytic subunit, but different regulatory proteins (34). Some of these regulatory proteins determine substrate specificity (35) or impose other special properties onto the catalytic subunit. One of these regulatory features is an altered sensitivity to inhibition by the heat-stable phosphatase inhibitor-1; this was reported to be controlled by a “deinhibitor” (36) or “G” subunit (14) of the glycogen-bound protein phosphatase. The catalytic form of this protein phosphatase contains a modulator subunit which together with the activating kinase Fα regulates the interconversion of the catalytic subunit between active and inactive conformations (1-4, 6, 7, 11, 15).

This suggests that there are at least two ways to lower the basal level of the ATP,Mg-dependent protein phosphatase activity. One involves the inactivation of the enzyme to the inactive ATP,Mg-dependent [Fc·M] form and is controlled by the modulator protein, while the second way is to inhibit the phosphatase activity by complexing it with the phosphoinhibitor-1. The results described in the present report show that these two mechanisms are not working in concert when using either the activated [Fc·M] complex or the active catalytic subunit as enzyme source. The phosphoinhibitor-1 stabilizes the catalytic subunit in its active conformation while keeping it in the inhibited state. Inhibitor-1 only counteracts the inactivating action of the modulator protein, but does not interfere in the kinase Fα-mediated activation of native or reconstituted [Fc·M] complexes.

The phosphorylation of inhibitor-1 is under hormonal control (20-22) and this is likely to impose a down-regulation upon all forms of ATP,Mg-dependent protein phosphatase. The inhibitory site on the catalytic subunit where phosphoinhibitor-1 exerts its action is not very well defined. It is possible that the phosphorylated inhibitor-1 may simply bind at the active site of the enzyme, and, by doing so, block the entrance to all exogenous substrates. It has been reported more than a decade ago (37) that tissue extracts contain a lot of latent or inhibited phosphorylase phosphatase, whose activity can be revealed by trypsin or ethanol treatment. Although the exact nature of the inhibitory moiety was never discovered, the phosphoinhibitor-1 would be a prime candidate as a structural component of this still unidentified latent enzyme(s).

The inactivation-activation cycle of the protein phosphatase may be restricted to the cytosolic modulator-containing forms of the ATP,Mg-dependent enzyme. This process could also be under hormonal control since the activating protein kinase Fα has been identified (38, 39) as an independent synthase kinase (GSK-3) which is able to revert some of the synthase dephosphorylations catalyzed by the activated ATP,Mg-dependent phosphatase (1, 40). The phosphorylation state of the kinase Fα (GSK-3) specific phosphorylation sites on glycogen synthase has been shown to be regulated by insulin (40).

We are not aware of any experimental evidence that would suggest an in vivo interconversion between the cytosolic and glycogen-bound forms of the ATP,Mg-dependent phosphatase (29). Different forms of the enzyme have different regulatory subunits attached to the same catalytic unit, and these regulatory proteins (specifically modulator or G-deinhibitor protein) were not shown to dissociate or exchange under normal physiological conditions (4, 14). We would like to suggest that one function of these regulatory subunits would be to determine the specific kind of up or down regulation of a certain
catalytic subunit in the hormonal and metabolic control of the cellular ATP,Mg-dependent phosphatase activity.

REFERENCES


ATP,Mg-dependent Protein Phosphatase

The ATP,Mg-dependent Protein Phosphatase

Regulation by Inhibitor-1 or Modulator Protein and Stabilization Role of Mg2+

Materials and Methods

Materials and methods were as described in (14, 21, 133). The inactive ATP,Mg-dependent phosphatase (pH 7) had a specific activity of 25,000 U/mg when measured after a 10 min preincubation at 30°C with 6 mM ATP and 8.5 mM Mg2+, using phosphorylase a as substrate. The specific activity of the inactive phosphatase substrate (free E)(f) was 6,850 U/mg in the presence and 3,160 U/mg in the absence of an optimal amount of modulator, when measured after activation by kinase E and ATP as outlined above. One unit of phosphatase released 1 pmol of 32P-phosphate/mg at 30°C from 32P-labeled protein substrates.

In order to measure the activation of the enzyme or the intrinsic activity of the phosphatase in the presence of inhibitory concentrations of phospho-inhibitor-1 or modulator, a tryptic treatment was performed using 50 μg of TPCK-treated trypsin (Sigma), to destroy the heat-stable phosphatase present after preincubation at 30°C. The proteinase K activity of trypsin was stopped by adding the effect of proteinase trypsin inhibitor (Sigma). Trypsin destroys the inactive enzyme, but does not impair the phosphatase activity (14, 21, 133).

The heat-stable modulator was isolated as in (6) and inhibitor-1 was as described in (14). Inhibitor-1 was phosphorylated by the cyclic AMP-dependent protein kinase (cAMP-dependent protein kinase) using non-radioactive cAMP, and freed of the kinase by an additional boiling step. The inhibitor-1 preparation showed one continuous blue protein staining band upon electrophoresis in the presence of sodium dodecyl sulfate (10% PAGE) and was essentially devoid of modulatory activity. The bovine heart cyclic AMP-dependent protein kinase catalytic subunit was a generous gift of Dr. A. Wallick (13). Rabbit skeletal muscle protein kinase (PKC) (2) and the active catalytic subunit of the cAMP-dependent protein kinase (PKA) were isolated as described in (14, 21, 133). Proteinase K- or phosphatase K2 electrophoresis in the presence of sodium dodecyl sulfo-}