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On the dephosphorylation of the ATP,Mg-dependent protein phosphatase modulator

Jackie R. Vandenheede, Carline Vanden Abeele and Wilfried Merlevede

Afdeling Biochemie, Faculteit Geneeskunde, Katholieke Universiteit te Leuven, B-3000 Leuven, Belgium

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The dephosphorylation of the modulator subunit is an essential step in the kinase F_A-mediated activation of the ATP,Mg-dependent protein phosphatase. Mg^{2+} is implicated in this autocatalytic dephosphorylation which is not effected by the addition of phosphoinhibitor-1. Dephosphorylation of free modulator by the catalytic subunit is also largely Mg^{2+}-dependent but can be abolished by phosphoinhibitor-1 in concentrations comparable to the amount of modulator used as substrate (micromolar). The phosphorylase phosphatase activity of the catalytic subunit is inhibited by nanomolar concentrations of phosphoinhibitor-1 and is completely independent of divalent cations.

ATP,Mg-dependent protein phosphatase; Modulator protein; Inhibitor-1; Protein kinase F_A; Mg^{2+}

1. INTRODUCTION

The ATP,Mg-dependent protein phosphatase comprises a family of enzyme forms with a broad substrate specificity, whose catalytic subunit can interconvert between an active and inactive conformation through the specific actions of protein kinase F_A and the modulator protein [1,2]. Inhibitor-1 is implicated in the hormonal regulation of glycogen metabolism [3-5] and inhibits the ATP,Mg-dependent phosphorylase phosphatase activity at physiological (nanomolar) concentrations [3].

The inactive ATP,Mg-dependent protein phosphatase is composed of a 38 kDa catalytic subunit and a 32 kDa modulator subunit [2]. The initial step in the phosphatase activation is the kinase F_A-mediated phosphorylation of the modulator subunit [6], which induces a conformational change in the catalytic unit [2]. A subsequent autocatalytic dephosphorylation of the modulator subunit is a prerequisite for the expression of the phosphatase activity towards exogenous substrates [7]. Mg^{2+} has been implicated in the transition of the catalytic subunit to the active form [8] as well as in the autodephosphorylation reaction [9,10].

Since the inactive ATP,Mg-dependent phosphatase as well as inhibitor-1 are both present in the cytosol [1], it seemed worthwhile to investigate the effect of inhibitor-1 on the kinase F_A-mediated activation of the phosphatase. We have also compared the autodephosphorylation of the activated enzyme with the dephosphorylation of free phosphomodulator by the isolated catalytic subunit.

Correspondence address: J.R. Vandenheede, Faculteit Geneeskunde, Katholieke Universiteit te Leuven, B-3000 Leuven, Belgium

2. MATERIALS AND METHODS

Materials and methods were essentially as described in [7,8,11,12]. The inactive ATP,Mg-dependent phosphatase [12] had a specific activity of 25 000 U/mg when measured after a 10 min preincubation at 30°C with kinase F_A, 0.1 mM ATP and 0.5 mM Mg^{2+}, using phosphorylase *a* (2 mg/ml) as substrate. One unit of phosphatase releases 1 nmol [^{32}P]phosphate per min at 30°C from ^{32}P-labeled protein substrates. Kinase F_A [13]

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and the active catalytic subunit of the ATP,Mg-dependent protein phosphatase with a specific activity of 10000 U/mg [14] were isolated as described.

In order to measure the kinase F_A-mediated activation of the phosphatase in the presence of inhibitor-1, trypsin treatment was performed using 50 μg/ml of TPCK-treated trypsin (Sigma) to destroy the inhibitor before phosphatase assay [15]. Trypsin destroys the inactive enzyme as well as the kinase F_A, but does not impair the activity of the activated phosphatase [7,8,12]. Proteolysis (2 min at 30°C) was stopped by the addition of excess soybean trypsin inhibitor (Sigma).

Inhibitor-1 [16], phosphorylated by the cyclic AMP-dependent protein kinase [17], was freed of the kinase by an additional boiling step. The bovine heart cyclic AMP-dependent protein kinase (catalytic subunit) was a generous gift of D.A. Walsh (USA).

3. RESULTS AND DISCUSSION

3.1. *Phosphorylation of modulator subunit during the kinase F_A-mediated activation of the ATP,Mg-dependent phosphatase*

Incubation of the inactive enzyme with kinase F_A and [^{32}P]ATP,Mg resulted in rapid and complete activation of the phosphatase. However, due to the transient nature of the modulator phosphorylation [6,13] the incorporation of [^{32}P]phosphate into the modulator subunit never exceeded 0.04 mol/mol (fig.1). Addition of phosphoinhibitor-1 at a concentration which completely abolished the phosphorylase phosphatase activity (the activity measured without trypsin treatment) did not result in increased [^{32}P]phosphate incorporation into the modulator subunit, and did not affect the activation of the enzyme (phosphatase activity measured after trypsin treatment as compared with activity observed in the absence of inhibitor-1). These results suggest that a 20-fold molar excess of inhibitor-1 over phosphatase did not block the autocatalytic dephosphorylation of the activated ATP,Mg-dependent phosphatase.

3.2. *Dephosphorylation of free modulator by the phosphatase catalytic subunit*

The modulator subunit of the inactive ATP,Mg-dependent phosphatase was dissociated from the complex by a boiling step and subsequently phosphorylated by kinase F_A and [^{32}P]ATP,Mg to a level of 0.3 mol [^{32}P]phosphate/mol modulator. The heat-stable modulator protein was reisolated by boiling the reaction mixture followed by Sephadex G-50 gel filtration. All radioactivity was localized in the 32 kDa modulator protein staining band on SDS-polyacrylamide gel electrophoresis (not shown).

The phosphatase activity of the active catalytic subunit was measured under different conditions with phosphorylase *a* or phosphorylated modulator as substrate. The phosphorylase phosphatase activity of the catalytic unit was independent of added Mg^{2+} and was completely blocked by the phosphoinhibitor-1 (fig.2A). Inhibitor-1 was not dephosphorylated or inactivated under the assay conditions used, with or without Mg^{2+} present (not shown). Using the phosphorylated modulator as substrate, the phosphatase activity was quite differently affected by Mg^{2+} and inhibitor-1. The dephosphorylation of modulator was greatly Mg^{2+}-dependent: 5 mM Mg^{2+} stimulated the phosphatase activity 4–5-fold (fig.2B). In addition, whereas the phosphorylase phosphatase activity was completely blocked by the addition of 50 ng inhibitor-1, much higher concentrations of inhibitor-1 were necessary to inhibit the modulator dephosphorylation significantly (fig.2B). The presence or absence of Mg^{2+} did not influence this sensitivity of the enzyme to inhibitor-1, using either one of the substrates.

The observation that such high concentrations of phosphoinhibitor-1 (5 μg/30 μl assay mixture containing 6 ng catalytic subunit) are necessary to block the dephosphorylation of modulator is easily explained if one assumes that modulator and inhibitor-1 compete for the same inhibitory binding site on the catalytic subunit. Phosphoinhibitor-1 and modulator (inhibitor-2) are about equally effective inhibitors of the phosphorylase phosphatase activity [18]. Since modulator, as substrate, is used at a concentration of about 3 μg/30 μl assay mixture, one would have to use comparable or even higher concentrations of phosphoinhibitor-1 in order to observe the expected inhibiton of the enzyme activity.

The effect of phosphoinhibitor-1 on the modulator dephosphorylation by the active catalytic

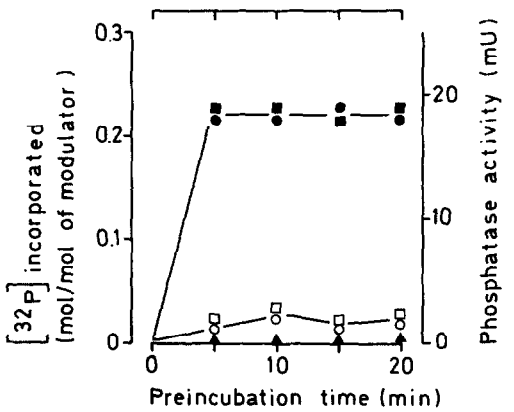

Fig.1. Incorporation of [^{32}P]phosphate into the modulator protein during the kinase F_A-mediated activation of the ATP,Mg-dependent phosphatase. 1 μg ATP,Mg-dependent phosphatase was incubated (20 μl) with 1 μg kinase F_A, 50 μM [^{32}P]ATP (spec. act. 10000 cpm/pmol) and 1 mM Mg^{2+}, in the presence (□,■,▲) or absence (○,●) of 10 μg phosphoinhibitor-1. At the times indicated, a 1 μl aliquot was taken to determine the phosphatase activity after (●,■) or without (▲) trypsin treatment and appropriate dilution and 2-μl aliquots were analyzed by polyacrylamide gel electrophoresis in the presence of SDS after addition of 1 μg unlabeled modulator. The modulator staining band was cut out of the gel to determine the ^{32}P label incorporated (○,□).

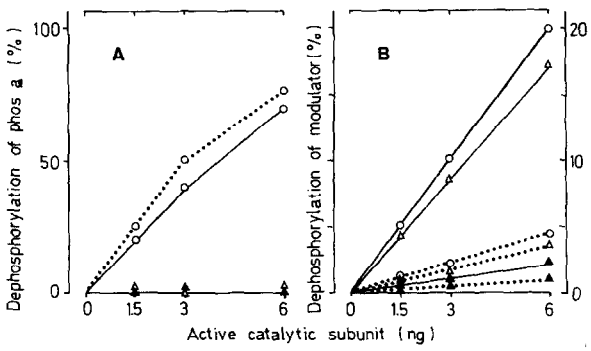

Fig.2. Dephosphorylation of free phosphomodulator by the active catalytic subunit of the ATP,Mg-dependent phosphatase. (A) Phosphorylase phosphatase activity of the catalytic subunit (10000 U/mg) in the presence (——) or absence (···) of 5 mM Mg^{2+}; control values (○) or activity in the presence of 50 ng (△) or 5 μg (▲) phosphoinhibitor-1. The assay mixture was 30 μl, and the assay time 10 min; 100% means total dephosphorylation of phosphorylase *a* (2 mg/ml). (B) Modulator (0.1 mg/ml) dephosphorylation by the active catalytic subunit (10000 U phosphorylase phosphatase activity/mg) in the presence or absence of Mg^{2+} and phosphoinhibitor-1. Symbols as in A.

subunit is not in agreement with the results presented above on the autodephosphorylation of the activated phosphatase. This discrepancy cannot be due to the modulator protein itself which originated from the same source and is phosphorylated by kinase F_A at a single threonine residue [6]. In the 'native' complex the modulator subunit could be dephosphorylated at an active site different from the site where exogenous substrates are dephosphorylated. Another explanation is that in the inactive phosphatase, the modulator subunit is bound in such a fashion that after phosphorylation its threonine-phosphate is presented to the active site of the enzyme in a specific manner, different from the way other phosphosubstrates, including exogenously added phosphomodulator, introduce their phosphorylated residues. It is conceivable that in the dephosphorylation assay of the modulator by the active catalytic subunit, the phosphorylated modulator binds at the general substrate binding site instead of at the original modulator subunit-binding site. In this hypothesis, the dephosphorylation of all exogenous phosphoproteins, including free phosphomodulator, would be inhibited by phosphoinhibitor-1. It should be noted that inhibitor-1 itself is not dephosphorylated by any form of the ATP,Mg-dependent phosphatase unless non-physiological concentrations of Mn^{2+} are included in the assay [18].

Tonks and Cohen [19] reported that dephosphorylation of modulator (0.6 μM) by the active catalytic subunit was not influenced by the addition of 0.2 μM inhibitor-1, and concluded that the modulator dephosphorylation process was insensitive to inhibitor-1; The results presented here agree with their experimental data but not with their conclusions, for the reasons stated above.

The stimulation of the modulator dephosphorylation by Mg^{2+} is in agreement with the Mg^{2+} requirement for autodephosphorylation of the activated ATP,Mg-dependent phosphatase [9,10]. The inactive catalytic subunit was also able to dephosphorylate the phosphomodulator upon addition of Mg^{2+} alone, without kinase F_A or ATP (not shown).

4. CONCLUSIONS

The inhibition characteristics of the isolated catalytic subunit suggest that the phosphoinhibitor-1 and modulator may compete for the inhibition site on the active catalytic subunit of the ATP,Mg-dependent protein phosphatase. This inhibitory site does not seem to be accessible to the phosphoinhibitor-1 in the native inactive enzyme, which contains a modulator subunit. The activation of the inactive phosphatase by kinase F_A involves a phosphorylation-dephosphorylation cycle of the modulator subunit, and this process is not affected by the phosphoinhibitor-1, although the resulting phosphorylase phosphatase activity (of the autodephosphorylated enzyme) is inhibitor-1-sensitive. Thus, the autocatalytic dephosphorylation of the modulator subunit, which results in phosphatase activity towards exogenous substrates, could also open up the 'inhibition site' for phosphoinhibitor-1. This may involve the translocation of the modulator to another site on the catalytic subunit. The catalytic subunit has been reported to contain two different modulator binding sites [7,12,19]. The inhibition site may well be localized at the active center of the enzyme, since both phosphorylated heat-stable proteins are dephosphorylated by the catalytic subunit in the presence of divalent metal ions ([9,10,18]; this work).

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