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THE ATP,Mg-DEPENDENT PHOSPHATASE: ROLE OF Mg IONS IN THE EXPRESSION OF THE PHOSPHORYLASE PHOSPHATASE ACTIVITY

Jackie R.Vandenheede*, Carline C.Vanden Abeele and Wilfried Merlevede

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

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The activation of the ATP,Mg-dependent phosphatase [F_CM] by kinase F_A has been shown to involve the phosphorylation or thiophosphorylation of the modulator subunit [M] and the consequent isomerization of the catalytic subunit [F_C] into the active conformation. The inactive catalytic subunit [free F_C] exhibits substantial activity in the presence of non-physiological concentrations of Mn ions whereas the Mn^{2+}-activation of the intact F_CM-enzyme requires the proteolytic destruction of the modulator subunit. The present study points to the importance of Mg^{2+} in the activation of the phosphatase. The inactive catalytic unit can be activated by millimolar concentrations of Mg^{2+} and the thiophosphorylated F_CM-enzyme only expresses its phosphorylase phosphatase activity after a subsequent trypsin treatment in the presence of Mg ions.

The <u>in vitro</u> activation of the ATP,Mg-dependent phosphatase [F_CM] is commonly achieved by two very different methods. One way to bring out the phosphatase activity is to proteolyze the inactive enzyme in the presence of metal ions such as Mn^{2+} or Co^{2+} (1). A more physiological but seemingly unrelated mechanism of activation is kinase F_A-mediated and involves phosphorylation of the modulator subunit [M] (1-3). This induces the transition of the enzyme's catalytic subunit into the active conformation (3) and is followed by a fast (auto) dephosphorylation of the modulator which allows the binding of exogenous phosphoprotein substrates to the activated enzyme (4). An inactive enzyme which is devoid of modulator [free F_C] cannot be activated by the kinase F_A but exhibits substantial phosphatase activity in the presence of non-

*To whom correspondence may be addressed.

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physiological concentrations of Mn^{2+} or Co^{2+} (5). This metal induced activation is prevented by the recombination of the inactive catalytic subunit with the modulator protein (5) suggesting that the modulator may block the metal binding site(s) on the catalytic unit. The results presented here provide a common element in the two above mentioned activation mechanisms by implying a metal incorporation during the kinase F_A-mediated phosphorylation of the phosphatase. Physiological concentrations of Mg^{2+} can activate the inactive catalytic subunit in the presence of detergent and the F_CM-enzyme which is thiophosphorylated by kinase F_A produces phosphorylase phosphatase activity after proteolytic destruction of the modulator only if Mg^{2+} is present. It has been reported (6) that Mg^{2+} is a necessary cofactor for the autodephosphorylation of the thiophosphorylated F_CM-enzyme.

MATERIALS AND METHODS

Materials and methods were as described in (3-5). The specific activity of the ATP,Mg-dependent phosphatase [F_CM], isolated as in (7) or (8), was about 20,000 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. The specific activity of the inactive catalytic subunit [free F_C] (5) was 18,000 U/mg in the presence and 1,300 U/mg in the absence of an optimal amount of modulator, when measured after activation by kinase F_A and ATP,Mg as outlined above. In the metal activation experiments, the phosphatase and metal ions were preincubated for 10 min at 30°C in the absence or presence of added modulator. Where indicated a subsequent trypsin treatment (2 min at 30°C) was performed using 50 µg/ml of TPCK-treated trypsin (Sigma), right before the phosphatase assay.

RESULTS AND DISCUSSION

1. Activation of the inactive catalytic subunit [free F_C]

The dependency of the kinase F_A-mediated activation of the (inactive) catalytic subunit [free F_C] upon exogenously added modulator [M] is shown in Fig. 1A. Full activation requires optimal amounts of [M] and excess modulator inhibits the phosphatase activity. No reversal of the activation takes place even in the presence of this excess modulator since an additional trypsin treatment can restore the full activity of the phosphatase as illustrated in Fig. 1B. The fundamental difference between inhibition and inactivation of the enzyme has been discussed in previous reports (4,9).

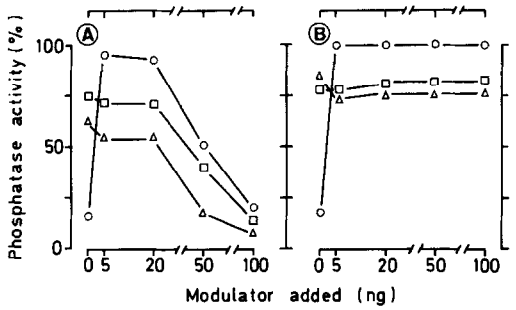

Fig. 1. *Activation of [free F_C]*
A: [free F_C] (50 mU of potential activity) was preincubated at 30°C for 10 min in the presence of 0.5 mM Mn^{2+} (□), 4 mM Mg^{2+} and 0.05 °/₀₀ SDS (△) or kinase F_A and ATP,Mg (o) in the absence or presence of increasing amounts of modulator. A 5 min phosphorylase phosphatase assay was performed.
B: Preincubations and symbols are as in (A) but a subsequent trypsin treatment was done before the phosphatase assay (see methods).

Mn ions alone bring out about 70% of the potential activity of the catalytic unit in the absence of modulator and kinase F_A and this activity is inhibited by the addition of high concentrations of [M] (Fig. 1A). Proteolysis restores the initial activity (Fig. 2B) which indicates that the metal

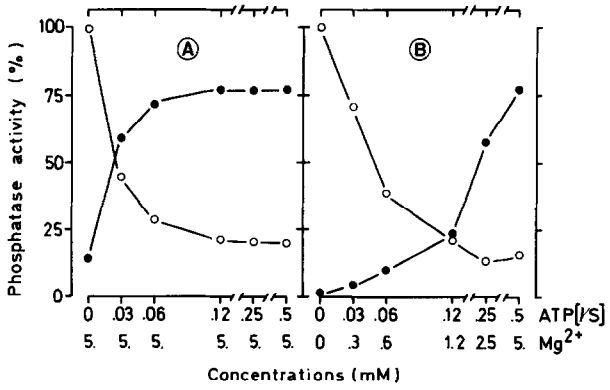

Fig. 2. *Thiophosphorylation of "native" [F_CM]*
A: "Native" [F_CM] (10 mU of potential activity) was preincubated for 10 min at 30°C with kinase F_A, 5 mM Mg^{2+} and increasing concentrations of ATP[γS]. The phosphorylase phosphatase activity was determined either after a subsequent trypsin treatment (●) or after another 10 min preincubation with kinase F_A, 0.5 mM ATP and 5 mM Mg^{2+} (o).
B: The same experimental set-up was used as in (A) except that the ATP[γS]/Mg^{2+} ratio was kept constant at 1/10 in the preincubation. The concentrations for ATP[γS] and Mg^{2+} are indicated on the abscissa, and symbols are as in (A).

ion stabilizes the active conformation of the catalytic unit. The observed inhibition by modulator in the presence of Mn ions confirms the earlier conclusion that inhibition and inactivation are unrelated phenomena controlled by two different binding sites for modulator on the catalytic subunit of the enzyme (9). Maximal activity was obtained with 0.5 mM Mn^{2+} which cannot be considered as physiologically relevant, but should simply be considered as an in vitro observation.

The modulator is a heat-stable protein whose activity can be measured after extraction from SDS-polyacrylamide gels (5). It had been noticed over the years that SDS-PAGE extracted fractions, which did not contain modulator, always produced a high background activity when added into a mixture of [free F_C, kinase F_A and ATP,Mg]. This constitutes the preincubation mixture for measuring the modulator activity (5). Concentrations of Mg^{2+} used in these determinations were usually low (0.5 mM) but when raised to millimolar concentrations, we were able to bring out a considerable portion of the potential phosphatase activity of [free F_C] in the absence of modulator. These observations led us to include low concentrations of SDS (0.05 °/₀₀) into our assay and it was observed that the inactive catalytic subunit [free F_C] could be activated by Mg^{2+} alone. Fig. 1A and B show the activating effect of 5 mM Mg^{2+} with and without an additional trypsin treatment. These results are quite similar to the activation seen with Mn^{2+} where no detergent is needed. This requirement for detergent may reflect the difference in size between the small Mn^{2+} and the relatively bulky Mg ion.

A difference between the Mn^{2+} and the Mg^{2+}-SDS activation is observed with the intact F_CM-enzyme. Whereas neither one of the two metal ions (with or without detergent) can produce phosphatase activity by themselves, Mn^{2+} activates the inactive phosphatase in the presence of trypsin (1), whereas Mg^{2+}-SDS cannot produce any significant activation under any conditions used (not shown). Presumably during the kinase F_A activation of the intact F_CM-enzyme, Mg^{2+} is incorporated during the phosphorylation of the modulator subunit. This hypothesis is substantiated by the results obtained with ATP[γ S] as thiophosphate donor.

2. Activation of "native" [F_CM] by kinase F_A and ATP[γS],Mg

We have reported in a previous publication (4) that ATP [γ S] can substitute for ATP in the activation of the F_CM-enzyme in as much as thiophosphorylation of the modulator by kinase F_A causes the transition of the inactive catalytic subunit into its active conformation. However, the thiophosphorylated enzyme cannot accomodate an exogeneous phosphoprotein as substrate and the expression of its phosphorylase phosphatase activity depends upon the proteolytic destruction of the thiophosphorylated modulator. The inactive enzyme itself is irreversibly destroyed by proteolysis (3) so that the phosphatase activity created by trypsin can be regarded as resulting only from "activated" catalytic subunits. Thiophosphorylation of [F_CM] prevents a subsequent activation by kinase F_A and ATP,Mg (4) so that an inverse correlation is expected between the amount of inactive F_CM-enzyme left over (which can still be activated by a subsequent kinase F_A and ATP,Mg treatment) and the amount of thiophosphorylated [F_CM] (whose activity is only expressed after proteolysis with trypsin). This is rightly so under conditions of high Mg^{2+} concentration such as used in (4) or as depicted in Fig. 2A which shows a dose response for ATP[γS] of the kinase F_A mediated phosphorylation in the presence of 5 mM Mg ions. Fig. 2B illustrates the same reaction where the ATP[γS]/Mg^{2+} ratio is kept constant at 1/10. It can be deduced from these results that the rate of thiophosphorylation, shown by the decrease in the amount of [F_CM] available for subsequent kinase F_A-ATP,Mg activation, does not depend upon high concentrations of Mg ions present (Fig. 2A and B). However, the phosphorylase phosphatase activity expressed after subsequent trypsin treatment seems to require millimolar concentrations of Mg^{2+} which suggests that the thiophosphorylated [F_CM] is only in the activated conformation in the presence of sufficient Mg^{2+}. This probably reflects a less efficient binding of Mg^{2+} to [γS]-triphosphate or protein thiophosphates than to ATP or protein phosphates.

When ATP,Mg is the phosphate donor, the metal ion is most likely transferred to the F_CM-enzyme during the kinase F_A-mediated phosphorylation reaction itself. The Mg ion is complexed to the β and γ phosphates in the ATP,Mg complex and a kinase reaction, which involves the dissociation of the β,γ-

phosphodiester bond, causes the migration of the Mg ion. In the case of the F_CM-activation reaction, Mg^{2+} is possibly transferred together with the γ-phosphate from ATP,Mg to the phosphatase. This may not be the case whith ATP [γS] where, because of the presence of the sulfur at the γ-phosphate, the Mg^{2+} may preferentially bind at the α,β-phosphodiester bond. This could result in an inefficient [Mg-thiophosphate] transfer. This difference in Mg^{2+}-binding may explain the rather slow rate of thiophosphorylation of proteins by protein kinases in general (10). The dephosphorylation of thiophosphorylated proteins by phosphatases has also been shown to require a surplus of Mg ions (6). The exact mode of action of Mg^{2+} in the activation of the phosphatase has to be determined by further studies.

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