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The ATP,Mg-dependent protein phosphatase: regulation by casein kinase-1

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The free modulator subunit of the ATP,Mg-dependent phosphatase is phosphorylated up to 1 mol per mol by casein kinase-1, up to 1.85 mol per mol after dephosphorylation by the PCS_{HI} phosphatase, but 10-fold less when purified in the presence of NaF, suggesting an in vivo phosphorylation of the casein kinase-1 sites. Peptide mapping of ^{32}P-modulator labeled by casein kinase-1 or -2 shows a different phosphorylation pattern. Phosphorylation of the inactive phosphatase by casein kinase-1 prevents the subsequent kinase F_A-mediated activation, while it does not impair the activated phosphatase.

ATP,Mg-dependent phosphatase; Casein kinase-1; Casein kinase-2; Modulator subunit

1. INTRODUCTION

The ATP,Mg-dependent protein phosphatase comprises a family of enzymes whose 38 kDa catalytic subunit interconverts between an active and an inactive conformation [1–5]. The exact subunit composition of the active enzyme forms is only now being unraveled [6–12]. Purification of the enzyme from rabbit skeletal muscle cytosol [2–4,13–16] invariably results in the isolation of an inactive phosphatase which is composed of the 38 kDa catalytic unit (F_c) and a 32 kDa modulator (M) protein. Phosphorylation of the M-subunit by protein kinase F_A on Thr-72 induces a conformational change in F_c which activates the enzyme [2–5,17].

Kinase F_A can exhibit two opposite activities [1]: it phosphorylates and inactivates glycogen synthase, while it also creates synthase phosphatase activity out of the inactive ATP,Mg-dependent phosphatase. A second protein kinase, casein kinase-2 (CK-2), is implicated in the regulation of the ATP,Mg-dependent phosphatase [18–22]: it phosphorylates in vitro Ser-120, Ser-121 and at a slower rate also Ser-86 of the M-subunit. Such phosphorylation does not elicit any phosphatase activity by itself, but does potentiate the kinase F_A-mediated activation [18]. The in vivo content of phosphate in the M-subunit is largely associated (up to 90%) with Ser residues [21] and among them Ser-86 is the site most extensively phosphorylated: up to 0.7 mol per mol [22]. The slow in vitro phosphorylation of this Ser-86 site by CK-2 is suggestive of the involvement of another protein kinase in the in vivo phosphorylation.

Since the in vivo content of phosphate in this site is not altered by hormone treatments [22] and Ser-86 is surrounded by aspartic and glutamic acid residues [20], we focussed our attention on CK-1, another 'independent' kinase which also requires acidic amino acids to recognize its target [23].

Presently we report the in vitro CK-1 induced

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formation of a 'latent' ATP,Mg-dependent phosphatase whose inactive catalytic subunit cannot be activated by the kinase F_A. The observations implicate a third kinase (CK-1) in the regulation of the ATP,Mg-dependent phosphatase and suggest that some of the cytosolic ATP,Mg-dependent phosphatase activity may be masked as a latent enzyme by the CK-1-mediated phosphorylation of the M-subunit.

2. MATERIALS AND METHODS

The inactive ATP,Mg-dependent phosphatase was purified from rabbit skeletal muscle essentially as described [3,13], except that the second polylysine column was replaced by a similar chromatography on aminohexyl-Sepharose 4B, which resulted in the isolation of an F_cM complex, without any 65 kDa protein present (spec. act. approx. 15000 U/mg, 1 U releasing 1 nmol [^{32}P]phosphate per min at 30°C in a 30 μl assay containing 1 mg/ml of ^{32}P-labeled phosphorylase prepared as in [24]). The polycation-stimulated (PCS_{H1}) protein phosphatase [25], M-subunit [26] and protein kinase F_A [27] were purified from rabbit skeletal muscle. 1 U kinase F_A generates 1 U phosphatase activity in 10 min of incubation at 30°C in buffer A (20 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol and 1 mg bovine serum albumin/ml). CK-1 and CK-2 were purified from rat liver cytosol essentially as in [28]; 1 U kinase incorporates 1 nmol phosphate per min in casein (2 mg/ml) at 37°C. Phosphorylase *b* kinase [29] was a gift from Dr D.A. Walsh (USA). Whole casein was prepared as in [30]. [γ-^{32}P]ATP was purchased from the Radiochemical Centre (Amersham), the molecular mass markers used in SDS-PAGE from Bethesda Research Laboratories, Dowex 1-X8 from Bio-Rad and *Staphylococcus aureus* V8 proteinase from Worthington. Autoradiograms were made and analyzed as in [19].

The M-subunit (0.1 mg/ml) was phosphorylated at 30°C in 50 μl incubation mixture containing 50 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 25 mM β-mercaptoethanol, 0.1 M NaCl, 5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (spec. act. 1000–2000 cpm/pmol), CK-1 (1.6 U/ml) or CK-2 (4 U/ml), unless specified otherwise. The reaction was stopped by addition of electrophoresis buffer (2% SDS, 1% β-mercaptoethanol, 10% glycerol and 0.5 M Tris-HCl, pH 8.3) to 10-μl aliquots, followed by SDS-PAGE (10% polyacrylamide) analysis according to Laemmli [31]. The previous dephosphorylation of the M-subunit by PCS_{H1} phosphatase was carried out as described [19].

For phosphoamino acid analysis the M-subunit was phosphorylated with kinase F_A and CK-1 in 20 μl of the phosphorylation medium in the presence of heparin (1.5 μg/ml). After 20 min the reaction was stopped, the remaining ATP was removed with Dowex 1-X8 as in [32] and the resulting samples processed as in [19].

Before *S. aureus* V8 proteinase digestion the M-subunit was phosphorylated with CK-1 or CK-2 for 3 min at 30°C. The reaction was stopped by boiling the samples for 3 min. After cooling 1 μg *S. aureus* V8 proteinase was added (enzyme/substrate wt ratio of 1:5) and digestion of the ^{32}P-labeled M-subunit was carried out at 37°C. At the time points indicated, 10-μl aliquots were

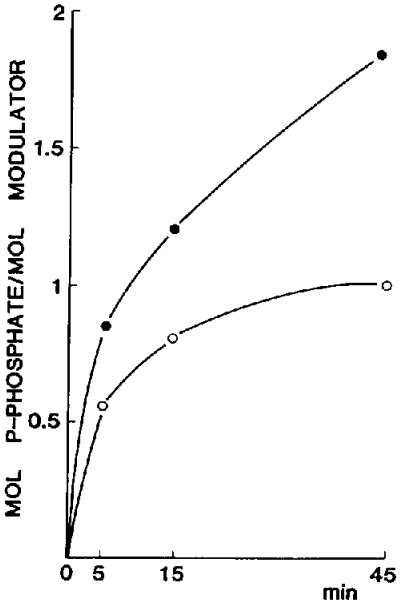


Fig.1. Time course of the phosphorylation by casein kinase-1 (1.6 U/ml) of M-subunit (0.1 mg/ml) as such (○—○) or previously dephosphorylated by PCS_{H1} phosphatase (10 U/ml) (●—●). The stoichiometry of phosphorylation was estimated from the radioactivity detected in the gel slices (SDS-PAGE) containing the M-subunit.

boiled for 3 min in the presence of electrophoresis buffer, subjected to 15% polyacrylamide slab gel electrophoresis [31], stained with 0.25% Coomassie brilliant blue, dried and autoradiographed.

3. RESULTS

3.1. *Phosphorylation of the isolated modulator subunit by casein kinase-1 and casein kinase-2*

CK-1 was able to incorporate up to 1 mol P_i per mol M-subunit (fig.1). As illustrated in the same figure previous dephosphorylation of the regulatory protein by PCS_{H1} phosphatase led to a maximum incorporation of 1.85 mol P_i per mol 32 kDa polypeptide, suggesting that the isolated M-subunit already contained some phosphate in the CK-1 site(s).

Phosphoamino acid analysis after acid hydrolysis demonstrated that only Ser residue(s) are phosphorylated by CK-1 and that CK-1 and kinase F_A brought about a synergistic increase in the amount of P_i incorporated by the latter protein kinase on threonine (fig.2). The latter observation cannot be attributed to contamination of CK-1 by CK-2, since phosphorylation was carried out in the presence of 1.5 μg heparin/ml which totally inhibits the CK-2 activity [33].

In order to distinguish between the sites phosphorylated by CK-1 and CK-2, the M-subunit was phosphorylated over a very short time period (3 min) and the ^{32}P-labeled protein was subjected to *S. aureus* V8 proteinase digestion followed by 15% SDS-PAGE of the phosphopeptides. As shown in fig.3 the two phosphorylation patterns clearly differ from each other in the presence of a 10 kDa phosphopeptide specifically in the CK-1 labeled digest. We would like to stress that the inclusion of heparin in the CK-1 phosphorylation medium did not change the digestion pattern (not shown).

M-subunit isolated in the presence of 50 mM NaF, to preserve the in vivo phosphorylation level, was a poor substrate for CK-1. Less than 0.1 mol ^{32}P per mol M-subunit could be incorporated (not

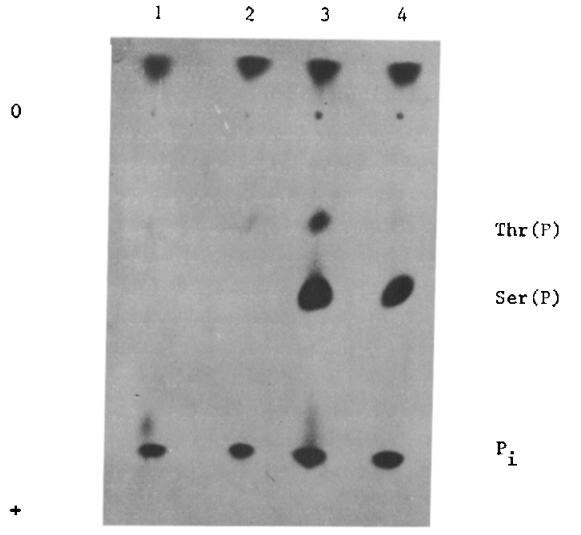


Fig.2. Phosphoamino acid analysis of the M-subunit phosphorylated by casein kinase-1 and kinase F_A. 1.2 μg M-subunit was phosphorylated in the absence (track 1) or presence of heparin (1.5 μg/ml) (tracks 2–4) by kinase F_A (1600 U/ml) alone (tracks 1,2), CK-1 (1.4 U/ml) alone (track 4) or CK-1 plus kinase F_A (track 3). Origin, O; anode, +; migrations of P_i, Ser-P and Thr-P markers as shown.

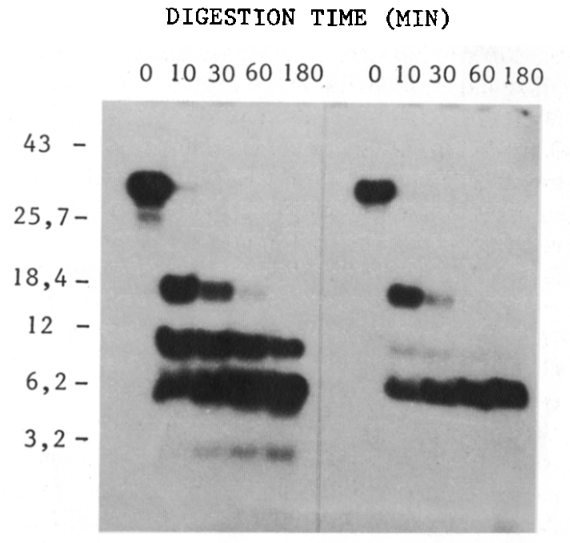


Fig.3. *Staphylococcus aureus* V_8 proteinase digests of M-subunit (0.1 mg/ml) phosphorylated (3 min) by casein kinase-1 (1 U/ml) (lanes 1–5) or casein-2 (1.6 U/ml) (lanes 6–10). The autoradiogram of the gel is shown with protein molecular mass standards and digestion times.

shown), which suggests that the CK-1 specific phosphorylation sites may already be occupied.

3.2. *Effect of casein kinase-1 and -2 on the activation of the ATP,Mg-dependent protein phosphatase*

Fig.4A shows the effect of CK-1 and CK-2 on the subsequent activation of the F_cM complex by protein kinase F_A. While at suboptimal concentrations of kinase F_A phosphorylation by CK-2 potentiates phosphatase activation, phosphorylation by CK-1 leads to a drastic reduction in the phosphatase activity produced. Moreover, phosphorylation by CK-1 prevents the potentiating effect of CK-2 when added simultaneously to the preincubation medium. No effect of CK-1 on the phosphorylase phosphatase activity of the F_cM complex could be detected when phosphorylation by CK-1 was carried out after kinase F_A activation (not shown), indicating that phosphorylation by CK-1 does not inhibit the activated phosphatase but rather prevents the kinase F_A-mediated activation. This is confirmed in the experiments illustrated in fig.4B, where the resulting phosphorylase phosphatase activities are measured after limited proteolysis with trypsin, which is known to degrade the M-subunit [1,17,34]. The trypsin treatment does not restore the activity of the CK-1/kinase F_A-treated F_cM complex to the control level reached after activation by kinase F_A alone (fig.4B). The increased phosphatase activity seen after trypsin treatment of the activated enzyme is a well-known phenomenon, probably due to the destruction of M-subunit bound at the inhibitory site of the activated enzyme [4,15,17,34].

Preliminary experiments have indicated that the dephosphorylation of the CK-1 site(s) can be an autocatalytic event: the kinase F_A-activated F_cM complex is able to remove the [^{32}P]phosphate subsequently incorporated by CK-1 (fig.5). This confirms our previous results which indicated that the kinase F_A-activated enzyme is not inhibited by

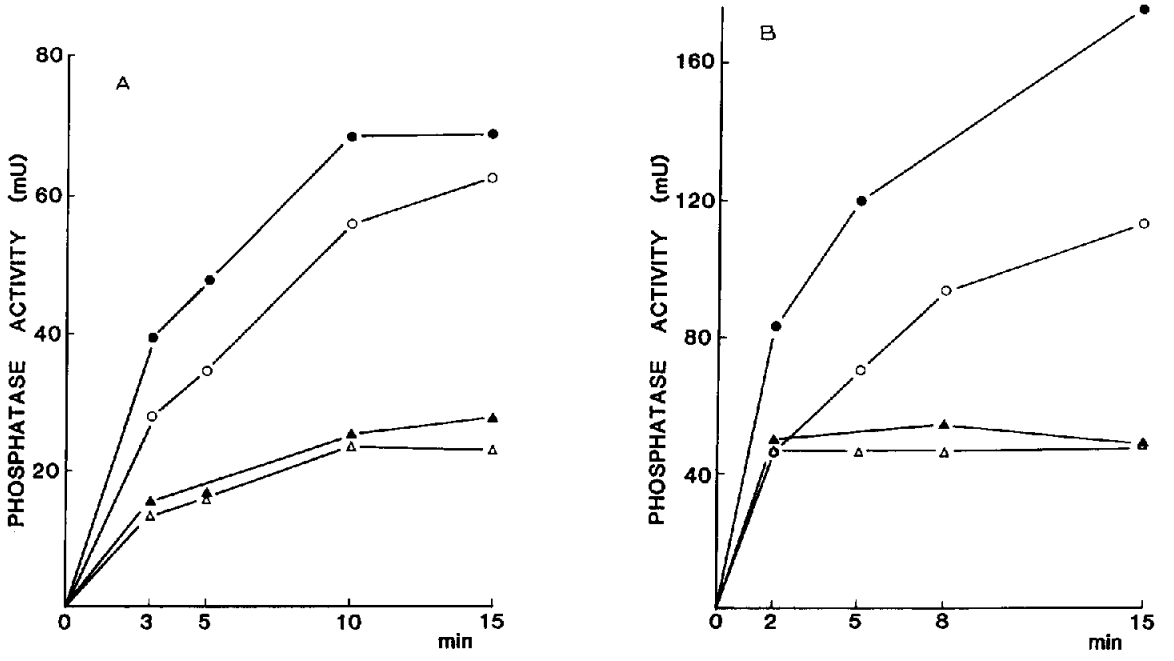


Fig.4. Time course of activation of the ATP,Mg-dependent phosphatase (630 mU). (A) The ATP,Mg-dependent phosphatase was incubated at 30°C alone (○—○), with CK-1 (2.4 U/ml) (▲—▲), CK-2 (4 U/ml) (●—●) or with CK-1 (2.4 U/ml) and CK-2 (4 U/ml) combined (△—△) in 20 μl containing 20 mM Tris-HCl, pH 7.4, 0.5 mM dithiothreitol, 1 mg bovine serum albumin per ml, 0.1 M NaCl, 200 μM ATP and 1.25 mM $MgCl_2$. After 10 min the samples were diluted 3-fold in buffer A before activation by kinase F_A (120 mU). At the indicated times 10-μl aliquots were assayed for phosphorylase phosphatase activity (1 min). (B) The same experimental conditions were used with the exception that trypsin treatment preceded the phosphorylase phosphatase assay as described in [17].

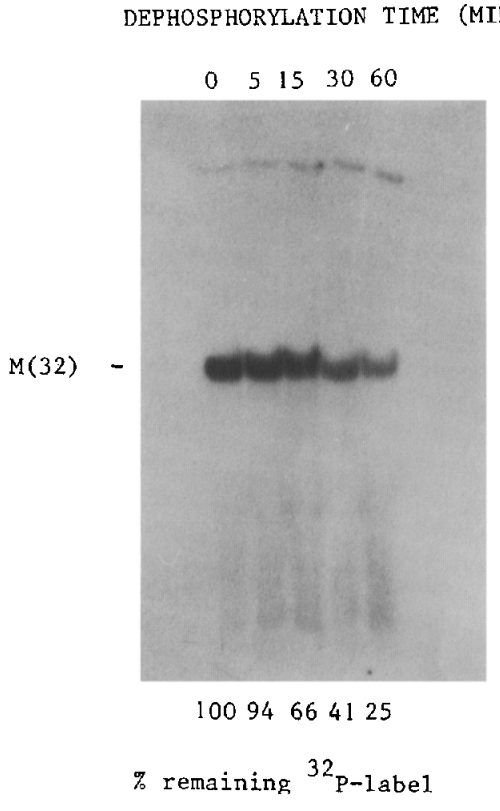


Fig.5. Autocatalytic dephosphorylation of the activated ATP,Mg-dependent phosphatase (3 μg) labeled by casein kinase-1 (1.3 U/ml). The ATP,Mg-dependent phosphatase was activated at 30°C by kinase F_A (4000 U/ml) in buffer A containing 10 μM ATP and 0.5 mM $MgCl_2$. After 15 min, CK-1 and [γ-^{32}P]ATP (25000 cpm/pmol) were added and incubated for another 15 min. The ^{32}P incorporation was stopped by isotopic dilution with 1 mM cold ATP and 5 mM $MgCl_2$. At the specified times 5-μl aliquots of the 30 μl reaction mixture were analyzed by SDS-PAGE (10% polyacrylamide). The autoradiogram of the gel is shown with the ^{32}P label (%) remaining in the M-subunit at the different time points, as measured by densitometric analysis.

a subsequent phosphorylation of the CK-1 specific site(s).

4. DISCUSSION

This study reports that CK-1 phosphorylates the M-subunit of the ATP,Mg-dependent phosphatase. This phosphorylation blocks the subsequent activation by kinase F_A while it does not prevent, but rather stimulates the phosphorylation by kinase F_A of the Thr-72 site. This suggests that the CK-1 specific serine phosphorylation of the M-subunit prevents the induction of the conformational change of the catalytic subunit otherwise produced by the phosphorylation of the threonine site. The overlapping phosphorylation sites of CK-1 and CK-2 suggested by the peptide mapping are reflected in the similar effects that both enzymes have in the synergistic phosphorylation of the Thr-72 site by kinase F_A. This effect is possibly the result of the phosphorylation of the Ser-120 and Ser-121 sites, which are readily phosphorylated in vitro by CK-2 [20]. The inhibitory effect of CK-1 on the kinase F_A-mediated activation of the ATP,Mg-dependent phosphatase contrasts with and obliterates the stimulatory action of CK-2. This either results from the phosphorylation of some as yet unidentified serine residues, or is due to the specific phosphorylation of the Ser-86 site. Since it has been conclusively shown that the only other amino acid which is highly phosphorylated in vivo [22] is Ser-86, which is a very poor substrate for CK-2, we would favour this last hypothesis. The in vivo occupancy of this 'inhibitory site' on the modulator may be one reason why it is difficult to detect any kinase F_A-mediated activation of the ATP,Mg-dependent protein phosphatase in freshly prepared tissue extracts. Furthermore, phosphorylation of this potential phosphatase by CK-1 may selectively control the two physiological but opposite effects of kinase F_A: phosphatase activation and synthase phosphorylation [1,27].

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