Reconstitution of a Mg-ATP-dependent protein phosphatase and its activation through a phosphorylation mechanism

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A Mg-ATP-dependent protein phosphatase has been reconstituted from the catalytic subunit of protein phosphatase-1 and inhibitor-2, and consists of a 1:1 complex between these proteins. Activation of this enzyme by glycogen synthase kinase-3 and Mg-ATP results from the phosphorylation of inhibitor-2 on a threonine residue(s) and is accompanied by the dissociation of the complex. The results prove that protein phosphatase-1 and the Mg-ATP-dependent protein phosphatase contain the same catalytic subunit, and that they are interconvertible forms of the same enzyme.

Protein phosphatase-1 *Glycogen synthase kinase* *Inhibitor-2* *Phosphorylase* *Thiophosphorylation*

1. INTRODUCTION

The Mg-ATP-dependent protein phosphatase, an enzyme first described in adrenal cortex by Merlevede and Riley [1], is now known to have a ubiquitous tissue distribution [2–4]. It consists of an inactive protein phosphatase that must first be preincubated with Mg-ATP and an activating protein in order to exhibit biological activity [2,3]. The activating protein was shown to be associated with glycogen synthase kinase activity [5], and purification to near homogeneity has established its identity as the enzyme glycogen synthase kinase-3 (GSK-3) [6]. This finding suggested that the activation process should involve a phosphorylation mechanism [6,7], but this idea has been disputed [8,9].

The substrate specificity and regulatory properties of the Mg-ATP-dependent protein phosphatase are remarkably similar to those of protein phosphatase-1 [10]. Furthermore, it has been reported that inhibitor-2, a thermostable protein that inhibits protein phosphatase-1 specifically [10], is involved in the inactivation and reactivation of the Mg-ATP-dependent protein phosphatase [11,12]. These findings led us to try to reconstitute the Mg-ATP-dependent protein phosphatase from homogeneous preparations of protein phosphatase-1 and inhibitor-2. We have now established that by preincubating these two proteins, a 1:1 complex is produced whose properties are indistinguishable from the Mg-ATP-dependent protein phosphatase (T.J.R., B.A.H., H.Y. Lim Tung, P.C., in preparation). Here, we demonstrate that the activation mechanism does involve a phosphorylation reaction.

2. MATERIALS AND METHODS

2.1. *Materials*

[^{35}S]Adenosine-5′-*O*-(3-thio) triphosphate [ATPγS] was purchased from New England Nuclear, and unlabelled ATPγS from Boehringer. [γ-^{32}P]ATP and [α-^{32}P]ATP were from Amersham International, Sephadex G-100 (Superfine grade) and blue Sepharose CL-6B from Pharmacia, and

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DEAE-cellulose (DE-52) from Whatman.

Phosphorylase *b* [13], [^{32}P]phosphorylase *a* [10,14], phosphorylase kinase [15] and GSK-3 [6,16] were purified from rabbit skeletal muscle by standard procedures.

2.2. *Isolation of protein phosphatase-1 and inhibitor-2 from rabbit skeletal muscle*

The M_r 33 000 catalytic subunit of protein phosphatase-1 was purified to homogeneity by a modification of the procedure in [17]. The procedure involved precipitation with ammonium sulphate and ethanol as in [17] followed by chromatography on DEAE-cellulose and polylysine–Sepharose at pH 7.5, and gel filtration on Sephadex-100. The preparation showed a single protein-staining band on SDS–polyacrylamide gels (app. M_r 33 000) and had spec. act. ~50 000 U/mg (section 2.5). Its properties were indistinguishable from protein phosphatase-1 isolated by other methods [10] as judged by the criteria in [18].

Inhibitor-2 was purified to homogeneity as in [19]. At the final chromatography on blue Sepharose, the protein was eluted at 0.1 M NaCl, rather than at 0.6 M NaCl as in [19]. The preparation showed a single protein staining band on SDS–polyacrylamide gels (app. M_r 33 000) and had spec. act. ~50 000 U/mg (section 2.5). Its properties were indistinguishable from protein phosphatase-1 isolated by other methods [10] as judged by the criteria in [18].

Inhibitor-2 was purified to homogeneity as in [19]. At the final chromatography on blue Sepharose, the protein was eluted at 0.1 M NaCl, rather than at 0.6 M NaCl as in [19]. The preparation showed a single protein staining band on SDS–polyacrylamide gels, M_r 30 500. Its specific activity, electrophoretic mobility and amino acid composition were essentially identical to those of inhibitor-2 isolated as in [20].

2.3. *Preparation of the Mg-ATP-dependent protein phosphatase and dephosphorylated inhibitor-2*

Equimolar proportions of protein phosphatase-1 and inhibitor-2 were incubated for 1–2 h at 30°C in 20 mM Tris–HCl (pH 7.0)–10% glycerol–0.1% (v/v) 2-mercaptoethanol. The solution was then applied to a 1.0 × 1.0 cm column of blue Sepharose equilibrated in the same buffer. The 1:1 complex of inhibitor-2 and protein phosphatase-1 passed through the column, while any uncombined inhibitor-2 and protein phosphatase-1 were retained. Full details of the preparation and characterisation of this reconstituted Mg-ATP-dependent protein phosphatase will be presented elsewhere (T.J.R., B.A.H., H.Y. Lim Tung, P.C., in preparation).

The prolonged incubation of protein phosphatase-1 and inhibitor-2 ensured that the latter protein was completely dephosphorylated. Aliquots of the preparation were therefore heated at 100°C for 10 min and centrifuged to remove protein phosphatase-1, which is completely denatured by this treatment. The supernatant was used as the source of dephosphorylated inhibitor-2 in the experiment in fig. 3 (section 3).

2.4. *Phosphorylation and thiophosphorylation of the Mg-ATP-dependent protein phosphatase*

The Mg-ATP-dependent protein phosphatase (0.02 mg/ml) was activated at 30°C in an incubation (0.15 ml) containing GSK-3, 50 mM Tris–HCl (pH 7.0) (25°C)–0.1 mM EDTA–0.1% (v/v) 2-mercaptoethanol–0.01% Brij 35, 2.0 mM magnesium acetate and 0.5 mM [γ-^{32}P]ATP (10^9 cpm/μmol). At various times, aliquots were removed and used to measure activation and phosphorylation. Thiophosphorylation was carried out in an identical manner except that [^{35}S]ATPγS was substituted for [γ-^{32}P]ATP. In calculating the phosphorylation or thiophosphorylation stoichiometry, the M_r of the Mg-ATP-dependent protein phosphatase was taken as 63 500 (protein phosphatase-1 = 33 000, inhibitor-2 = 30 500) and protein concentrations were determined as in [21] using bovine serum albumin, $A^{1\%}_{280\ nm}$ = 6.5, as a standard.

2.5. *Assay of the Mg-ATP-dependent protein phosphatase and protein phosphatase-1*

Both enzymes were assayed at 30°C by the release of $^{32}P_i$ from [^{32}P]phosphorylase *a*. The assays (0.06 ml) contained phosphorylase *a* (1.0 mg/ml), 50 mM Tris–HCl pH 7.0 (25°C), 0.1 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 5 mM caffeine and 0.6 mg bovine serum albumin/ml. Assays of the Mg-ATP-dependent protein phosphatase also contained 1.25 mM

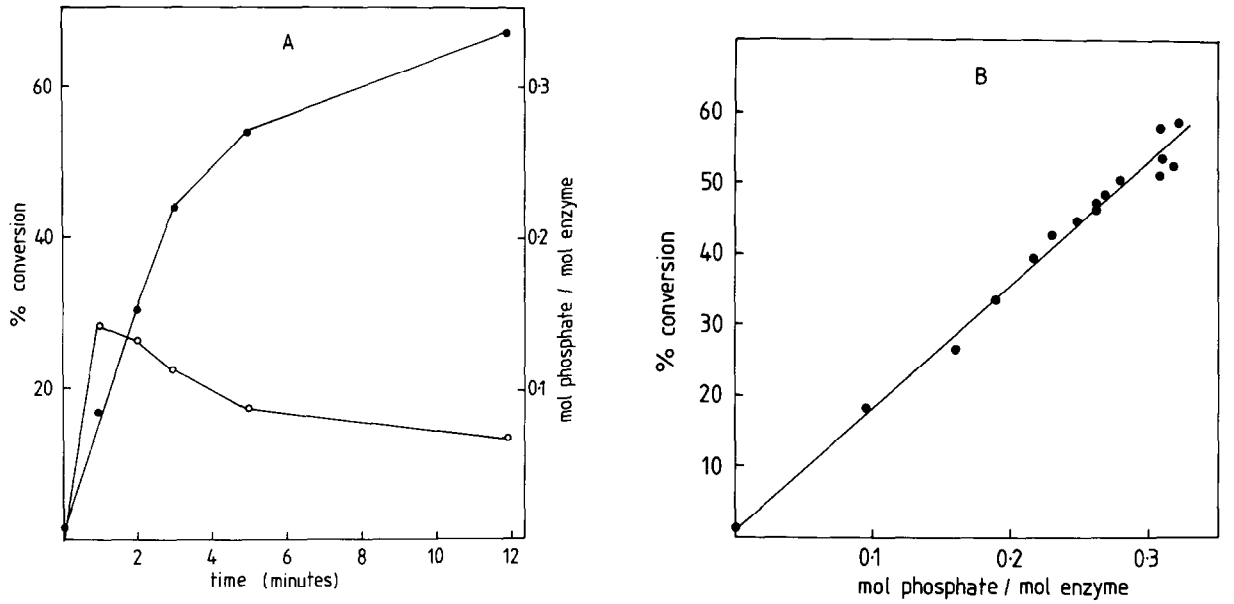

Fig. 1. Activation and phosphorylation of the Mg-ATP-dependent protein phosphatase. The Mg-ATP-dependent protein phosphatase, GSK-3 and [γ-^{32}P]ATP were incubated at 30°C as in section 2.4. The reactions were initiated with Mg-ATP, and at the times indicated aliquots were diluted with 50 mM Tris–HCl pH 7.0 (25°C), 0.5 mM EDTA, 0.1% (v/v) 2-mercaptoethanol, 1.0 mg bovine serum albumin/ml. The diluted enzyme was assyed directly and after maximal activation by GSK-3 and Mg-ATP in order to determine the percentage conversion to the active form (●—●). Further aliquots were analysed for the incorporation of ^{32}P radioactivity (○—○): (A) 20 μg Mg-ATP-dependent protein phosphatase were incubated with 0.3 μg of GSK-3, and the reactions diluted 200-fold before assay; (B) 20 μg Mg-ATP-dependent protein phosphatase were incubated for 1 min with different concentrations of GSK-3 (0–1.1 μg), and the reactions diluted 20-fold before assay.

magnesium acetate–0.125 mM ATP and GSK-3. The Mg-ATP-dependent protein phosphatase was assayed as in [6] and protein phosphatase-1 as in [20]. Reactions were terminated and analysed as in [20]. One unit of protein phosphatase was that amount which catalysed the release of 1.0 nmol phosphate/min in the above assays. Further details are given in the legends to the figures presented in section 3.

3. RESULTS

3.1. *Phosphorylation of the reconstituted Mg-ATP-dependent protein phosphatase*

The enzyme was completely inactive until incubated with GSK-3 and Mg-ATP. GSK-3 (0.3 μg) (6 pmol [6]) was capable of activating 20 μg (300 pmol) of Mg-ATP-dependent protein phosphatase (fig. 1A) demonstrating that GSK-3 was acting catalytically.

To investigate the activation mechanism, experiments were carried out using [^{32}P]-labelled ATP. These studies showed that activation was accompanied by a rapid incorporation of [^{32}P]-radioactivity into the preparation using [γ-^{32}P]ATP (fig. 1A) but not [α-^{32}P]ATP (not shown). All the ^{32}P radioactivity was incorporated into inhibitor-2 and ~90% was present as phosphothreonine. Phosphoserine accounted for ~10% of the radioactivity, while phosphotyrosine was completely absent (not shown).

A linear relationship between phosphorylation and activation could be demonstrated during the first minute of activation, when incubations were performed using a range of GSK-3 concentrations (fig. 1B). At later time points, however, the level of phosphorylation declined, while activation continued to increase. The lack of correlation between phosphorylation and activation is explained in section 3.4.

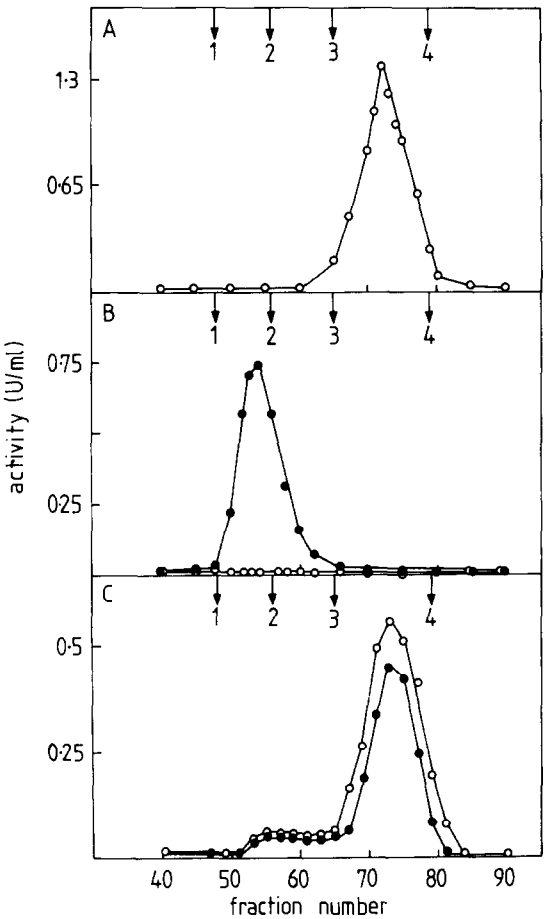

Fig. 2. Gel filtration of protein phosphatase-1 (A), inactive Mg-ATP-dependent protein phosphatase (B) and activated Mg-ATP-dependent protein phosphatase (C) on Sephadex G-100 Superfine (45 × 1.7 cm). The column was equilibrated in 50 mM Tris–HCl (pH 7.0)–0.1% (v/v) 2-mercaptoethanol–10% (v/v) glycerol–2.0 mM magnesium acetate–0.2 mM ATP, except for (A) in which Mg-ATP was omitted. Samples (0.5 ml) containing 10–20 U protein phosphatase activity were applied to the column, and fractions of 1.0 ml collected at 8 ml/h. Fractions were assayed, without further dilution, for protein phosphatase activity in the absence (○—○) or presence (●—●) of Mg-ATP and GSK-3 as in section 2.5. The arrows 1–4 denote the elution positions of the marker proteins yeast alcohol dehydrogenase M_r = 148 000, bovine serum albumin M_r = 68 000, ovalbumin M_r = 43 000 and carbonic anhydrase M_r = 29 500, respectively.

3.2. *Dissociation of the Mg-ATP-dependent protein phosphatase*

Protein phosphatase-1 and the reconstituted Mg-ATP-dependent protein phosphatase migrated as single components on Sephadex G-100 with app. M_r of 33 000 and 87 000, respectively (fig. 2A,B). An app. M_r of 87 000 would be expected, since inhibitor-2 is a protein with little ordered structure that yields an app. M_r of 65 000 on Sephadex G-100 [20,22]. Activation of the Mg-ATP-dependent protein by GSK-3 and Mg-ATP was accompanied by its dissociation to protein phosphatase-1, M_r 35 000 (fig. 2C). No dissociation occurred if GSK-3 was omitted from the incubations (fig. 2B). The dissociated protein phosphatase-1 (fig. 2C) could be reconverted to the Mg-ATP-dependent protein phosphatase by reincubation with inhibitor-2 (not shown).

Table 1

Inactivation of the Mg-ATP-dependent protein phosphatase by thiophosphorylation of inhibitor-2

Time (min)	Thiophosphate incorporated (mol/mol inhibitor-2)	Phosphatase inactivation (%)
0	0	0
2	0.37	25
5	0.47	50
10	0.68	62
20	0.77	66

The Mg-ATP-dependent protein phosphatase (32 μg), GSK-3 (0.5 μg) and [^{35}S]ATPγS were preincubated at 30°C in 1.0 ml total vol., as in section 2.4. The reaction was initiated with [^{35}S]ATPγS, and at the times indicated, aliquots were analysed for incorporation of ^{35}S-radioactivity. Further aliquots were diluted 250-fold, and the diluted samples assayed directly and after maximal activation by GSK-3 and Mg-ATP. The inactivation of the enzyme was calculated by reference to a control preincubation in which GSK-3 was omitted

3.3. *Thiophosphorylation of inhibitor-2*

It has been reported that ATPγS cannot substitute for ATP in the activation of the Mg-ATP-dependent protein phosphatase [9], an observation confirmed here. However, further experiments using [^{35}S]ATPγS showed that GSK-3 catalysed the thiophosphorylation of inhibitor-2, and that thiophosphorylation prevented the Mg-ATP-dependent protein phosphatase from being activated by GSK-3 and Mg-ATP (table 1). The thiophosphorylation stoichiometry approached

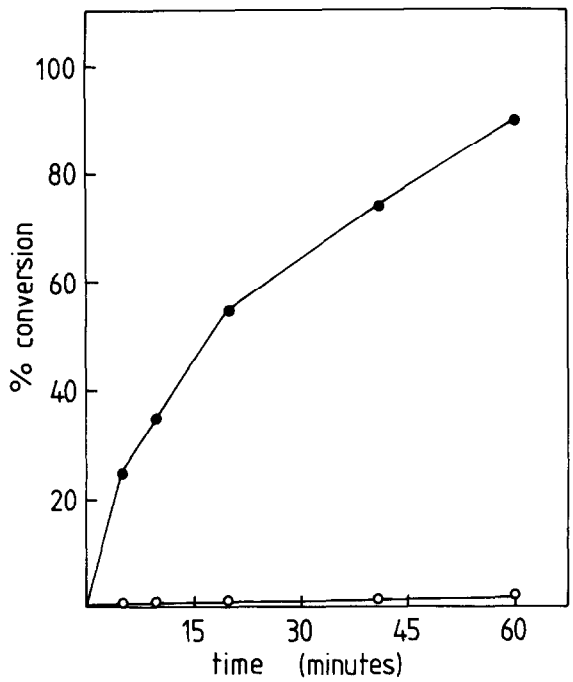

Fig. 3. Conversion of protein phosphatase-1 to the Mg-ATP-dependent protein phosphatase by incubation with dephosphorylated inhibitor-2 (section 2.2). Protein phosphatase-1 (1.3 μg) was incubated at 30°C with inhibitor-2 (1.3 μg) in 0.1 ml 50 mM Tris–HCl (pH 7.0)–0.5 mM EDTA–0.1% (v/v) 2-mercaptoethanol–1.0 mg bovine serum albumin/ml (●—●). At various times aliquots were diluted 10-fold and assayed for conversion to the Mg-ATP-dependent protein phosphatase as in section 2.5: (○—○) control incubation in which inhibitor-2 was omitted.

1.0 mol/mol after prolonged incubation (table 1). These experiments demonstrate that phosphorylation of inhibitor-2 is essential for activation, although why thiophosphorylation does not mimic phosphorylation in this system is unclear.

3.4. *Mechanism of activation and inactivation of the Mg-ATP-dependent protein phosphatase*

The conversion of protein phosphatase-1 to the Mg-ATP-dependent protein phosphatase is relatively slow under the conditions tested, occurring with a $t_{1/2}$ of ~15 min, even using dephosphorylated inhibitor-2 (fig. 3). This slow reassociation accounts for the lack of correlation between phosphorylation and activation (fig. 1), and the simplest model that explains the results is illustrated in fig. 4. Activation of the Mg-ATP-dependent protein phosphatase is initiated by the phosphorylation of inhibitor-2, which causes its dissociation to protein phosphatase-1 (fig. 2C). The activated phosphatase can then analyse the dephosphorylation of inhibitor-2, the phosphorylation state of this protein being determined by the relative activities of GSK-3 and protein phosphatase-1. However, provided the reassociation of protein phosphatase-1 and dephosphorylated inhibitor-2 is rate-limiting, the extent of activation of the Mg-ATP-dependent phosphatase will continue to increase, as each inhibitor-2 molecule undergoes a phosphorylation–dephosphorylation cycle (fig. 4).

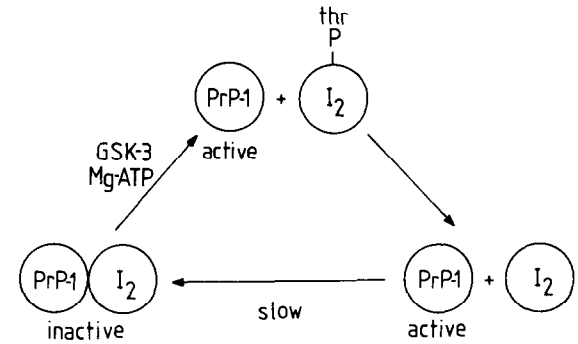

Fig. 4. Model for activation of the Mg-ATP-dependent protein phosphatase, and its reformation from protein phosphatase-1 (PrP-1) and inhibitor-2 (I_2).

4. DISCUSSION

The present finding, that activation of the Mg-ATP-dependent protein phosphatase is triggered by the phosphorylation of inhibitor-2 is at variance with the conclusions of Merlevede and coworkers. This group purified the Mg-ATP-dependent protein phosphatase to a state where it showed one major protein, M_r 70 000 on SDS–polyacrylamide gels, and reported that no ^{32}P-radioactivity was incorporated into this band following incubation with GSK-3 and Mg-[γ^{32}P]ATP [8,9]. They recently suggested that activation and inactivation might involve the addition and removal of Mg^{2+}, the activated form of the phosphatase being a Mg^{2+}-metalloenzyme [9]. It was envisaged that GSK-3 catalysed the insertion of Mg^{2+} into the phosphatase from Mg-ATP. This idea stemmed from their report that the Mg-ATP-dependent protein phosphatase could be partially activated by Mn^{2+}, even in the absence of GSK-3 and Mg-ATP

[9]. However, the reconstituted Mg-ATP-dependent protein phosphatase used in this study cannot be activated by Mn^{2+} (unpublished). The conversion of protein phosphatase-1 to Mn^{2+}-dependent forms is well documented [23,24], and is a phenomenon unrelated to the formation of the Mg-ATP-dependent protein phosphatase. It seems likely that the enzyme isolated by Merlevede and coworkers also contained a Mn^{2+}-dependent form of protein phosphatase-1.

The nature of the M_r 70 000 protein isolated in [8] is unclear. One possibility is that the M_r 70 000 protein represents an undegraded form of protein phosphatase-1, the M_r 33 000 species being an active fragment of the enzyme. Alternatively the M_r 70 000 protein could be an impurity, since the specific activities of the preparations, 10 000 U/mg [8,12], were lower than that of the M_r 33 000 form of protein phosphatase-1 (~50 000 U/mg, section 2.2). Further work is needed to distinguish between these two possibilities. Nevertheless, the finding that phosphorylation of inhibitor-2 underlies the activation of the Mg-ATP-dependent protein phosphatase explains why phosphorylation of the M_r 70 000 protein was not detected previously.

Recent studies of the phosphorylation of glycogen synthase in vivo suggest that GSK-3 activity is under the control of insulin. This raises the possibility that activation of the Mg-ATP-dependent protein phosphatase is also under hormonal control. It will be of considerable interest to examine the phosphorylation state of inhibitor-2 in vivo and how it changes in response to insulin and other hormones.

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