Isolation of an active form of the ATP + Mg²⁺-dependent protein phosphatase stimulated by the deinhibitor protein and by *p*-nitrophenyl phosphate

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A 75 kDa + 37 kDa-subunit high-*M_r* form of the ATP + Mg²⁺-dependent phosphatase, containing no modulator or inhibitor-1 and specific towards the β -subunit of phosphorylase kinase, was isolated from dog liver. The phosphorylase phosphatase activity is stimulated by the deinhibitor protein, by *p*-nitrophenyl phosphate or by trypsin treatment. The sensitivity to modulator or inhibitor-1 can be increased dramatically by partial proteolysis. The enzyme inactivation by modulator after trypsin treatment can be reversed by protein kinase F_A. The effects of *p*-nitrophenyl phosphate and the deinhibitor protein are synergistic, and *p*-nitrophenyl phosphate does not influence the sensitivity of the phosphatase to the heat-stable inhibitor proteins. These observations confirm that *p*-nitrophenyl phosphate stimulation is a suitable criterion for the identification of the active enzyme forms of the ATP + Mg²⁺-dependent phosphatase.

INTRODUCTION

In most tissues the major phosphorylase phosphatase activity has been identified as the ATP + Mg²⁺-dependent protein phosphatase, purified mainly as an inactive form from the cytosol and as a spontaneously active enzyme from the glycogen particle [1,2]. The interconversion between inactive F_cM and active F_c*M is possible *in vitro* through the action of protein kinase F_A, the modulator (M) protein being an essential subunit in the activation–inactivation of the catalytic subunit F_c [3,4]. The activity state of the different forms of ATP + Mg²⁺-dependent phosphatase *in vivo* is debatable, since little is known about their native subunit structure, specificity *in vivo* and equilibria between the different enzyme forms.

Addition of the deinhibitor (DI) protein to a gel-filtered postmitochondrial supernatant leads to a several-fold increase in phosphorylase phosphatase activity [5]. On the basis of the known effects of the DI [6], this would suggest that the ATP + Mg²⁺-dependent phosphatase in crude extracts is present as an inhibited ‘active’ enzyme. Full expression of this activity necessitates elimination of the inhibition rather than the activation by the kinase F_A. This could be initiated by dephosphorylation of the DI protein, which is shown to be active only in its dephospho form [7]. The increase of phosphorylase phosphatase activity by trypsin treatment of crude preparations [8,9] favours this hypothesis. Indeed, trypsin treatment destroys the inhibitors and the active form of the ATP + Mg²⁺-dependent phosphatase is very resistant to the action of trypsin [1]. Since the inactive form of this enzyme [10] as well as the different polycation-stimulated (PCS) phosphatases [11] are trypsin-labile, stimulation by trypsin cannot be due to activation of one of these enzymes.

Investigating the stimulation of phosphorylase

phosphatase activity by the DI, we purified from dog liver a novel high-*M_r* DI-stimulated phosphorylase phosphatase, which is a form of the ATP + Mg²⁺-dependent protein phosphatase containing no modulator (M) or inhibitor-1 (I-1), resistant to inhibition by both I-1 and M, stimulated by *p*-nitrophenyl phosphate (*p*NPP), and consisting of a 37 kDa catalytic and a 75 kDa regulatory subunit. *p*NPP stimulation is further discussed as a new criterion for the identification of any active form of ATP + Mg²⁺-dependent phosphatase.

MATERIALS AND METHODS

Materials

Polylysine, protamine hydrochloride, phenylmethanesulphonyl fluoride, *N*^α-*p*-tosyl-L-lysylchloromethane, benzamidine and soya-bean trypsin inhibitor were obtained from Sigma Chemical Co.; DEAE-cellulose was from Whatman; Ultrogel AcA-34 was from LKB; phenyl-Superose HR 5/5, Mono Q HR 5/5 and Superose 6 HR 10/30 columns and CNBr-activated Sepharose-4B were from Pharmacia; *p*NPP and *p*-nitrophenol were from Merck; α -naphthol phosphate, β -naphthol phosphate and dithiothreitol were from Janssen Chimica; the silver staining kit was from Bio-Rad Laboratories; [γ -³²P]ATP was from The Radiochemical Centre; trypsin (tosyl-lysylchloromethane-treated) was from Worthington Biochemical Corp; the *M_r* marker proteins used in SDS/PAGE [myosin H chain (200 kDa), phosphorylase *a* (97.4 kDa), bovine serum albumin (68 kDa), ovalbumin (43 kDa), α -chymotrypsinogen (25.7 kDa), β -lactoglobulin (18.4 kDa) and cytochrome *c* (12 kDa)] were from Bethesda Research Laboratories; the *M_r* marker proteins used in gel filtration [ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa),

Abbreviations used: F_c and F_c*, inactive and active catalytic subunits of the ATP + Mg²⁺-dependent protein phosphatase; DI, deinhibitor; M, modulator; I-1, inhibitor-1; PCS, polycation-stimulated; *p*NPP, *p*-nitrophenyl phosphate; PAGE, polyacrylamide-gel electrophoresis.

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bovine serum albumin (68 kDa) and ovalbumin (45 kDa) were from Pharmacia; the ^{14}C -labelled ovalbumin (43 kDa, 3.6 S) used in the sucrose-density-gradient centrifugation was from The Radiochemical Centre.

Polylysine-Sepharose [12], phosphorylase *b* [13], I-1 [14], M [15], DI [6,16], F_c^* [6,17] and F_cM [12], protein kinase F_A [18], the different PCS phosphatases [17,19], classified as high (PCS_H), medium (PCS_M) and low (PCS_L) phosphatases according to their native M_r values, and their catalytic subunit (PCS_c), were purified according to published methods. Phosphorylase *b* kinase [20] and bovine heart cyclic AMP-dependent protein kinase [21] were generously given by Professor D. A. Walsh (University of California, Davis, CA, U.S.A.). ^{32}P -phosphorylase *a* [22], ^{32}P -I-1 [23] and ^{32}P -phosphorylase kinase [24] labelled by cyclic AMP-dependent protein kinase to a phosphorylation level of 0.5 mol of P/mol of α -subunit and 0.7 mol of P/mol of β -subunit were prepared as described in the references cited.

Assay procedures

Phosphorylase phosphatase was assayed as described in ref. [19] and in the presence of 10 μg of protamine/ml or 2 mM-*p*NPP, or after preincubation for 10 min at 30 °C with 100 munits of DI/30 μl , which is at least twice the concentration necessary for full stimulation of 15–30 munits of phosphatase ($A_{50} = 10$ munits). F_cM was measured after activation by kinase F_A [12]; M, after boiling, was measured as the activator of F_c [4]. I-1 was assayed after boiling and activation by cyclic AMP-dependent protein kinase as inhibitor of F_c^* [25]. Alkaline phosphatase was measured in the presence of 20 mM-*p*NPP and 20 mM- Mg^{2+} [26]. Phosphorylase kinase phosphatase was measured as described in ref. [24].

Other procedures

PAGE [27] was carried out, in the presence or in the absence of SDS, with 12.5 % polyacrylamide in 0.8 mm

mini slab gels (10 cm $\times$ 8 cm). The gels were stained with 0.25 % Coomassie Brilliant Blue followed by silver staining according to the Bio-Rad protocol. Ultrogel AcA-34 and Superose were calibrated as described in ref. [19]. Proteins were determined according to the Bradford method [28], with bovine serum albumin as the standard. Autoradiograms were made after drying the gel between two cellophan sheets with the use of intensifying screens and Agfa-Gevaert Curix RP1 X-ray film. Optical-density scans of autoradiograms or silver-stained gels were made with a LKB Density Laser Ultrosan and quantified by cutting out the scan paper record and weighing the peaks. Sucrose-density-gradient centrifugation was performed as described in ref. [12] and sedimentation coefficients were calculated as described in ref. [29].

Purification of the DI-stimulated protein phosphatase

The liver (800–1300 g) of a dog, fed on a carbohydrate-rich diet the day before being killed, was homogenized in 2 vol. of 100 mM-Tris/HCl buffer, pH 7.8, containing 0.25 M-sucrose, 1 mM-EDTA, 1 mM-EGTA, 0.5 mM-dithiothreitol, 1 mM-benzamidine, 0.1 mM-phenylmethanesulphonyl fluoride and 0.1 mM-*N*-*p*-tosyl-L-lysylchloromethane. Unless otherwise stated all procedures were carried out at 4 °C. The homogenate was centrifuged at 10000 *g* for 15 min, the supernatant was filtered through glass-wool and mixed with 3.7 litres of settled DEAE-cellulose, equilibrated in the homogenization buffer without sucrose and containing 20 mM-Tris (buffer A). The suspension, filtered batchwise through a Buchner sintered-glass filter and, after reapplication of the filtrate, washed six times with 1 litre of buffer A, was taken up in 2 litres of the same buffer, poured into the column (8 cm $\times$ 75 cm bed volume after settlement) and washed with a further 1 litre of buffer A. The column was developed with 10 litres of a linear 0–0.4 M-NaCl gradient in buffer A. The DI-stimulated phosphatase is eluted in the first phosphorylase phosphatase activity peak,

Fig. 1. Polylysine-Sepharose 4B chromatography

The pooled fractions from the Ultrogel AcA-34 gel-filtration step were applied to a polylysine-Sepharose 4B column (1.5 cm $\times$ 20 cm) and eluted as described in the Materials and methods section. Panel (a) shows the phosphorylase phosphatase activity without addition (x), after preincubation with DI (●) and after preincubation with *p*NPP (▼)., A_{280} ; — — —, NaCl gradient. The elution of F_cM (and M, measured after boiling) is indicated by the arrow. Panel (b) shows the phosphorylase phosphatase activity in the presence of protamine (▲) and the alkaline phosphatase activity (□).

Fig. 2. Mono Q f.p.l.c. of the DI-stimulated phosphatase

The pooled flow-through fractions of the phenyl-Superose column were dialysed as described in the Materials and methods section and applied to a Mono Q f.p.l.c. column. After being washed with 0.2 M-NaCl in buffer C, the chromatogram was developed with 24 ml of a 0.2–0.6 M-NaCl gradient in buffer C. Fractions (15 drops) were collected and assayed for phosphorylase phosphatase activity without addition ($\times$), in the presence of pNPP ($\blacktriangledown$) and after preincubation with DI ($\bullet$). $\cdots\cdots$, A_{280} ; ----, NaCl gradient.

together with the PCS_H phosphatase and F_cM. Solid $(\text{NH}_4)_2\text{SO}_4$ was slowly added to the pooled peak to obtain 60% saturation, and after 30 min at 4 °C and centrifugation for 20 min at 15000 *g* the pellet was dissolved in a minimal volume (100 ml) of buffer A and dialysed twice against the same buffer for 1 h.

The dialysed sample was centrifuged at 15000 *g* for 10 min and the supernatant applied to an Ultrogel Aca-34 column (5 cm $\times$ 95 cm) equilibrated in 20 mM-Tris/HCl buffer, pH 7.4, containing 0.5 mM-dithiothreitol, 1 mM-EDTA, 1 mM-EGTA, 1 mM-benzamidinium and 0.1 M-NaCl (buffer B). A partial separation was obtained between PCS_H phosphatase (360 kDa), DI-stimulated phosphatase (260 kDa), eluted at the same position as a PCS phosphatase, and F_cM (140 kDa).

After pooling and the addition of glycerol (final concn. 2%, v/v), the DI-stimulated phosphatase was applied on to a polylysine-Sepharose 4B column (1.5 cm $\times$ 20 cm) equilibrated with buffer B containing 2% (v/v) glycerol (buffer C), washed with 120 ml of the same buffer and eluted with 800 ml of a 0.1–0.7 M-NaCl gradient in buffer C (Fig. 1). The DI-stimulated phosphatase, well separated from the PCS phosphatase, F_cM and alkaline phosphatase, was pooled, diluted 2-fold in buffer C without 0.1 M-NaCl and applied to a second polylysine-Sepharose 4B column (0.9 cm $\times$ 3 cm) equilibrated in buffer C. After the column had been washed with 0.2 M-NaCl in buffer C, the DI-stimulated phosphatase

was flushed off with 0.7 M-NaCl in buffer C. The phosphatase was further concentrated by dialysis against 60% (v/v) glycerol in buffer B without 0.1 M-NaCl and stored at –20 °C.

In order to lower the viscosity, the sample was diluted 5-fold in 20%-satd. $(\text{NH}_4)_2\text{SO}_4$ and applied to an f.p.l.c. phenyl-Superose column equilibrated with 20%-satd. $(\text{NH}_4)_2\text{SO}_4$ in buffer C. The DI-stimulated phosphatase was not retained on the column, whereas the remaining contaminating PCS- and alkaline phosphatase bind to the column and could be recovered with a descending 20–0%-satd. $(\text{NH}_4)_2\text{SO}_4$ gradient in buffer C. The flow-through fractions were concentrated by dialysis against 20% (w/v) poly(ethylene glycol) and then against 60% (v/v) glycerol in buffer B without 0.1 M-NaCl, applied to a f.p.l.c. Mono Q column equilibrated with buffer C. After elution as described in the legend to Fig. 2, the active fractions were dialysed against 60% (v/v) glycerol in buffer B and stored at –20 °C.

RESULTS AND DISCUSSION

Purification of the DI-stimulated phosphatase

Addition of DI to crude liver extracts leads to a several-fold increase of phosphorylase phosphatase activity [5]. The degree of stimulation is not linear and is primarily dependent on the dilution of the extract, presumably by diluting out phosphatase inhibitor(s) such as I-1 and M, responsible to a large extent for the DI effect. DI-stimulated phosphatase activity was mainly localized in the cytosol, although a substantial amount was also found in the microsomal fraction, the glycogen-bound enzyme being only slightly stimulated [30]. Therefore no high-speed centrifugation was done before the com-

Fig. 3. SDS/PAGE of the DI-stimulated phosphatase (lane 1) and the catalytic subunit (lane 2)

SDS/PAGE of 4 μ g of DI-stimulated phosphatase (fraction 32 of the Mono Q column represented in Fig. 2) or 1 μ g of the catalytic subunit (fraction 23 of the Mono Q column after dialysis and freeze-drying) and the Coomassie Brilliant Blue/silver staining were carried out as described in the Materials and methods section.

Table 1. Purification of the DI-stimulated phosphatase

The starting material was 900 g liver. For experimental details see the Materials and methods section.

Purification step	Volume (ml)	Protein (mg)	Total activity (units)			Sp. activity of control (units/mg)
			Control	+ DI	+ <i>p</i> NPP	
DEAE-cellulose pool	875	3200	11000	18500	22300	3.4
(NH ₄) ₂ SO ₄ precipitation	92	2100	21000	27800	25800	10
Ultrogel AcA-34	350	620	10140	16200	19775	16
Polylysine 1	90	32	3690	5500	8300	115
Polylysine 2	7	21	1660	3200	3490	79
Phenyl-Superose	17	1.8	1400	1820	2930	778
Mono Q						
Peak 1	0.5	0.05	940	1030	1410	18800
Peak 2	0.5	0.32	430	750	910	1344

pulsory large-scale (8 cm × 75 cm column) DEAE-cellulose chromatography, resulting in less than 10 % loss of DI-stimulated phosphatase activity in the flow-through.

During DEAE-cellulose chromatography of the crude liver extract, DI- and *p*NPP-stimulated phosphorylase phosphatase activity were co-eluted with F_cM, M and the PCS_H phosphatase (0.2 M-NaCl), well separated from I-1 (0.12 M-NaCl). The DI-stimulated phosphatase activity was separated from F_cM through Ultrogel AcA-34 gel filtration followed by polylysine-Sepharose 4B affinity chromatography (Fig. 1). These results suggested that DI stimulation of the phosphorylase phosphatase activity can also be independent of any inhibition by I-1 or M. The PCS phosphatase activity, with apparent *M_r* of 360000 and 260000, was partially separated from the DI-stimulated phosphatase during the Ultrogel AcA-34 gel filtration, but more efficiently by polylysine-Sepharose 4B chromatography (Fig. 1). However, although no PCS phosphatase activity was observed on assaying the column fractions containing the DI-stimulated phosphatase, a PCS phosphatase activity could still be separated in the subsequent phenyl-Superose chromatography step. In this step the DI-stimulated phosphatase does not bind to the column in 20 %-satd. (NH₄)₂SO₄, but the residual PCS phosphatase activity can be recovered well beyond in the reverse-salt-gradient elution. In the last purification step (Fig. 2) two *p*NPP-stimulated phosphatases are separated. The major activity peak is not stimulated by DI and represents F_c*, on the basis of its specific activity, its sensitivity to I-1 and M, as well as the *M_r* in SDS/PAGE (Fig. 3). The second peak (about one-third of the total activity) represents the purified DI-stimulated phosphatase further characterized in this study. The partial dissociation of the holoenzyme is probably due to hydrophobic interaction with phenyl-Superose. Nevertheless this step is mandatory, since if omitted the PCS phosphatase activity and the DI-stimulated phosphatase co-migrate during the Mono Q f.p.l.c.

A typical purification of the DI-stimulated phosphatase is summarized in Table 1. The *p*NPP-stimulated activity is also included, since it co-purifies with the DI-stimulated activity. Yields are difficult to evaluate throughout the purification, since the DI-stimulated activity is also due to inhibitory M and I-1 initially present, and the basal activity could be due to con-

taminating or complexed PCS phosphatase, or to F_c*. On some occasions in the gel-filtration step up to 30 % of the enzyme activity was recovered as F_c*, stimulated by *p*NPP but not by the DI. The total amount of DI-stimulated phosphatase thus isolated represents a major fraction of the ATP + Mg²⁺-dependent type of protein phosphatase. From the Ultrogel AcA-34 step onwards it is reasonable to assume that stimulation by the DI is due to the presence of DI-stimulated phosphatase, since I-1 was separated during the DEAE-cellulose chromatography and most of the F_cM was separated during the Ultrogel AcA-34 gel filtration. The amount of DI-stimulated phosphatase recovered at this stage almost equals the amount of F_cM. With a final specific activity of 1340 units/mg, and taking into account the potential 12–15-fold stimulation by *p*NPP and DI (see below), probably cancelling the inhibitory constraint imposed by the 75 kDa subunit, as well as the fact that the catalytic subunit represents only one-third of the enzyme protein, the specific activity thus obtained is among the highest ever observed for this type of enzyme.

Several authors reported the co-purification of an alkaline (*p*NPP-hydrolysing) phosphatase activity with some forms of the ATP + Mg²⁺-dependent and PCS phosphatases [26,31–36]. During the DEAE-cellulose chromatography two major alkaline phosphatase activities were separated from the DI-stimulated phosphatase: one, probably representing calcineurin, early in the gradient, the other being co-eluted with the PCS_L phosphatase. In the Ultrogel AcA-34 gel-filtration step most of the contaminating *p*-NPP phosphatase migrated with a lower apparent *M_r* than F_cM and the DI-stimulated phosphatase. In the polylysine affinity chromatography step most of the remaining contaminating alkaline phosphatase co-migrated with the PCS phosphatase activity (Fig. 1). Trace amounts of *p*-NPP-hydrolysing activity still present after the second polylysine chromatography was removed completely in the phenyl-Superose step, being co-eluted with the PCS phosphatase activity at the end of the gradient. No *p*NPP-hydrolysing activity could be detected in the purified and concentrated DI-stimulated phosphatase preparation even in the presence of Mn²⁺ or after trypsin treatment in the presence or in the absence of Mn²⁺. Hence the DI-stimulated phosphatase is different from the Mn²⁺-stimulated high-*M_r* enzyme, also identified as a

form of ATP + Mg²⁺-dependent phosphatase [34], the *p*NPP-hydrolysing activity of which is apparently expressed after trypsin treatment in the presence of Mn²⁺ [34], and from the human erythrocyte high-*M_r* type-1 protein phosphatase [36], purified on the basis of the *p*NPP-hydrolysing activity. We could not detect any *p*-nitrophenyl phosphatase activity in purified F_cM stimulated by Mn²⁺ or activated by kinase F_A even after trypsin treatment in the presence or absence of Mn²⁺.

Physical properties

The purified DI-stimulated phosphatase migrated as a single 160 kDa protein peak during Superose 12 gel filtration, whereas earlier in the purification procedure it migrated as a 260 kDa protein in Ultrogel AcA-34 gel filtration. The reason for this change in apparent *M_r* is not clear. It was observed that the PCS phosphatases, which moreover still co-migrated with the DI-stimulated phosphatase during Ultrogel AcA-34 gel chromatography, showed a similar change in apparent *M_r* after the Ultrogel AcA-34 gel filtration [19]. Parallel observations were made by other investigators early in the purification of a native high-*M_r* phosphatase from liver [8] or skeletal muscle [35]. SDS/PAGE of the DI-stimulated phosphatase showed a 75 kDa and a 37 kDa protein band after Coomassie Brilliant Blue and silver staining (Fig. 3), present in a 1:1 molar ratio as judged from a densitometric analysis of the stained gel. SDS/PAGE of the enzyme eluting as peak I during the Mono Q f.p.l.c. (Fig. 3) showed only the 37 kDa subunit, and in sucrose-density-gradient centrifugation this phosphatase migrated as a 39 kDa (3.4 S) activity peak, whereas the DI-stimulated phosphatase migrated as a 75 kDa (4.4 S) enzyme. SDS/PAGE of the individual fractions showed that the 75 kDa protein band peaked with the DI-stimulated phosphatase, well separated from a 70 kDa silver-staining band (6.06 S in the sucrose-density-gradient centrifugation), present in some preparations.

After PAGE in the absence of SDS, only one Coomassie Brilliant Blue-stained band with an *R_f* value of 0.21 was observed. If parallel lanes of a slab gel were cut into 1 mm sections and eluted overnight in buffer, the DI- and *p*NPP-stimulated phosphatase had the same *R_f* value as the protein band. SDS/PAGE in the second dimension after freeze-drying showed the same two subunits present in a 1:1 molar ratio, as judged from densitometric analysis of the silver-stained gel.

The DI-stimulated phosphatase has therefore very similar physical and enzymic properties to the phosphoprotein phosphatase from skeletal muscle isolated by Khatra [37], with a sedimentation coefficient of 4.4 S and a Stokes radius of 4.4 nm, or by Cohen and co-workers [38,39], who found a sedimentation coefficient of 4.4 S and a Stokes radius of 5.4 nm for protein phosphatase-1_G. This phosphatase contains a large G subunit of 103 kDa, responsible for the binding of the 37 kDa catalytic subunit to glycogen. Just like the DI protein, the G protein influences the sensitivity of the catalytic subunit to the heat-stable protein inhibitors, and can be inactivated through phosphorylation by the cyclic AMP-dependent protein kinase. Whereas Khatra [37] represents the high-*M_r* phosphatase as a dimer, explaining the divergent Stokes radius by the use of Sephacryl S-300 versus Sepharose CL-6B, Cohen and co-workers [38,39] suggest that the former preparation contains the 103 kDa G subunit (or a fragment of it) but

not noticed since it stains very poorly with Coomassie Brilliant Blue. We found 4.4 S as the sedimentation coefficient for the DI-stimulated phosphatase and a subunit composition (37 kDa + 75 kDa) as found by Khatra before the sucrose-density-gradient centrifugation in which most of the 75 kDa band separated from the 38 kDa band and was therefore considered to be unrelated to the phosphatase [37]. This could be the 70 kDa protein observed in some of our preparations. Our observations indicate that in sucrose-density-gradient centrifugation the 37 kDa protein of the DI-stimulated phosphatase is associated with another 75 kDa component, different from the 103 kDa G subunit or its fragments (see below). This 75 kDa component has some inhibitory action on the catalytic activity, which can be relieved by the DI or by trypsin treatment. We cannot exclude the possibility that the 75 kDa subunit also contains catalytic activity nor that it could represent a larger precursor of the 38 kDa subunit, as proposed by Brautigan *et al.* [40]. The DI-stimulated phosphatase also shows some similarities to the erythrocyte high-*M_r* type-1 protein phosphatase, showing a 62 kDa + 36 kDa subunit structure in a 1:2 ratio, migrating as a 6.1 S enzyme in glycerol-gradient centrifugation [36]. However, this phosphatase appears to be different from the DI-stimulated phosphatase as mentioned above.

DI-stimulated phosphatase is an active form of the ATP + Mg²⁺ dependent phosphatase

The sensitivity of the DI-stimulated phosphatase to I-1 or M is largely suppressed: 870 nM-I-1 inhibits 30 munits of DI-stimulated phosphatase by only 30% and 640 nM-I-1 by only 60%, whereas after partial proteolysis of the DI-stimulated phosphatase the *I*₅₀ values for M and I-1 are 5 and 2 nM respectively. The relative resistance of the DI-stimulated phosphatase to I-1 or M is not due to the presence of DI or G subunit or one of its fragments, since (1) no DI activity was found under conditions [23] that allow for its detection in the DI-phosphatase complex of the glycogen pellet, (2) the sensitivity to the heat-stable inhibitors does not change after preincubation with cyclic AMP-dependent protein kinase, as would be expected for the DI [7] and the G subunit [38], (3) the DI-stimulated phosphatase is not phosphorylated by cyclic AMP-dependent protein kinase, since no ³²P label is incorporated, as judged from autoradiography, (4) no DI-stimulated phosphatase precipitates with added particulate glycogen in conditions where 70% of the phosphatase containing the G subunit would have been precipitated, partial fragmentation of the G subunit not affecting its interaction with glycogen [39], (5) in contrast with the G subunit [38] or its fragments [39] the 75 kDa subunit can be silver-stained, and (6) contrary to the DI-containing phosphatase [6] the DI-stimulated phosphatase can be further stimulated by the DI.

Limited trypsin digestion of the DI-stimulated phosphatase leads to a 2–3-fold increase of the phosphorylase phosphatase activity. This activation is due to an increase in *V*_{max} and is not observed with the catalytic unit separated in the last purification step. The resulting catalytic activity is also remarkably resistant to the action of trypsin, as known for the other forms of ATP + Mg²⁺-dependent phosphatase [1], in contrast with the PCS phosphatases [11]. As a result of the partial

Fig. 4. Conversion into the inactive form of ATP+Mg²⁺-dependent phosphatase by M after trypsin treatment

DI-stimulated phosphatase was incubated with (▲ and ●) or without (△ and ○) 25 μg of trypsin/ml for 3 min at 30 °C and the reaction was stopped by addition of trypsin inhibitor (500 μg/ml final concn.). The phosphatase was subsequently incubated with the indicated concentration of modulator for 10 min at 30 °C, further incubated for 10 min at 30 °C, in the presence (○ and ●) and in the absence (△ and ▲) of the kinase F_A, ATP+Mg²⁺ and DI, and phosphatase activity was measured. Results are expressed as percentages of the control measured in the absence of M (280 munits/ml of assay medium without trypsin, 610 munits/ml of assay medium with trypsin treatment). No re-activation by DI was obtained in the absence of kinase F_A and ATP+Mg²⁺ (results not shown).

proteolysis, the DI-stimulated phosphatase is not stimulated any more by the DI (results not shown) and becomes as sensitive to the heat-stable inhibitors as F_c^{*}; moreover, after inactivation of the trypsin-treated form by M, the phosphatase activity can be restored by kinase F_A in the presence of ATP+Mg²⁺ and DI, the definite proof that the DI-stimulated phosphatase is an active form of the ATP+Mg²⁺-dependent phosphatase (Fig. 4). As expected for this type of enzyme [41], the DI-stimulated phosphatase is specific for the β-subunit of phosphorylase b kinase (results not shown).

pNPP stimulation of phosphorylase phosphatase activity as a feature of the ATP+Mg²⁺-dependent phosphatases

Although none of the different forms of ATP+Mg²⁺-dependent phosphatase can dephosphorylate pNPP at any detectable rate under the standard assay conditions, even in the presence of Mg²⁺, the phosphorylase phosphatase activity of the ATP+Mg²⁺-dependent phosphatase is stimulated several-fold by pNPP at an optimal concentration of 1 mM. The property of being stimulated by pNPP is shared by all the active forms of the enzyme, and the inactive form can only be stimulated after activation by kinase F_A in the presence of ATP and Mg²⁺. The pNPP stimulation is also specific for the type of enzyme: whereas the different active forms of the ATP+Mg²⁺-dependent phosphatase are stimulated by pNPP, the five different PCS phosphatases (PCS_{H1}, PCS_{H2}, PCS_M, PCS_L, and PCS_C) are inhibited by pNPP

Fig. 5. Synergistic effects of DI and pNPP

The DI-stimulated phosphatase was preincubated in the presence of the indicated concentrations of DI during 10 min at 30 °C and assayed for phosphorylase phosphatase in the presence (●) and in the absence (▲) of 2 mM-pNPP.

with very similar I₅₀ values (about 1 mM). From the DEAE-cellulose chromatography on, pNPP stimulation is only observed in those fractions that are also stimulated by the DI; only F_c^{*} is stimulated by pNPP but not by the DI. F_c^{*}, prepared by a method [17] based on the traditional ethanol procedure, from the glucogen pellet without any harsh treatment [6], or from the DI-stimulated phosphatase by limited trypsin digestion, all show the stimulation by pNPP. The DI-phosphatase complex and the DI-free phosphatase from dog liver [23] both show pNPP stimulation, and in no other fraction of the DEAE-cellulose chromatogram is pNPP stimulation observed.

From these results it can be concluded that all the active, and only these, forms of the ATP+Mg²⁺-dependent phosphatase are stimulated by 1 mM-pNPP. pNPP stimulation of phosphorylase phosphatase is therefore an easy criterion for identifying a phosphatase as an active ATP+Mg²⁺-dependent phosphatase. pNPP and DI act in different ways since (1) the effects of both factors are synergistic (Fig. 5), resulting in an up to 15-fold stimulation of the DI-stimulated phosphatase, (2) pNPP does not influence the sensitivity to the heat-stable inhibitors, and (3), in contrast with pNPP, the DI does not stimulate F_c^{*} but only the 75 kDa+37 kDa holo-enzyme; in addition, it neutralizes the inhibition by M and I-1 [6].

CONCLUSION

We described a new spontaneously active form of the ATP+Mg²⁺-dependent phosphatase, whose phosphorylase phosphatase activity is largely suppressed by a regulatory subunit and which is relatively insensitive to inhibition by I-1 and M. This subunit is different from M, I-1 and the G subunit. Whether and how this enzyme can exchange its catalytic subunit with other pools of the ATP+Mg²⁺ dependent phosphatase require further investigation.

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