REGULATION OF LIVER PHOSPHORYLASE PHOSPHATASE

ATP-Mg-mediated activation of the partially purified dog-liver enzyme

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Received 22 June 1980

1. Introduction

An ATP-Mg-dependent phosphorylase phosphatase has been described in bovine adrenal cortex [1], dog liver [2], pigeon skeletal muscle [3] and *Neurospora crassa* [4]. The ATP-Mg-dependent phosphatase present in dog liver cytosol constitutes an important fraction of the total phosphorylase phosphatase activity [5]. Its activity is controlled by heat-stable protein inhibitors, one of which is activated by cyclic AMP-dependent protein kinase [6]. The activation of the liver enzyme results from the interaction of two protein fractions (F_A,F_C) which can be separated by DEAE-cellulose chromatography [7]. These observations were extended to other tissues and animals and a similar resolution of the ATP-Mg-dependent phosphatase into a two protein component system has been observed in liver, heart- and skeletal-muscle from rat and rabbit [8]. We now report the partial purification of F_C as the ATP-Mg-dependent phosphorylase phosphatase from dog liver and describe some characteristics of the activation reaction.

2. Materials and methods

Phosphorylase phosphatase was assayed as in [8]. Prior to assay of the ATP-Mg-dependent phosphatase the enzyme preparation was preincubated for 15 min in the presence or absence of saturating concentrations of F_A in 20 mM Tris–HCl (pH 7.4), 1 mM dithiothreitol and 1.5 mg/ml bovine serum albumin with or without 0.2 mM ATP and 1 mM $MgSO_4$, as indicated. To follow the time course of activation of the phosphatase, a 2 min phosphatase assay was used.

A partially purified preparation of ATP-Mg-dependent phosphorylase phosphatase was obtained from dog liver cytosol as in [7]. The peak fractions of the Sephadex G-200 column eluate were pooled and applied to a second DEAE-cellulose column (1.5 × 13 cm) equilibrated with 20 mM Tris–HCl (pH 7.8) and 1 mM dithiothreitol. After washing with 30 ml buffer, the proteins were eluted with 400 ml of a linear 0–0.4 M NaCl gradient in the same buffer. F_C eluted in a single activity peak, and the fractions containing the ATP-Mg-dependent phosphatase were pooled, dialyzed overnight against 20 mM Tris–HCl (pH 7.8) and 1 mM dithiothreitol, and applied to a histone–Sepharose-4B column (1 × 3 cm) equilibrated in 20 mM Tris–HCl (pH 7.8) and 1 mM dithiothreitol. After washing with 20 ml of the same buffer the ATP-Mg-dependent phosphatase was eluted in a single symmetrical peak, with 60 ml of a linear 0–0.4 M NaCl gradient in the same buffer. The pooled fractions were concentrated over a small DEAE-cellulose column (1 × 1 cm) and eluted with 0.3 M NaCl in 20 mM Tris–HCl (pH 7.8) and 1 mM dithiothreitol). The pooled phosphatase fractions were dialyzed overnight against 60% (v/v) glycerol in 20 mM Tris–HCl (pH 7.4) and 1 mM dithiothreitol. Although a substantial increase in specific activity of the ATP-Mg-dependent phosphatase was obtained by this histone–Sepharose purification step, yields were usually low and quite variable (<30% recovery from the previous step).

The catalytic subunit of the cyclic AMP-dependent

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protein kinase was isolated following [9] as modified for rabbit skeletal muscle [10]. It was assayed by following the incorporation of [^{32}P]phosphate from [γ-^{32}P]ATP into histone H2B (1.0 mg/ml) in a 0.06 ml assay containing 20 mM Tris–HCl (pH 7.4), 1.0 mM dithiothreitol, 1.0 mM [γ-^{32}P]ATP and 5.0 mM $MgSO_4$. One unit was that amount which incorporated 1.0 nmol phosphate/min at 30°C. F_A was prepared as in [7]. The specific heat stable inhibitor of cyclic AMP-dependent protein kinase was prepared according to [11], up to the DEAE-cellulose chromatography step. This preparation (0.35 mg protein/ml) was free of phosphatase inhibitors. Histone–Sepharose 4B was prepared according to [12].

Calmodulin prepared from bovine brain with the method using organic solvents [13] was kindly provided by Dr R. J. A. Grand (University of Birmingham), f_{2b} (H2B) histones were obtained from Sigma Chemical Co. (USA); lyophylised trypsin (200 U/mg) from Worthington (England); CNBr-activated Sepharose from Pharmacia (Sweden); DEAE (DE 52)-cellulose from Whatman (England). Other techniques, as well as the source of chemicals have been described [8].

3. Results

The purification of the ATP-Mg-dependent phosphatase (F_C) is summarized in table 1. Throughout the preparation the enzyme activity was measured as spontaneously active or after activation in the presence of 0.2 mM ATP, 1 mM $MgSO_4$ and a saturating concentration of F_A. It is difficult, however, to evaluate recoveries and specific activities during the initial purification steps. The choice of assay conditions is made extremely difficult in view of the existence of different forms of the phosphatase, characterised by different degrees of stimulation or inhibition by either low molecular weight ligands such as nucleotides and divalent ions [5], or heat-stable proteins such as the protein inhibitors-1, -2α and -2β [6] and the protein deinhibitor [14]. The crude extracts and high speed supernatant fractions were filtered over a small Sephadex G-25 column before assay to avoid interference of low molecular weight ligands.

The characteristics of the phosphatase resulting from the interaction of these two protein fractions in the presence of ATP-Mg cannot be distinguished from those ascribed to the ATP-Mg-dependent

Table 1
Purification of the ATP-Mg-dependent phosphorylase phosphatase from dog liver[a]

Purification step	Total protein (mg)	Phosphorylase phosphatase activity		
		Without ATP-Mg	With ATP-Mg	
		Total act. (units)	Total act. (units)	Spec. act. (units/mg)
Crude extract	36 400	4925[b]	11 600[b]	–
High speed supernatant	25 200	2152[b]	7010[b]	–
30–50% $(NH_4)_2SO_4$ fraction	4790	1458[b]	3735[b]	–
First DEAE-cellulose eluate	440	340	2769	6.3
Sephadex G-200 eluate	72	183	2291	31.8
Second DEAE-cellulose eluate	17.8	83	2006	112.7
Histone–Sepharose 4B eluate	1.59	n.d.	441	277.1

[a] The amount of liver used was 350 g. The values are calculated for the total amount of liver tissue used

[b] Subject to over- or under-estimation (see section 3)

n.d., not detectable

phosphorylase phosphatase [5,6]: in both cases the K_a for ATP in the presence of 0.1 mM $MgSO_4$ was 0.3 μM, the activation was time-dependent and reversible upon gel filtration and the phosphatase was equally sensitive to the protein inhibitors-1, -2α and -2β. Both F_A and the purified ATP-Mg-dependent phosphatase were thermolabile and non-dialysable and were inactivated by urea (6 M), ethanol (5 vol. 95% ethanol at room temp.) and trypsin (0.7 mg/ml for 15 min at 30°C). The M_r 138 000 as determined by gel filtration for the purified ATP-Mg-dependent phosphatase (F_C) was identical to the molecular weight attributed to the enzyme in the more crude preparations [5].

Fig.1 shows the amount of active phosphatase formed as a function of time during preincubation of one concentration of F_A with different amounts of purified F_C. When care was taken to choose the F_A concentration sufficiently high, different plateaus of phosphatase activity were obtained, and the final activity level reached was proportional to the amount of purified F_C used. On the other hand, when a constant amount of F_C was preincubated with different quantities of F_A the resulting phosphatase activity was not proportional to the amount of F_A used (fig.2). At low concentrations of F_A, the time-dependent phosphatase formation leveled off at intermediate values, but from a certain F_A concentration on upwards, there was a maximal phosphatase activity that could be produced. These results indicate that the potential phosphatase activity is localized in the F_C fraction, and that F_A contains a factor which activates the phosphatase. We therefore refer to the purified F_C fraction as the ATP-Mg-dependent phosphatase. The production of phosphatase activity was dependent upon the period of time that the ATP-Mg-dependent phosphatase, F_A and ATP-Mg were all incubated together; a preincubation of any 2 of the 3 factors, did not abolish the time dependency of the phosphatase activation upon addition of the third (not shown).

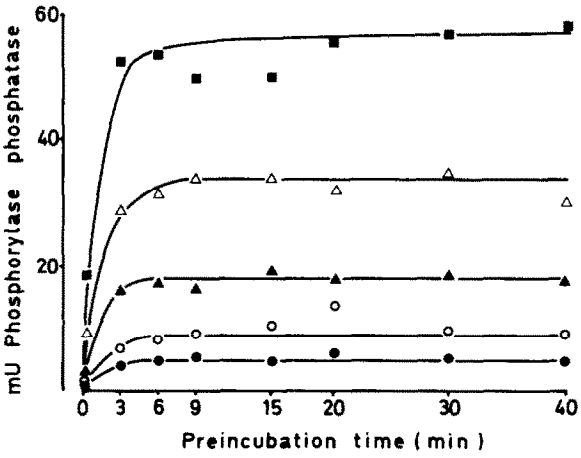

Fig.1. Time-dependent activations of the ATP-Mg-dependent phosphatase using saturating concentrations of F_A and different amounts of F_C. The total preincubation mixture was 240 μl containing 12 μg F_A, and, respectively: 0.32 μg (•), 0.5 μg (○), 1 μg (▲), 2 μg (△), and 4 μg (■) of F_C (second DEAE-cellulose eluate). At various time intervals, a 20 μl aliquot was assayed for phosphatase activity. Activities are expressed as mU phosphatase produced in this 20 μl aliquot.

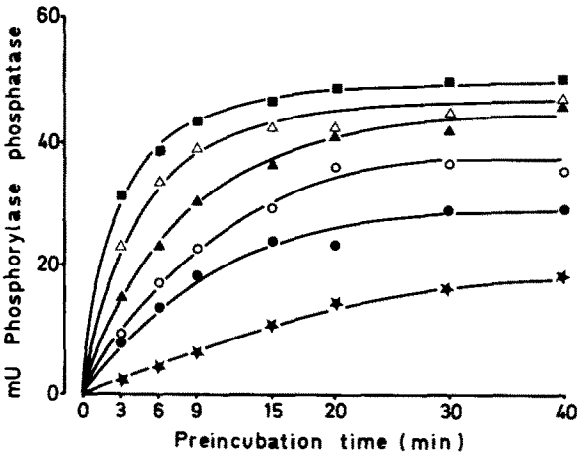

Fig.2. Time-dependent activations of the ATP-Mg-dependent phosphatase using a constant amount of a purified F_C preparation, and different amounts of F_A. Phosphatase activity was assayed after preincubation as in fig.1, except that in the 240 μl preincubation mixture, 5 μg F_C and, respectively: 0.7 μg (⋆), 2.4 μg (•), 3 μg (○), 6 μg (▲), 12 μg (△), and 24 μg (■) of F_A were used.

When high concentrations of the purified catalytic subunit of cyclic-AMP-dependent protein kinase were preincubated, under identical conditions, with the ATP-Mg-dependent phosphatase and ATP-Mg, a similar phosphatase activation was achieved as when an excess of F_A was used (fig.3). Heat-stable protein kinase inhibitor (added before or after the activation reaction) caused a drastic reduction in the resulting phosphatase activity level when the cyclic AMP-dependent protein kinase was used, but had no effect on the activation or the final activity level produced by F_A (fig.3). The effect of the heat-stable protein

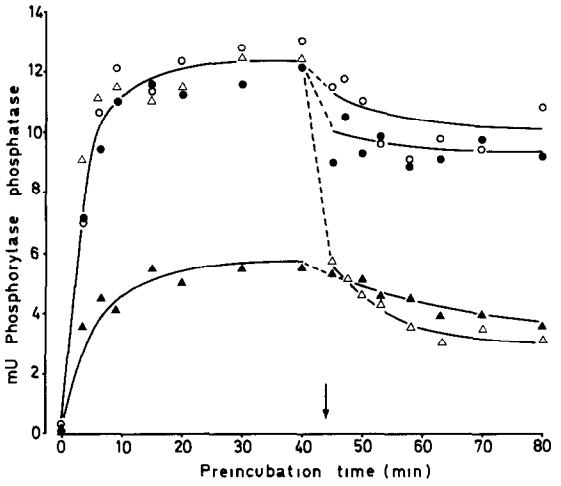

Fig.3. Effect of the heat-stable protein kinase inhibitor on the activation of the ATP-Mg-dependent phosphatase by F_A and by the catalytic subunit of cyclic AMP-dependent protein kinase. The 240 μl preincubation mixture contained 3 μg ATP-Mg-dependent phosphatase (second DEAE-cellulose eluate) and either 28 μg F_A (○,●) or 4 units catalytic subunit of cyclic AMP-dependent protein kinase (△,▲) in the presence (●,▲) or absence (○,△) 10 μg protein kinase inhibitor. After 40 min preincubation (arrow) 7 μg (20 μl) protein kinase inhibitor (○,△) or the same volume of 20 mM Tris–HCl buffer (pH 7.4) (●,▲) are added to the 170 μl remaining preincubation mixture. At regular time intervals, 10 μl aliquots were removed for the determination of phosphatase activity. The activities are expressed as mU phosphatase produced in this 10 μl aliquot.

kinase inhibitor was proportional to the concentration used (not shown).

Phosphorylase *b* kinase does not seem to be implicated in the activation of the ATP-Mg-dependent phosphatase by F_A since this activation was not affected by 0.2 mM EGTA or 20 μg/ml calmodulin in the presence or absence of 0.1 mM Ca^{2+} (not shown). Neither did F_A contain any detectable phosphorylase *b* kinase activity towards purified rabbit skeletal muscle phosphorylase *b*, and homogeneously purified rabbit muscle phosphorylase *b* kinase [15,16] or the partially purified rat liver enzyme [10] could not mimic the F_A activity.

4. Discussion

The ATP-Mg-dependent phosphatase activity, present in dog liver was found to result from the interaction of at least two protein factors, F_A and F_C in the presence of ATP-Mg. The catalytic activity was shown to reside in the F_C protein. It was extensively purified in an inactive form which, at all stages of purification, could be activated by a preincubation with F_A and ATP-Mg. Since the catalytic subunit of cyclic AMP-dependent protein kinase can activate F_C, and the F_A preparations contain a cyclic AMP- and Ca^{2+}-independent glycogen synthase kinase activity [17], it is possible that the activation of F_C involves its phosphorylation. However, no [^{32}P]phosphate could be incorporated into the partially purified F_C preparation when it was incubated with either F_A or the catalytic subunit of cyclic AMP-dependent protein kinase.

Although a pure preparation of F_C is clearly required to answer definitively the question of whether phosphorylation is involved in its activation, the easy reversibility of activation upon removal of the ATP-Mg [18] has made us hypothesize before that the appearance of phosphatase activity is the result of a ligand–enzyme interaction. It remains possible that the ATP-Mg-dependent phosphatase becomes activated by a protein–protein interaction with a kinase in the presence of ATP-Mg without phosphorylation. With the cyclic AMP-dependent protein kinase, the heat-stable inhibitor not only prevents, but also abolishes the phosphatase activation. Thus, the reversibility of the activation by cyclic AMP-dependent protein kinase can be explained in two ways:

(1) A phosphorylation–dephosphorylation reaction (possibly an autocatalytic dephosphorylation) could be invoked, so that with a certain amount of kinase activity present, the activation will take place until an equilibrium is reached between the kinase and the phosphatase activity.

This would explain the effect of the heat-stable inhibitor when it is added after the activation has reached a plateau: it brings the phosphatase activity level down to a different plateau, determined by the residual amount of active kinase.

(2) The heat-stable protein kinase inhibitor may disrupt a protein–protein interaction, between the catalytic subunit of the cyclic AMP-dependent protein kinase and the ATP-Mg-dependent phosphatase.

Although high concentrations of cyclic AMP-dependent protein kinase are able to mimic the F_A-mediated activation of the phosphatase, it is not likely

that this has any physiological significance, since crude preparations of dog liver, which contain ATP-Mg-dependent phosphatase, F_A and the cyclic AMP-dependent protein kinase, have been shown to produce ATP-Mg-dependent phosphatase activations which were independent of cyclic AMP, and unaffected by the heat stable protein kinase inhibitor [6]. This would suggest that in those preparations, F_A activity greatly exceeds that of the endogenous cyclic AMP-dependent protein kinase.

Acknowledgements

The authors are grateful to R. Verbiest and R. Bollen for expert technical assistance. This work was supported by the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek. J. R. V. is Bevoegdverklaard Navorser van het NFWO.

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