Regulatory subunit of type II cAMP-dependent protein kinase as substrate and inhibitor of protein phosphatase-1 and -2A

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The dissociated regulatory subunit (R^{II}) of autophosphorylated cAMP-dependent protein kinase II was dephosphorylated by the catalytic subunits of protein phosphatase-1 and -2A (phosphatase-1_c and -$2A_c$) and by a high-M_r polycation-dependent form of phosphatase-2A ($2A_o$) with K_m values of 5, 0.3 and 1 μM, respectively. Dissociation of protein kinase by cAMP preferentially increased the dephosphorylation of R^{II} by phosphatase-1_c, whereas polycations (histone HI or polybrene) markedly stimulated phosphatase-$2A_c$ and -$2A_o$ even in the absence of cAMP. Thiophosphorylated R^{II} inhibited the dephosphorylation of phosphorylase *a* by these phosphatases with half-maximum inhibitory concentrations of 0.1–0.36 μM.

cyclic AMP dependence *Protein kinase* *Phosphorylase* a *Protein phosphatase* *Polybrene*

1. INTRODUCTION

Autophosphorylation of the cAMP-dependent protein kinase II and its physiological role have been intensively studied [1,2]. There are also data about its dephosphorylation [3–6], however, the role and regulation of some protein phosphatases in this process are not clear yet. Many proteins phosphorylated through cAMP-mediated processes can be dephosphorylated by two types of protein phosphatases designated phosphatase-1 and phosphatase-2A [7]. Phosphatase-1 is inhibited by heat-stable inhibitors [8] or heparin [9], whereas phosphatase-2A is insensitive to these inhibitors.

Here we studied the dephosphorylation of the regulatory subunit (R^{II}) of cAMP-dependent protein kinase by the catalytic subunits of protein phosphatase-1 and -2A (phosphatase-1_c and phosphatase-$2A_c$) and by a high-M_r polycation-activated form of phosphatase-2A (phosphatase-$2A_o$) [10]. The effect of cAMP, polycations and Mn^{2+} on the dephosphorylation reaction is also presented. In addition, thiophosphorylated R^{II} was found to be a potent inhibitor of all phosphatases investigated, using phosphorylase *a* as substrate, suggesting that autophosphorylation of protein kinase II is an important factor in the regulation of various phosphatases.

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2. MATERIALS AND METHODS

2.1. *Materials*

Histone H1 and cAMP were purchased from Sigma, heparin-Sepharose CL-6B from Pharmacia, adenosine-5′-*O*-(3-thiotriphosphate) (ATP-γ-S) and polybrene from Serva, cAMP-Sepharose from PL-Biochemicals and [8-^{3}H]cAMP from Amersham. [γ-^{32}P]ATP was prepared as in [11]. All chemicals used were of reagent grade.

2.2. *Preparation of enzymes*

The catalytic subunit (C) [12] and R^{II} subunit of cAMP-dependent protein kinase II were prepared

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from bovine heart [13], with the following modifications: elution of R^{II} from cAMP-Sepharose was performed with 7 M urea [14] and the holoenzyme was dephosphorylated with 10 μg/ml protein phosphatase-$2A_c$ in the presence of 1 mM $MnCl_2$, 50 μg/ml polybrene and 20 mM benzamidine before chromatography on cAMP-Sepharose. The protein kinase II holoenzyme was reconstituted in the dephospho form and autophosphorylated with either [γ-^{32}P]ATP [2], resulting in a specific activity of 2000–3000 cpm/pmol R^{II}, or ATP-γ-S. Rabbit skeletal muscle phosphorylase *a* was purified as in [15]. Protein phosphatase-1_c and -$2A_c$ were isolated from rabbit skeletal muscle [9]. Protein phosphatase-$2A_o$ from rabbit liver [10] was further purified to apparent homogeneity, as will be published elsewhere.

2.3. *Dephosphorylation reactions*

The autophosphorylated protein kinase II holoenzyme (0.1–5 μM) or phosphorylase *a* (120–240 μg/ml) was incubated with various phosphatases at 30°C (5–20 min) in 50 μl reaction mixture containing 50 mM Tris-HCl (pH 7.0), 5 mM dithiothreitol and 2 mg/ml bovine serum albumin. Additives (histone H1, polybrene, cAMP or $MnCl_2$) were preincubated with protein kinase for 5 min. Phosphorylase *a* was dephosphorylated in the presence of 2 mM caffeine. Reactions were started by the addition of phosphatase, diluted so that no more than 30–40% of the substrate was converted. ^{32}P release was determined from the supernatant after precipitation of proteins by 20% trichloroacetic acid. Dephosphorylation of phosphorylase *a* was followed by phosphorylase *a* activity measurements as in [16].

3. RESULTS

3.1. *Effect of cAMP, polycations and Mn^{2+} on the dephosphorylation of protein kinase II*

Table 1 shows the relative rates of dephosphorylation of ^{32}P-protein kinase II by phosphatase-1_c and -$2A_c$ in the presence of various effectors. Under the conditions used the holoenzyme proved to be a poor substrate of phosphatases. Addition of cAMP greatly enhanced the activity of phosphatase-1_c but caused only a 2-fold increase in the activity of phosphatase-$2A_c$. In the presence of polybrene (a synthetic polycation) or histone H1

Table 1

Effects of cAMP, polycations and Mn^{2+} on the dephosphorylation of protein kinase II (holoenzyme)

Effector	Activity (%)	
	Phosphatase-1_c	Phosphatase-$2A_c$
None	8	11
cAMP	86	22
Histone Hl	24	42
Polybrene	26	46
cAMP + polybrene	100	100
cAMP + Mn^{2+}	75	237
cAMP + Mn^{2+} + polybrene	100	380

Concentration of ^{32}P-protein kinase was 1 μM, cAMP 2.5 × 10^{-5} M, histone Hl 100 μg/ml, polybrene 50 μg/ml and Mn^{2+} 1 mM. Details of dephosphorylation are described in section 2. Phosphatase activities measured in the presence of cAMP + polybrene were taken as 100%. The means of 3 experiments are shown

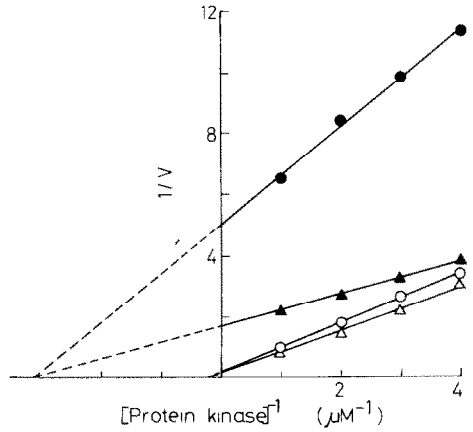

Fig.1. Effect of substrate concentration on the activity of phosphatase-1_c and -$2A_c$ in the presence or absence of polybrene. ^{32}P-protein kinase II was dissociated by 2.5 × 10^{-5} M cAMP. The dephosphorylation was catalyzed by phosphatase-1_c (open symbols) or $2A_c$ (closed symbols) in the absence (○,●) and presence of 50 μg/ml polybrene (△,▲) as described in section 2. Initial velocities of ^{32}P release were expressed in arbitrary units.

both phosphatase-1_c and -$2A_c$ became partially active towards the holoenzyme. However, the activation of phosphatase-$2A_c$ by polybrene was more pronounced, especially in the presence of cAMP. Mn^{2+} had a further activating effect on phosphatase-$2A_c$, which was potentiated by polybrene.

Analysing the effect of substrate concentration on the phosphatase activities (fig.1) different K_m values were obtained in the presence of cAMP for phosphatase-1_c and -$2A_c$ (5 and 0.3 μM, respectively). Also, as seen in fig.1, the stimulation of phosphatase-$2A_c$ by polybrene is due to an increase in V_{max}.

According to fig.2, phosphatase-$2A_o$ proved to be totally polycation-dependent in dephosphorylating ^{32}P-protein kinase II in either the presence or absence of cAMP. Polybrene was a more potent activator than histone H1. The K_m value for protein kinase in the presence of 50 μg/ml polybrene and 2.5×10^{-5} M cAMP was 1 μM (not shown).

3.2. *Effect of protein kinase II on the dephosphorylation of phosphorylase a*

In preliminary experiments autophosphorylated protein kinase was found to inhibit phosphorylase phosphatase activity of phosphatase-1_c. This inhibition became 5-fold greater after its dissociation by cAMP (not shown). The increased inhibition was attributed to the dissociated P-R^{II}, since neither cAMP nor the isolated C subunit of protein kinase had any inhibitory effect. To complete these experiments, the susceptibility of phosphatase-$2A_c$ and -$2A_o$ to the inhibition by P-R^{II} was also investigated. Thiophosphorylated protein kinase was used to avoid dephosphorylation of the P-R^{II} subunit (fig.3).

As seen in fig.3, dissociated R^{II} in the dephospho form moderately inhibited phosphatase-1_c, but thiophosphorylated R^{II} was a potent inhibitor of all phosphatases tested, with half-maximum inhibitory concentrations of 0.1–0.36 μM. Polybrene was necessary to test phosphatase-$2A_o$ but it did not influence the results with phosphatase-1_c or -$2A_c$ (not shown).

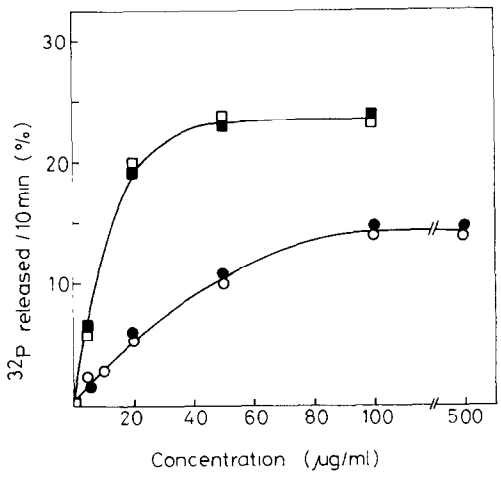

Fig.2. Polycation dependency of phosphatase-$2A_o$ using ^{32}P-protein kinase as substrate. Reactions were run as described in section 2 in the presence of 0.08 μM ^{32}P-protein kinase and phosphatase-$2A_o$. Histone HI (○,●) or polybrene (□,■) was added without (open symbols) or with 2.5×10^{-5} M cAMP (closed symbols). ^{32}P released in 10 min was expressed as % of total.

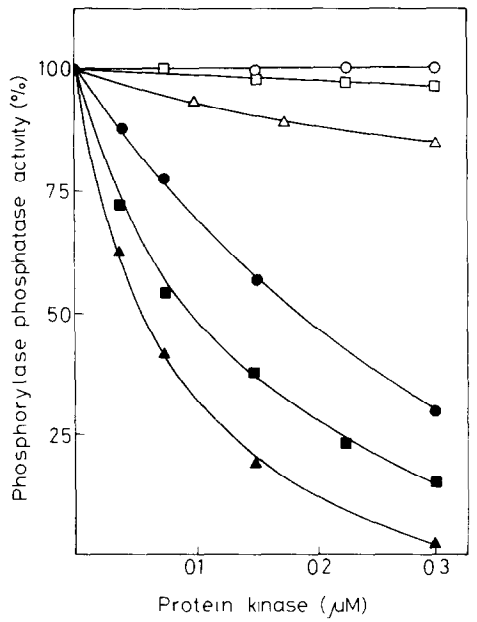

Fig.3. Effect of dissociated dephospho and thiophosphorylated protein kinase II on phosphorylase phosphatase activity of various protein phosphatases. Dephosphorylation of 120 μg/ml phosphorylase *a* was carried out as described in section 2 with phosphatase-1_c (△,▲); phosphatase-$2A_c$ (□,■); or phosphatase-$2A_o$ (○,●) in the presence of dephospho protein kinase (open symbols), or thiophosphorylated protein kinase (closed symbols). Reaction mixtures contained 2.5×10^{-5} M cAMP and in experiments with protein kinase-$2A_o$ also 50 μg/ml polybrene. Phosphatase activities without protein kinase were taken as 100%.

4. DISCUSSION

Previous observations, using various protein phosphatases [3-6], demonstrated the cAMP-dependency of the dephosphorylation of auto-phosphorylated protein kinase. This was proved for phosphatase-1_c, whereas the phosphatase-2A catalyzed dephosphorylation was stimulated by polycations either in the presence or absence of cAMP, offering another regulatory device. The K_m value of phosphatase-2A for R^{II} is close to the concentrations of R^{II} in the heart [17], raising the possibility of its in vivo effectiveness.

The inhibition of phosphorylase phosphatase activity by R^I was described by Gergely and Bot in 1977 [16]. Recently Khatra et al. [18] have shown the inhibitory effect of R^{II} on a high-M_r form of phosphatase-1 which was increased upon thiophosphorylation of R^{II}. According to our results thiophosphorylated R^{II} inhibits not only phosphatase-1_c but also type 2A phosphatases well within the intracellular concentration of protein kinase II. This finding further suggests that the phosphorylation state of R^{II} can play a role in the regulation of type 1 and 2A phosphatases, switching out dephosphorylating processes simultaneously with the cAMP-induced phosphorylations, preventing futile cycles.

The present results show similarities between R^{II} and the heat-stable inhibitor-1 of phosphatase-1, including their increasing inhibitory potency upon phosphorylation and preferential dephosphorylation by phosphatase-2A (figs 1,3 and [7]). However, after being heated at 90°C (5 min) R^{II} or phosphorylated R^{II} lost the ability of inhibiting phosphatase-1_c (not shown). A detailed investigation of the mechanism of inhibition exerted by R^{II} on phosphatase-1 and -2A is in progress.

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