THE CONTROL OF PHOSPHORYLASE PHOSPHATASE BY cAMP-DEPENDENT PROTEIN KINASE

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1. Introduction

The enzymes of glycogen metabolism are regulated by interconversion between phosphorylated and dephosphorylated forms. Contrary to our well-defined knowledge of the phosphorylation processes of different enzymes and their role in glycogen breakdown and synthesis, the phosphatases catalyzing the reverse processes are only now being understood. The regulation of dephosphorylation reactions is still unclear.

Phosphorylase phosphatase converts 'active' phosphorylase *a* (EC 2.4.1.1) into 'inactive' phosphorylase *b* by cleavage of the phosphate groups from the serine residues. For the control of phosphorylase phosphatase the following mechanisms are possible:

(i) By ligands which affect the substrate phosphorylase *a* (or phosphatase).
(ii) By proteins which affect phosphorylase *a* (or phosphatase).
(iii) By enzymatic modification of phosphatase.

It was reasoned that ligands (e.g. glucose, glucose 6-phosphate, glycogen, AMP and caffeine) influence phosphorylase phosphatase reaction by changing the conformation of the substrate phosphorylase *a* or by shifting the tetramer $\rightleftharpoons$ dimer equilibrium of phosphorylase *a* [1–8].

It is known that different proteins can mediate the phosphorylase phosphatase reaction. We have shown that phosphorylase kinase inhibits the dephosphorylation of phosphorylase *a* and the inhibition is competitive in nature [9]. The phosphorylation (or thiophosphorylation) of phosphorylase kinase increases its inhibitor constant [10].

Another protein isolated from different mammalian tissues which inhibits the phosphatase reaction is heat-stable [11,12]. This heat-stable inhibitor protein is phosphorylated by cAMP-dependent protein kinase [13]. Recently, Tóth et al. demonstrated the reversible phosphorylation and dephosphorylation of this heat-stable inhibitor protein in vivo [14]. Huang and Glinsmann demonstrated the existence of two heat-stable inhibitor proteins for phosphatase [15,16].

Enzymic chemical modification of phosphorylase phosphatase has not been established as a means of regulation [17].

In the present communication the effect of cAMP-dependent protein kinase on the phosphatase reaction (i.e., on the dephosphorylation of phosphorylase *a*) has been investigated. It is demonstrated that protein kinase in the presence of cAMP inhibits the dephosphorylation of the phosphorylase *a*. The experiments show that the inhibitory effect is due to the regulatory subunit. Since the regulatory subunit loses its ability to inhibit the phosphatase reaction on heat-treatment it is distinct from the heat-stable inhibitors isolated by Huang and Glinsmann [15,16] and Cohen et al. [13].

2. Methods

Rabbit skeletal muscle phosphorylase *a* was prepared as previously described [8]. Phosphorylase activity was assayed by the procedure of Illingworth and Cori [18]. Specific activity of phosphorylase *a* was 54 units.mg^{-1} in the presence of 0.016 M glucose 1-phosphate and in the absence of AMP.

Phosphorylase phosphatase was prepared according to the method of Brandt et al. [19]. Phosphorylase

phosphatase assay was carried out as described previously [8].

cAMP-dependent protein kinase was prepared from rabbit skeletal muscle according to Beavo et al. [20]. The separation of regulatory and catalytic subunits of protein kinase was carried out on a blue-dextran–Sepharose column [21]. Enzymic activity of protein kinase was determined by the method of Hofmann et al. [22]. The isolated protein kinase holoenzyme, the separated regulatory and catalytic subunits were homogenous by 0.1% SDS–7.5% polyacrylamide gel electrophoresis. The gel electrophoresis experiments were carried out in the manner described by Weber and Osborn [23]. The preparations (phosphorylase *a*, phosphorylase phosphatase, cAMP-dependent protein kinase and its regulatory subunit) were free of phosphorylase kinase and did not contain heat-stable inhibitors.

The [^{32}P]tetradecapeptide incorporating the phosphorylated site was isolated after chymotryptic digestion of [^{32}P]phosphorylase *a* by the method of Nolan et al. [24]. Dephosphorylation of the [^{32}P]tetradecapeptide by phosphorylase phosphatase was measured at 30°C in 0.04 M Tris/0.002 M EDTA/0.0005 M dithiothreitol buffer, pH 7.4 [5].

Protein was determined by the modification of the biuret procedure [25].

Inhibition of phosphorylase phosphatase reaction by cAMP-dependent protein kinase was assayed by the following manner: 1.0 mg/ml phosphorylase *a* was incubated in 0.04 M Tris/0.002 M EDTA/0.0005 M dithiothreitol buffer (pH 7.4) with phosphorylase phosphatase in the presence of various concentrations of protein kinase or regulatory/catalytic subunit. The reaction mixtures were incubated at 30°C, aliquots (50 μl) were removed at different points of incubation and the reaction was stopped by the addition of 0.1 M NaF/0.04 M glycerophosphate/0.002 M EDTA, pH 6.8, to attain a dilution in which the activity of residual phosphorylase *a* could still be measured.

3. Results and discussion

The effect of cAMP-dependent protein kinase on the dephosphorylation of phosphorylase *a* by phosphatase is shown in fig.1. It is seen that protein kinase in the absence of cAMP does not affect the rate of the dephosphorylation process. However, protein kinase in the presence of cAMP strongly inhibits the dephosphorylation of phosphorylase *a*. The greater the concentration of protein kinase in the presence of cAMP the greater is the inhibitory effect on the phosphatase reaction.

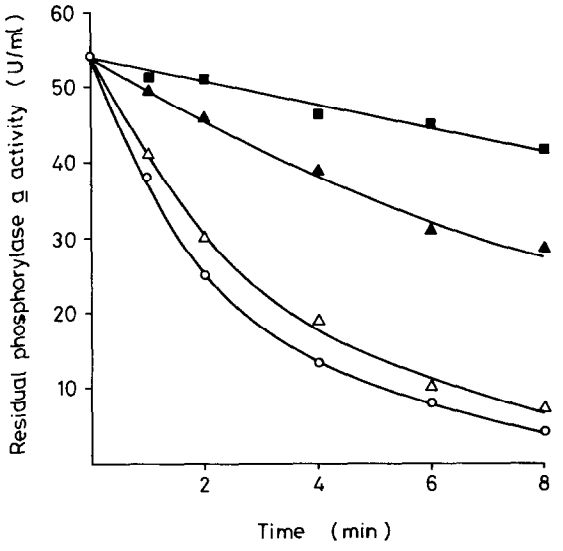

Fig.1. Effect of cAMP-dependent protein kinase on the dephosphorylation of phosphorylase *a*. Experimental conditions are described in Methods. Activity of residual phosphorylase *a* in the absence of protein kinase (○——○), in the presence of 0.65 mg/ml protein kinase (△——△), 0.65 mg/ml protein kinase + 5.10^{-6} M cAMP (▲——▲) and 1.1 mg/ml protein kinase + 5.10^{-6} M cAMP (■——■).

It is known that cAMP dissociates the protein kinase holoenzyme into regulatory and catalytic subunits. The inhibition of phosphatase reaction observed only in the presence of cAMP indicates that the inhibition could be due to the free subunits and not to the holoenzyme. (cAMP, alone does not effect the rate of phosphorylase *a* dephosphorylation, not documented.) Therefore the inhibitory effectiveness of isolated regulatory and catalytic subunits were also investigated (fig.2).

It can be seen that the presence of catalytic subunit of cAMP-dependent protein kinase does not influence the dephosphorylation of phosphorylase *a*. Whereas the presence of regulatory subunit considerably moderates the dephosphorylation reaction. Increasing the concentration of regulatory subunit increases the

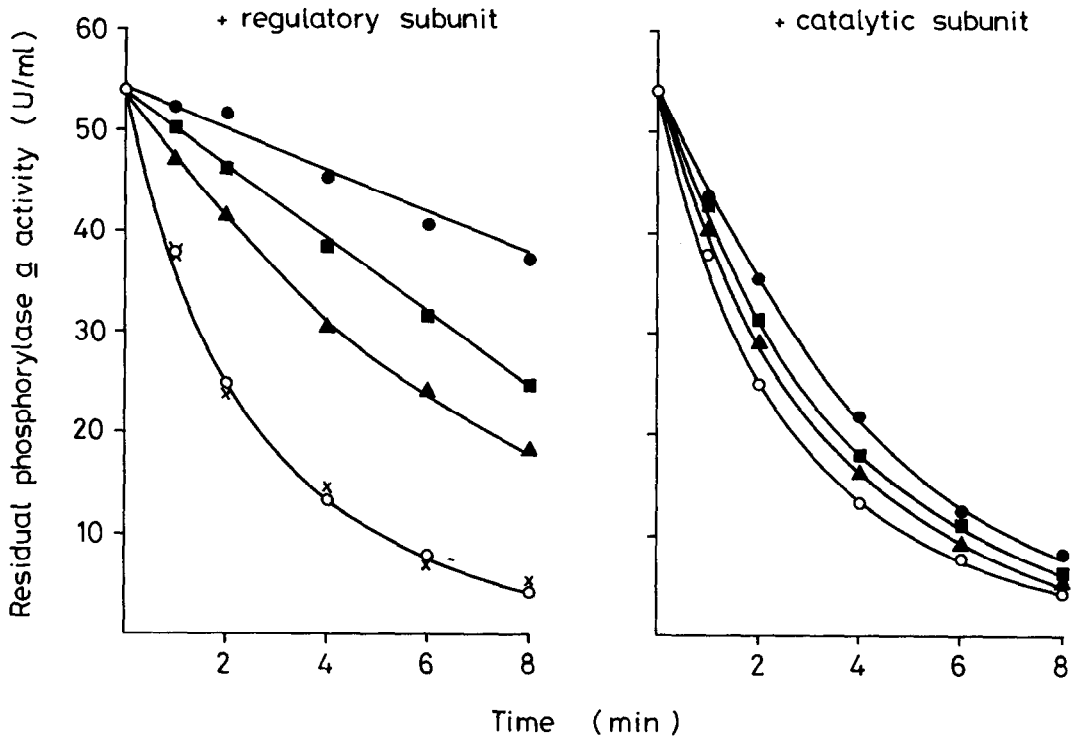

Fig.2. Dephosphorylation of phosphorylase *a* in the presence of regulatory and catalytic subunits. Experimental conditions are described in Methods. Activity of residual phosphorylase *a* in the absence (o——o) and in the presence of 0.23 mg/ml (▲——▲), 0.46 mg/ml (■——■), 0.7 mg/ml (●——●) regulatory or catalytic subunits, respectively. Activity of phosphorylase *a* in the presence of heat-treated regulatory subunit (x——x). The heat-treatment was carried out according to Brandt et al. [12] at 90°C for 5 min and the protein concentration after this procedure was 0.37 mg/ml.

inhibition. The data presented in fig.1 and 2 demonstrate that only the regulatory subunit is a potent inhibitor in the dephosphorylation process of phosphorylase *a*, the catalytic subunit and the protein kinase holoenzyme have no inhibitory function. The regulatory subunit lost its inhibitory effectiveness after heat-treatment (fig.2). This observation suggests that the inhibition caused by the regulatory subunit is distinct form the different heat-stable inhibitors.

The inhibition of phosphorylase phosphatase reaction by the regulatory subunit can be explained by two different mechanisms. On the one hand, the regulatory subunit can modify the conformation of the substrate (phosphorylase *a*). On the other hand, it can influence the enzyme (phosphorylase phosphatase) itself. To distinguish these two supposed mechanisms [^{32}P]tetradecapeptide was used as a substrate of the phosphatase reaction. Phosphorylase phosphatase can dephosphorylate the [^{32}P]tetradecapeptide but this peptide does not show conformational change in contrast to phosphorylase *a*.

Figure 3 demonstrates the dephosphorylation of [^{32}P]tetradecapeptide by phosphatase. It is seen that neither protein kinase in the presence of cAMP nor the isolated regulatory subunit do alter the rate of the dephosphorylation. These results show that the inhibition of phosphatase reaction by the regulatory subunit is substrate directed.

Our observations point to a new feature of cAMP-dependent protein kinase, i.e., not only its catalytic subunit has an important control role in the glycogen metabolism but its regulatory subunit, too. The results of this paper provide evidence for the regulation of phosphorylase *a*–*b* interconversion by the regulatory subunit of the cAMP-dependent protein kinase.

In light of experimental data presented here the

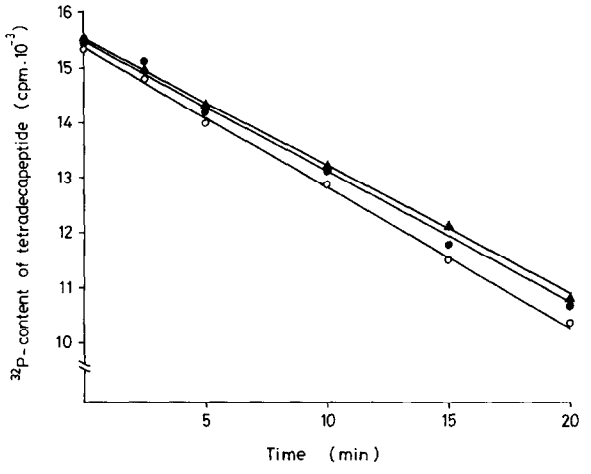

Fig.3. Effect of protein kinase and regulatory subunit on the dephosphorylation of [^{32}P]tetradecapeptide by phosphorylase phosphatase. Conditions of the dephosphorylation process are described in Methods. Dephosphorylation of [^{32}P]tetradecapeptide by phosphorylase phosphatase in the absence (o———o) and in the presence of 1.2 mg/ml protein kinase + 5.10^{-6} M cAMP (•———•) and 1.1 mg/ml regulatory subunit (▲———▲).

events taking place during glycogen breakdown might be explained as follows. The phosphorylation enzyme cascade begins with the activation of cAMP-dependent protein kinase by cAMP. cAMP binds to the regulatory subunit resulting in the release of the active catalytic subunit. Catalytic subunit triggers a phosphorylation sequence resulting in the formation of the active phosphorylase *a* and the inactive glycogen synthase. During this time the regulatory subunit inhibits the dephosphorylation of phosphorylase *a* prolongating the glycogen breakdown. When the concentration of cAMP decreases the catalytic and regulatory subunits associate, thereby allowing the dephosphorylation of phosphorylase *a*.

Acknowledgement

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