Role of Phosphorylase Kinase and Cyclic AMP-dependent Protein Kinase in the Regulation of Phosphorylase Phosphatase

PÁL GERGELY and GYÖRGY BOT

Institute of Medical Chemistry, University of Medicine, H-4026 Debrecen, Bem tér 18/B, Hungary

The enzymes of glycogen metabolism are regulated by interconversion between phosphorylated and dephosphorylated forms. In contrast with our well-defined knowledge of the phosphorylation processes of different enzymes and their role in glycogen breakdown and synthesis, the phosphatases catalysing 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 (EC 3.1.3.17) some mechanisms are possible, namely ligands that affect the substrate phosphorylase \( a \) or phosphatase; proteins that affect phosphorylase \( a \) (or phosphatase); or enzymic modification of phosphatase.

It is known that different proteins can influence the phosphorylase phosphatase reaction. The activation of phosphorylase with synchronous inhibition of phosphorylase phosphatase was observed in the protein–glycogen complex isolated from rabbit skeletal muscle (Haschke et al., 1970). No clear explanation has been offered for the transient inhibition of phosphorylase phosphatase during the 'flash activation'. Haschke et al. (1972) attributed the inhibition to a protein–protein interaction caused by an unknown protein component of the complex. This assumption was supported by frontal gel filtration of muscle extract and purified enzymes showing a strong association between phosphorylase, phosphorylase kinase and phosphatase (Gergely et al., 1974, 1975).

A heat-stable protein isolated from different mammalian tissues also inhibits the phosphatase reaction (Brandt et al., 1974, 1975a). This heat-stable inhibitor protein is phosphorylated by cyclic AMP-dependent protein kinase (Cohen et al., 1977). Tóth et al. (1977) demonstrated the reversible phosphorylation and dephosphorylation of this heat-stable inhibitor protein in vivo. Huang & Glinsmann (1975, 1976a,b) demonstrated the existence of two heat-stable inhibitor proteins for phosphatase.

In the present study the effect of phosphorylase kinase and cyclic AMP-dependent protein kinase on the regulation of phosphorylase phosphatase has been investigated. Rabbit skeletal-muscle phosphorylase \( a \) was prepared and assayed as previously described (Bot et al., 1977). The specific activity of phosphorylase \( a \) was 55 units/mg in the presence of 0.016 M-glucose 1-phosphate and in the absence of AMP. Non-activated phosphorylase kinase was prepared from rabbit skeletal muscle, and its activity was measured, as described by Cohen (1973). Thiophosphate-activated phosphorylase kinase was prepared by the method of Gergely et al. (1976). Cyclic AMP-dependent protein kinase was prepared from rabbit skeletal muscle by the method of Beavo et al. (1974). The separation of regulatory and catalytic subunits of protein kinase was carried out on a Blue Dextran/ Sepharose column (Witt & Roskoski, 1975). The isolated protein kinase holoenzyme, the separated regulatory and catalytic subunits, phosphorylase \( a \) and phosphorylase kinase were homogeneous by sodium dodecyl sulphate (0.1\%) / polyacrylamide-gel (7.5\%) electrophoresis. The gel-electrophoresis experiments were carried out as described by Weber & Osborn (1969).

Phosphorylase phosphatase was prepared by method of Brandt et al. (1975b). Inhibition of the phosphorylase phosphatase reaction by phosphorylase kinase and cyclic AMP-dependent protein kinase was assayed in the following manner. Phosphorylase \( a \) (1.0 mg/ml) was incubated in 0.04 M-Tris/2 mM-EDTA/0.5 mM-dithiothreitol buffer (pH 7.4) with phosphorylase phosphatase in the presence of various concentrations of phosphorylase kinase or cyclic AMP-dependent protein kinase or its regulatory/catalytic subunit. The reaction mixtures were incubated at 30°C, portions (50 µl) were removed at various times, and the reaction was stopped by the addition of 0.1 M-NaF/
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Fig. 1. Influence of non-activated and thiophosphate-activated phosphorylase kinase on the $K_m$ of phosphatase

Data were taken from the Lineweaver–Burk plots reported by Bot et al. (1975) and Gergely et al. (1976). Changes in the $K_m$ of phosphorylase phosphatase for phosphorylase $a$ in the presence of non-activated (○) and thiophosphate-activated phosphorylase kinase (△) are shown.

40 mM-glycerophosphate/2 mM-EDTA (pH 6.8) to attain a dilution in which the activity of residual phosphorylase $a$ could still be measured (Gergely & Bot, 1977).

We showed previously (Bot et al., 1975) that non-activated phosphorylase kinase inhibits the dephosphorylation of phosphorylase $a$ and that the inhibition is competitive. Fig. 1 demonstrates the change in the affinity of phosphatase for phosphorylase $a$ in the presence of non-activated and thiophosphate-activated phosphorylase kinase. The $K_m$ of phosphatase for phosphorylase $a$ increases to about sixfold at the highest non-activated phosphorylase kinase concentration applied. The inhibitory effect of non-activated phosphorylase kinase does not explain the transient inhibition of phosphatase during the 'flash activation', since it may cause a permanent inhibition. There have been reports of the transient phosphorylation of phosphorylase kinase in the protein–glycogen complex (Yeaman & Cohen, 1975; Gergely et al., 1976). Therefore the effect of activated (phosphorylated) phosphorylase kinase was also investigated.

In the present experiments we used thiophosphate-activated phosphorylase kinase, since it is resistant to the action of phosphatase. It was found that the inhibition of phosphatase reaction caused by thiophosphate-activated phosphorylase kinase was also competitive (Gergely et al., 1976). The change in the $K_m$ values of phosphatase is much larger in the presence of thiophosphate-activated phosphorylase kinase than in the presence of the non-activated one (Fig. 1). The inhibitor constants were also calculated as described by Dixon (1953) and found to be 110 μg/ml for non-activated and 40 μg/ml for thiophosphate-activated phosphorylase kinase (Bot et al., 1975; Gergely et al., 1976). On the basis of these experiments, the transient inhibition of phosphatase observed in the protein–glycogen complex could be attributed to the formation of activated phosphorylase kinase. That activated phosphorylase kinase is a stronger inhibitor than the non-activated one, explains the reversible inhibition of phosphatase.

Cyclic AMP-dependent protein kinase plays an important role in glycogen metabolism, since the phosphorylation sequence begins with the activation of this enzyme.

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Protein kinase phosphorylates glycogen synthetase and phosphorylase kinase; the latter in turn phosphorylates (activates) phosphorylase and inhibits phosphatase. We decided to investigate the effect of cyclic AMP-dependent protein kinase on the dephosphorylation of phosphorylase a by phosphatase (Fig. 2). Protein kinase in the presence of cyclic AMP strongly inhibits the dephosphorylation of phosphorylase a. However, protein kinase in the absence of cyclic AMP does not affect the rate of the dephosphorylation process.

It is known that cyclic AMP dissociates the protein kinase holoenzyme into regulatory and catalytic subunits. The inhibition of the phosphatase reaction observed only in the presence of cyclic AMP indicates that the inhibition could be due to the free subunits and not to the holoenzyme. Cyclic AMP alone does not affect the rate of dephosphorylation (results not shown). Therefore the inhibitory effectiveness of isolated regulatory and catalytic subunits was also investigated (Fig. 3).

The presence of the catalytic subunit of cyclic AMP-dependent protein kinase does not influence the dephosphorylation of phosphorylase a, whereas the presence of the regulatory subunit considerably moderates the dephosphorylation reaction. The results presented in Figs. 2 and 3 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.

Our observations point to a new feature of cyclic AMP-dependent protein kinase, namely that not only has its catalytic subunit an important control role in the glycogen metabolism, but so has its regulatory subunit (Gergely & Bot, 1977). In the light of the present experimental data the events taking place during glycogen breakdown might be explained as follows. The phosphorylation enzyme cascade begins with the dissociation of cyclic AMP-dependent protein kinase by cyclic AMP. Cyclic AMP binds to the regulatory subunit, resulting in the release of the active catalytic subunit. The catalytic subunit triggers a phosphorylation sequence, resulting in the formation of active phosphorylase a and the inactive glycogen synthetase. During this time the cyclic AMP-
Fig. 3. Dephosphorylation of phosphorylase a in the presence of regulatory and catalytic subunits of cyclic AMP-dependent protein kinase

Experimental conditions are given in the text. Activities of residual phosphorylase a in the absence (○) and in the presence of 0.7 mg of catalytic subunit/ml (□) and in the presence of 0.7 mg of regulatory subunit/ml (■) are shown.

regulatory-subunit complex inhibits phosphorylase phosphatase, prolonging the glycogen breakdown. When the concentration of cyclic AMP decreases, the catalytic and regulatory subunits of protein kinase associate, thereby allowing the dephosphorylation of phosphorylase a.

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Mammalian Phosphorylase Phosphatase

E. Y. C. LEE, R. L. MELLGREN, J. H. AYLWARD and S. D. KILLILEA

Department of Biochemistry, University of Miami School of Medicine, Miami, FL33152, U.S.A.

Phosphorylase phosphatase converts phosphorylase a into phosphorylase b. It was the first interconverting enzyme to be discovered (Cori & Green, 1943) and is the best characterized of the protein phosphatases involved in the regulation of glycogen metabolism, although its enzymology is still incompletely understood (for a review see Lee et al., 1976).

We have developed a procedure for the isolation of phosphorylase phosphatase. Rabbit liver phosphorylase phosphatase was purified to homogeneity and the rabbit skeletal-muscle enzyme was extensively purified by this procedure (Brandt et al., 1975a). More recently, we have used the same procedure to isolate phosphorylase phosphatase from bovine heart as an essentially homogeneous preparation (Lee et al., 1977). The properties of the rabbit liver, rabbit skeletal muscle and bovine heart enzyme preparations are shown in Table 1. All three enzymes are proteins of relative mol.wt. 35000. Thus phosphorylase phosphatase of both liver and muscle may be isolated as a form of mol.wt. 35000. This 35000-mol.wt. form of phosphorylase phosphatase we have termed protein phosphatase C (Lee et al., 1977). Khandewal et al. (1976) have also isolated protein phosphatase C as well as a lower specific activity form of mol.wt. 30500 from rabbit liver. Rabbit liver phosphatase C appears to be a general protein phosphatase, and thus far appears to be capable of the dephosphorylation of all proteins phosphorylated by either cAMP-dependent protein kinase or phosphorylase kinase (Killilea et al., 1976a; Khandewal et al., 1976; Lee et al., 1977), although it may be noted that phosphorylase a is more rapidly dephosphorylated than any of the other substrates.

Table 1. Properties of phosphorylase phosphatase prepared by the procedure of Brandt et al. (1975a)

Data for the rabbit liver and rabbit muscle enzymes are taken from Brandt et al. (1975a). The bovine heart enzyme was purified as by Brandt et al. (1975a), except that the acid-precipitation step was omitted. One unit of phosphorylase phosphatase activity converts 1 nmol of rabbit muscle phosphorylase a dimer into phosphorylase b/min.

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<thead>
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<th>Source</th>
<th>Specific activity (units/mg)</th>
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