THE CONTROL OF PHOSPHORYLASE KINASE PHOSPHATASE BY "SECOND SITE PHOSPHORYLATION"; A NEW FORM OF ENZYME REGULATION

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1. Introduction

The activation of phosphorylase kinase by calcium ions is the mechanism by which phosphorylase *b* to phosphorylase *a* conversion and glycogenolysis takes place when muscle is stimulated electrically [1–3]. However the activity of phosphorylase kinase in the presence of calcium can vary by a factor of 50 with the degree of phosphorylation of the enzyme, which is determined at any instant by the relative activities of cyclic AMP dependent protein kinase [4, 5] and phosphorylase kinase phosphatase [6].

Rabbit skeletal muscle phosphorylase kinase is formed from three types of polypeptide chain [7–10] and has the structure $(\alpha\beta\gamma)_4$ where the molecular weights of the three subunits α, β and γ are 145 000, 128 000 and 45 000 daltons, respectively [8]. The cyclic AMP dependent activation of the enzyme was found to correlate with the phosphorylation of a unique site on the β-subunit, although a second site, on the α-subunit, also became rapidly labelled after a short lag period [8, 10], without any apparent effect on enzyme activity [8].

Several lines of evidence suggested that the phosphorylation of the α-subunit might play some role in the regulation of phosphorylase kinase activity. Firstly, the potential activity of the cyclic AMP dependent protein kinase in skeletal muscle is sufficient to phosphorylate half the α-subunits within 5–10 sec. Secondly, specificity studies with cyclic AMP dependent protein kinase have shown that the rate of α-subunit phosphorylation is more than two orders of magnitude faster than the rate of phosphorylation of any other enzyme in the glycolytic pathway tested [11]. Thirdly, the α- as well as the β-subunit becomes rapidly phosphorylated and dephosphorylated during a reversible activation of phosphorylase kinase demonstrable in glycogen particles [11], which are thought to resemble a functional complex in skeletal muscle [3, 12].

In this communication, it is demonstrated that following the phosphorylation of the α-subunit, there is a great enhancement of the rate of dephosphorylation of the β-subunit and enzyme inactivation, catalysed by phosphorylase kinase phosphatase, suggesting that this second site phosphorylation may function in the regulation of the reversal of phosphorylase kinase activation.

2. Materials and methods

2.1. *Enzymes*

Phosphorylase *b* was prepared by the method of Fischer and Krebs [13]. Phosphorylase kinase *a* (slightly active at pH 6.8 in the presence of calcium ions) was purified and assayed as described previously [8]. The cyclic AMP dependent protein kinase was the peak II "isozyme" from DEAE-cellulose [8].

Abbreviations:
cyclic AMP; adenosine 3'5' monophosphate;
EGTA; ethyleneglycol bis (2-amino-ethylether)-N,N'-tetra-acetic acid.

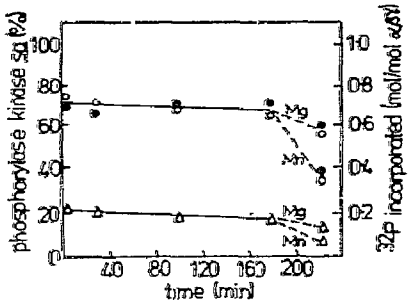

Fig. 1. Influence of phosphorylase kinase phosphatase at pH 7.0 on phosphorylase kinase (2 mg/ml) containing 0.98 moles of phosphate per $\alpha\beta\gamma$ ($\alpha = 0.23$ $\beta = 0.75$): phosphorylase kinase activity (•—•—•), β-subunit phosphorylation (○—○—○) and α-subunit phosphorylation (△—△—△). The activation reaction was terminated at time = 0 min as described under Methods. The temperature was 20°C, except for the first 10 min in which the enzyme was kept in ice. The broken lines indicate the effect of adding 10 mM Mg^{2+} or 1 mM Mn^{2+} at 150 min.

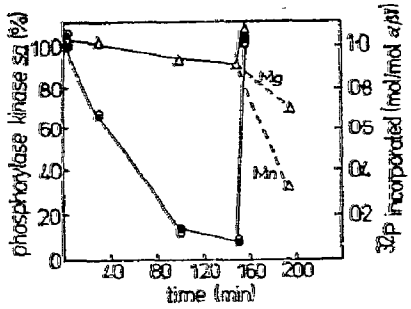

Fig. 2. Influence of phosphorylase kinase phosphatase at pH 7.0 on phosphorylase kinase (2 mg/ml) containing 2.1 moles of phosphate per $\alpha\beta\gamma$ ($\alpha = 1.05$, $\beta = 1.05$). Phosphorylase kinase activity (•—•—•), β-subunit phosphorylation (○—○—○) and α-subunit phosphorylation (△—△—△). After 150 min ATP-Mg^{2+}, cyclic AMP and cyclic AMP dependent protein kinase were added. The broken lines indicate the effect of 10 mM Mg^{2+} or 1 mM Mn^{2+} at 150 min. Other conditions as in fig. 1.

2.2. *Activation of phosphorylase kinase*

The conversion of phosphorylase kinase *a* to phosphorylase kinase *sa* (superactive at pH 6.8 in the presence of calcium ions [8]) was carried out at 20°C, pH 6.8, in the following reaction mixture: 10 mM sodium glycerophosphate, 0.4 mM EDTA, 0.1 mM EGTA, 0.2 mM [γ-^{32}P]ATP of specific radioactivity 25 Ci per mole, 2.0 mM magnesium acetate, 0.01 mM cyclic AMP, and cyclic AMP dependent protein kinase. The concentration of the last component was adjusted to produce a 50% conversion of phosphorylase kinase *a* to *sa* and β-subunit phosphorylation in 30 sec. Essentially complete conversion required 5 min. The half time for α-subunit phosphorylation was about 2 min, and the labelling of this component was complete in 20–30 min. The proportion of phosphorylase kinase *sa* at any instant was computed from the basal activity of phosphorylase kinase *a* and the plateau of phosphorylase kinase *sa* activity between 5 and 30 min.

Activation reactions were terminated by addition of an equal volume of 0.01 M EDTA pH 7.0 containing 80% saturated ammonium sulphate (time = 0). The solutions were kept in ice for 10 min and then centrifuged at 15 000 *g* for 2 min. The precipitate was resuspended at 20°C in Tris-HCl, I = 0.02, 1.0 mM EDTA, 1.0 mM dithiothreitol, pH 7.0, containing 30% saturated ammonium sulphate. The suspension was recentrifuged, and the precipitate redissolved in the same buffer without ammonium sulphate (step A). The solution was dialysed against this buffer for 1 hr at 20°C (step B) and then stored for a further hour (step C). The solutions were analysed at time = 0, and after steps A, B and C for phosphorylase kinase activity at pH 6.8 and for total covalently bound phosphate [5]. The covalently bound phosphate in the α- and β-subunits was computed from the total phosphate incorporated into the enzyme and the radioactivity *ratios* determined after separation of the subunits by acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate [8].

3. Results and discussion

Phosphorylase kinase activation reactions were initially terminated when one covalently bound phosphate molecule had been incorporated per $\alpha\beta\gamma$. As expected from previous results [8] the enzyme was predominantly labelled in the β-subunit ($\beta = 0.75$, $\alpha = 0.23$) and activation and phosphorylation of the β-subunit correlated with one another (fig. 1). There was a very slow loss of phosphate from both the α- and β-subunits during the following 150 min but addition of magnesium or manganese ions at this point stimulated both a decrease in activity and a loss of

radioactivity from the α- and β-subunits (fig. 1), suggesting that purified phosphorylase kinase is contaminated with some phosphorylase kinase phosphatase. The product of this reaction was established as inorganic phosphate by high voltage paper electrophoresis at pH 6.5.

Fig. 2 shows the results of an analogous experiment to that described in fig. 1 in which the activation reaction was continued until both α- and β-subunit phosphorylation were complete (α = 1.05, β = 1.05 per αβγ). In complete contrast to the results shown in fig. 1, there was an immediate and specific dephosphorylation of the β-subunit in the *absence* of divalent cations. The perfect correlation between enzyme inactivation and loss of phosphate from the β-subunit conclusively demonstrated that the reversible activation of phosphorylase kinase catalysed by cyclic AMP dependent protein kinase and phosphorylase kinase phosphatase depends only on the reversible phosphorylation of a unique site on the β-subunit. This therefore led to the formation of an enzyme which had almost returned to the basal activity of phosphorylase kinase *a* but still contained close to one covalently bound phosphate molecule in the α-subunit. At this time the readdition of cyclic AMP dependent protein kinase, cyclic AMP, ATP and magnesium ions led to a reactivation of the enzyme and rephosphorylation of the β-subunit (fig. 2). This showed that prior phosphorylation of the α-subunit did not prevent the reactivation process, and again confirmed that the original loss in radioactivity from the β-subunit was caused by the release of inorganic phosphate, and not the removal of a small peptide containing the phosphorylated site by trace proteolytic activity which might have been present in the preparation. Addition of Mg^{2+} and Mn^{2+} after 150 min stimulated a dephosphorylation of the α-subunit (fig. 2) at a rate similar to that obtained for the β-subunit at the comparable time in fig. 1, although this had no further effect on enzyme activity (not illustrated). The K_m of activation for the Mg^{2+} and Mn^{2+} stimulated dephosphorylation was 5–10 mM for both the α- and β-subunits.

The endogenous phosphorylase kinase phosphatase activity could be completely inhibited by including 50 mM sodium fluoride in the buffer used to terminate the activation reaction and at all subsequent steps (not illustrated), and less than 1% of the covalently bound phosphate was released per week at 4°C.

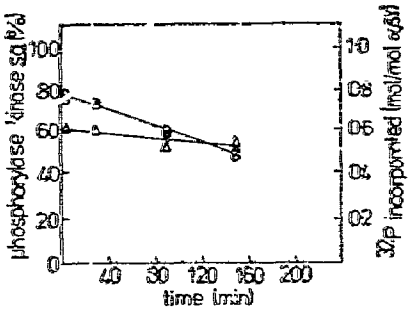

Fig. 3. Influence of phosphorylase kinase phosphatase at pH 7.0 on phosphorylase kinase 2 mg/ml containing 1.4 moles of phosphate per αβγ (α = 0.6, β = 0.8). Phosphorylase kinase activity (●—●—●), β-subunit phosphorylation (○—○—○) or α-subunit phosphorylation (△—△—△). Other conditions as in fig. 1.

Mn^{2+} (but not Mg^{2+}) restores the activity to 70% of that measured in the absence of fluoride. Therefore phosphorylase kinase *a* labelled with one phosphate in the α- and one phosphate in the β-subunit and stored in the presence of 50 mM fluoride may be used as a substrate for assay of phosphorylase kinase phosphatase during its isolation (Antoniw and Cohen, unpublished work).

The experiments shown in figs. 1 and 2 clearly show that provided the α-subunit is not significantly labelled, phosphorylase kinase phosphatase is hardly able to dephosphorylate the β-subunit and inactivate phosphorylase kinase unless divalent cations are added. On the other hand a complete labelling of the α-subunit allows the phosphatase to rapidly and specifically dephosphorylate the β-subunit with accompanying enzyme inactivation. The simplest explanation to account for these findings is that the phosphorylation of the α-subunit alters the conformation of the phosphorylase kinase molecule in such a way as to facilitate the action of phosphorylase kinase phosphatase on the primary phosphorylated site on the β-subunit responsible for enzyme activation.

An extension of these findings is illustrated in fig. 3. In this experiment phosphorylase kinase was phosphorylated to an intermediate extent, 1.4 moles per αβγ (α = 0.6, β = 0.8). Although 60% of the α-subunits were phosphorylated, the decline in enzyme activity and β-subunit dephosphorylation after 150 min was only 30%. Likewise in fig. 1, the loss of phosphate from the β-subunit over 150 min was only 12% although the total α-subunit phosphorylation was 23%.

These results, which have been confirmed with a number of enzyme preparations, suggest that the transition of phosphorylase kinase to the form which is highly susceptible to β-subunit dephosphorylation is a co-operative process requiring the phosphorylation of at least 2 α-subunits per active enzyme molecule, $(\alpha\beta\gamma)_4$. It should also be recalled that, earlier in this activation reaction catalysed by cyclic AMP dependent protein kinase, the phosphorylation of the α-subunit only occurs rapidly after approx. 2 β-subunits have been phosphorylated per $(\alpha\beta\gamma)_4$ [8, 10].

While the enhancement of β-subunit dephosphorylation by α-subunit phosphorylation represents an example of a new type of enzyme regulation by covalent modification, it is only possible to speculate at present about the precise role it might play in the regulation of glycogenolysis. If however it is assumed that free divalent cations available to phosphorylase kinase phosphatase are low, then immediately following an adrenalin pulse and cyclic AMP formation, the cyclic AMP dependent protein kinase would rapidly phosphorylate the β-subunit of phosphorylase kinase, with initially little competing effect from phosphorylase kinase phosphatase. The half time for this reaction may be estimated as approx. 1 sec based on *in vitro* activities [11]. Then provided calcium ions have reached the required threshold level of approx. 10^{-6} M [2, 3], phosphorylase kinase *sa* will be activated and convert phosphorylase *b* to *a* with a half time of approx. 0.5 sec, again based on *in vitro* activities [11]. The phosphorylation of the α-subunit which would presumably occur after 5 sec of cyclic AMP dependent protein kinase action on this time scale, would then represent the point at which phosphorylase kinase phosphatase activity is turned on. If at this point the adrenalin pulse has ceased, cyclic AMP has been hydrolysed by phosphodiesterase and protein kinase has therefore become inactivated, it may be calculated from the phosphorylase kinase phosphatase activity in skeletal muscle extracts (which are several hundred times higher relative to phosphorylase kinase than in the purified preparations of the latter) that the half time for reconversion of phosphorylase kinase *sa* to *a* would be within 2 sec.

On the other hand it is interesting to speculate that the activation of phosphorylase kinase phosphatase by α-subunit phosphorylation might represent part of an automatic shutdown process for phosphorylase kinase after prolonged hormonal stimulation, although for this to be effective there would have to be a synchronous inhibition of the cyclic AMP dependent protein kinase at the time α-subunit phosphorylation nears completion. If however cyclic AMP dependent protein kinase did become inactive at this point, by for instance the combined action of the two protein factors which inhibit cyclic AMP dependent protein kinase [14] and activate cyclic AMP phosphodiesterase [15], respectively, and whose function is not yet known, then the rapid dephosphorylation of phosphorylase kinase that would ensue might serve two functions. Firstly provided phosphorylase phosphatase is synchronously inhibited as phosphorylase kinase becomes activated, for which there is some evidence [16], phosphorylase *a* levels would be stable and there would be no further need to maintain phosphorylase kinase in the superactive form. Secondly it is possible that if both phosphorylase kinase *sa* and cyclic AMP dependent protein kinase were active for too long, then random non-specific phosphorylation of a number of other proteins might well occur causing both losses of ATP and disruption to other cellular functions.

Finally, the possibility that enzyme regulation by "second site phosphorylation" might exist in other enzyme systems controlled by phosphorylation and dephosphorylation should be considered. Thus glycogen synthetase which is inactivated and reactivated by the same kinase and phosphatase as phosphorylase kinase [17, 18], and pyruvate dehydrogenase, have both been reported to become phosphorylated at more than one site [19, 20]. It is therefore possible that inactivation is determined by the phosphorylation of a unique site and that the reactivation of these enzymes depends upon the dephosphorylation of this site, a reaction triggered by the phosphorylation of a second site which may or may not be under separate control.

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