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# PARTIAL PHOSPHORYLATION OF MUSCLE PHOSPHORYLASE

## I. FORMATION OF A HYBRID PHOSPHORYLASE IN VITRO

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SUMMARY

The conversion of rabbit skeletal muscle phosphorylase *b* (1,4-α-D-glucan: orthophosphate α-glucosyltransferase, EC 2.4.1.1) to phosphorylase *a* by phosphorylase *b* kinase has been investigated. In agreement with previous reports it was established that during the interconversion a partially phosphorylated hybrid phosphorylase is formed. Hybrid phosphorylase was detected both by the incorporation of $^{32}P$ from γ-labelled ATP and by a new assay of phosphorylase activity. This assay is based upon the observation that the activity of hybrid phosphorylase is equally increased by AMP in the presence or the absence of caffeine, whereas AMP-induced activity of phosphorylase *b* is inhibited by caffeine.

The extent of phosphorylation is controlled by the relative amount of phosphorylase *b* and of phosphorylase *b* kinase. Increasing the concentration of phosphorylase *b* or decreasing the concentration of phosphorylase *b* kinase lead to the formation of hybrid phosphorylase.

## INTRODUCTION

Hurd et al. [1] and Fischer et al. [2] observed, in the course of in vitro conversion of phosphorylase *b* to phosphorylase *a* and vice versa, the formation of a hybrid phosphorylase which was sensitive to inhibition by glucose 6-phosphate, when the activity was measured in the presence of 100 mM glucose 1-phosphate.

Livanova et al. [3] reported the possibility of the formation of a hybrid phosphorylase in a mixture of phosphorylase *a* and *b* monomers after reassociation by mercaptoethanol. Heilmeyer et al. [4] observed that the "flash activation" of phosphorylase bound to glycogen particles of rabbit muscle produced a phospho–dephosphohybrid, too.

The isolation of a stable hybrid phosphorylase failed because the hybrid molecules rearranged to form phosphorylated and non-phosphorylated enzymes in aqueous solution [2]. Feldmann et al. [5] succeeded in isolating and stabilizing the hybrid phosphorylase by attachment to Sepharose activated by cyanogen bromide [5].

In the present work we introduce an appropriate method to assay the activities

of non-phosphorylated, partially and fully phosphorylated phosphorylases and we investigate the conditions of formation of hybrid phosphorylase in vitro.

In the following paper we discuss the in vivo formation of hybrid phosphorylase and the regulation of its activity.

## MATERIALS AND METHODS

Phosphorylase *b* was prepared from rabbit skeletal muscle and recrystallized three times [6]. AMP was removed by Norit–cellulose treatment [7] reaching a ratio of $A_{260\ nm}:A_{280\ nm} \leq 0.58$.

Phosphorylase *b* kinase was prepared from rabbit skeletal muscle by the procedure of Krebs et al. [8] with the modification of DeLange et al. [9]. The activity of phosphorylase *b* kinase was assayed according to the method of Krebs et al. [8] at pH 6.8 and 8.2. One unit of phosphorylase *b* kinase is defined as that amount of kinase which catalyzes the formation of 100 units of phosphorylase *a* per ml of reaction mixture in 5 min under stated conditions [8, 10]. The ratio of activity at pH 6.8 to that at 8.2 is considered an index of degree of kinase activation [9]. Specific activity of kinase at pH 8.2 was 86 units/mg protein and the ratio of pH 6.8/8.2 was 0.08.

Phosphorylase *a* was prepared from crystalline phosphorylase *b* with purified phosphorylase *b* kinase according to the method of Fischer and Krebs [6]. After Norit–cellulose treatment the ratio of $A_{260\ nm}:A_{280\ nm}$ was 0.52.

Phosphorylase activity was assayed in the direction of glycogen synthesis by the method of Illingworth and Cori [11]. The unit of enzyme activity is defined as the amount of enzyme causing the release of 1 $\mu$mole of $P_i$ from glucose 1-phosphate per min at 30 °C, pH 6.8. Specific activity of phosphorylase *b* ranged from 50 to 55 units/mg in the presence of 1 mM AMP and 16 mM glucose 1-phosphate. Specific activity of phosphorylase *a* was 59.5 units/mg in the presence of 16 mM glucose 1-phosphate and in the absence of AMP.

Protein was determined by the procedure of Lowry et al. [12], or by measuring the absorbance at 280 nm using an absorbance index of $A^{1\%}_{280\ nm} = 12.5$ [13]. Pyridoxal 5′-phosphate content was measured spectrophotometrically after release of pyridoxal 5′-phosphate from phosphorylases by precipitating the protein with $HClO_4$ (final concentration 0.3 M) [14]. A molar extinction coefficient of 6250 $M^{-1} \cdot cm^{-1}$ was used [14].

*Conversion of phosphorylase b into phosphorylase a*

Phosphorylase *b* was converted into phosphorylase *a* according to the method of DeLange et al. [9] using non-activated phosphorylase *b* kinase, in the presence of ATP and $Mg^{2+}$ at pH 8.2. When $^{32}P$ incorporation was measured during the conversion using $\gamma$-labelled [$^{32}P$]ATP (obtained from The Radiochemical Centre, Amersham, and used without further purification), aliquots were removed and precipitated with trichloroacetic acid. The precipitate was dissolved in 0.1 M NaOH, reprecipitated and washed once more with trichloroacetic acid, and finally redissolved in 0.25 M NaOH. The radioactivity was measured by counting an aliquot using the Cerenkov effect of $^{32}P$ with a Packard Tri-Carb scintillation spectrometer [15, 16].

## RESULTS AND DISCUSSION

*Preparation of hybrid phosphorylase*

The hybrid phosphorylase was prepared from phosphorylase *b* upon incubation at 30 °C of a solution containing 400 units of phosphorylase *b* and 0.4 unit of phosphorylase *b* kinase per ml, 3 mM ATP, 10 mM $Mg^{2+}$, and 0.04 M glycerophosphate and 0.01 M mercaptoethanol, pH 8.2. The reaction was started by the addition of ATP/$Mg^{2+}$. Aliquots were removed at various times, diluted with a buffer solution containing 0.04 M glycerophosphate–0.01 M mercaptoethanol–0.004 M EDTA (pH 6.8), and tested for the activity of phosphorylase *a* in the presence of 16 and 100 mM glucose 1-phosphate. The kinase reaction was stopped by the addition of EDTA, when the activity of phosphorylase *a* in the presence of 100 mM glucose 1-phosphate reached the maximum, and was twice as high as the activity in the presence of 16 mM glucose 1-phosphate. Indeed, it is known that the activity of hybrid phosphorylase in the presence of 16 mM glucose 1-phosphate will be doubled in the presence of 100 mM glucose 1-phosphate [1, 2].

The incubation mixture was then chromatographed on DEAE-cellulose column for the purification of hybrid phosphorylase. Fig. 1 shows the elution profiles of the incubation mixture. At the beginning of the kinase reaction the total amount of phosphorylase can be eluted by 5 mM glycerophosphate (Fig. 1A). It is known that phosphorylase *b* is eluted by low concentration (2–5 mM) of glycerophosphate [17]. Accordingly, these fractions contain phosphorylase *b* and they have activity only in the presence of AMP. In the 30th minute of the kinase reaction (Fig. 1B) only very slight amount of phosphorylase gets into the fractions eluted by 5 mM glycerophosphate. The main part of phosphorylase could be eluted by 0.1 M glycerophosphate, and it is active in the absence of AMP. The activity of these fractions is twice as great in the presence of 100 mM glucose 1-phosphate, as in the presence of 16 mM glucose 1-phosphate, verifying the existence of hybrid phosphorylase.

The peak fractions were collected and used without further purification.

*Measurement of activity of hybrid phosphorylase in the presence of caffeine and AMP*

It is known that caffeine inhibits both the activities of phosphorylase *b* and of phosphorylase *a* [18]. Its inhibitory effect on phosphorylase *a*, however, is counteracted by AMP [19].

In our experiments we have studied the effect of caffeine on the activity of hybrid phosphorylase, phosphorylase *a* and phosphorylase *b*. Fig. 2 shows that caffeine almost entirely abolishes the activating effect of AMP on phosphorylase *b*. AMP increases the activity of hybrid phosphorylase about twofold but this activation is only slightly diminished by caffeine. AMP has practically no effect on phosphorylase *a* and, moreover, caffeine does not inhibit its activity in the presence of AMP.

The inhibitory effect of caffeine, which in the presence of AMP is limited to phosphorylase *b*, permits the determination of hybrid phosphorylase and phosphorylase *a* in the presence of phosphorylase *b*. With earlier methods only the hybrid phosphorylase and phosphorylase *a* could be assayed together [1, 2]. According to our results, the activity in the absence of AMP corresponds to the total amount of phosphorylase *a* and to one half of the hybrid phosphorylase. The increase in activity in the presence of AMP and caffeine corresponds to the other half of hybrid phos-

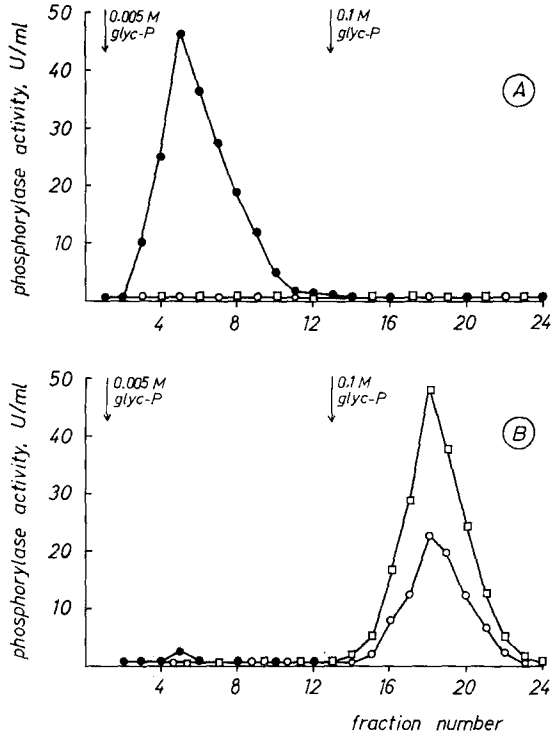


Fig. 1. Elution patterns of the kinase reaction mixtures. Kinase reaction mixture, composition in the text, was chromatographed on a column, 1 cm × 15 cm, of DEAE-cellulose and eluted in 2-ml fractions at the rate 10 ml/h with a buffer containing 0.005 or 0.1 M glycerophosphate–0.04 M NaF–0.01 M mercaptoethanol–0.002 M EDTA, pH 6.8. ○—○, □—□, phosphorylase activity in the presence of 16 and 100 mM glucose 1-phosphate, respectively; ●—●, activity in the presence of 16 mM glucose 1-phosphate and 1 mM AMP. Elution at the beginning (A) and at 30th min (B) of the reaction.

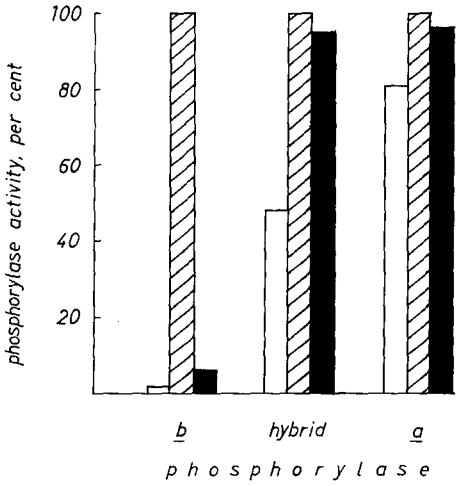


Fig. 2. Effect of caffeine and AMP on the activity of phosphorylase *b*, hybrid phosphorylase and phosphorylase *a*. The activity measurements of phosphorylases are described in the Methods. Open columns: activity without AMP; hatched columns: activity with AMP (1 mM). Closed columns: activity with AMP + caffeine (1 and 5 mM, respectively). Activities are expressed in percent of activity with AMP (1 mM).

phorylase. The amount of phosphorylase *b* is equal to the difference between the activity measured in the presence of AMP and that measured in the presence of AMP and caffeine. ATP has a similar effect as caffeine. The activity of hybrid phosphorylase is equally increased by AMP in the presence or the absence of ATP, whereas AMP-induced activity of phosphorylase *b* is inhibited by ATP. The above effect of ATP could be of a physiological importance therefore it is discussed in the second paper [20].

*Influence of the ratio of phosphorylase b to phosphorylase kinase on the formation of hybrid phosphorylase*

We have found that the formation of hybrid was dependent upon the relative amounts of phosphorylase *b* and of phosphorylase kinase present in the incubation mixture. We show in Fig. 3 that when 200 units of phosphorylase *b* were incubated with 0.4 unit of phosphorylase kinase, nearly all the substrate was converted to phosphorylase *a* after 70 min. One remarks however that the activity with AMP and caffeine (phosphorylase *a* + hybrid) increases much faster than the activity without AMP (phosphorylase *a* + 50% of the hybrid). The difference between these two measurements is indicative of a rapid formation of the hybrid followed by its progressive conversion into phosphorylase *a*.

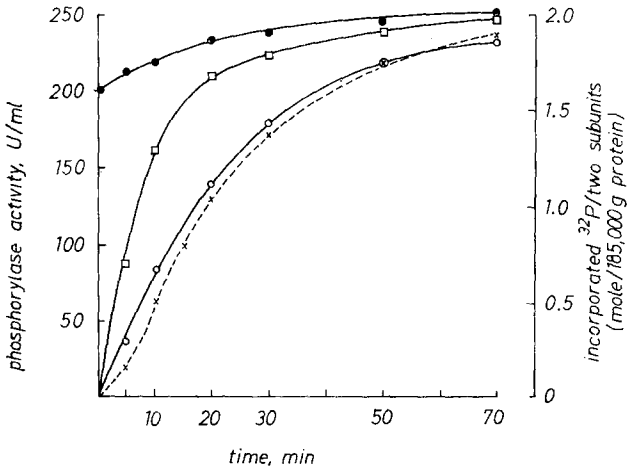


Fig. 3. Conversion of phosphorylase *b* into phosphorylase *a*. Conversion of 200 units/ml rabbit muscle phosphorylase *b* into *a* catalyzed by 0.4 unit/ml phosphorylase *b* kinase at pH 8.2. Conditions are described in Methods. Activity of phosphorylase: ○—○, without AMP; □—□, with AMP + caffeine (1 and 5 mM, respectively); ●—●, with AMP (1 mM). ×---×, Incorporated $^{32}P$ mole per 185 000 g protein).

During the interconversion the incorporation of $^{32}P$ from labelled ATP into phosphorylase also shows that the partially phosphorylated enzyme forms only at the beginning of the transformation. During the further conversion the amount of $^{32}P$ incorporated into phosphorylase increases gradually and in the 70th minute reaches a value of approximately 2 moles incorporated $^{32}P$ per 185 000 g protein.

Using a larger amount (400 units/ml) phosphorylase *b* and the same amount of phosphorylase kinase, the interconversion leads to the accumulation of hybrid

phosphorylase (Fig. 4). The data of the interconversion show that the activity measured in the presence of AMP and caffeine is nearly twice as high as the activity in the absence of AMP during the total process and even at the end of the reaction. This shows that hybrid phosphorylase was formed and accumulated, which could not be converted into fully phosphorylated phosphorylase *a* even if the total amount of phosphorylase *b* has already been converted.

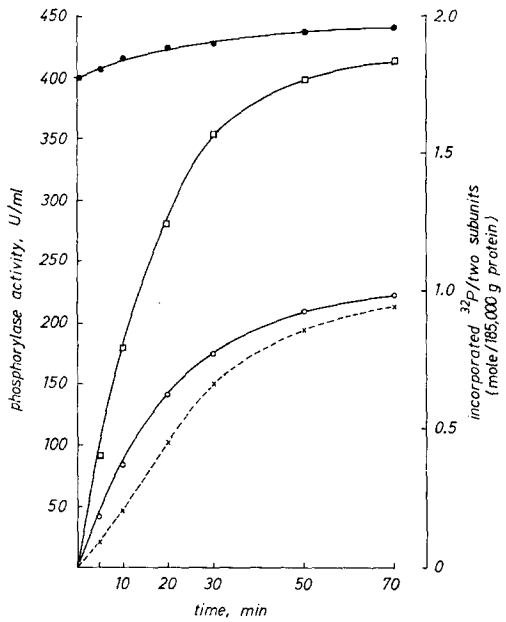


Fig. 4. Conversion of phosphorylase *b* into hybrid phosphorylase. Conversion of 400 units/ml phosphorylase *b* into *a* catalyzed by 0.4 unit/ml kinase at pH 8.2. Conditions are described in Methods. Activity of phosphorylase: ○—○, without AMP; □—□, with AMP + caffeine (1 and 5 mM, respectively); ●—●, with AMP (1 mM). ×---×, incorporated $^{32}P$ (mole per 185 000 g protein).

The incorporation of $^{32}P$ also shows the formation of hybrid phosphorylase, because the two subunits contain only one mole $^{32}P$ at the end of the conversion. This incorporation of $^{32}P$ can not be attributed to the formation of a mixture of phosphorylase *b* and totally phosphorylated *a*, because practically no phosphorylase *b* is present from the 50th minute of the reaction (caffeine does not inhibit the phosphorylase activity in the presence of AMP). The absence of phosphorylase *b* was also verified chromatographically in a similar way as it was demonstrated in Fig. 1 (not documented).

According to the above experiments the partial and total phosphorylation of phosphorylase *b* is controlled by the amount of substrate (Figs 3 and 4). However, the formation of fully or partially phosphorylated phosphorylase is also influenced by the amount of the converting enzyme, phosphorylase *b* kinase. Decreasing the amount of kinase in the presence of 200 units/ml phosphorylase *b* changed the extent of phosphorylation.

Fig. 5 illustrates the converting effect of a smaller amount of kinase on such quantity of phosphorylase *b* (200 units/ml) which had been converted into totally phosphorylated *a* by a larger amount of kinase. (As shown in Fig. 3, fully phosphorylated *a* was formed by 0.4 unit/ml kinase). It can be seen that 0.2 unit/ml kinase results only in the formation of hybrid phosphorylase verified by activity assays and by incorporation of $^{32}P$ (Fig. 5).

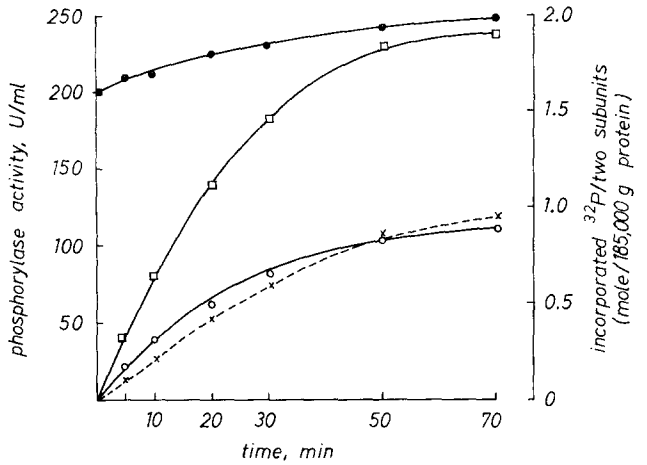


Fig. 5. Effect of the amount of phosphorylase *b* kinase on the conversion of phosphorylase *b*. Conversion of 200 units/ml phosphorylase *b* into *a* catalyzed by 0.2 unit/ml kinase at pH 8.2. Conditions are described in Methods. Symbols are the same as in Fig. 3.

However we can change the proportion of the kinase to the phosphorylase by decreasing or increasing their concentration, but also by modification of the pH value, because the activity of non-activated kinase decreases to a great extent by changing the pH value from 8.2 to 6.8 [8, 9]. It was observed that 0.4 unit/ml kinase results only in hybrid phosphorylase at pH 6.8 (not documented), while the same amount of kinase resulted in a fully phosphorylated phosphorylase at pH 8.2 (Fig. 3).

According to our results phosphorylation of phosphorylase *b* takes place in two steps: first a partially phosphorylated hybrid phosphorylase is formed. The phosphorylation of one subunit is followed by the phosphorylation of the second subunit, causing the formation of totally phosphorylated *a*.

The extent of phosphorylation has been controlled by the proportion between the substrate phosphorylase *b* and the converting enzyme kinase. Both the increase of the amount of phosphorylase *b* and the decrease of kinase result in a partial phosphorylation.

The physiological importance and the regulatory role of hybrid phosphorylase will be discussed in the second paper.

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