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PARTIAL PHOSPHORYLATION OF MUSCLE PHOSPHORYLASE

II. FORMATION OF A HYBRID PHOSPHORYLASE IN VIVO

PÁL GERGELY, GYÖRGY BOT and EDIT F. KOVÁCS
Institute of Medical Chemistry, University of Medicine 4026 Debrecen, Bem Place 18/B (Hungary)
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SUMMARY

The conversion of phosphorylase *b* (1,4-α-D-glucan:orthophosphate α-glucosyltransferase, EC 2.4.1.1) to phosphorylase *a* has been investigated in rabbit and frog skeletal muscle in vivo. It was found that hybrid phosphorylase (containing phosphorylated and non-phosphorylated subunits) is formed in vivo through the effects of hormonal and electrical stimulation. The stimulation in all cases leads to the conversion of the total amount of phosphorylase *b* to hybrid phosphorylase, i.e. phosphorylase *b* does not remain in the stimulated muscle.

Hybrid phosphorylase is an allosteric sensitive enzyme species, it may be activated by AMP in the absence and in the presence of ATP, too. Therefore AMP and ATP may play a regulatory role, because ATP inhibits the activity of phosphorylase *b* in resting muscle but it does not inhibit the activity of hybrid phosphorylase. The possibility of this mechanism will be also discussed in this paper.

INTRODUCTION

In the preceding paper, it was shown that the extent of the phosphorylase phosphorylation is controlled in vitro by the proportion of the substrate phosphorylase *b* to the converting enzyme phosphorylase *b* kinase [1]. It may be assumed that hybrid phosphorylase could also be formed in vivo and it may play a role in the regulation of glycogen utilization [2]. This is also apparent from the previous work on the formation of hybrid phosphorylase in muscle. It was observed that maximally 40–50% of phosphorylase *b* was converted into phosphorylase *a* if the activity of phosphorylase *a* was assayed in the presence of 16 mM glucose 1-phosphate without AMP [3, 4]. The explanation of this observation may be the formation of a hybrid phosphorylase because its activity could be doubled in the presence of 100 mM glucose 1-phosphate, therefore this may represent 80–100% of the total phosphorylase.

Other authors observed the formation of a larger amount of phosphorylase *a* but the assays were carried out in the presence of 100 mM glucose 1-phosphate [5, 6]. It appears that in these cases also hybrid phosphorylase was formed but its activity was twice higher in the presence of 100 mM glucose 1-phosphate than in the presence

of 16 mM glucose 1-phosphate. Formation of a hybrid phosphorylase was also observed in glycogen particles during the "flash activation" [7].

In this report we show that hybrid phosphorylase is formed in rabbit and frog skeletal muscle in vivo through the effect of epinephrine or electrical stimulation. Furthermore the total amount of phosphorylase *b* can be converted to hybrid species.

METHODS

Stimulation of rabbit muscle

The animals were anesthetized with pentobarbital sodium and the hind limbs were then skinned. Epinephrine of 200 μg per kg body weight was injected intraperitoneally. 2–20 min after the injection biopsy samples of the muscle (300–400 mg) were removed with Wollenberger forceps and rapidly plunged into liquid air. Then the frozen muscle samples were powdered and approximately 100 mg of powdered muscle was homogenized at 0 °C with 3 vol. of a buffer containing 0.04 M NaF–0.04 M glycerophosphate–0.01 M mercaptoethanol–0.002 M EDTA, pH 6.8 [5–8]. The homogenate was centrifuged 20 min at 3000 $\times$ *g* and the supernatant fluid was assayed for phosphorylase.

Phosphorylase activity was measured in the direction of glycogen synthesis by the method of Illingworth and Cori [9]. One unit of activity is defined as the amount of enzyme causing the release of 1 μmole P_i from glucose 1-phosphate per min at 30 °C, pH 6.8.

Total phosphorylase activity

This was measured in the homogenate (tissue dilution 1:100) in the presence of 1 mM AMP.

Hybrid phosphorylase assay

Hybrid phosphorylase assay is based upon the observation that the activity of hybrid is equally increased by 1 mM AMP in the presence or the absence of 5 mM caffeine, whereas AMP-induced activity of phosphorylase *b* is inhibited by caffeine [1].

In order to determine whether the muscle extract contents significant amounts of AMP which would cause an error in the determination of phosphorylase *b*, the homogenate was treated in 1:100 dilution with Norit (4 mg Norit per ml at 0 °C, 10 min). Norit treatment resulted in a decrease of total phosphorylase activity but did not change the ratio of the activity $-$AMP/$+$AMP, compared it with the untreated homogenate, therefore Norit treatment was not necessary in the experiments.

Electrical stimulation of frog musculus gastrocnemius

Female *Rana pipiens* weighing approximately 60–80 g were used. After decapitating the *m. gastrocnemius* with *nervus ischiadicus* was carefully dissected and transferred to 20 °C Ringer's solution for 20 min. (Composition of Ringer's solution: 0.0025 M KCl–0.0115 M NaCl–0.00215 M Na_2HPO_4–0.00085 M NaH_2PO_4–0.0018 M $CaCl_2$, pH 6.8). The muscle was stimulated with platinum electrodes through *n. ischiadicus*. Stimulation was by 12-V shocks, 1.2 ms duration with 40–50 shocks per s at 20 °C which tetanized the muscle. After the electrical stimulation *m. gastrocnemius*

was removed with Wollenberger forceps and plunged into liquid air. Homogenate was made from muscle sample as described in the case of rabbits. The homogenate in 1:100 dilution was also treated with Norit for the removing of AMP but the ratio of activity −AMP/+AMP did not differ from the untreated homogenate. Therefore phosphorylase assays were carried out without Norit treatment as described above.

It was shown that our method for the determination of phosphorylase *a*, hybrid phosphorylase and phosphorylase *b* based on the combined effect of AMP and caffeine [1] can be applied to frog skeletal muscle phosphorylase. Frog phosphorylase *b*, prepared according to Metzger et al. [10] was converted into phosphorylase *a* by rabbit muscle phosphorylase *b* kinase. Hybrid phosphorylase was obtained as described in the preceding paper [1].

RESULTS

Formation of hybrid phosphorylase under the action epinephrine

The effect of an intraperitoneal injection of epinephrine on the activity of phosphorylase in rabbit skeletal muscle is shown in Table I. The activity measured in the absence of AMP reached only 40–50% of the activity measured in the presence

TABLE I

EFFECT OF EPINEPHRINE ON THE ACTIVITY OF RABBIT SKELETAL MUSCLE PHOSPHORYLASE

Biopsy samples were taken at the indicated intervals after epinephrine injection (200 μg per kg body weight) given intraperitoneally. Homogenates were made from the samples and after 1:100 dilution phosphorylase was measured in the absence of AMP and in the presence of AMP + caffeine (1 and 5 mM, respectively). Total phosphorylase was measured in the presence of 1 mM AMP. Results represents the average of 5 experiments indicating the standard errors.

Time after epinephrine injection (min)	Phosphorylase activity in percent of total phosphorylase	
	− AMP	+ AMP + caffeine
0	7.5 ± 1.4	17.2 ± 2.3
2	19.3 ± 2.1	34.6 ± 2.8
5	30.4 ± 2.7	58.1 ± 3.3
10	40.6 ± 3.6	77.3 ± 4.3
15	45.2 ± 3.7	92.4 ± 5.2
20	36.3 ± 3.9	75.1 ± 4.8

of 1 mM AMP with 5 mM caffeine. The stimulation due to AMP is indicative of the presence of an hybrid since neither phosphorylase *b* nor phosphorylase *a* are stimulated by the nucleotide in the presence of caffeine. We estimate that about 92% of phosphorylase *b* have been converted to the hybrid form when the stimulation was maximal.

Formation of hybrid phosphorylase by electrical stimulation

The effect of electrical stimulation on the formation of hybrid phosphorylase was also studied. Fig. 1 shows the effect of stimulation on the conversion of phos-

phorylase *b* in frog *m. gastrocnemius*. The activity measured in the absence of AMP was only 50% of that measured in the presence of the nucleotide with caffeine, and also of that measured in the presence of 100 mM glucose 1-phosphate (not shown). We calculate that at the peak of the stimulation 90% of phosphorylase *b* was converted to the hybrid.

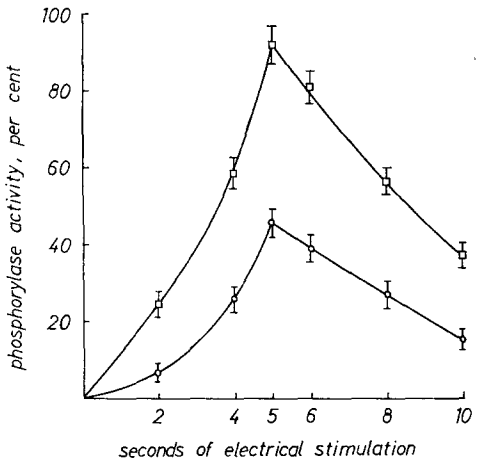

Fig. 1. Formation of hybrid phosphorylase in frog skeletal muscle on the effect of electrical stimulation. Electrical stimulation of frog musculus gastrocnemius, the homogenization of the muscle and the activity measurements of phosphorylase are described in Methods. Phosphorylase activities are expressed in percent of total phosphorylase (activity with AMP, 1 mM). ○—○, activity without AMP; □—□, activity with AMP + caffeine (1 and 5 mM, respectively). Each point represents the average of 8 experiments and the vertical lines the standard errors.

The above conclusion was confirmed by chromatographic separation of the various forms of phosphorylase present in the frog muscle, before and after stimulation (Fig. 2). It is shown that before electrical stimulation the major part of phosphorylase could be eluted with 5 mM glycerophosphate and that this fraction was active only in the presence of AMP. These properties are those of phosphorylase *b* [11]. In Fig. 2B it appears that only a slight amount of the enzymes was eluted by 5 mM glycerophosphate whereas the main part of the enzyme came out of the column with 0.1 M glycerophosphate. This fraction was active in the absence of AMP but its activity was doubled by the addition of AMP and caffeine. These properties are those of the hybrid phosphorylase.

Effect of AMP and ATP on hybrid phosphorylase

It may be assumed that hybrid phosphorylase has the properties of both phosphorylase *b* and phosphorylase *a*, and that these properties could be important in the regulation of its activity in vivo. First of all, the effect of AMP and ATP may be important in respect of the regulation. The concentrations of these nucleotides in muscle are high enough (0.5 mM AMP and 7 mM ATP [12]) to influence the activity of phosphorylase. It is known that AMP (0.2 mM) activates phosphorylase *b* and that ATP (8 mM) completely counteracts this activating effect [13, 14]. On the other

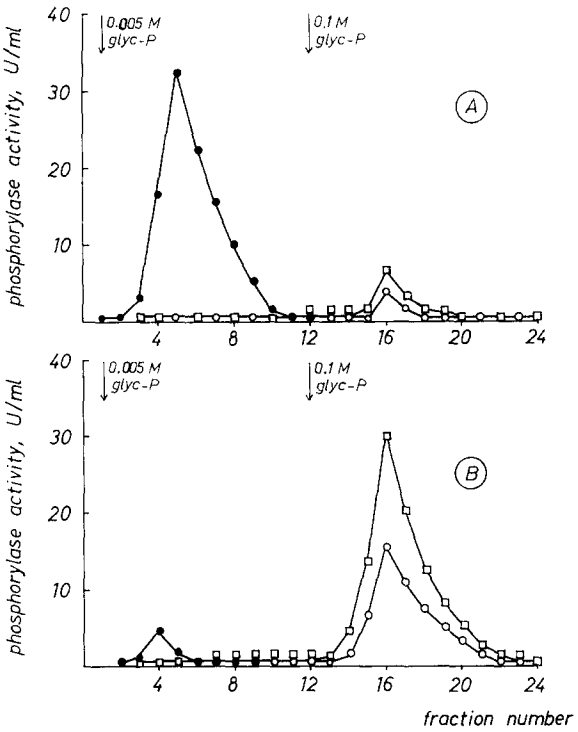

Fig. 2. Distribution of frog skeletal muscle phosphorylase before (A) and after (B) electrical stimulation. Frog musculus gastrocnemius before and after electrical stimulation were frozen in liquid air. Homogenates were made from the muscles as described in Methods, with a buffer consisting of 5 mM glycerophosphate, 0.04 M NaF, 0.01 M mercaptoethanol and 2 mM EDTA, pH 6.8. The homogenate (about 240 units phosphorylase, assayed with 1 mM AMP) was then chromatographed on a column, 1 cm × 15 cm, of DEAE-cellulose and eluted in 2-ml fractions at the rate 10 ml/h with buffer containing 5 mM or 0.1 M glycerophosphate, 0.04 M NaF, 0.01 M mercaptoethanol and 2 mM EDTA, pH 6.8. Phosphorylase activity: ○—○, without AMP; □—□, with AMP + caffeine (1 and 5 mM, respectively); ●—●, with AMP (1 mM).

hand, the activity of phosphorylase *a* is slightly increased by AMP and is not inhibited by ATP. There are no data available on the effect of AMP and ATP on hybrid phosphorylase. Therefore we investigated the effect of AMP in the absence and in the presence of ATP on the activity of hybrid as well as of phosphorylase *b* and phosphorylase *a* (Table II).

Table II shows that AMP doubles the activity of hybrid phosphorylase in the presence or the absence of ATP. The most significant difference between phosphorylase *b* and hybrid phosphorylase is that AMP does not activate phosphorylase *b* in the presence of ATP, but it is able to double the activity of hybrid phosphorylase under the same conditions. According to our results the hybrid form resembles phosphorylase *b* because AMP can activate it, and also resembles phosphorylase *a* because ATP can not inhibit its activity. This new property of hybrid species can play an important role in the regulation of enzyme activity in vivo. Therefore we investigated the effect of AMP and ATP at physiological concentrations on hybrid phosphorylase activity formed by electrical stimulation (Fig. 3).

TABLE II

EFFECT OF AMP AND ATP ON THE ACTIVITIES OF HYBRID PHOSPHORYLASE, PHOSPHORYLASE *a* AND *b*

Hybrid phosphorylase, phosphorylase *a* and *b* were prepared as described in Methods of the first part of this paper [1]. The activity of phosphorylase was measured in the presence or in the absence of effectors, the concentrations of effectors: 0.2 mM AMP or 0.2 mM AMP + 8 mM ATP. The activity assays were carried out according to the method of Illingworth and Cori [9]. Phosphorylase activities are expressed in percent of the activity measured in the presence of 0.2 mM AMP.

Effector	Phosphorylase activity (%)		
	Phosphorylase *a*	Hybrid phosphorylase	Phosphorylase *b*
–	85.1	54.2	0.0
+ AMP	100.0	100.0	100.0
+ AMP + ATP	98.9	96.8	4.2

Fig. 3 shows that the activity of phosphorylase was doubled in the presence of AMP and ATP (0.2 and 8 mM, respectively) during the whole time of stimulation. This fact supports the formation of hybrid phosphorylase.

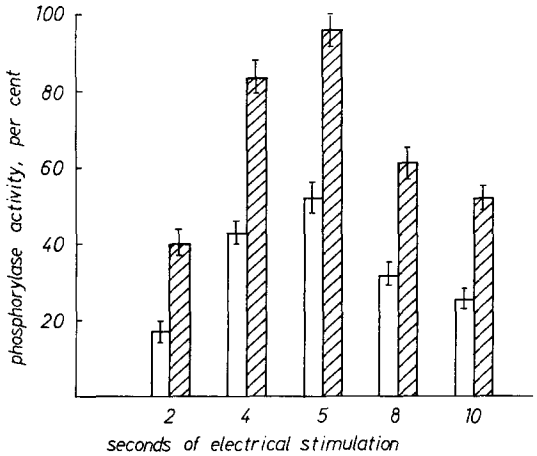

Fig. 3. Effect of AMP and ATP on the activity of hybrid phosphorylase formed in frog, musculus gastrocnemius. Electrical stimulation, the homogenization of the muscle are described in Methods. Open columns: activity without AMP and without ATP; hatched columns: activity with AMP + ATP (0.2 and 8 mM respectively). Phosphorylase activities are expressed in percent of total phosphorylase (activity with AMP, 1 mM).

DISCUSSION

The fact that after hormonal or electrical stimulation, phosphorylase was active in the absence of AMP and that this activity could still be increased by the addition of the nucleotide, had been previously interpreted as indicative of the presence of a mixture of phosphorylase *a* and of phosphorylase *b*. Our data indicate that this is not the case and that after maximal stimulation, phosphorylase *b* has

completely disappeared and has been converted into a hybrid whereas no phosphorylase *a* is present.

Hybrid phosphorylase formed in vivo permits a variety of regulatory mechanism because it remains an allosteric sensitive form. Hybrid phosphorylase can be activated by AMP and is simultaneously released from the inhibitory effect of ATP. Since hybrid phosphorylase consists of phosphorylated and non-phosphorylated subsubunits it could interact with both phosphorylase *b* kinase and phosphorylase phosphatase.

REFERENCES

1 Bot, G., Kovács, E. F. and Gergely, P. (1974) Biochim. Biophys. Acta 370, 70–77
2 Fischer, E. H. and Krebs, E. G. (1966) Fed. Proc. 25, 1511–1520
3 Mayer, S. E. and Krebs, E. G. (1970) J. Biol. Chem. 245, 3153–3160
4 Stull, J. T. and Mayer, S. E. (1971) J. Biol. Chem. 246, 5716–5723
5 Danforth, W. H., Helmreich, E. and Cori, C. F. (1962) Proc. Natl. Acad. Sci. U.S. 48, 1191–1199
6 Helmreich, E., Danforth, W. H., Karpatkin, S., and Cori, C. F. (1965) in Control of Energy Metabolism, (Chance, B., Estrabrook, R. W. and Williamson, J. R., eds), pp. 299–312, Academic Press, New York
7 Heilmeyer, L. M. G., Meyer, F., Haschke, R. H. and Fischer, E. H. (1970) J. Biol. Chem. 245, 6649–6656
8 Danforth, W. H. (1965) in Control of Energy Metabolism, (Chance ,B., Estrabrook, R. W. and Williamson, J. R., eds), pp. 287–297, Academic Press, New York
9 Illingworth, B. and Cori, C. F. (1953) in Biochemical Preparations, (Snell, E. E., ed.), Vol. 3, pp. 1–9, John Willey and Sons, Inc., New York
10 Metzger, B. E., Glaser, L. and Helmreich, E. (1968) Biochemistry, 7, 2021–2036
11 Davis, C. H., Schliselfeld, L. H., Wolf, D. P., Leavitt, C. A. and Krebs, E. G. (1967) J. Biol. Chem. 242, 4824–4833
12 Fischer, E. H., Heilmeyer, L. M. G. and Haschke, R. H. (1971) in Current Topics in Cellular Regulation, (Horecker, B. L. and Stadtman, E. R., eds), Vol. 4, pp. 211–251, Academic Press, New York
13 Morgan, H. E. and Parmeggiani, A. (1964) J. Biol. Chem. 239, 2435–2439
14 Morgan, H. E. and Parmeggiani, A. (1964) J. Biol. Chem. 239, 2440–2445