HYBRIDIZATION OF PHOSPHORYLASE *a* AND *b*

N.B. LIVANOVA, T.B. ERONINA and G.V. SILONOVA
Bakh Institute of Biochemistry, USSR Academy of Sciences, Moscow V-71, USSR

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1. Introduction

Muscle glycogen phosphorylase (EC 2.4.1.1) exists in two enzymatically interconvertible forms: phosphorylase *b*, the non-phosphorylated form with molecular weight 200,000 [1], which is inactive in the absence of AMP, and phosphorylase *a*, the phosphorylated form with molecular weight 400,000 [1], which can be fully active without AMP. The conversion of phosphorylase *a* into *b* form is catalysed by phosphorylase *a* phosphatase (EC 3.1.3.17), whereas the reverse reaction is catalysed by phosphorylase *b* kinase (EC 2.7.1.38) and requires Mg^{2+} and ATP [2,3].

Fischer et al. [4,5] proposed that phosphorylation and dephosphorylation of phosphorylase do not proceed as all-or-none reactions, but are step-wise processes in which partially phosphorylated intermediates are produced. Fischer et al. demonstrated that these partially phosphorylated hybrids are catalitycally active when assayed at high G1P * concentration and inactive at low G1P or when G6P is present.

We have studied the possibility of such partially phosphorylated hybrid formation in the mixture of phosphorylase *a* and *b* monomers.

2. Materials and methods

Crystalline phosphorylase *b* was prepared from rabbit skeletal muscle according to Fischer and Krebs [6] with some modifications [7]. Phosphorylase *a* was prepared from the four-times crystallized preparation of phosphorylase *b* with purified phosphorylase *b* kinase, Mg^{2+} and ATP [8]. Phosphorylase *a* was recrystallised three times. Protein concentrations were determined spectrophotometrically using the absorbancy index $A_{280}^{1\%} = 13.1$ [1,9]. Phosphorylase activity was assayed in the direction of glycogen synthesis by the method of Illingworth and Cori [10]. The enzyme activity was expressed as micromoles of P_i released per min per mg of enzyme.

G1P, G6P and AMP were obtained from Reanal, Hungary and were chromatographically pure. Sodium β-glycerophosphate was obtained from Merck and β-mercaptoethanol from Fluka. Rabbit liver glycogen was purified by passing it though a charcoal column [11].

Before hybridization the crystals of the two phosphorylases were collected by centrifugation, dissolved in 40 mM β-glycerophosphate pH 7.0 and dialyzed exhaustively against several portions of the same buffer for at least 36 hr at 2–4° to remove mercaptoethanol. Phosphorylase *b* was passed before dialysis through a charcoal column to remove nucleotides [12]. The solutions of phosphorylase *b* and *a* were mixed in the proportion 1:1. The protein concentration in the resulting mixture was 6–7 mg/ml. 1 ml of the solution containing 130 μmoles of glycerophosphate, 540 μmoles of KCl and 5 μmoles of *p*-CMB was added to 3 ml of the mixture of phosphorylase *a* and *b* and the final solution was allowed to stand for 3 hr at 20° to cause the dissociation of both phosphorylases into monomers [13]. Examination in the ultracentrifuge revealed practically complete monomerization of phosphorylase *a* and *b*. For reassociation and hybridization of the enzyme, mercaptoethanol was added to the mixture of

* Abbreviations: G1P, glucose 1-phosphate; G6P, glucose 6-phosphate.

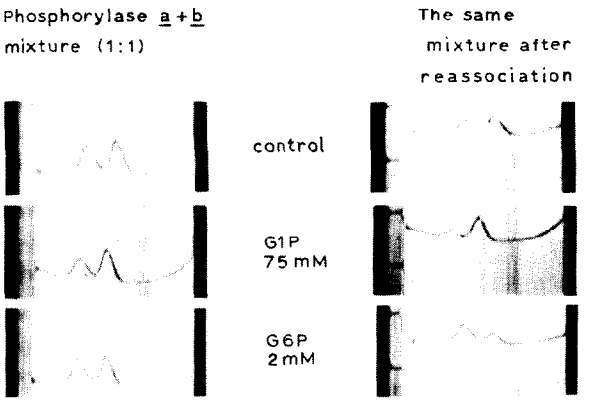

Fig. 1. Effect of G1P and G6P on sedimentation patterns of a mixture of phosphorylase *a* and *b* (left) or the same mixture after hybridization (right) at protein concentration 5–6 mg/ml in 50 mM glycerophosphate – 30 mM mercaptoethanol, pH 7.0. The pictures were taken 40 min after a speed of 52,640 rpm was reached at 20°C; bar angle, 60°; sedimentation from left to right.

monomers to a final conc. of 50 mM, and the solution was frozen and thawed several times. Then the solution was dialysed overnight against 30 mM mercaptoethanol – 40 mM β-glycerophosphate, pH 7.0 at 2–4°.

3. Results and discussion

After hybridization the mixture contained two components: of S_{20} = 8.6 and 13.2, though the proportion of the fast sedimenting component was slightly increased as compared with the initial mixture (fig. 1, upper patterns).

The behaviour of the initial mixture of phosphorylase *a* and *b* considerably differed from that of the same mixture after hybridization in the ultracentrifuge in the presence of G1P or G6P. When G1P or G6P was added to the initial mixture of phosphorylase *a* and *b* only a slight change occurred in the dimer to tetramer ratio either in favour of tetramer or dimer, respectively (fig. 1, left). This disproportionation may result from hybridization between the dimers of phosphorylase *a* and *b* (a_2b_2) *. These

* The subscripts show the numbers of monomers of phosphorylase *a* and *b*, respectively, in dimers or tetramers.

Table 1
The activity of the initial and reassociated mixture of phosphorylase *a* and *b* in the presence of 18 mM and 100 mM G1P *

Expt.	G1P (mM)	Activity (μmoles P_i/min/mg of protein)	
		The initial mixture ** of phosphorylase *a*+*b*	The same mixture after reassociation
1	18	11.0	15.3
	100	10.7	26.6
2	18	7.1	9.5
	100	7.1	15.0

* Assay was carried out in the absence of AMP in 50 mM glycerophosphate pH 6.8, containing 1% glycogen, the protein concentration in the sample was 25 μg per ml.
** The activity was tested without AMP, so only the activity of phosphorylase *a* could be detected in the initial mixture.

changes are much more pronounced in the mixture after reassociation (fig. 1, right). This seems to show that hybridization occurs between monomers of phosphorylase *a* and *b*, because such partially phosphorylated hybrids (*ab*) easily aggregate into tetramers on addition of G1P ($(ab)_2$; $(ab)b_2$; $(ab)a_2$) while on addition of G6P they mostly form dimers [4].

The activity assay also revealed that some formation of the hybrids took place in the mixture of phosphorylase *a* and *b* during reassociation. After reassociation a pronounced activation of the mixture with high concentration of substrate, G1P, is observed (table 1, right), while in the initial mixture there is no activation with high G1P concentrations (table 1, left).

The plots of initial velocity against G1P concentration for phosphorylase *a* in the original mixture (fig. 2, curve 1) and for the same mixture after hybridization (fig. 2, curve 2) are substantially different.

G6P is known to have no effect on the activity of phosphorylase *a* [14]; so it is quite natural that in the initial mixture in the absence of AMP it does not inhibit the enzymatic activity (fig. 3, curve 1). A different situation is observed in the same mixture after hybridization. The activity of this mixture at high concentration of G1P (75 mM) is strongly inhibited by G6P (fig. 3, curve 2).

The activation of the reassociated mixture with high concentrations of G1P and its inhibition with

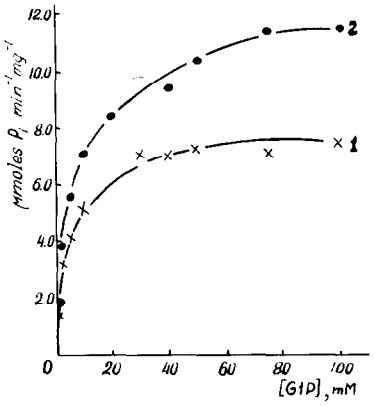

Fig. 2. The initial velocity of the original (curve 1) and reassociated (curve 2) mixture of phosphorylase $a+b$ as a function of the G1P concentration. Experimental conditions as described in the legend to table 1.

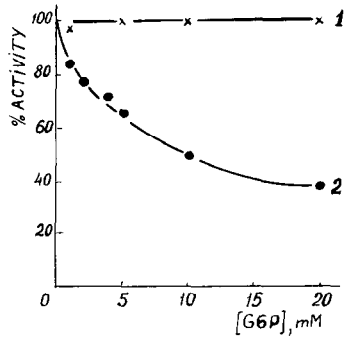

Fig. 3. Inhibition of the initial (curve 1) and reassociated (curve 2) mixture of phosphorylase $a+b$ by G6P. Experimental conditions are described in the legend to table 1; the concentration of G1P was 75 mM.

G6P seems to indicate that this mixture contains half phosphorylated dimers (ab). Such dimers appear to have a slightly lower affinity for G1P than does phosphorylase a, but higher than that of phosphorylase b [4], which is inactive in the absence of AMP. The hybrid dimers, unlike phosphorylase a, are sensitive to the inhibiting action of G6P.

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