PROBABLE FORMATION OF PARTIALLY PHOSPHORYLATED INTERMEDIATES IN THE INTERCONVERSIONS OF PHOSPHORYLASE a AND b*

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The conversion of phosphorylase a to b (EC 2.4.1.1) catalyzed by phosphorylase phosphatase (EC 3.1.3.17) results in a change in molecular weight from 500,000 to 250,000 (Keller and Cori, 1953) and the release of 4 moles of protein-bound phosphate (Graves et al., 1960). When this reaction was originally followed both by release of radioactivity from [^{32}P]-phosphorylase a and by loss of phosphorylase a activity, essentially identical rates were obtained (Graves et al., 1960). Re-examination of this reaction using purified phosphorylase phosphatase now indicates, however, that it can follow quite a different course if conditions for the assay of phosphorylase a are altered: when this enzyme is measured in the direction of glycogen synthesis at high concentrations of α-D-glucose 1-phosphate (G1P)†, almost 50% of the bound radioactivity could be released before phosphorylase a activity starts to decline.

The present note describes the influence of G1P and G6P on the interconversions of phosphorylase b and a catalyzed by either phosphorylase phosphatase or phosphorylase b kinase (EC 2.7.1.38; Krebs et al., 1956). The data presented herein seem to indicate that

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† Abbreviations used are as follows: G1P, α-D-glucose 1-phosphate; G6P, D-glucose 6-phosphate; AMP, adenosine 5'-phosphate.

during these interconversions, partially phosphorylated intermediate forms of the enzyme are produced whose enzymatic activity and molecular properties are neither those of phosphorylase b nor those of a, in that they are greatly affected by the concentration and relative proportion of the sugar esters.

METHODS

Phosphorylase b was a four-times crystallized preparation obtained from rabbit muscle by the method of Fischer and Krebs (1958). It was converted to phosphorylase a by the use of Mg^{++}, [^{32}P] ATP, and purified phosphorylase b kinase (Krebs et al., 1964) and the [^{32}P]-labelled phosphorylase a obtained (4,000 cpm/μmole enzyme monomer) was recrystallized three times. Phosphorylase b and a were assayed in the direction of glycogen synthesis by the method of Hedrick and Fischer (1965); final concentration of substrates in the assay was 2% glycogen, 0.1 M Na maleate, 2 X 10^{-3} M AMP when added and G1P at the concentrations indicated, all at pH 6.8. The phosphorylase phosphatase was a 2,000-fold purified preparation obtained from rabbit muscle (specific activity > 2 X 10^{4} umoles phosphorylase a "monomer" converted/minute/mg protein); its enzymatic activity was determined both by disappearance of phosphorylase a activity (measured in the absence of AMP) or by release of radioactivity (^{32}P) following trichloroacetic acid precipitation of the protein.

RESULTS AND DISCUSSION

Figure 1A shows a time course of the conversion reaction of phosphorylase a to b. When measured either by release of ^{32}P or by loss of phosphorylase a activity (assayed at low G1P concentration), both rates were essentially the same. If, however, phosphorylase a was assayed at concentrations of G1P > 0.05 M, no loss could be seen until approximately 50% of the protein-bound ^{32}P had been released. Figure 1B shows that addition of as little as 0.001 M G6P to the G1P

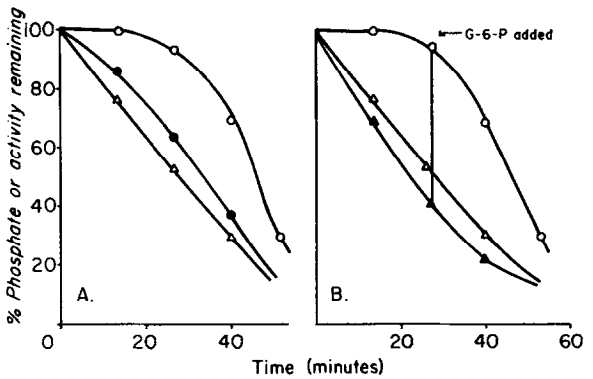

Figure 1.

A. Effect of G1P on phosphorylase activity during a to b conversion. Δ—Δ release of ^{32}P; phosphorylase a activity measured at .075 M G1P (o—o); and .015 M G1P (•—•).

B. Effect of G6P on phosphorylase activity during a to b conversion. Δ—Δ release of ^{32}P; phosphorylase a activity measured at .075 M G1P (o—o); and .075 M G1P + .001 M G6P (▲—▲).

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also suppressed the high initial enzymatic activity, bringing it to a level even below that of the bound phosphate. There is no effect of G6P on the activity of phosphorylase a itself. In these experiments, the sugar esters were not added during the phosphatase reaction, but only at the time of the phosphorylase assay.

Identical results were obtained whether the disappearance of phosphorylase a was measured at high enzyme dilution (in a glycero-glycerophosphate-cysteine rather than a maleate-mercaptoethanol buffer) or on concentrated enzyme solutions — in a 30-second "rapid assay" system — as long as the concentration of G1P was maintained above 0.05 M.

When the sugar esters were added to the phosphorylase a to b reaction itself, the release of bound phosphate was inhibited by G1P and accelerated by G6P (Figure 2). It is interesting to note that in the latter case, acceleration occurred only after 15 to 20% of the

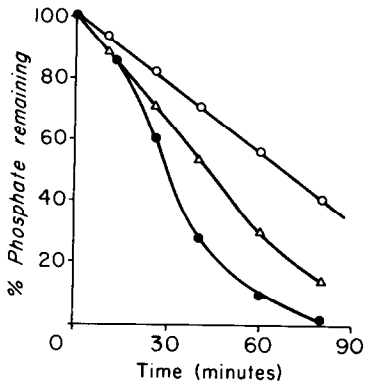

Figure 2. Effect of sugar esters on release of ^{32}P from phosphorylase a. Δ—Δ control; o—o .010 M G1P; ●—● .001 M G6P.

^{32}P had been released. Behavior of this sort could be explained if one assumed that intermediates formed during the reaction were more susceptible to phosphatase attack than the native molecule, perhaps due to dissociation to lower aggregates.

The following interpretation is proposed for the above results: the phosphorylase a to b reaction does not proceed in an all-or-none fashion in which all four phosphate groups present in phosphorylase a are hydrolyzed simultaneously. This kind of reaction would yield mixtures of fully phosphorylated molecules of a, and totally dephosphorylated molecules of b, in varying proportion. Rather, one could assume that removal of the four phosphate groups proceeds in a stepwise fashion, to give mixtures of molecules containing 4, 3, 2, 1 or no phosphate — in whatever state of aggregation they may be. When the reaction mixture is assayed whether the active species are in the tetrameric form as originally assumed for phosphorylase a, or in the dimeric form as proposed by Wang and Graves (1964), the partially phosphorylated intermediates will be catalytically active when assayed at high G1P concentration, but inactive at low G1P or if G6P is present. In other words, their apparent affinity for G1P would be intermediate between that of phosphorylase a (which is high) and that of b (which is so low that this form of the enzyme is inactive in the absence of

AMP); consequently, they would be detected enzymatically only at high substrate concentration.

The following evidence is consistent with the above assumption. a) The same species active at high G1P concentration can also be observed in the conversion of phosphorylase b to a catalyzed by phosphorylase b kinase (Figure 3). Full phosphorylase a activity is already attained at half ^{32}P incorporation and here again, this high activity is abolished by addition of G6P at 1/100 the amount of G1P. b) If the phosphorylase a to b reaction is interrupted half-way by the addition of .05 M NaF and the resulting mixture examined in the ultracentrifuge (Figure 4, right) it does not behave like a simple 1:1 mixture of crystalline phosphorylase a and b (Figure 4, left). Although there is some evidence of hybridization between phosphorylase b and a (increase in the proportion of the fast sedimenting component with G1P, and, conversely, decrease with G6P) this disproportionation is far less pronounced than observed with the mixture obtained at midpoint in the a→b reaction. Clearly, then this solution contains protein components different than those present in a mixture of phosphorylase a and b.

It remains to be determined whether or not these intermediates are produced in vivo, and if so, the extent to which they are involved in

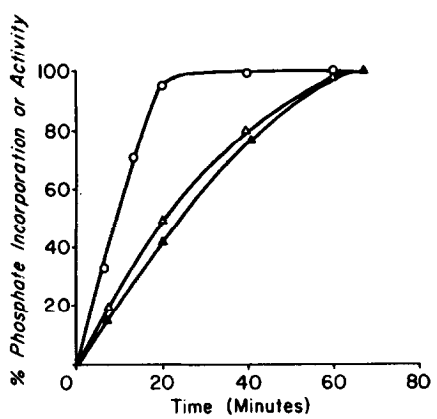

Figure 3. Effect of sugar esters on phosphorylase activity during b to a conversion. ▲—▲ ^{32}P incorporation; phosphorylase a activity measured at .10 M G1P (o—o); and .10 M G1P + .001 M G6P (▲-▲).

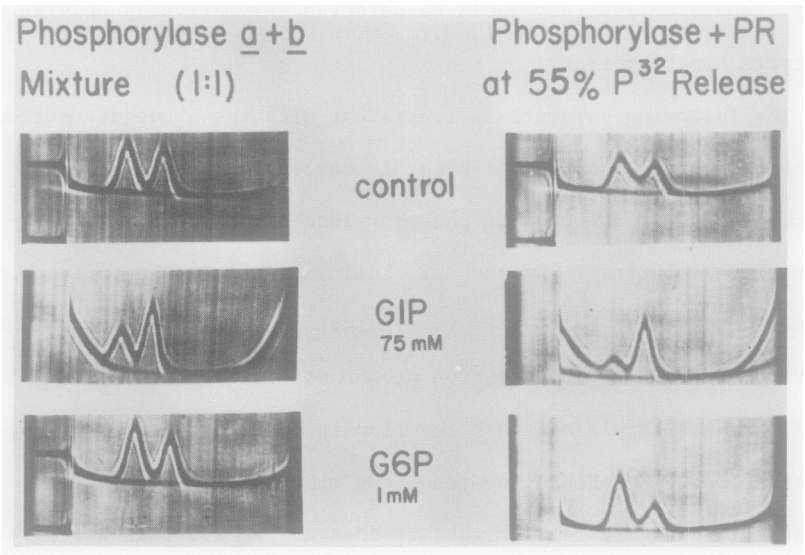

Figure 4. Effect of G1P and G6P on sedimentation patterns of mixture of pure phosphorylase b and a (left) or enzyme components obtained at 55% phosphate released during a b conversion catalyzed by phosphorylase phosphatase (right). Temperature, 24°; time, 24 minutes; bar angle, 65°; maximum xpeed, 59,780 rpm.

the process of glycogen breakdown. If they are physiologically operative, then their differential response to the relative concentrations of G1P, G6P and AMP might allow for a sensitive and effective control of glycogen utilization.

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