THE EFFECT OF GLUCOSE ON THE CONVERSION OF MUSCLE PHOSPHORYLASE a INTO b OR b'

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Summary : The stimulatory effect of glucose on the conversion of muscle phosphorylase a into b by phosphorylase phosphatase or b' by trypsin is AMP dependent at 30°, but not at 13°. These findings explain previous disagreement concerning the effect of the hexose on the inactivation of muscle phosphorylase a by its phosphatase. This glucose effect appears to be due to the binding of the hexose to phosphorylase a.

Holmes and Mansour (1) have shown that the conversion of muscle phosphorylase a into b by a crude diaphragm extract is stimulated by glucose. However, Torres and Chelala (2) did not observe this effect of glucose when using as a source of converting enzyme a muscle extract that had been filtered through Sephadex G-25. Furthermore, according to the work of Cori and Cori (3), purified muscle phosphorylase phosphatase is insensitive to glucose. On the other hand, an important stimulatory effect of glucose on the inactivation of liver phosphorylase by its phosphatase has been observed both in a liver Sephadex filtrate and in a partially purified preparation (4). Glucose has been found also to stimulate the inactivation of muscle phosphorylase a by a thyroid extract (5).

Trypsin converts phosphorylase a into b' by removing an oligopeptide that contains the serine phosphate (6). The reaction is inhibited by AMP (6) and stimulated by glucose, and the glucose effect is greater at 20° than at 30° (7,8). The similarities between the attack of phosphorylase a by its phosphatase and by trypsin have prompted us to study the effect of glucose on the two reactions in a parallel manner.

While this work was in progress, we have been informed that a similar study has been undertaken in the laboratory of Dr. W.J. Whelan at the University of Miami, Florida.

Material and Methods

Muscle phosphorylase a (30 I.U./mg at 30°) was obtained by activation of the crystalline b enzyme (9) with purified phosphorylase kinase (10), and was crystallized twice; when necessary, contaminating AMP was removed by treatment with Norit A (9). Phosphorylase phosphatase was purified from rabbit muscle (11). Extracts from mouse muscle were prepared and filtered on Sephadex G-25 as described for mouse liver (12). Crystalline trypsin, soybean trypsin inhibitor, and Norit A were purchased from Sigma Chemical Company. AMP was measured by the cycling procedure of Breckenridge (13) as modified by Van den Berghe et al.(14). The dimer-tetramer transition of phosphorylase a was measured by the fluorescence of the 2-methylanilino-naphtalene-6-sulphonate (MNS)-phosphorylase complex (15).

Conversion of phosphorylase a into b or b' : phosphorylase a, dissolved in 0.1 M glycylglycine, pH 7.4, was preincubated for 10 min at 30° or 30 min at 13°, in the presence of AMP and glucose, as indicated; a preparation of phosphorylase phosphatase or trypsin was then added, the concentration of phosphorylase a being at that moment 0.2 mg/ml. The reaction was stopped by adding one volume of either 0.2 M NaF or 0.1 % trypsin inhibitor; these solutions contained appropriate amounts of AMP and glucose to reach a uniform final concentration of each reagent within the same experiment. The mixtures were then incubated at 30° for 10 min in order to ensure full activity of phosphorylase a (16). Phosphorylase a was assayed at 30° as previously described (17), except that the substrate contained 1 mM caffeine and no AMP; the b and b'enzymes were completely inactive under these conditions.

Results

The effect of AMP on the sensitivity of the phosphorylase phosphatase reaction to glucose.

At 30°, the inactivation of muscle phosphorylase a by the phosphatase present in a muscle extract was 2- to 3-fold faster in the presence of 0.5 % glucose than in its absence (Fig. 1A); the reaction proceeded at a much faster rate when the extract had been previously filtered on Sephadex G-25, and then glucose was almost without effect (Fig. 1B and 2A). The filtration had therefore removed an inhibitor of the phosphatase as well as a micromolecule that renders the reaction sensitive to glucose. AMP, a well-known inhibitor of phosphorylase phosphatase (18), is rapidly formed in a muscle extract by hy-

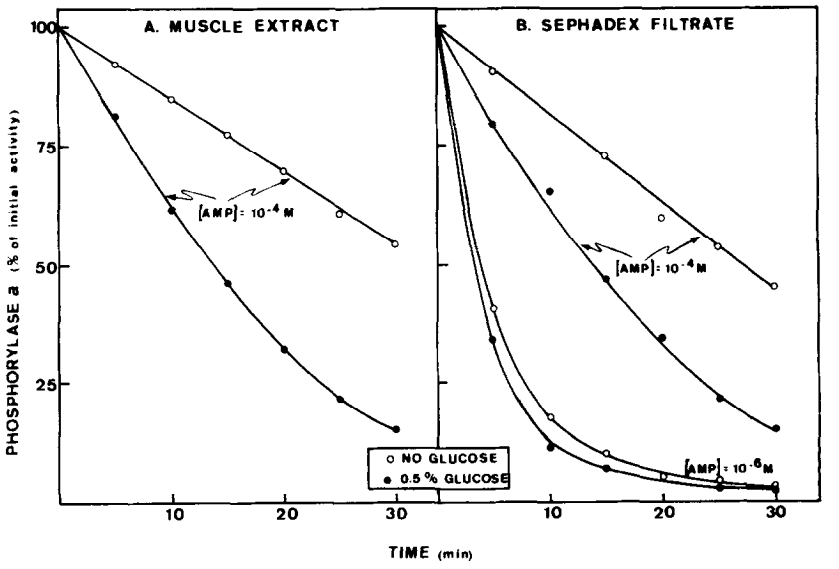

Fig. 1. The interaction of glucose and AMP in the conversion of phosphorylase a into b at 30°. A muscle extract (0.7 mg protein/ml) or a Sephadex filtrate (0.5 mg/ml), were used as a source of converting enzyme.

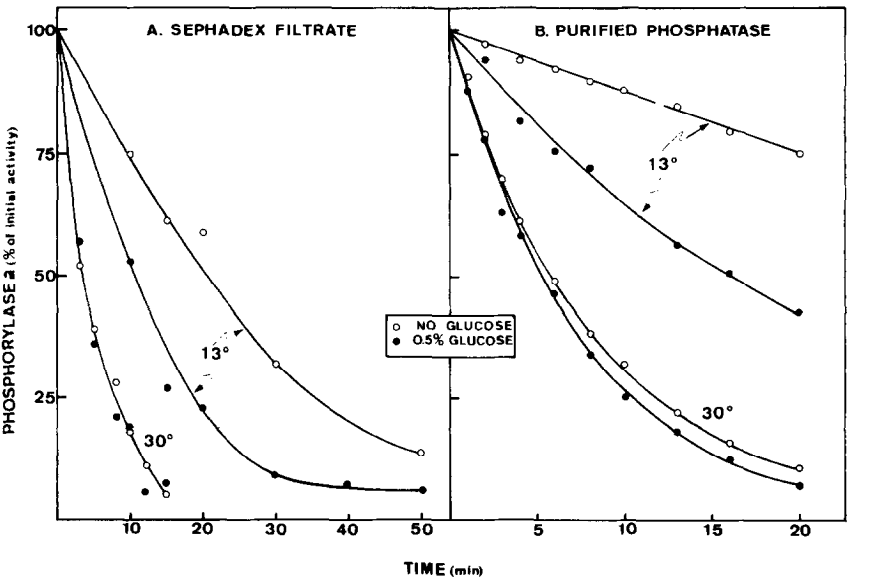

Fig. 2. The interaction of glucose and temperature in the conversion of phosphorylase a into b. A muscle Sephadex filtrate (0.6 mg protein/ml at 30°; 1,8 mg/ml at 13°) or purified phosphorylase phosphatase (5 µg/ml at 30°; 10 µg/ml at 13°), were used as a source of converting enzyme. The final concentration of AMP was 10^{-6}M in part A, 5 x 10^{-8}M in part B.

drolysis of endogenous ATP. Its concentration in the incubation medium was reduced from 10^{-4} M to 10^{-6} M by gel filtration of the extract. In the presence of 10^{-4} M AMP, the filtrate gave results similar to those observed with the original extract (Fig. 1B).

Similar results were obtained with purified phosphorylase phosphatase : at 30°, the reaction was almost insensitive to glucose (Fig. 2B) unless AMP was added (not shown). With this purified preparation, the requirement for AMP was one order of magnitude smaller than with the gel filtrate; this is probably due to binding of the nucleotide to other proteins in the latter preparation.

The influence of temperature on the sensitivity of the phosphorylase phosphatase reaction to glucose.

When the phosphorylase phosphatase reaction was run at 20°, the glucose effect was obtained even in the absence of added AMP. It was still more apparent at lower temperature. At 13°, a clear effect could be demonstrated both with a muscle Sephadex filtrate (Fig. 2A) and with the purified phosphorylase phosphatase (Fig. 2B). In the latter experiment, the concentration of AMP was 5×10^{-8} M.

The influence of glucose on the conversion of phosphorylase a into b'.

We show in Fig. 3A that at 30° and at a concentration of

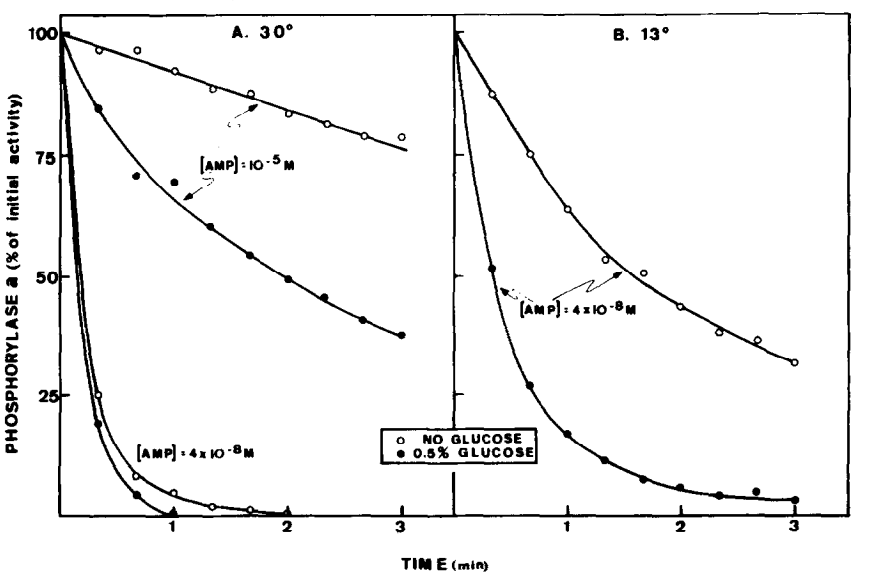

Fig. 3. The interaction of temperature, glucose and AMP in the conversion of phosphorylase a to b'. The concentration of trypsin was 20 µg/ml.

AMP equal to 4 x 10^{-8} M, glucose had little effect on the tryptic attack of phosphorylase a. The reaction was strongly inhibited by 10^{-5}M AMP but was then stimulated by glucose. At 13°, the glucose effect was obtained without addition of the nucleotide (Fig. 3B). 10^{-5}M AMP inhibited the reaction and enhanced the glucose effect (not shown).

The influence of temperature, glucose and AMP on the tetramer-dimer transition of phosphorylase a.

The fluorescence of the MNS-phosphorylase a complex was measured at the same protein concentration and ionic conditions as used in the enzymatic assays. Taking the fluorescence as an index of the state of aggregation (15), we have observed (Table 1) that raising the temperature from 13° to 30° induced the dissociation of the tetramer into dimer. The addition of glucose or of AMP at 30° had no effect, indicating that the dissociation of the enzyme at this temperature was complete. At 13°, glucose or AMP caused a partial dissociation.

Table 1. The effect of temperature, glucose and AMP on the fluorescence of the MNS-phosphorylase a complex. The fluorescence of MNS (20 µM) in the presence of phosphorylase a (0.2 mg/ml) and of 0.1 M glycylglycine at pH 7.4 was measured against a blank without phosphorylase. In each experiment, the same mixture was brought to a different temperature or received an addition of glucose (final concentration : 0.5 %) or of AMP (final concentration : 10^{-5}M) in the order as indicated. The changes of volume due to these additions did not exceed 0.1 %. The results are expressed as percent of the initial fluorescence at 13°.

Experimental Conditions		Relative Fluorescence			
Temperature	Addition	Expt. nr 1	Expt. nr 2	Expt. nr 3	Expt. nr 4
13°	-	100	100	100	100
30°	-	38	38	37	-
30°	Glucose	-	38	-	-
30°	AMP	-	-	37	-
13°	-	100	70	61	-
13°	AMP	-	69	-	54
30°	-	-	36	-	-
30°	Glucose	-	-	39	-

Discussion

The inactivation of phosphorylase a by its phosphatase at 30° was stimulated by glucose only in the presence of AMP. This requirement for the nucleotide explains why the glucose effect has been observed by Holmes and Mansour (1) working at 24° with a crude muscle extract as a source of phosphatase, but not by Cori and Cori(3) with a purified phosphatase at 27° or by Torres and Chelala (2) with a Sephadex filtrate at 30°.

There are several striking similarities between the inactivation of phosphorylase a by phosphorylase phosphatase and by trypsin. Both reactions are inhibited by AMP and stimulated by glucose; at 30°, the glucose effect was dependent upon the presence of AMP, whereas at 13° it was obtained in the virtual absence of the nucleotide. These similarities suggest strongly that glucose, like AMP, interferes in these reactions by binding to the substrate, phosphorylase a. Indeed, glucose is known to inhibit phosphorylase a (19) by binding to the glucose 1-phosphate site (20); on the other hand, glucose displaces the equilibrium between the dimeric and tetrameric forms towards the dimer (16). Raising the temperature to 30° has a similar effect (16).

In our experimental conditions the dissociation at 30°, as measured by the fluorescence method , was the same in the presence or absence of glucose. At 13°, part of the tetrameric species dissociated upon the addition of glucose. The glucose effect on the conversion of phosphorylase a into b and b' could therefore be explained by assuming that both the phosphatase and trypsin act better on the dimer than on the tetramer. Such a conclusion has been reached by Graves et al. (8) for the tryptic attack of phosphorylase a. However, the effects of AMP do not agree with this interpretation. The nucleotide appears to favor dissociation of phosphorylase a at 13°, and nevertheless inhibits the inactivation of the enzyme. Also, the antagonistic effects of AMP and of glucose on the inactivation of phosphorylase a at 30° are not reflected in changes in MNS fluorescence. Furthermore, glucose stimulates also the inactivation, whether by phosphatase (4) or by trypsin (21), of liver phosphorylase which seems not to form aggregates (22). These data suggest that the effects of glucose on dissociation and on enzymatic inactivation of phosphorylase a are not causally related.

At 30°, phosphorylase a is predominantly dimeric (16) and the effect of glucose on the enzymatic inactivation is to counteract

the inhibitory action of AMP. This antagonistic effect of the two ligands might be related to their specific affinity for two conformational states of the enzyme (20). It has also been studied at 20° by Wang and Black (23), who proposed a more complex model.

Since no free glucose occurs inside the muscle cell (24), the stimulation of phosphorylase phosphatase by the hexose has presumably little physiological implication. We will describe elsewhere (21) that, in the liver, the binding of glucose to its receptor, phosphorylase a, accounts for the regulation of glycogen metabolism by the level of glycemia.

Acknowledgements

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