Biochimica et Biophysica Acta, 452 (1976) 392—397

BBA 67967

A NOTE ON THE DUAL ROLE OF GLUCOSE IN THE PROTECTION OF GLUCOKINASE AGAINST INACTIVATION

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(Received February 25th, 1976)
(Revised manuscript received July 13th, 1976)

Summary

Protection against thermal denaturation, urea denaturation and tryptic inactivation of rat liver glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2) is provided by glucose and to a lesser degree sorbitol.

2. Protection by glucose exhibits two distinct stages, one occurring at the physiological substrate levels, while the other occurring well above that necessary for enzyme saturation. Sorbitol protection increases uniformly with increasing sorbitol concentration.

3. Several other enzymes also are protected against heat inactivation by high concentrations of glucose or sorbitol.

Introduction

Several workers have reported a protective influence of the substrate glucose on glucokinase (ATP:D-glucose 6-phosphotransferase, EC 2.7.1.2) and hexokinase activities [1—3]. In our studies on the purification of glucokinase from rat liver, we noted a protective effect of glucose on the heat denaturation of glucokinase in crude liver extracts [4]. The protective effect became significant only at glucose concentrations well above that necessary for saturation of the enzyme. Although the partially purified enzyme preparation could be heated at 48.5°C for 30 min, no improvement in specific activity was obtained, suggesting a general stabilizing effect of high concentrations of glucose on protein.

A widely recognized, though little understood and sparsely studied phenomenon is the remarkable stabilizing influence of polyhydric alcohols on enzymes

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[5—7]. It has been suggested that polyhydric alcohols might promote the formation of a more stable enzyme conformation [5] or interfere with subunit dissociation [7]. In view of the role of glucose as substrate and possible role of glucose as polyhydric alcohol in stabilizing glucokinase, we have extended our studies to investigate the influence of glucose on the stability of purified glucokinase from rat liver. D-sorbitol, a polyhydric alcohol, was shown to be neither a substrate, nor a competitive inhibitor of glucokinase [4]. Accordingly, we have compared the effect of D-sorbitol and glucose on stabilizing glucokinase against inactivation by trypsin and denaturation by heat and urea.

Experimental

Materials

Hexokinase type I (300 units/mg, 2 mg/ml), glucose-6-phosphate dehydrogenase from yeast, (350 units/mg, 5 mg/ml) and pyruvate kinase (200 units/mg, 2 mg/ml) from rabbit muscle were obtained from Sigma Chemical Co. Trypsin (11 000 units/mg) was purchased from P-L Biochemicals, Inc. Nutritional Biochemicals Corp. supplied the D-sorbitol. Pure glucokinase was prepared as described previously [4].

Methods

Assay of glucokinase [4], hexokinase [8], pyruvate kinase [9], glucose-6-phosphate dehydrogenase [10], aldolase [11] and lactate dehydrogenase [12], were by methods previously described. Purified glucokinase was kept in buffer consisting of 0.02 M potassium phosphate, 0.1 M KCl, 0.001 M EDTA, 0.01 M $MgSO_4$ and 0.01 M mercaptoethanol, pH 7.0. The same buffer was used for all the experiments described here, unless indicated otherwise. The specific activity of pure glucokinase is 80 units/mg, 0.5 units/ml.

Results

Effects on inactivation by trypsin

Trypsin rapidly inactivates partially purified glucokinase in the absence of high concentrations of glucose [1]. Pure glucokinase is also susceptible to trypsin inactivation but protection is afforded by glucose or sorbitol (Fig. 1a). This effect becomes more pronounced at concentrations of glucose well above that required for saturation of purified glucokinase (Fig. 1b). Increased resistance to trypsin inactivation is also observed with increasing amounts of sorbitol but to a lesser degree than that observed with glucose. Glucose does not interfere with the action of trypsin [2].

Effects on inactivation by urea

It has been reported [1] that high concentrations of glucose protect glucokinase against denaturation by urea. In the presence of 0.5 M glucose, glucokinase retains half its activity when incubated in 5 M urea for 5 min (Fig. 2a). Sorbitol, at a concentration of 0.5 M also affords some protection against urea denaturation. The lower levels (0.025 M) of glucose or sorbitol have only slight protective influence against denaturation by urea. Fig. 2b documents the in-

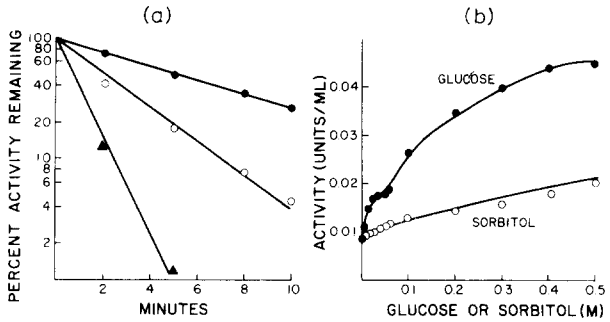

Fig. 1. Influence of glucose and sorbitol on the inactivation of glucokinase by trypsin. (a) Pure glucokinase (0.625 μg), trypsin (0.5 μg) and 0.5 M glucose (●), 0.5 M sorbitol (○) or 0.02 M potassium phosphate, 0.1 M KCl, 0.001 M EDTA 0.01 M $MgSO_4$, 0.01 M mercaptoethanol, pH 7.0 buffer (▲) in a final volume of 0.13 ml, were held at 22°C for the indicated time after which an 0.1-ml aliquot was removed for assay. (b) Pure glucokinase (0.625 μg) trypsin (0.5 μg) and glucose (●) or sorbitol (○) in a total volume of 0.13 ml, were incubated for 2.0 minutes at 22°C and 0.1-ml aliquot assayed.

creased protective effect of sorbitol and glucose with increasing concentration. Again the resistance to denaturation of glucokinase by urea is retarded by increasing concentrations of glucose and to a lesser extent by sorbitol.

Effects on inactivation by heat

At 48.5°C, in the presence of 0.5 M glucose, glucokinase exhibits remarkable stability (Fig. 3a). Sorbitol also retards thermal inactivation of glucokinase but not as effectively as glucose. In Fig. 3b, it is shown that at all concentrations glucose is a better protective agent than sorbitol but it is still noteworthy that activity is three times greater in the presence of 0.5 M sorbitol than in its absence after heating at 48.5°C for 15 mim.

Effects on other enzymes

Owing to the observed stability of crude protein solutions in the presence of high concentrations of glucose [4], several purified enzymes were examined for

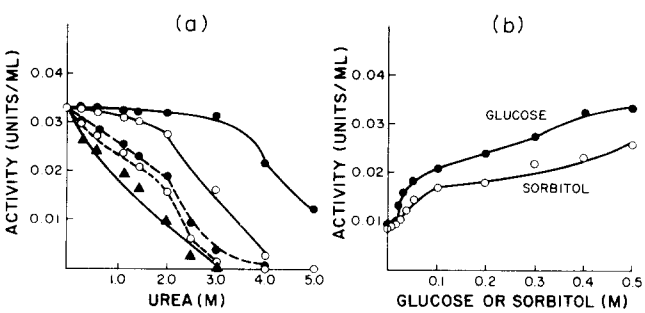

Fig. 2. Influence of glucose and sorbitol on the inactivation of glucokinase by urea. (a) Pure glucokinase (0.625 μg), urea and 0.5 M glucose (●————●), 0.025 M glucose (● - - - - - - ●), 0.5 M sorbitol (○————○), 0.025 M sorbitol (○ - - - - - - ○) or 0.02 M potassium phosphate, 0.1 M KCl, 0.001 M EDTA, 0.01 M $MgSO_4$, 0.01 M mercaptoethanol, pH 7.0 buffer (▲————▲) in a final volume of 0.24 ml were incubated for 5 minutes at 22°C and 0.2-ml aliquot assayed. (b) pure glucokinase (0.625 μg), urea (2.2 M) and varying concentrations glucose (●) or sorbitol (○) were treated as in 2 (a).

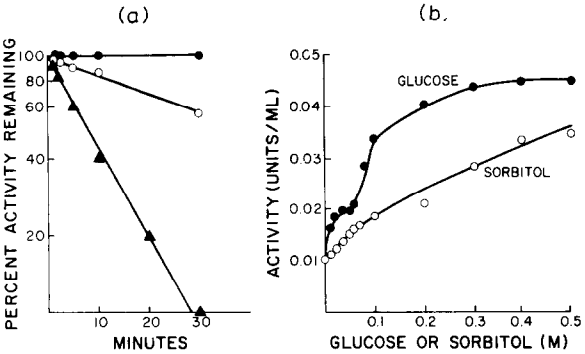

Fig. 3. Influence of glucose and sorbitol on the inactivation of glucokinase by heat. (a) Pure glucokinase (0.625 μg) and 0.5 M glucose (○), 0.5 M sorbitol (●) or 0.02 M potassium phosphate, 0.1 M KCl, 0.001 M EDTA, 0.01 M $MgSO_4$ and 0.01 M mercaptoethanol, pH 7.0 buffer (▲) in a final volume of 0.24 ml were held at 48.5°C for the times indicated after which 0.08 ml was assayed. (b) Pure glucokinase (0.625 μg) and varying amounts of glucose (○) or sorbitol (●) were held at 48.5°C for 10 min after which 0.08 ml was assayed.

the influence of glucose and sorbitol on their thermal stability. As shown in Fig. 4, hexokinase from yeast, muscle pyruvate kinase and glucose-6-phosphate dehydrogenase from yeast are all protected to varying degrees against loss of activity due to heating. Hexokinase protection by glucose is probably influenced by a substrate effect. Not shown but giving similar results were rabbit muscle aldolase and chicken heart lactate dehydrogenase.

Discussion

The observed stabilizing influence of glucose on glucose on glucokinase activity appears to be the result of two phenomena, one a substrate-protecting in-

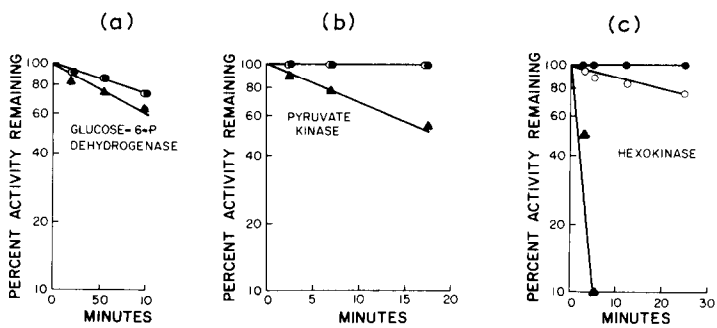

Fig. 4. Influence of glucose or sorbitol on the heat inactivation of other enzymes. (a) Glucose-6-phosphate dehydrogenase: Incubation mixtures consisting of 3.5 units of glucose-6-phosphate dehydrogenase plus 0.5 M glucose (●), 0.5 M sorbitol (○) or 0.05 M potassium phosphate buffer, pH 7.0 (▲) in a total volume of 0.4 ml were held at 50.1°C after which 0.005-ml samples were assayed. (b) Pyruvate kinase: Incubation mixtures consisting of 0.4 units of pyruvate kinase plus 0.5 M glucose (●), 0.5 M sorbitol (○) or 0.05 M potassium phosphate buffer, pH 7.0 (▲) in a total volume of 0.4 ml were held at 48.2°C after which 0.01 ml samples were assayed. (c) Hexokinase from yeast: Incubation mixtures consiting of 15 units of hexokinase plus 0.5 M glucose (●), 0.5 M sorbitol (○) or 0.05 M potassium phosphate buffer, pH 7.0 (▲) in a total volume of 1.0 ml were held at 47.2°C after which 0.05-ml samples were assayed.

fluence and the other a polyhydric-alcohol-stabilizing effect. The fact that concentrations of glucose well above that required for saturation are most effective and that sorbitol also has a protective influence suggests that the hydroxyl groups on glucose may play a role in stabilization. The greater efficiency of glucose over sorbitol and other polyhydric alcohols particularily at lower concentration, however, indicates that substrate binding at the active site has a significant protective effect.

It is noteworthy that the protective effect of glucose against denaturation by urea and heat and inactivation by trypsin appears to occur in two distinct stages. One occurs at glucose levels up to about 50 mM and the second above 50 mM. For all three types of inactivation, the protective influence changes little in the concentration range of 30 mM to 70 mM. Since the K_m for glucokinase is approximately 11 mM [4], the increase in protection up to 50 mM may reflect saturation of the active site, while above 50 mM, the protective effect may be attributed to a stabilization of conformation by glucose acting as a polyhydric alcohol.

Susceptibility of glucokinase and the other hexokinases to trypsin is well documented and at one time it was thought that the multiple hexokinases were the results of proteolytic activity [13]. The instability of glucokinase activity in crude liver extracts may result from the susceptibility of the enzyme to proteolytic inactivation. Indeed, the appearance of two forms of yeast hexokinase can be prevented if the purification buffers contain phenylmethylsulfonylfluoride [14].

Studies have shown that the low-K_m hexokinases from mammalian sources are also subject to glucose protection against rapid inactivation by trypsin [13] or urea [3]. In those studies relatively high (100—1000 mM) concentration of glucose were used. In one study [3] it was concluded that the protective influence of glucose was not related to its substrate role at all since the non-substrate sucrose was an effective protector but the substrate fructose was without significant influence.

We provide no direct evidence that stabilization is due in part to a direct interaction between glucose and free glucokinase. However, many enzymes are known to be stabilized by substrates or polyhydric alcohols although the mechanism of this stabilization is uncertain [5—7,15].

The initial interests in the protective influence of glucose arose as a result of purification studies on rat liver glucokinase [4]. It was shown that inclusion of 0.1 M glucose in polyacrylamide gels during electrophoresis allowed for the retention of glucokinase activity [16]. It was also demonstrated that glucokinase was stable to lengthy heating if sufficient glucose were present [4]. Improvements in specific activity were insignificant, however, owing to the lack of precipitation of protein in general. It is noteworthy that prevention of activity losses in the presence of glucose is also exhibited by pyruvate kinase, glucose-6-phosphate dehydrogenase, aldolase, lactate dehydrogenase, and hexokinase from yeast, although stabilization of hexokinase from yeast is to a good measure a substrate effect. Therefore the dual influence of high concentrations of polyhydric alcohols which are substrates for other enzymes may be a generalized phenomena which may prove useful in protein isolation and stabilization.

Acknowledgment

The author wishes to thank Dr. Van R. Potter and Dr. H. Campbell for their helpful discussions during the course of this work. Support was provided for this work by Training Grant T01-CA5002 and Project Grant CA-07175 from USPHS. The author is the recipient of a postdoctoral fellowship from the National Institutes of Health (IF22CA04033-01).

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