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Binding of sorbitol 6-phosphate and of fructose 1-phosphate to the regulatory protein of liver glucokinase

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Using a binding assay in which the ligand–protein complex is separated from free ligand by precipitation with poly(ethylene glycol) 6000, we found that the regulatory protein of rat liver glucokinase bound close to 1 mol of radiolabelled sorbitol 6-phosphate, a negative effector, or of fructose 1-phosphate, a positive effector, per mol of regulatory protein. Scatchard plots were linear, the dissociation constant being 0.3 μM for both phosphate esters. Sorbitol 6-phosphate and fructose 1-phosphate competed with each other for the binding. Competition was also observed with psicose 1-phosphate, ribitol 5-phosphate, arabitol 5-phosphate and 3-phosphoglycerate, all of which are known to affect the inhibition exerted by the regulatory protein. At a concentration of 10%, poly(ethylene glycol) 6000 decreased the concentration of regulatory protein causing 50% inhibition to a larger extent in the absence (12-fold) than in the presence (3-fold) of a saturating concentration of fructose 6-phosphate, another negative effector. Furthermore, it increased by about 3-fold the apparent affinity for inhibitory phosphate esters, indicating that it induced conformational changes of the regulatory protein.

INTRODUCTION

Rat liver contains a regulatory protein, which inhibits glucokinase (hexokinase IV or D) competitively with respect to glucose [1–3]. The inhibition exerted by this protein is greatly reinforced by fructose 6-phosphate, which promotes the association of the regulatory protein with glucokinase, and released by fructose 1-phosphate, which causes dissociation of the complex [2]. Several phosphate esters have effects similar to those of fructose 6-phosphate or fructose 1-phosphate. The most potent inhibitor that we found is sorbitol 6-phosphate, which acts at concentrations 4-fold lower than fructose 6-phosphate, whereas fructose 1-phosphate is the most powerful activator (or, strictly speaking, deinhibitor) [3]. We have postulated that the effect of these compounds is mediated by their binding to the regulatory protein, on the basis that they have no effect on glucokinase in the absence of regulatory protein [1]. Although there are kinetic and other data in support of this interpretation [2,3], no formal proof of the binding has yet been provided. In this paper, we show, by a method based on the precipitation of proteins with poly(ethylene glycol), that radiolabelled sorbitol 6-phosphate and fructose 1-phosphate bind to the regulatory protein.

MATERIALS AND METHODS

Materials

Poly(ethylene glycol) 6000 from UCB (Brussels, Belgium) was used; some batches from other companies were found to be contaminated by P_i ($\approx$ 3 μmol/g), known to have fructose 1-phosphate-like effects on the regulatory protein [3]. Fructose 1-[^{32}P]phosphate was synthesized by incubating [γ-^{32}P]ATP (10^9 c.p.m.) with 5 mM-fructose and 2 units of fructokinase/ml for 30 min at 20 °C in 0.5 ml, and purified by anion-exchange chromatography [3]. Glucose 6-[^{32}P]phosphate was synthesized as above with 1 mM-glucose and 7 units of yeast hexokinase/ml. It was purified and converted into sorbitol 6-[^{32}P]phosphate as in [3]. The regulatory protein was purified as previously described [2] up to the stage of hydroxyapatite chromatography and gel filtration on Sephadex G-25. Glucokinase was purified as in [2]. The source of other materials was as described previously [1–3].

Measurement of binding

The following reagents were successively added in an Eppendorf tube: 80 μl of a mixture containing 32 mM-Hepes, pH 7.1, 125 mM-potassium acetate, 0.25 g of poly(ethylene glycol) 6000/ml, 1.25 mg of defatted BSA/ml and 12.5% (v/v) ethanol; approx. 10^5 c.p.m. of sorbitol 6-[^{32}P]phosphate or fructose 1-[^{32}P]phosphate; the indicated concentrations of unlabelled phosphate esters; 8 units (as defined in [1]) or the indicated amount of regulatory protein. The volume was adjusted to 100 μl with water. Unless otherwise indicated, the mixture was incubated for 90 min at 19 °C. The tubes were then kept for 15 min at 0 °C and centrifuged at 10000 g for 10 min at 0–4 °C. The supernatant was carefully pipetted off, and the tubes were submitted to a brief ($\approx$ 10 s; 5000 g) centrifugation. The remaining supernatant was removed with a thin-tip pipette (Crystal, Eppendorf). The pellet was taken in 50 μl of water and the tube was washed with 100 μl of water. The two fractions were combined and counted for radioactivity in the presence of 5 ml HiSafe 2 (Pharmacia–LKB). Blanks were performed with 1 mM-sorbitol 6-phosphate or -fructose 1-phosphate and represented about 2% of the total radioactivity.

One of the critical steps in the procedure described above is the complete removal of the supernatant. Using ^3H_2O, we consistently found that the amount of water contained in the pellet represented about 2% of the total water. Another potential difficulty was in the preparation of the reaction medium: to obtain a homogeneous mixture, we first dissolved poly(ethylene glycol) in the Hepes/potassium acetate buffer; BSA was then dissolved in the resulting mixture, and ethanol was added last. The incubation was carried out at temperatures < 20 °C, since, at higher temperatures, the regulatory protein lost irreversibly its ability to inhibit glucokinase (e.g. 75% in 20 min at 25 °C). This loss was due to the presence of ethanol in the incubation mixture.

Enzymic measurements

Glucokinase was measured as previously described [2,3]. The

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regulatory protein was assayed by its inhibitory effect on glucokinase as described previously [1,2]. The dissociation constant of the regulatory protein–inhibitor complex (K_i) was determined kinetically as in [3], by using four different concentrations of regulatory protein ranging from 0.7 to 3 units/ml. The results shown are representative of 2–7 experiments that gave comparable results.

RESULTS

Binding studies

Because the available quantities of regulatory protein were limited, it was decided to use a binding assay method in which the ligand–protein complex is separated from free ligand. Gel filtration on spun columns [4] proved unsatisfactory because the recovery of regulatory protein was poor. No binding of radioactive sorbitol 6-phosphate or fructose 1-phosphate could be detected by using nitrocellulose filters [5] or hydroxyapatite [6], presumably because the complex dissociated during the washing step.

Precipitation of the ligand–protein complex with poly(ethylene glycol) [7,8], a protein precipitant thought to act by steric exclusion [9], was found to be suitable. At a final concentration of 20% (w/v), the polymer precipitated approx. 40% of the regulatory protein in the absence of ethanol, and > 97% in the presence of 10% ethanol. With this separation method, we found that the regulatory protein bound fructose 1-[^{32}P]-phosphate and sorbitol 6-[^{32}P]phosphate. Since the preparation used for this study was not homogeneous, it was decided to test if the binding of these phosphate esters was specific for the regulatory protein. Fig. 1 shows that, when the regulatory protein was chromatographed on Mono-Q, it co-eluted perfectly with the protein that bound sorbitol 6-phosphate and fructose 1-phosphate.

The time course of the binding of sorbitol 6-phosphate and fructose 1-phosphate at 19 °C is illustrated in Fig. 2; half-maximal binding occurred after 15 and 6.5 min at 0.2 μM- and 2 μM-sorbitol 6-phosphate respectively, and after 24 and 20 min for the same concentrations of fructose 1-phosphate. Fig. 3 shows that the Scatchard plot was linear for both phosphate esters. The dissociation constant was $0.31 \pm 0.05\,\mu$M for sorbitol 6-phosphate and $0.31 \pm 0.06\,\mu$M for fructose 1-phosphate (means $\pm$ S.E.M. for 7 observations) and the number of binding sites were 4.2 ± 0.3 and 3.7 ± 0.5 pmol/unit of regulatory protein (Fig. 3) respectively. Because of the slowness of the binding, these values might slightly underestimate the actual binding capacity of the regulatory protein.

As shown in Fig. 4, fructose 1-phosphate competed with the binding of sorbitol 6-phosphate, and reciprocally. Dixon plots [10] were linear, indicating complete inhibition (not shown). Table 1 summarizes the results of similar competition experiments performed with various phosphate esters acting as either inhibitor or deinhibitor of glucokinase in the presence of regulatory protein. All compounds tested completely competed with the binding of sorbitol 6-phosphate and fructose 1-phosphate with similar affinities. For the compounds with fructose 1-phosphate-like effects (fructose 1-phosphate, ribitol 5-phosphate, psicose 1-phosphate, 3-phosphoglycerate), the values derived from the binding studies were close to the dissociation constants (K_a) derived from the kinetic data [3]. In contrast, for the inhibitory compounds (sorbitol 6-phosphate, fructose 6-phosphate, arabitol 5-phosphate), the values obtained from the binding data were several-fold lower than the K_i. Glucose 6-phosphate, glucose 1-phosphate and *sn*-glycerol 3-phosphate, which do not affect the inhibition exerted by the regulatory protein [1,3], did not compete the binding when tested at 0.2 mM (results not shown).

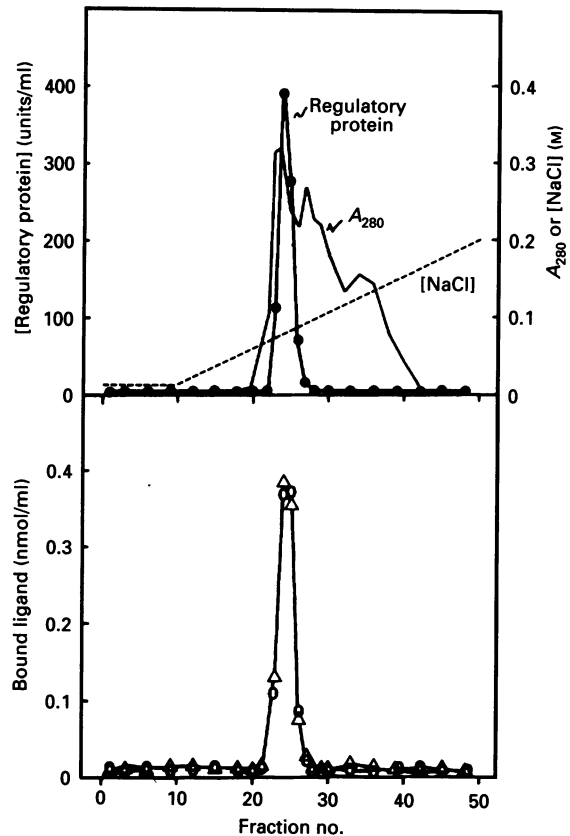

Fig. 1. Co-elution of the regulatory protein with a binding protein for sorbitol 6-phosphate and fructose 1-phosphate

A sample of purified regulatory protein (900 units in 500 μl) was diluted 5-fold with a buffer containing 25 mM-Hepes, pH 7.1, 10 mM-NaCl, 1 mM-dithiothreitol, 10 μg of antipain/ml and 10 μg of leupeptin/ml and applied to a Mono Q column (HR 5/5) equilibrated in the same buffer. The column was washed with 5 ml of dilution buffer and the retained protein was eluted with a NaCl gradient programmed in a f.p.l.c. system (Pharmacia); 1 ml fractions were collected. Binding was measured with 0.2 μM-sorbitol 6-[^{32}P]-phosphate (○) or fructose 1-[^{32}P]phosphate (△) on 10 μl of each fraction.

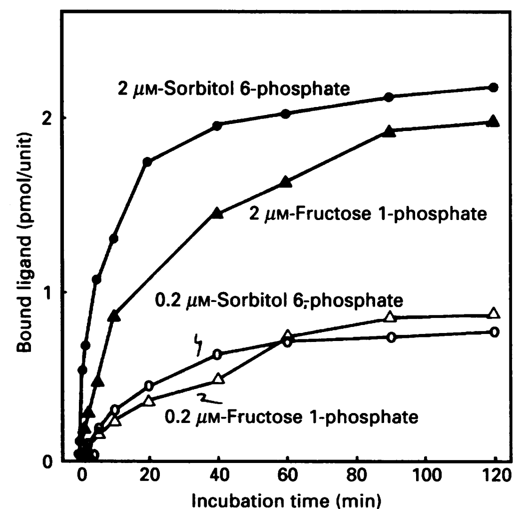

Fig. 2. Time course of the binding of sorbitol 6-[^{32}P]phosphate and fructose 1-[^{32}P]phosphate to the regulatory protein

The concentration of ligand was as indicated in the Figure. Results are expressed per unit of regulatory protein.

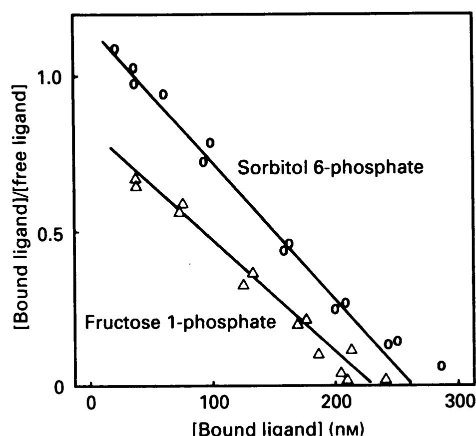

Fig. 3. Scatchard plot of the binding of sorbitol 6-[^{32}P]phosphate and fructose 1-[^{32}P]phosphate to purified regulatory protein

The regulatory protein was incubated for 30 min in the presence of sorbitol 6-[^{32}P]phosphate or for 60 min in the presence of fructose 1-[^{32}P]phosphate.

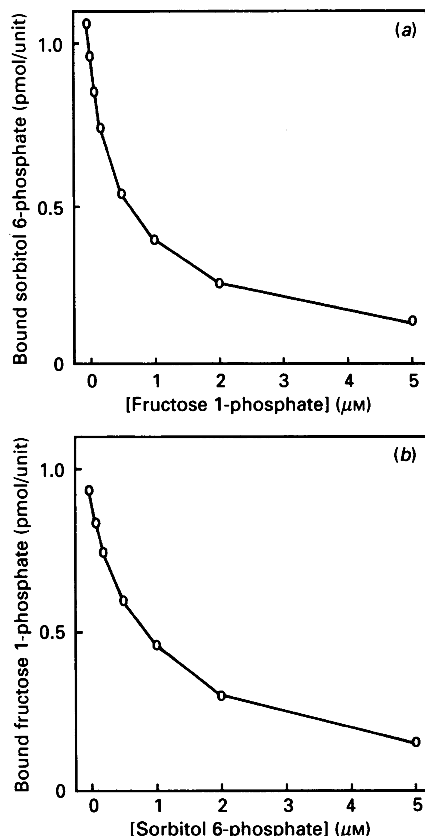

Fig. 4. Competition between sorbitol 6-phosphate and fructose 1-phosphate for binding to the regulatory protein

The binding of 0.2 μM-sorbitol 6-[^{32}P]phosphate (*a*) or -fructose 1-[^{32}P]phosphate (*b*) was measured in the presence of the indicated concentrations of the competitor.

To test the possibility that the binding of sorbitol 6-phosphate or of fructose 1-phosphate was covalent, the regulatory protein was incubated with radioactive ligand as described in the Materials and methods section, except that poly(ethylene glycol) and ethanol were omitted, and that the incubations were arrested by quenching in dilute H_3PO_4 as in [11]. No radioactivity was found to be associated with protein.

Table 1. Competition of the binding of radiolabelled sorbitol 6-phosphate or fructose 1-phosphate by various effectors of the regulatory protein

The same experimental conditions were used as in Fig. 4. The concentrations causing 50 % inhibition were estimated by Dixon [8] plots. K_a values are taken from [3]. K_i values marked * were calculated by using eqn. (10) of [3]. n.d.: not determined.

Competitor	[Competitor] (μM) causing 50 % inhibition of the binding of: Sorbitol-6-*P*	Fructose-1-*P*	K_a (μM)	K_i (μM)	K_i [+10 % poly(ethylene glycol)] (μM)
Fructose-1-*P*	0.6	–	0.5	–	–
Psicose-1-*P*	2.0	1.8	1.4	–	–
Ribitol-5-*P*	1.5	1.5	1.6	–	–
3-*P*-glycerate	100	110	73	–	–
Sorbitol-6-*P*	–	0.7	–	5 (*)	1.5
Arabitol-5-*P*	1.8	2.2	–	5 (*)	n.d.
Fructose-6-*P*	5.0	5.5	–	20 (*)	7.0

Kinetic measurements

Because of the discrepancy between the results obtained with the kinetic method and those obtained with the binding assay, it was decided to test the effect of poly(ethylene glycol) on the inhibition exerted by the regulatory protein. The polymer could not be used at a final concentration of 20 % because it increased the turbidity of the reaction mixture. At 10 %, it stimulated the glucokinase activity by about 50 % and decreased the concentration of regulatory protein required to cause 50 % inhibition from 16 units/ml to about 1.3 units/ml in the absence of fructose 6-phosphate and from 1 unit/ml to 0.3 unit/ml in the presence of 200 μM-fructose 6-phosphate (results not shown). Table 1 shows that inclusion of 10 % poly(ethylene glycol) in the assay also decreased by about 3-fold the K_i for fructose 6-phosphate and for sorbitol 6-phosphate.

DISCUSSION

The results show that the regulatory protein binds sorbitol 6-phosphate and fructose 1-phosphate, and thus support the mechanism of action of the regulatory protein that we previously proposed [2,3]. We have calculated that 1 unit of regulatory protein corresponded to 4.6 pmol of the 62 kDa monomer [2], a value close to that (5.0 pmol) derived from the specific activity of regulatory protein purified to homogeneity (3250 units/mg of protein; A. Vandercammen, unpublished work). The finding that up to ≈ 4 pmol of ligand can bind per unit of regulatory protein, together with the fact that the Scatchard plots were linear, indicates a binding stoichiometry of 1 mol of fructose 1-phosphate or sorbitol 6-phosphate per mol of regulatory protein.

The mutually exclusive binding of sorbitol 6-phosphate and of fructose 1-phosphate is consistent with the fact that these compounds exert competitive effects on the regulatory protein [1,3]. Furthermore, other compounds that act as inhibitors (fructose 6-phosphate) or as activators (psicose 1-phosphate, ribitol 5-phosphate, 3-phosphoglycerate) competed for the binding of the two radioactive ligands with almost identical affinities. These results suggest that sorbitol 6-phosphate and fructose 1-phosphate bind to a common site on the regulatory protein,

although we cannot formally exclude the existence of two distinct sites that, owing to steric hindrance or conformational constraints, cannot be accessible at the same time.

The constants calculated for the inhibitors from the competitions were significantly lower than the values derived from the kinetic data. The difference is most likely due to the presence of poly(ethylene glycol) in the binding assay, since the polymer increased the apparent affinity for sorbitol 6-phosphate and for fructose 6-phosphate by a factor of about 3. This is probably the consequence of a conformational change, which is further indicated by the fact that poly(ethylene glycol) increased to a larger extent the inhibition exerted by the regulatory protein in the absence of fructose 6-phosphate than in its presence.

The method used in this and in other studies (see, e.g., [7,8]) to measure the binding of ligand is potentially applicable to any protein which can be separated from free ligand by precipitation with poly(ethylene glycol). The procedure is rather simple and economical. Furthermore, washing can be avoided, thus preventing underestimation of binding owing to ligand leakage. One drawback, however, is that poly(ethylene glycol) can affect the value of the dissociation constants. Furthermore, the high viscosity of the medium is presumably responsible for the slowness of the binding (see Fig. 2).

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