Investigation of the mechanism by which glucose analogues cause translocation of glucokinase in hepatocytes: evidence for two glucose binding sites

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Glucokinase translocates between the cytoplasm and nucleus of hepatocytes where it is bound to a 68 kDa protein. The mechanism by which glucose induces translocation of glucokinase from the nucleus was investigated using glucose analogues that are not phosphorylated by glucokinase. There was strong synergism on glucokinase translocation between effects of glucose analogues (glucosamine, 5-thiogluco-*se*, mannoheptulose) and sorbitol, a precursor of fructose 1-phosphate. In the absence of glucose or glucose analogues, sorbitol had a smaller effect than glucose on translocation. However, sorbitol potentiated the effects of glucose analogues. In the absence of sorbitol the effect of glucose on glucokinase translocation is sigmoidal with a Hill coefficient of 1.9 suggesting involvement of two glucose-binding sites. The effects of glucosamine and 5-thiogluco-*se* were also sigmoidal but with lower Hill Coefficients. In the presence of sorbitol, the effects of glucose, glucosamine and 5-thiogluco-*se* were hyperbolic. Mannoheptulose, unlike the other glucose

analogues, had a hyperbolic effect on glucokinase translocation in the absence of sorbitol suggesting interaction with one site and was synergistic rather than competitive with glucose. The results favour a two-site model for glucokinase translocation involving either two glucose-binding sites or one binding-site for glucose and one for fructose 1-phosphate. The glucose analogues differed in their effects on the kinetics of purified glucokinase. Mannoheptulose caused the greatest decrease in co-operativity of glucokinase for glucose whereas *N*-acetylglucosamine had the smallest effect. The anomalous effects of mannoheptulose on glucokinase translocation and on the kinetics of purified glucokinase could be explained by a second glucose-binding site on glucokinase.

Key words: glucokinase, glucose analogues, translocation, hepatocytes.

INTRODUCTION

Glucokinase (hexokinase IV, EC 2.7.1.1) is the predominant glucose phosphorylating enzyme in hepatocytes of most mammals. It differs from the other hexokinase isoenzymes (I–III) in its lower molecular mass (50 compared with 100 kDa), its low affinity for glucose and in having sigmoidal kinetics with respect to glucose [1,2]. It is not inhibited by physiological concentrations of glucose 6-phosphate, but it is inhibited by a 68 kDa regulatory protein [3–5], which is present predominantly in the nucleus of hepatocytes [6]. Glucokinase shuttles between the nucleus and cytoplasm depending on the substrate conditions [6]. Translocation of glucokinase in response to substrates and hormones has been studied by immunostaining [6] and by a digitonin-release assay, which measures the distribution of enzyme between bound and free states [7]. Since the regulatory protein, unlike glucokinase, does not change its distribution between bound and free states [8], it is inferred that the digitonin-release assay measures changes in glucokinase binding to the regulatory protein. Because there is close agreement between changes in binding of glucokinase, determined by the digitonin-release assay [7], and the distribution of enzyme between the nucleus and cytoplasm [6], it is assumed that the free glucokinase activity represents the enzyme in the cytoplasmic compartment. Substrate-induced dissociation of glucokinase from the regulatory protein has a major role in the acute control of hepatic glycogen synthesis [7] and accounts for the high control coefficient of glucokinase on hepatic glycogen synthesis [9–11]. Dissociation of glucokinase from the regulatory

protein in the intact cell [7], or translocation to the cytoplasm [6], can be induced either by an increase in glucose concentration or by low concentrations of fructose or other precursors of fructose 1-phosphate (fructose 1-P). Whilst the latter effect is explained by the decrease in binding affinity of the regulatory protein for glucokinase by fructose 1-P [12], the effect of glucose on translocation has not been adequately explained.

Kinetic studies on the combined effects of the regulatory protein and *N*-acetylglucosamine, a competitive inhibitor of hexokinases, on the activity of purified glucokinase showed that these two inhibitors can bind to glucokinase simultaneously, indicating that the regulatory protein does not bind to the catalytic site [13]. Glucokinase is a monomeric enzyme with a single catalytic site [14] and is thought not to have additional (non-catalytic) glucose binding sites [2,14]. Consequently, effects of glucose on glucokinase translocation could be due to interaction of glucose with the catalytic site of glucokinase or with a glucose-binding site on the regulatory protein or the heterodimeric complex. Alternatively, metabolites of glucose could affect the affinity of the regulatory protein for glucokinase.

To distinguish between these possibilities we investigated the effects of glucose analogues that are not phosphorylated by glucokinase on the translocation of glucokinase. The results support a model whereby glucose induces translocation by interacting with two glucose-binding sites, one of which most likely represents an allosteric glucose-binding site on glucokinase that binds mannoheptulose. This questions the current view that glucokinase has only one glucose binding-site.

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; fructose 1-P, fructose 1-phosphate; MEM, Minimum Essential Medium.

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Figure 1 Effects of glucose and glucose analogues on glucokinase translocation

Hepatocyte monolayers were incubated for 30 min with the additions indicated: (A) varying concentrations of either glucose or glucose analogues; (B) varying concentrations of glucose either alone (○) or in the presence of 5 mM mannoheptulose (■), 2 mM 5-thioglucose (▼) or 20 mM glucosamine (◆). Free and bound glucokinase activities were determined as described in the Materials and methods section and free activity is expressed as a percentage of total activity. The results are representative of the experiments summarized in Table 1.

MATERIALS AND METHODS

Materials

D-Glucosamine, D-mannoheptulose, *N*-acetyl-D-glucosamine, 5-thio-D-glucose, 2-deoxy-D-glucose, bovine hexokinase, DEAE-Sephacrose, 6-aminohexanoic acid-Sephacrose 4B, 1-ethyl-3-(3-dimethylpropyl)carbodiimide were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Yeast hexokinase was from Boehringer-Mannheim (Mannheim, Germany). Glucose (Analar) was from BDH, Poole (Dorset). Sources of other materials were as in [11].

Hepatocyte monolayers

Hepatocytes were isolated from male Wistar rats (150–250 g body wt.) fed *ad libitum* by collagenase perfusion of the liver [15]. They were suspended in Minimum Essential Medium (MEM)

containing 7% (v/v) newborn calf serum and seeded either in 24-well plates (6×10^4 cells/cm²) for determination of glucokinase binding or on gelatin-coated (1 mg/ml) glass coverslips for immunostaining. After cell attachment (~4 h), the medium was replaced by serum-free MEM containing 10 nM dexamethasone [11].

Glucokinase compartmentation and glucose phosphorylation

After culture for 16 h, hepatocyte monolayers were incubated in fresh medium containing the additions indicated. The experiments in Figure 5 used MEM with the additions indicated. All other experiments used glucose-free Dulbecco's modified Eagle's medium (DMEM) supplemented with the additions indicated. Different batches of glucose were tested for contamination by fructose and sorbitol and a batch that was found to have no detectable contamination as determined by gas chromatography and mass spectrometry was used. For determination of glucose phosphorylation, the medium contained [2-³H]glucose (1.5 μ Ci/ml). On termination of the incubations the medium was collected in 0.1 M HCl for determination of ³H₂O [16]. Glucokinase distribution between free (cytoplasmic) and bound (nucleus) states was determined as in [10]. Hepatocyte monolayers were washed once in 150 mM NaCl and then permeabilized with digitonin for 8 min in medium containing 300 mM sucrose, 2 mM DTT, 5 mM MgCl₂, 0.04 mg/ml digitonin, pH 7.2 for determination of free glucokinase activity. Bound glucokinase activity was determined by extraction in 150 mM KCl, 3 mM Hepes, 2 mM DTT, 0.05 mg/ml digitonin, pH 7.2 [10]. The free glucokinase activity is expressed as a percentage of total activity. The total activity of glucokinase was 10–35 milliunits per mg of protein.

Glucokinase immunostaining

Hepatocyte monolayers on glass coverslips were cultured for 16 h and then incubated in DMEM with the substrates indicated. On termination of the incubation the coverslips were washed once in phosphate-buffered saline (PBS) and fixed for 30 min in 4% (w/v) paraformaldehyde in PBS and washed in PBS. Cells were treated first with 1 mg/ml sodium borohydride (10 min) then with 0.2% Triton X-100 (10 min) followed by 0.2% Triton X-100/1% BSA (10 min) all in PBS. The coverslips were washed in PBS after each treatment. The cells were incubated with primary antibody (sheep anti-glutathione S-transferase glucokinase fusion protein [17], kindly given by Dr. M. Magnuson, University of Tennessee) at a dilution of 1:100 in 0.1% Triton X-100/1% BSA overnight at 4 °C, followed by three washes (10 min each) with PBS. Incubation with secondary antibody (donkey anti-sheep FITC; Jackson Immunoresearch, Westgrove, PA, U.S.A.) was for 1 h at room temperature at a dilution of 1:100 in 0.1% Triton X-100/1% BSA followed by three 10 min washes in PBS and three quick washes in water and 100% ethanol. After drying, the coverslips were mounted onto microscope slides using Mowiol R-4088 (Aldrich, Gillingham, Dorset, U.K.) containing 2.5% diazabicyclo[2.2.2]octane (Sigma, St. Louis, MO, U.S.A.). Confocal images were obtained using a Bio-Rad scanning laser microscope (Bio-Rad, CA, U.S.A.) with a $\times 60$ oil immersion objective. The 488 nm line of an argon/krypton laser was used to excite the FITC fluorophore. For each field a series of 1 μ m optical sections were combined to produce a single image. Final rendering of the images was obtained using Microsoft Powerpoint Software.

Table 1 Effects of glucose analogues on glucokinase translocation

Hepatocytes were incubated for 30 min in medium containing a range of concentrations of the first analogue in the absence or presence of a second analogue at the concentration indicated. Free and bound glucokinase were determined by permeabilization with digitonin. The $S_{0.5}$ (for the first analogue) was determined from Hill plots (h = Hill coefficient) of the free glucokinase activity (see Figure 1). Values are means $\pm$ S.E.M. for the number of experiments shown in parentheses. Statistical analysis: $\dagger P < 0.05$, $\ddagger P < 0.005$ relative to glucose; $*P < 0.05$, $**P < 0.01$ effect of second analogue relative to the corresponding controls.

Second analogue	First analogue ...	Glucose	Mannoheptulose	5-Thiogluucose	Glucosamine
None					
$S_{0.5}$		12.2 $\pm$ 1.3 (12)	7.2 $\pm$ 0.4 $\dagger$ (21)	3.2 $\pm$ 0.2 $\ddagger$ (21)	8.1 $\pm$ 1.0 $\dagger$ (8)
h		1.9 $\pm$ 0.2	1.0 $\pm$ 0.05 $\ddagger$	1.3 $\pm$ 0.1 $\ddagger$	1.8 $\pm$ 0.3
10 mM glucose					
$S_{0.5}$		—	1.9 $\pm$ 0.1* (6)	5.3 $\pm$ 0.1 (5)	—
h		—	0.9 $\pm$ 0.08	1.9 $\pm$ 0.3	—
5 mM mannoheptulose					
$S_{0.5}$		6.6 $\pm$ 0.9* (5)	—	—	3.3 $\pm$ 0.7* (5)
h		1.4 $\pm$ 0.5	—	—	1.5 $\pm$ 0.2
2 mM 5-thiogluucose					
$S_{0.5}$		21.8 $\pm$ 3.4* (6)	—	—	5.0 $\pm$ 1.1** (4)
h		1.3 $\pm$ 0.4	—	—	1.9 $\pm$ 0.4
10 mM glucosamine					
$S_{0.5}$		—	3.0 $\pm$ 0.6* (4)	3.6 $\pm$ 1.2 (6)	—
h		—	1.1 $\pm$ 0.08	0.9 $\pm$ 0.1	—
20 mM glucosamine					
$S_{0.5}$		18.1 $\pm$ 3.6* (3)	—	—	—
h		1.6 $\pm$ 0.1	—	—	—
30 mM 2-deoxyglucose					
$S_{0.5}$		—	8.3 $\pm$ 1.1 (7)	9.3 $\pm$ 0.9** (5)	18.2 $\pm$ 2.6 (7)
h		—	1.1 $\pm$ 0.06	1.2 $\pm$ 0.1	0.8 $\pm$ 0.3
50 μ M sorbitol					
$S_{0.5}$		4.7 $\pm$ 0.5** (6)	1.3 $\pm$ 0.2* (5)	0.9 $\pm$ 0.2* (3)	—
h		1.1 $\pm$ 0.2	1.3 $\pm$ 0.2	1.0 $\pm$ 0.5	—

Glucokinase purification for enzyme kinetics

Rat liver glucokinase was overexpressed in hepatocytes using a recombinant adenovirus containing rat liver glucokinase cDNA, under the control of the cytomegalovirus promoter [18]. Hepatocyte monolayers in 6-well plates were treated with high titres (30 p.f.u./cell) of adenovirus for 1 h after cell attachment [10] and were then cultured in fresh MEM containing 10 nM dexamethasone and 10 nM insulin for 2–4 days. Glucokinase was extracted by permeabilizing the hepatocytes with digitonin (0.05 mg/ml) in 80 mM KCl, 20 mM potassium phosphate, 1 mM MgCl₂, 1 mM EDTA, 5 % glycerol, 2 mM DTT, 100 mM sucrose, pH 7.0. The glucokinase was purified by affinity chromatography using N-hexylglucosamine-Sepharose [19] (prepared by coupling D-glucosamine to CH-Sepharose 4B and diluted with unsubstituted Sepharose 4B [20]), followed by DEAE-Sepharose chromatography. The preparations had a specific activity $\sim$ 200 units/mg protein (using BSA as standard).

Glucokinase assays

For determination of free and bound glucokinase activity in the digitonin permeabilization assay, glucokinase was assayed spectrometrically coupled to glucose 6-phosphate dehydrogenase [10]. Either of two assays were used for the kinetic studies on purified glucokinase. For determination of the phosphorylation of the glucose analogues (mannose, 2-deoxyglucose, 5-thiogluucose, mannoheptulose) activity was determined by coupling ADP production to oxidation of NADH using pyruvate kinase and lactate dehydrogenase. The final assay contained 100 mM

KCl, 10 mM Hepes, 2 mM MgCl₂, 3 mM Mg-ATP, 0.25 mM phosphoenolpyruvate, 0.15 mM NADH, 1 mM KH₂PO₄, 4 U/ml pyruvate kinase and 10 U/ml lactate dehydrogenase pH 7.6 and varying concentration of the glucose analogues. For all other kinetic assays using glucose as substrate for glucokinase, yeast hexokinase or bovine heart hexokinase the glucose 6-phosphate dehydrogenase (*Leuconostoc mesenteroides*) linked assay was used. The final assay contained: 50 mM KCl, 25 mM Hepes, 2.5 mM ATP-Mg²⁺, 1 mM MgCl₂, 0.5 mM NAD, 1 mM DTT, 0.05 mg/ml BSA, 1.5 units/ml glucose 6-phosphate dehydrogenase pH 7.8 and varying glucose concentration. Inhibition constants (K_i) for yeast hexokinase and bovine heart hexokinase were determined over the glucose concentration range of 20–300 μ M and with four inhibitor concentrations. The apparent kinetic constants K_m^{app}/V^{app} at individual values of I were determined from primary plots of s/v against s (Hanes–Woolf plots). The competitive inhibition constant K_i was determined from secondary plots of K_m^{app}/V^{app} against inhibitor concentration (I) from the intercept on the I axis. For both yeast and bovine heart enzymes, the primary and secondary plots were linear indicating classical linear competitive inhibition. For rat liver glucokinase the inhibition constants were determined over the glucose concentration range of 7.5–20 mM glucose. These concentrations were used because of non-linearity of the plots at lower concentrations.

For determination of $S_{0.5}$ and Hill coefficients for substrate-induced translocation and for the kinetics of purified glucokinase at varying glucose concentration, the Fig. P (DOS version) Software was used (Biosoft, Cambridge, U.K.). Results are presented as means $\pm$ S.E.M. for the number of experiments indicated. Statistical analysis was by the Student's paired t -test.

RESULTS

Glucokinase inhibitors mimic the effect of glucose on glucokinase translocation

Previous studies showed that the glucokinase inhibitors mannoheptulose and glucosamine exert stimulatory and inhibitory effects, respectively, on glucokinase release in the presence of glucose [12,21]. Since mannoheptulose is a substrate for fructokinase [22], the possibility that its effects are due to binding of its phosphorylated derivative to the fructose 1-P site on the regulatory protein could not be excluded. In the experiments in Figure 1A the effects of mannoheptulose and glucosamine on glucokinase release were determined in the absence of glucose. Both inhibitors caused translocation of glucokinase. Their maximal effects were $71 \pm 6\%$ and $50 \pm 3\%$, respectively, of the effect of glucose ($n = 5$). 5-Thioglucose, a close analogue of glucose with the oxygen of the pyranose ring replaced by sulphur, also caused translocation with a maximal effect that was $82 \pm 2\%$ ($n = 5$) relative to glucose. The affinities of the glucose analogues are summarized in Table 1. The affinity for 5-thioglucose ($S_{0.5}$ 3.2 mM) was greater than for glucose, glucosamine or mannoheptulose. The effect of glucose was sigmoidal with a Hill coefficient of 1.9 and the effects of 5-thioglucose and glucosamine were also sigmoidal though with lower ($P < 0.05$) Hill coefficients. The effect of mannoheptulose in contrast was hyperbolic with a Hill coefficient of unity. The interactions of the glucokinase inhibitors with the effects of glucose on glucokinase release are shown in Figure 1B (and Table 1). Mannoheptulose, but not 5-thioglucose or glucosamine increased ($P < 0.05$) the affinity for glucose. It is noteworthy that the values for $S_{0.5}$ and Hill coefficients of the glucose analogues on glucokinase release (Table 1) are independent of variations in assay conditions such as digitonin concentration. However, the percentage of enzyme released in the absence or presence of glucose analogues is dependent on assay variables, such as the ratio of [digitonin]/cell protein, probably because the release of cytoplasmic glucokinase is incomplete. Accordingly, comparisons of differences in the maximum enzyme release for different analogues are valid for comparisons within the same sets of experiments.

The glucokinase inhibitors are not phosphorylated by glucokinase

Previous studies showed that 5-thioglucose is a linear competitive inhibitor of rat brain hexokinase and bovine heart hexokinase [23,24] and is poorly phosphorylated by brain hexokinase I [24]. Using purified rat liver glucokinase and the ADP-coupled assay we found no detectable phosphorylation of 5-thioglucose, at concentrations up to 30 mM. Using the same assay conditions, the phosphorylation kinetics of glucose ($S_{0.5}$ 8.3 mM, Hill coeff. 1.5), mannose ($S_{0.5}$ 19.0 mM, Hill coefficient 1.45) and 2-deoxyglucose ($S_{0.5}$ 140 mM, Hill coefficient 1.0) were in agreement with previous data [2]. Glucosamine and mannoheptulose were also not phosphorylated by glucokinase in agreement with previous findings [2]. This does not exclude the possibility that they may be phosphorylated by other kinases in the hepatocyte. However, it establishes that glucokinase-catalysed phosphorylation is not a pre-requisite for translocation by these analogues.

Synergism between glucose analogues and sorbitol on glucokinase translocation

Previous studies showed that sorbitol and fructose cause translocation of glucokinase in the presence of 5 mM glucose [8,12,21] through formation of fructose 1-P, which decreases the affinity of the regulatory protein for glucokinase [12]. Figure 2 shows that

Figure 2 Effects of sorbitol on the affinity for glucose analogues on translocation

Hepatocyte monolayers were incubated for 30 min with varying concentrations of glucose (A), 5-thioglucose (B), or mannoheptulose (C) in the absence (○, □, ▽) or presence (●, ■, ▼) of 50 μM sorbitol. Free glucokinase activity is expressed as a percentage of total activity. Means $\pm$ S.E.M. for 3–6 experiments. The affinity data are summarized in Table 1.

50 μM sorbitol markedly increases the affinities for glucose (~ 2.5 -fold), 5-thioglucose (~ 3 -fold) and mannoheptulose (~ 5 -fold) (affinity data are summarized in Table 1). Similarly, the glucose analogues markedly increased the affinity for sorbitol (Figure 3) by 2.5–11-fold [control (14) 33 ± 4 μM sorbitol; 10 mM glucose (7) $7 \pm 2^*$; 10 mM glucosamine (5), $14 \pm 2^*$; 5 mM 5-thioglucose (5), $3 \pm 1^*$; 10 mM mannoheptulose, $13 \pm 3^*$, means $\pm$ S.E.M., (n) $*P < 0.05$ relative to controls], indicating strong synergism between the effects of sorbitol and all glucose analogues tested. The maximum enzyme release in the presence

Figure 3 Effects of glucose analogues on the affinity for sorbitol on translocation

Hepatocyte monolayers were incubated for 30 min with the concentrations of sorbitol indicated either in the absence (○) or presence of 1 mM fructose (●), 5 mM 5-thioglucose (▼), 10 mM mannoheptulose (■), 10 mM glucosamine (◆). Free glucokinase activity is expressed as a percentage of total activity.

of saturating concentrations of sorbitol and glucose analogues was greater than with sorbitol alone (Figure 3). Glucose (10 mM) had a similar effect on glucokinase release in the presence of sorbitol (results not shown) as mannoheptulose and 5-thioglucose (Figure 3). This establishes that the greater effect of fructose and sorbitol shown previously in the presence of glucose [7,12,21] can be explained by the synergism between glucose and precursors of fructose 1-P.

As expected the effects of fructose and sorbitol were not additive with sorbitol (Figure 3). The additive effects of mannoheptulose and sorbitol establish that the effect of mannoheptulose is not due to its phospho derivative binding to the fructose 1-P site on the regulatory protein. Immunostaining for glucokinase in hepatocytes incubated with sorbitol (500 μM) or glucose (35 mM) either separately or in combination (Figure 4), confirmed the findings of the digitonin release assay that translocation of glucokinase from the nucleus is greater in the combined presence of glucose and sorbitol rather than with sorbitol alone and it was also greater than with 35 mM glucose alone.

Lack of correlation between glucokinase translocation and inhibition of glucose phosphorylation

To determine whether the affinity of the glucokinase inhibitors on translocation (Figure 1) correlates with their affinity for the catalytic site of glucokinase, the effects of these inhibitors on

Figure 4 Immunostaining of glucokinase in hepatocyte monolayers

Hepatocyte monolayers were incubated for 60 min in glucose-free DMEM either with no additions (a) or with 500 μM sorbitol (b), 35 mM glucose (c) or with 35 mM glucose + 500 μM sorbitol (d). They were fixed and stained for glucokinase as described in the Materials and methods section. The bar represents 25 μm.

Figure 5 The effects of glucose analogues on glucose phosphorylation and glucokinase translocation

Hepatocytes were incubated for 2 h in MEM containing $[2\text{-}^3\text{H}]\text{glucose}$ and either 7.5 mM glucose (A, D) or 7.5 mM glucose + 200 μM sorbitol, (B, E) or 35 mM glucose (C, F) and concentrations of inhibitors indicated (mannoheptulose ■, 5-thioglucose ▼, glucosamine ◆). Glucose phosphorylation (A–C) determined from detritiation of $[2\text{-}^3\text{H}]\text{glucose}$ is expressed as nmol of glucose/min per mg of protein. Free glucokinase activity is expressed as a percentage of total activity. Values are means $\pm$ S.E.M. for three experiments.

glucokinase translocation and glucose phosphorylation were determined in parallel in three substrate conditions: 7.5 mM glucose; 7.5 mM glucose + 200 μM sorbitol and 35 mM glucose (Figure 5). 5-Thioglucose was the most potent and mannoheptulose the least potent inhibitor of glucose phosphorylation in the three substrate conditions (Figure 5A–C). However, mannoheptulose was the most potent at causing translocation in incubations with glucose alone (7.5 mM or 35 mM) and it was equally potent as 5-thioglucose in incubations with sorbitol. Glucosamine, which caused a greater inhibition of glucose phosphorylation than mannoheptulose had little effect on translocation at 7.5 mM glucose and partly counteracted the effect of 35 mM glucose. The lack of correlation between inhibition of glucose phosphorylation and translocation suggests that the latter cannot be explained by the affinity of these compounds for the catalytic site. The effects of *N*-acetylglucosamine (5–35 mM) on glucokinase translocation and glucose phosphorylation were also tested. There was no effect of *N*-acetylglucosamine on glucokinase translocation determined in either the absence or presence of 5 mM glucose without or with 50 μM sorbitol (results not shown). In the latter situation inhibition of glucose phosphorylation (determined from detritiation of $[2\text{-}^3\text{H}]\text{glucose}$) by 35 mM *N*-acetylglucosamine was 20% compared with 70% inhibition by 35 mM glucosamine. The small inhibition by *N*-acetylglucosamine (despite its low K_i , see below) is consistent with previous findings that *N*-acetylglucosamine is poorly transported by hepatocytes [25].

Interactions of glucose analogues on glucokinase translocation

Since mannoheptulose but not the other inhibitors increased the affinity for glucose (Figure 1B and Table 1) the interactions of other glucose analogues were determined. The affinity for mannoheptulose was increased by 10 mM glucose (Figure 6A) and by

10 mM glucosamine (Figure 6B), whereas the affinity for 5-thioglucose was unaffected by glucose or glucosamine (Table 1). The affinity for glucosamine was increased by mannoheptulose and 5-thioglucose but it was decreased by 2-deoxyglucose. 2-Deoxyglucose had only a small effect on glucokinase translocation in the absence of other sugars (less than 10% of the effect of glucose). However, 30 mM 2-deoxyglucose partially counteracted ($P < 0.05$, for maximum enzyme release) the effects of glucose and 5-thioglucose but not mannoheptulose. In the presence of 2-deoxyglucose the effects of glucose did not fit either hyperbolic or sigmoidal curves and the affinity constant could not be determined.

Effects of glucose analogues on the co-operativity of purified glucokinase for glucose

The differences between mannoheptulose and the other glucose analogues on glucokinase translocation as regards hyperbolic rather than sigmoidal effects and synergistic rather than competitive effects with glucose raise the question whether mannoheptulose may be interacting by a different mechanism or through a different binding site. The effects of *N*-acetylglucosamine on the kinetics of glucokinase have been studied extensively [2,26]. Dixon plots are non-linear with a downward curvature at glucose concentrations less than 2 mM tending towards lack of inhibition by *N*-acetylglucosamine at decreasing glucose concentration. However, there have been few kinetic studies on the other analogues [2]. To investigate whether mannoheptulose differs from the other analogues in its interactions with glucokinase we compared the effects of these glucokinase inhibitors on the kinetics of purified glucokinase at varying glucose concentration. Because of the variation in 'apparent K_i ' of these inhibitors with glucose concentration [2], we compared these inhibitors by

Figure 6 Interactions of mannoheptulose and 5-thioglucoase with glucose or glucosamine on glucokinase translocation

Hepatocyte monolayers were incubated for 30 min with varying concentrations of mannoheptulose or 5-thioglucoase in either the absence ($\square$, ∇) or presence ($\blacksquare$, $\blacktriangledown$) of 10 mM glucose (A) or 10 mM glucosamine (B). Free glucokinase activity is expressed as a percentage of total activity. Results are representative of the experiments summarized in Table 1.

determining their effects on the $S_{0.5}$ for glucose and Hill coefficient at the concentrations of inhibitors shown in Table 2. Figure 7 shows the relation between the Hill coefficient and the $S_{0.5}$ for glucose for the different inhibitors. This shows that 5-thioglucoase and glucosamine cause a greater decrease in Hill coefficient (for a corresponding increase in $S_{0.5}$) than *N*-acetylglucosamine, whereas mannoheptulose causes the greatest decrease in the Hill coefficient.

Effects of inhibitors on bovine heart hexokinase and yeast hexokinase

Glucokinase shows similarities with the other mammalian hexokinases and with yeast hexokinase in terms of substrate affinities [27,28] and it shows greater similarities with yeast hexokinase in affinity labelling [14,29]. Because of the differences between mannoheptulose and the other glucose analogues on trans-

Table 2 Effects of glucokinase inhibitors on $S_{0.5}$ and Hill coefficients of glucokinase with respect to glucose

The activity of purified rat liver glucokinase was determined at different glucose concentrations (2.5, 5, 7.5, 10, 15, 30 and 100 mM) under the assay conditions described in the Materials and methods section and without or with the concentrations of *N*-acetylglucosamine (NAG), glucosamine (GCS), 5-thioglucoase (5TG) and mannoheptulose (MH) indicated. The Hill coefficient (h) and $S_{0.5}$ (mM, glucose) were determined from Hill plots. The values in the absence of inhibitors were $S_{0.5}$ 8.2 ± 0.07 mM glucose, h , 1.7 ± 0.02 . Values shown are means $\pm$ S.E.M. for four (NAG), five (GCS), three (5TG) and five (MH) determinations.

[Inhibitor]	NAG	GCS	5TG	MH
1 mM				
$S_{0.5}$	26 ± 2	12 ± 0.3	21 ± 0.2	13 ± 0.6
h	1.51 ± 0.02	1.56 ± 0.05	1.43 ± 0.05	1.35 ± 0.03
2.5 mM				
$S_{0.5}$	40 ± 1	19 ± 0.8	36 ± 0.4	24 ± 0.4
h	1.39 ± 0.04	1.46 ± 0.01	1.35 ± 0.01	1.22 ± 0.04
5 mM				
$S_{0.5}$		33 ± 3	63 ± 1	36 ± 2
h		1.34 ± 0.03	1.23 ± 0.01	1.18 ± 0.02

Figure 7 Effects of glucokinase inhibitors on the kinetics of purified glucokinase with respect to glucose

Values for $S_{0.5}$ (mM glucose) and Hill coefficients (h) were from Table 2 (mannoheptulose $\blacksquare$, 5-thioglucoase $\blacktriangledown$, glucosamine $\blacklozenge$, *N*-acetylglucosamine $\bullet$).

Table 3 Inhibition constants K_i (mM) for yeast hexokinase, bovine heart hexokinase and rat liver glucokinase

Inhibitor	Yeast hexokinase (K_i)	Heart hexokinase (K_i)	Rat glucokinase (apparent K_i)
<i>N</i> -acetylglucosamine	3.5	0.2	0.4
Glucosamine	1.4	0.3	0.9
5-Thioglucoase	1.3	0.2	0.5
Mannoheptulose	0.4	0.2	1.7

location and on the co-operativity of glucokinase with respect to glucose we compared the inhibition constants (K_i) for these analogues on yeast hexokinase and bovine heart hexokinase. The apparent K_i for rat liver glucokinase was determined at glucose

concentrations greater than 7.5 mM, because of non-linearity of the plots at lower glucose concentrations [2]. For rat liver glucokinase the apparent inhibition constants were lower for *N*-acetylglucosamine and 5-thiogluco-*se* than for glucosamine and mannoheptulose (Table 3). The inhibition constants for bovine heart hexokinase (K_m for glucose, 40 μ M) were 5–10-fold lower than for glucokinase and were similar for the four analogues. For yeast hexokinase (K_m for glucose, 200 μ M) the inhibition constant was 10-fold lower for mannoheptulose than for *N*-acetylglucosamine.

DISCUSSION

Translocation of glucokinase from a kinetically inactive state bound to the regulatory protein [7,8] in the nucleus [6] to the cytoplasm has a major role in the acute control of hepatic glucose metabolism and glycogen synthesis and accounts for the high control strength of glucokinase on glycogen synthesis [10,11]. The current view on the translocation of glucokinase between the nucleus and the cytoplasm is that the enzyme is retained in the nucleus in metabolic conditions that favour its binding to the 68 kDa protein and it translocates to the cytoplasm in conditions that favour dissociation from the regulatory protein [12]. Translocation of glucokinase by fructose and sorbitol [7,21] has been explained by the formation of fructose 1-P, which decreases the affinity of the regulatory protein for glucokinase [12]. However, less is known about the mechanism by which glucose causes translocation. The results of the present study support a model whereby translocation of glucokinase from the nucleus can be induced either by glucose alone acting through two glucose binding sites or by the combined effects of precursors of fructose 1-P (fructose or sorbitol) and glucose acting synergistically. It is suggested that one of the two glucose binding sites binds mannoheptulose and represents an allosteric glucose binding site on glucokinase that might also be involved in the co-operative kinetics of glucokinase with respect to glucose. This challenges the current view that the only glucose binding-site on glucokinase is the catalytic site. The assumption that glucokinase has only one glucose binding site was the basis for the kinetic models (mnemonic and slow transition) that were proposed to explain the sigmoidal kinetics of glucokinase with respect to glucose [2]. However, binding of two moles of affinity label to glucokinase has been reported [14].

The significance of the marked synergism between glucose and precursors of fructose 1-P found in the present study is that in the absence of glucose the effect of the latter compounds is smaller than the effect of glucose. Accordingly, since glucose alone (or the non-metabolizable analogue, 5-thiogluco-*se*) can cause greater translocation than saturating concentrations of sorbitol, it can be argued that glucose is the main regulator for glucokinase translocation and the role of fructose 1-P is to increase the affinity for glucose.

The proposed model that glucose causes translocation by interaction with two binding sites is supported by the following findings. First, that glucose analogues, that are not phosphorylated by glucokinase to a significant extent (e.g. 5-thiogluco-*se*) also cause translocation of glucokinase, indicating that the mechanism does not involve metabolites of glucose or glucokinase-catalysed phosphorylation. Second, the lack of correlation between the effects of glucokinase inhibitors on translocation and inhibition of glucose phosphorylation suggests that the effects of these compounds on translocation cannot be explained simply by their affinity for the catalytic site. Third, that in the absence of precursors of fructose 1-P, the effect of glucose is sigmoidal with a Hill coefficient of 1.9 suggesting involvement

of two glucose binding sites. Fourth, the additive effects of mannoheptulose and sorbitol and the hyperbolic effects of mannoheptulose indicate that its effect is independent of the fructose 1-P binding site on the regulatory protein and involves a single binding-site. Finally, the greater effect of mannoheptulose on the co-operative kinetics of purified glucokinase with respect to glucose compared with the other glucokinase inhibitors is consistent with a model whereby mannoheptulose interacts predominantly with an allosteric glucose binding site on glucokinase rather than with the catalytic site and that this site may have a role in the co-operativity of glucokinase. The low K_i of yeast hexokinase for mannoheptulose is also consistent with a model where the mannoheptulose binding site represents a hexokinase site.

A study of the effects of various alkylating agents on the covalent inactivation of glucokinase showed that glucokinase shares closer similarities with yeast than with mammalian hexokinase II. Like yeast hexokinase, glucokinase was not inactivated by *N*-bromoacetyl glucosamine which is a potent reversible inhibitor of these hexokinases [14,29]. However, glucokinase was irreversibly inactivated by the affinity label *N*-(*N*-bromoacetyl)-6-aminohexanoyl glucosamine [14]. Interestingly, glucokinase bound two moles of this affinity label. One of these sites also bound *N*-acetylglucosamine and this site accounted for the inactivation of glucokinase by the affinity label. Binding of the affinity label to the second site did not inactivate glucokinase, thus providing clear evidence that glucokinase has only one catalytic site and that *N*-acetylglucosamine (but not the affinity label) binds only to the catalytic site [14]. The significance of the second site that bound the affinity label was not investigated. It has since been assumed that this second site is non-specific [2].

A plausible explanation for the greater effect of mannoheptulose compared with *N*-acetylglucosamine on the co-operativity of purified glucokinase is that mannoheptulose and *N*-acetylglucosamine bind to different sites on glucokinase. Whereas *N*-acetylglucosamine binds only to the catalytic site [14], mannoheptulose may bind predominantly or exclusively to an allosteric site, and possibly the same site that bound the second molecule of affinity label [14]. The finding that 5-thiogluco-*se* and glucosamine, which are closer structural analogues of glucose than mannoheptulose and *N*-acetylglucosamine, respectively, had a larger effect on the co-operativity than *N*-acetylglucosamine (though a smaller effect than mannoheptulose) suggests that like glucose they may also bind to the allosteric or second site.

Previous findings that the regulatory protein can bind to glucokinase simultaneously with *N*-acetylglucosamine [13], but not with mannoheptulose [12], are consistent with the hypothesis that these compounds bind to different sites on glucokinase. An alternative explanation would be that they both bind to the catalytic site but have different effects on the conformation of the protein such that mannoheptulose but not *N*-acetylglucosamine decreases the affinity of glucokinase for the regulatory protein. Various kinetic models have been proposed to explain the sigmoidal kinetics of glucokinase with respect to glucose [2]. The slow transition model predicts that glucokinase exists in two conformational states [26]. One conformation represents a more thermodynamically stable state with a low affinity for glucose and predominates at low glucose concentration and the other conformation has a higher affinity for glucose and is favoured by high glucose concentration. The suppression of the co-operativity of glucokinase with respect to glucose by *N*-acetylglucosamine can then be explained if the inhibitor favours one conformation. Based on the observation that *N*-acetylglucosamine activates glucokinase at very low glucose concentration, the inhibitor is predicted to favour the conformation with higher affinity for

glucose [26]. An extension of this argument would be that mannoheptulose also suppresses the co-operativity by favouring one conformation. In this study we confirmed that *N*-acetylglucosamine (and to a lesser extent glucosamine) activates glucokinase at low glucose. However, similar behaviour was not observed for mannoheptulose. This suggests that if the slow transition model is applicable, then mannoheptulose may favour the less active conformation. If the hyperbolic kinetics of glucokinase release with mannoheptulose were due to trapping of enzyme in one conformation (the low affinity conformation) such a mechanism would not explain the synergism between mannoheptulose and glucose on glucokinase release (evident from the four-fold increase in affinity for mannoheptulose in the presence of glucose), since these ligands are predicted to favour different rather than the same conformation. A more plausible explanation for the synergism between glucose and mannoheptulose on glucokinase release is that mannoheptulose decreases the affinity of glucokinase for the regulatory protein (regardless of whether mannoheptulose binds to an allosteric or the catalytic site) consistent with the findings of Niculescu and colleagues that the regulatory protein and mannoheptulose cannot bind glucokinase simultaneously [12]. The simplest explanation for the hyperbolic kinetics of glucokinase release with mannoheptulose but not with the other glucose analogues is that mannoheptulose (but not glucose, 5-thioglucose or glucosamine) binds to only one site.

The extensive sequence similarity between yeast hexokinase, glucokinase (hexokinase IV) and the N-terminal and C-terminal halves of the mammalian 100 kDa hexokinases suggested that the latter enzymes evolved by duplication and fusion of an ancestral gene resembling the present-day yeast hexokinase [27,28]. The inhibition of the 100 kDa hexokinases by low concentrations of glucose 6-phosphate was suggested to be due to an allosteric site rather than the catalytic based on the lack of correlation between the ability of various hexoses to act as substrates for these enzymes and the inhibition by the corresponding hexose 6-phosphate [30]. Subsequent studies provided supportive evidence that the hexose 6-phosphate binding site was on the N-terminal half and the catalytic site on the C-terminal half. It was assumed therefore that the allosteric site on the N-terminal half represented the 'duplicated' catalytic site. However, later work showed that the isolated C-terminal half of hexokinase is also sensitive to hexose 6-phosphate and that both the N-terminal and C-terminal halves of hexokinase II each have a catalytic site and hexose 6-phosphate sensitivity [31]. Thus if hexose 6-phosphate sensitivity is due to a distinct allosteric site, then the 100 kDa isoenzymes may have evolved from an ancestral enzyme with two sugar binding sites. Accordingly, a second hexose binding site on glucokinase (hexokinase IV) is not inconsistent with this model.

Glucosamine and mannoheptulose are widely used as competitive inhibitors of glucokinase in studies on hepatocytes. Although *N*-acetylglucosamine is a potent competitive inhibitor of glucokinase it is inappropriate for studies on intact cells

because it is not taken up readily by hepatocytes [25]. 5-Thioglucose is not generally used as an inhibitor of glucokinase. Based on the present findings of the effects of mannoheptulose and 5-thioglucose on glucokinase translocation, glucose phosphorylation and on the kinetics of purified glucokinase, it is suggested that 5-thioglucose is a more appropriate competitive inhibitor of glucokinase for studies on intact hepatocytes. It should be the preferred alternative to mannoheptulose which is currently no longer available commercially.

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