Analysis of the structural requirements of sugar binding to the liver, brain and insulin-responsive glucose transporters expressed in oocytes

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We have expressed the liver (GLUT 2), brain (GLUT 3) and insulin-responsive (GLUT 4) glucose transporters in oocytes from *Xenopus laevis* by microinjection of *in vitro*-transcribed mRNA. Using a range of halogeno- and deoxy-glucose analogues, and other hexoses, we have studied the structural basis of sugar binding to these different isoforms. We show that a hydrogen bond to the C-3 position is involved in sugar binding for all three isoforms, but that the direction of this hydrogen bond is different in GLUT 2 from either GLUT 1, 3 or 4. Hydrogen-bonding at the C-4 position is also involved in sugar recognition by all three isoforms, but we propose that in GLUT 3 this hydrogen bond plays a less significant role than in GLUT 2 and 4. In all transporters we propose that the C-4 position is directed out of the sugar-binding pocket. The role of the C-6 position is also discussed. In addition, we have analysed the ability of fructopyranose and fructofuranose analogues to inhibit the transport mediated by GLUT 2. We show that fructofuranose analogues, but not fructopyranose analogues, are efficient inhibitors of transport mediated by GLUT 2, and therefore suggest that GLUT 2 accommodates D-glucose as a pyranose ring, but D-fructose as a furanose ring. Models for the binding sites of GLUT 2, 3 and 4 are presented.

INTRODUCTION

The facilitated diffusion of glucose across the plasma membranes of animal cells is mediated by a family of homologous, yet genetically distinct, glucose transporters. cDNAs encoding several members of this family have been identified, and each has been shown to exhibit a pronounced tissue-specific pattern of expression [for reviews, see Bell et al. (1990), Gould and Bell (1990), Mueckler (1990) and Waddell et al. (1992)]. To date, five glucose transporters (GLUTs) have been identified: the erythrocyte-type transporter (GLUT 1), which is expressed at highest levels in fetal tissues and is also found in placenta, erythrocytes, brain micro-vessels, kidney and colon (Mueckler et al., 1985; Birnbaum et al., 1986; Flier et al., 1987); the liver-type transporter (GLUT 2) expressed in the liver, pancreatic β-cells, kidney and small intestine (Thorens et al., 1988; Fukumoto et al., 1988; Orci et al., 1989); the brain-type transporter (GLUT 3) the mRNA for which is widely expressed in humans, but restricted to brain and neural cells in rodents (Kayano et al., 1988; Yano et al., 1991; Nagamatsu et al., 1992; Gould et al., 1992); the insulin-responsive transporter (GLUT 4) expressed only in tissues which exhibit acute insulin-sensitive glucose transport (fat, skeletal muscle and heart) (James et al., 1989; Birnbaum, 1989; Charron et al., 1989; Kaestner et al., 1989; Fukumoto et al., 1989); and the most recent addition to the family, the hepatic microsomal glucose transporter (GLUT 7), which is unique among the transporter family by virtue of its targeting to the endoplasmic-reticulum membrane. This transporter functions to allow the movement of glucose generated during glycogenolysis in the endoplasmic-reticulum lumen by the action of glucose-6-phosphatase into the cytoplasm (Waddell et al., 1992). An additional member of this family, GLUT 5, has also been identified and found to be expressed predominantly in the small intestine and at low levels in many other tissues (Kayano et al., 1990; Shepherd et al., 1992). However, recent studies have demonstrated that this protein is a high-affinity fructose transporter and its role in glucose transport is at present uncertain (Burant et al., 1992). GLUT 6 is an expressed pseudogene-like sequence (Kayano et al., 1990). These proteins exhibit between 39 and 65% amino acid identity (50–76% nucleotide similarity) and are predicted to possess identical overall structures and orientations in the plasma membrane. These transporters have all been functionally expressed either in oocytes or cell-culture systems (Gould and Lienhard, 1989; Keller et al., 1989; Gould et al., 1991; Waddell et al., 1992; Colville et al., 1993).

We have recently presented evidence that, although GLUT 1–4 all function to transport glucose, the nature of the glucose-binding sites on these proteins is subtly different (Gould et al., 1991; Colville et al., 1993). In addition, we have demonstrated that GLUT 2 (the liver-type glucose transporter) is unique among the transporters in that it is able to mediate the transport of D-fructose as well as D-glucose; in addition, GLUT 1, 2 and 3 all transport D-galactose (Gould et al., 1991; Colville et al., 1993). The unique ability of GLUT 2 to transport fructose and glucose, and the (relatively) high affinity of GLUT 3 for galactose, suggested to us that the nature of the interaction of the transported sugars with the transport proteins may be different between isoforms.

In a pioneering study, Barnett and his colleagues used a series of specifically substituted analogues of D-glucose to investigate the nature of the interaction of D-glucose with the erythrocyte-type transporter, GLUT 1 (Barnett et al., 1973, 1975). Those authors demonstrated that glucose interacted with the protein probably via hydrogen bonding at the 1, 3 and 4 positions of the glucose, and also by a potential hydrophobic interaction via the C-6 position. A similar model was also proposed independently (Kahlenberg and Dolansky, 1972). This approach has also been used successfully to examine the nature of the binding site for glucose in insulin-stimulated rat adipocytes (GLUT 4) (Holman et al., 1981; Holman and Rees, 1981).

Abbreviation used: GLUT, glucose transporter.
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Here, we present the results of a similar analysis of GLUT 2, 3 and 4 expressed in oocytes, and propose models for the interaction of the substrate D-glucose with these different transporter isoforms. In addition, we propose that GLUT 2 accepts D-fructose in the furanose, but not in the pyranose, form.

MATERIALS AND METHODS

Materials

Xenopus laevis were from the African Xenopus Facility (Noordhoek, South Africa). Restriction enzymes, SP6 polymerase and nucleotides were from Promega (Southampton, U.K.) and 5′ cap analogues were from Pharmacia (Milton Keynes, U.K.). β-D-Glucose, β-D-fructose, L-glucose, D- and L-mannose, D- and L-xylose, D- and L-arabinose and phloretin were from Sigma Chemical Co. (Poole, Dorset, U.K.). D- and L-fucose, D-allose, L-sorbose, maltose, lactose and cellobiose were from Pfanstiehl Laboratories (Waukegan, IL, U.S.A.). Recrystallized 4,6-*O*-ethylidene-D-glucose was a gift of Professor G. E. Lienhard (Dartmouth Medical School, Hanover, NH, U.S.A.). 3-*O*-Propylglucose, 1-deoxy-D-glucose, 6-deoxy-D-glucose, 6-*O*-propylgalactose, 6-fluorogalactose, 6-*O*-methylgalactose and 3-fluoroglucose were the gift of Dr. G. D. Holman (University of Bath, Bath, U.K.). All other sugars and analogues were generously given by Professor Mohammed Akhtar (University of Southampton, Southampton, U.K.). [2,6-^{3}H]-2-deoxy-D-glucose was from DuPont/NEN. All other reagents were as described by Gould et al. (1991).

Oocyte isolation and injection

Stage V and VI oocytes were individually dissected and injected with mRNA encoding the different glucose transporter isoforms as described by Gould et al. (1991). The oocytes were incubated in Barths buffer [88 mM NaCl/1 mM KCl/2.4 mM $NaHCO_3$/0.82 mM $MgSO_4$/0.41 mM $CaCl_2$/0.33 mM $Ca(NO_3)_2$/5 mM Hepes/NaOH (pH 7.6)/penicillin (10 μg/ml)/streptomycin (10 i.u./ml)] for 48–72 h before assay.

Plasmid construction and synthesis of mRNA

The human glucose transporter constructs used for the preparation of synthetic mRNA were described previously (Kayano et al., 1990). Linearized plasmid DNA was used as a template for mRNA synthesis which was performed as described (Gould and Lienhard, 1989; Gould et al., 1991).

Hexose transport in oocytes

Transport of deoxyglucose

Groups of six oocytes were incubated in 0.45 ml Barths buffer at pH 7.4 in 13.5 ml centrifuge tubes. Transport measurements were initiated by the addition of an aliquot of [2,6-^{3}H]deoxy-D-glucose to the concentration indicated in the Figures and Tables. The reaction was stopped after the requisite time interval by quickly aspirating the media and washing the oocytes with 3 ml of ice-cold PBS (150 mM NaCl/10 mM sodium phosphate, pH 7.4) containing 0.1 mM phloretin, a potent transport inhibitor (Krupka, 1971). The oocytes were washed in this fashion a further two times and dispensed to scintillation vials (1 oocyte/vial). These three washes were completed within 30 s; 0.5 ml of 1% SDS was added to each scintillation vial, and the vials incubated at room temperature for 1 h with agitation before addition of OptiPhase scintillant and measurement of radioactivity.

D-Fructose and D-galactose transport

Transport of these sugars was determined exactly as for 2-deoxyglucose. Groups of oocytes (typically five to ten) were incubated in Barths buffer at pH 7.4 in 13.5 ml centrifuge tubes and the transport rate measurement initiated by the addition of the appropriate sugar/radiolabel to the media. Uptake was stopped by three washes in ice-cold PBS containing 0.1 mM phloretin, and the radiolabel in each oocyte determined as for 2-deoxyglucose transport.

In all transport assays, the transport in control (non-injected) oocytes was measured in parallel, and the rate of uptake in injected oocytes was corrected for this background rate, which typically amounted to between 2 and 10% of the injected rate, depending on the population of oocytes used (see Thomas et al., 1993).

RESULTS

In all the following analysis, we have measured the effects of different sugar analogues on the transport of 2-deoxy-D-glucose into oocytes expressing GLUT 2, 3 or 4. The uptake of 2-deoxyglucose (100 μM) was determined for 30–60 minutes; owing to the low level of transport activity measured in oocytes expressing GLUT 4, we have used longer uptake times (i.e. 60 min) with this population (Thomas et al., 1993). We have previously shown that, under these conditions, the transport of 2-deoxyglucose is rate-limiting, and not its subsequent phosphorylation by hexokinase (Gould et al., 1991; Colville et al., 1993). The choice of 2-deoxy-D-glucose as the substrate for this analysis was based upon our observation that D-mannose (the C-2 position epimer of D-glucose) is as effective an inhibitor of transport by GLUT 2, 3 and 4 as D-glucose itself (Gould et al., 1991). This observation, together with our previous demonstration that 2-deoxy-D-glucose is transported with a lower K_m than other sugar analogues such as 3-*O*-methyl-D-glucose (Gould et al., 1991; Colville et al., 1993), all point to an insignificant role of hydrogen-bonding at the C-2 position in the recognition of the transported sugar (see below). This has previously been suggested for the erythrocyte-type transporter, GLUT 1 (Kahlenberg and Dolansky, 1972; Barnett et al., 1973). Moreover, the use of 2-deoxyglucose rather than 3-*O*-methyl-D-glucose permits an analysis of the outward-facing binding site of the transporter specifically, since 2-deoxyglucose is rapidly phosphorylated by hexokinase, and thus, unlike 3-*O*-methyl-D-glucose, is trapped inside the oocyte resulting in a negligible efflux of sugar.

Effects of C-1 and C-2 position analogues on 2-deoxyglucose transport

We have examined the ability of 1-deoxy-D-glucose to inhibit the transport of 2-deoxyglucose mediated by GLUT 2, 3 and 4. In addition the effects of a series of 2-position analogues (β-D-mannose, 2-chloroglucose and 2-deoxyglucose) were examined. The results of this analysis are presented in Table 1.

Effects of C-3 position analogues on 2-deoxyglucose transport

The effects of C-3 position analogues on the transport of 2-deoxyglucose by GLUT 2, 3 and 4 is shown in Table 2. The

Table 1 Effects of C-1 and C-2 position analogues on 2-deoxyglucose transport by GLUT 2, 3 and 4

Groups of six oocytes expressing the transporter of interest were incubated in Barths buffer containing the competing sugar for approx. 5 min before the addition of [2,6-^{3}H]2-deoxyglucose (100 μM; 0.25 μCi/assay). Transport was determined for 30–60 min. After this time, the oocytes were washed and the radiolabel associated with each oocyte determined as described in the Materials and methods section. The results shown below are from a typical experiment, repeated at least twice for each analogue/transporter. Rates are expressed as a percentage of the rate measured in the presence of 10 mM L-glucose, and are the means ± S.D. for six oocytes and for each analogue. Note that the inclusion of L-glucose did not alter the transport rate compared with oocytes assayed in the absence of any other sugars. Values which are statistically different from the L-glucose rates are indicated by *. Transport rates were between 3 and 5 pmol/min per oocyte for GLUT 2 and 3, and between 0.5 and 1 pmol/min per oocyte for GLUT 4.

Competing sugar (all at 10 mM)	Relative 2-deoxyglucose transport rate (%)		
	GLUT 2	GLUT 3	GLUT 4
L-Glucose	100	100	100
D-Glucose	24 ± 4*	10 ± 2*	13 ± 4*
1-Deoxyglucose	109 ± 10	104 ± 12	79 ± 12
2-Chloroglucose	76 ± 5*	42 ± 3*	40 ± 7*
2-Deoxyglucose	20 ± 2*	12 ± 2*	14 ± 9*
D-Mannose	29 ± 3*	14 ± 2*	13 ± 7*

Table 2 Effects of C-3 position analogues on 2-deoxyglucose transport by GLUT 2, 3 and 4

The results shown below are from a typical experiment, repeated at least twice for each analogue/transporter. In all cases competing sugars were at 10 mM in the assay mixture, and the assay conditions were as described in the legend to Table 1. Rates are expressed as a percentage of the rate measured in the presence of 10 mM L-glucose and are the means ± S.D. for six oocytes and for each analogue. Note that the inclusion of L-glucose did not alter the transport rate compared with oocytes assayed in the absence of any other sugars. Values which are statistically different from the L-glucose rate are indicated by *. Transport rates were between 3 and 5 pmol/min per oocyte for GLUT 2 and 3, and between 0.5 and 1 pmol/min per oocyte for GLUT 4.

Competing sugar (all at 10 mM)	Relative 2-deoxyglucose transport rate (%)		
	GLUT 2	GLUT 3	GLUT 4
L-Glucose	100	100	100
D-Glucose	18 ± 3*	8 ± 2*	18 ± 4*
3-Deoxyglucose	103 ± 12	85 ± 7	106 ± 12
3-Bromoglucose	95 ± 10	75 ± 4*	79 ± 12
3-Fluoroglucose	85 ± 5*	14 ± 2*	40 ± 6*
3-*O*-Methyl-D-glucose	73 ± 6*	17 ± 3*	41 ± 7*
3-*O*-Propyl-D-glucose	100 ± 12	80 ± 7	72 ± 8*
D-Allose	59 ± 6*	96 ± 7	96 ± 7

analogues studied were D-allose (the C-3 epimer of D-glucose), 3-deoxyglucose, 3-bromo-3-deoxyglucose, 3-fluoro-3-deoxyglucose, 3-*O*-methyl-D-glucose and 3-*O*-propyl-D-glucose.

Effects of C-4 and C-6 position analogues on 2-deoxyglucose transport

Table 3 shows the effect of D-galactose, maltose, cellobiose, lactose and ethylideneglucose on the transport of 2-deoxyglucose by GLUT 2, 3 and 4. It is established that all three of these

Table 3 Effects of C-4 position analogues on 2-deoxyglucose transport by GLUT 2, 3 and 4

The results shown below are from a typical experiment repeated at least twice for each analogue/transporter. Rates are expressed as a percentage of the rate measured in the presence of 10 mM L-glucose and are the means ± S.D. for at least six oocytes and for each analogue. Values which are statistically different from the L-glucose rates are indicated by *. Note that the inclusion of L-glucose did not alter the transport rate compared with oocytes assayed in the absence of any other sugars. Transport rates were between 3 and 5 pmol/min per oocyte for GLUT 2 and 3, and between 0.5 and 1 pmol/min per oocyte for GLUT 4.

Competing sugar	Concn. (mM)	Relative 2-deoxyglucose transport rate (%)		
		GLUT 2	GLUT 3	GLUT 4
L-Glucose	10	100	100	100
D-Glucose	10	17 ± 2*	9 ± 2*	10 ± 4*
D-Galactose	10	110 ± 12	57 ± 5*	96 ± 12
Ethylideneglucose	10	102 ± 9	73 ± 6*	82 ± 7*
Maltose	20	105 ± 8	75 ± 7*	99 ± 9
Lactose	20	110 ± 9	112 ± 9	107 ± 12
Cellobiose	20	113 ± 12	82 ± 6*	88 ± 9

Table 4 Effects of C-6 position analogues on 2-deoxyglucose transport by GLUTS 2, 3 and 4

The results shown below are from a typical experiment repeated at least twice for each analogue/transporter. Rates are expressed as a percentage of the rate measured in the presence of L-glucose, and are the means for six oocytes and for each analogue (S.D. was typically between 5 and 15%). Note, however, that the concentration of competing sugars used in these analyses were 50 mM for GLUT 2, and 25 mM for GLUT 3 and 4. Values which are statistically different from the L-glucose rates are indicated by *. Transport rates were between 3 and 5 pmol/min per oocyte for GLUT 2 and 3, and between 0.5 and 1 pmol/min per oocyte for GLUT 4. Abbreviation: n.d., not determined.

Competing sugar	Relative 2-deoxyglucose transport (%)		
	GLUT 2	GLUT 3	GLUT 4
L-Glucose	100	100	100
D-Glucose	24 ± 5*	10 ± 1*	22 ± 4*
6-Deoxyglucose	33 ± 9*	46 ± 7*	44 ± 7*
D-Xylose	106 ± 9	78 ± 6*	75 ± 6*
L-Arabinose	138 ± 22	63 ± 7*	90 ± 8
D-Fucose	65 ± 18*	25 ± 2*	51 ± 6*
D-Galactose	63 ± 7*	16 ± 2*	96 ± 8
6-*O*-Methyl-D-galactose	57 ± 10*	109 ± 11	49 ± 6*
6-*O*-Propyl-D-galactose	n.d.	93 ± 17	78 ± 8*
6-*O*-Fluoro-D-galactose	50 ± 12*	30 ± 9*	48 ± 12*

transporters transport galactose (Gould et al., 1991; Burant and Bell, 1992), but note that the disaccharides are not transported substrates, neither is 4,6-*O*-ethylidene-D-glucose (Barnett et al., 1973; Rees and Holman, 1981). Table 4 shows the effects of a range of C-6 position analogues on 2-deoxyglucose transport.

Analysis of fructose transport by GLUT 2

The transport of fructose by GLUT 2 is a more complex consideration, since it is not clear whether fructose is transported in the pyranose or furanose ring forms by this transporter, since in aqueous solution fructose is about 30% in the furanose form. In an effort to address this, we have examined the ability of L-sorbose (a fructose analogue with > 98% in the pyranose ring

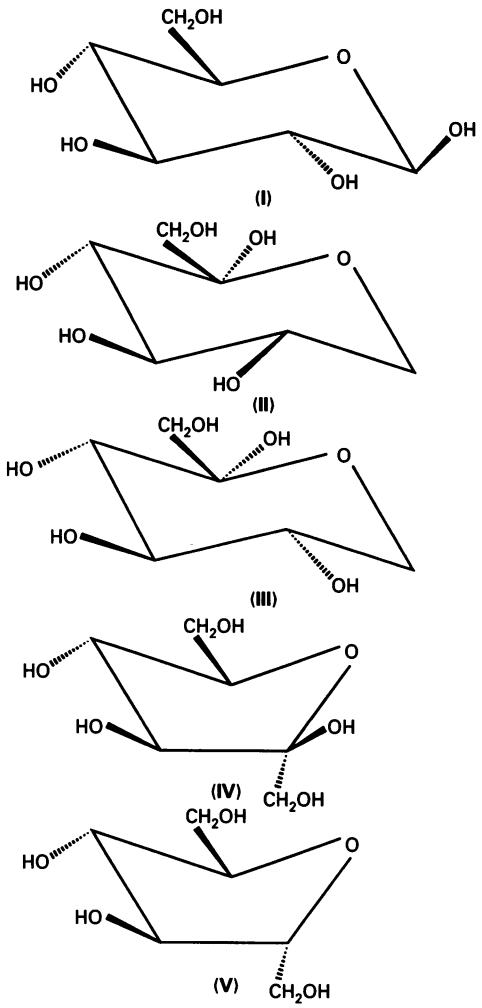

Figure 1 Structures of pyranose and furanose sugars

Structural comparisons of pyranose and furanose sugars used in the analysis of substrate binding to the transporters. Compound (**I**) is β-D-glucose, compound (**II**) is β-D-fructopyranose, compound (**III**) is α-L-sorbopyranose, compound (**IV**) is β-D-fructofuranose and compound (**V**) is 2,5-anhydro-D-mannitol.

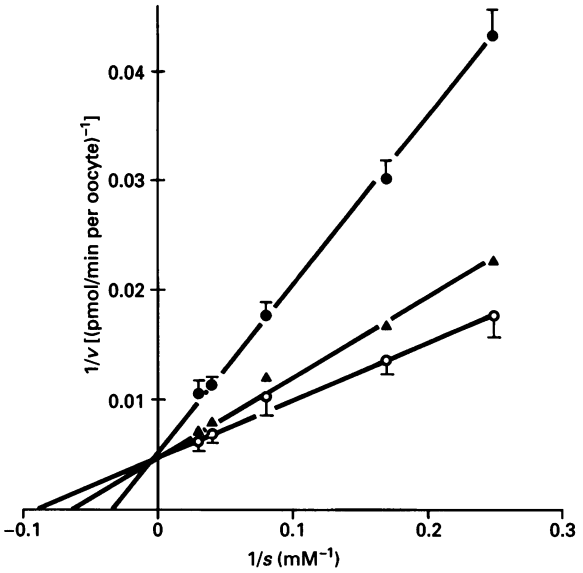

Figure 2 Lineweaver–Burk plot of 2-deoxyglucose transport mediated by GLUT 2 in the presence of L-sorbose or 2,5-anhydro-D-mannitol

The transport rates of 2-deoxyglucose into oocytes expressing GLUT 2 was determined over a range of substrate concentrations either in the absence (○) or in the presence of either 50 mM L-sorbose (▲) or 50 mM 2,5-anhydro-D-mannitol (●). The results were corrected for the transport rates measured in control oocytes (typically less than 10% at all concentrations) and are presented in the form of a Lineweaver–Burk plot. The data presented are from a typical experiment, each point representing the mean ± S.D. for six oocytes.

form; see Figure 1) and 2,5-anhydro-D-mannitol (a fructose analogue locked in the furanose form; see Figure 1) to inhibit 2-deoxyglucose transport mediated by this isoform. The results of this analysis are presented in Figure 2.

DISCUSSION

The interaction of the transported sugar with the different glucose transporters involves an equilibrium between solvated sugar in bulk solution and sugar bound to the transporter. In addition, it is possible that the transporter may also have water molecules associated with the sugar-binding site. The association between sugar and transporter most likely involves hydrogen bonds. If the hydroxy groups on the sugar are in the appropriate stereochemical configuration for binding, then the equilibrium will lie towards transporter–sugar complex formation, as the hydroxy groups will occupy the correct regions in space for interaction with the appropriate groups within the transporter binding site. Thus if a hydroxy group important for binding is replaced by a hydrogen atom (i.e. the deoxy analogue), then the affinity of this analogue for the transporter will be significantly reduced. Replacement of the hydroxy group with another electronegative atom such as fluorine may restore the affinity of the sugar for the carrier provided that it is the protein which donates the hydrogen atom, but not if it was the hydroxy group that originally donated the hydrogen atom (assuming that the stereochemistry is retained and the substituent is not too large). Thus we have examined the effects of fluoro, alkoxy, chloro and bromo substitutions [decreasing order of effectiveness in hydrogen-bond participation (Pauling, 1940)] at specific sites in the hexose ring on the efficacy of these analogues to inhibit transport by GLUT 2, 3 and 4.

The small amounts of some sugar analogues available for the present study has precluded an exhaustive analysis of the K_i values of each sugar analogue. However, we have measured the percentage inhibition of 2-deoxyglucose transport by these analogues, and compared the extent of inhibition relative to the inhibition elicited by L-glucose within the same experiment. The extent of inhibition is a reflection of the K_i for the different analogues. When the K_i is high, the affinity is low and the percentage inhibition will be small. It has previously been shown that, at positions where hydrogen bonds appear important for the binding of glucose to GLUT 1 in erythrocytes, substitution of a hydroxy group for a hydrogen atom results in a $\sim$ 10-fold increase in K_i (Kahlenberg and Dolansky, 1972; Barnett et al., 1973).

We have interpreted the results of this analysis to reflect sugar interaction to the outward-facing sugar-binding site of the transporter molecules. This assumption is based upon the fact that we are measuring changes in the rates of 2-deoxyglucose transport. Since this glucose analogue is phosphorylated by hexokinase and hence trapped inside the oocyte, the rate of sugar

efflux is assumed to be minimal. Under conditions of deoxyglucose transport at concentrations far in excess of the 100 μM used in this analysis, we have observed no significant accumulation of non-phosphorylated deoxyglucose in the oocyte, indicating that hexokinase is not rate-limiting under these conditions (Gould et al., 1991; Colville et al., 1993).

The potential that some of the sugar analogues used in this analysis may produce the inhibitions in transport rate observed by directly inhibiting hexokinase is worthy of comment. Given that insufficient amounts of some of the sugar analogues are available for direct examination of this issue, we have not undertaken this analysis directly. However, we would point out that if a given analogue were producing the observed inhibition of uptake by inhibiting hexokinase, it could be expected that the effects of that analogue would be identical for all transporter isoforms. This result is not observed for any sugar except D-glucose. Moreover, the stereochemistry of glucose binding to hexokinase is quite distinct from that of sugar binding to GLUT 1 [see, for example, Wohlheuter and Plagemann (1987)]. Thus, although we cannot state definitively that the observed effects are a reflection of the inhibition of the transporter, we believe that this interpretation is reasonable.

Binding at the C-1 position

1-Deoxyglucose at 10 mM did not inhibit transport to any significant extent by GLUT 2, 3 or 4 (Table 1). This suggests the presence of a hydrogen bond at this position in the transporters which is important for substrate recognition/binding. 1-Deoxyglucose at 20 mM produced a small level of inhibition (less than 20%) in all transporters (results not shown). 1-Deoxyglucose is a competitive inhibitor of transport (results not shown), indicating that, as was expected based on studies of GLUT 1 and 4 (Barnett et al., 1973; Holman and Rees, 1981) the pyranose form of glucose binds to the carriers. This interpretation is based on the fact that the 1-deoxy-D-glucose (1,5-anhydro-D-glucitol) ring cannot open, thus the compound may be viewed as locked in the pyranose form.

Binding at the C-2 position

We have chosen to use 2-deoxyglucose for the analysis of the sugar-binding properties of these transporters for several reasons. Firstly, it is easier and requires less oocytes to assess the effects of analogues on 2-deoxyglucose transport than for 3-*O*-methyl-D-glucose, the other commonly used sugar analogue. In addition, studies of GLUT 1 have suggested that the C-2 position is not important for hydrogen-bonding (Kahlenberg and Dolansky, 1972; Barnett et al., 1973). Moreover, we have shown that the affinity of 2-deoxyglucose for all the transporters is higher than that of 3-*O*-methyl-D-glucose, and that β-D-mannose (the C-2 epimer of glucose) is as effective as glucose in inhibiting transport by all isoforms studied here (Table 1; see also Gould et al., 1991). Taken together, we therefore believe that the C-2 position is not important for binding of the sugars to any of the isoforms. This result is further confirmed by the demonstration that the replacement of the 2-deoxy with 2-chloro did not inhibit transport to a greater extent than isotopic dilution of the transported sugar with itself (Table 1), suggesting that hydrogen-bonding at this position is unlikely to play a significant role in sugar binding.

Binding at the C-3 position

The results of the C-3 position analysis are presented in Table 2. 3-Deoxyglucose was found to be a poor inhibitor of transport by GLUT 2, indicating that hydrogen-bonding at the C-3 position is important for sugar binding to this isoform. However, comparison of the extent of inhibition recorded by the same concentration of either the 3-deoxy or 3-fluoro analogues indicated that the 3-fluoro sugar did not exhibit a significantly higher affinity for the GLUT 2 transporter than the 3-deoxy analogue (Table 5). One interpretation of this result would therefore be that the hydrogen bond formed at position C-3 is formed by donation of the hydrogen atom from the sugar hydroxy group, not from the transporter. However, an alternative explanation should be considered. It is possible that the replacement of the hydroxy group with a fluoro substituent has rendered the sugar sterically unable to approach the binding site. Although we cannot rule this out, the observation that 3-halogeno substituted sugars are excellent inhibitors of GLUT 1, 3 and 4 (i.e. are better inhibitors than 3-deoxyglucose; see below) would suggest that this is unlikely, and also the fluoro group is itself not much bigger than a hydrogen atom. Rather, we would propose that the C-3 position is involved in hydrogen-bonding of the transported sugar, but the nature of this hydrogen-bonding is unique in GLUT 2.

Table 5 Comparison of the inhibitions of 2-deoxyglucose transport mediated by GLUT 2 and 3 induced by 3-substituted sugars

Transport of 2-deoxyglucose was measured in oocytes expressing either GLUT 2 or GLUT 3 as described in the text. The extent of inhibition at two concentrations of the 3-fluoroglucose or 3-deoxyglucose analogues was determined. Results from a representative experiment are shown, each value representing the mean ± S.D. for six oocytes at each point.

		Relative 2-deoxyglucose transport (%)			
		GLUT 2		GLUT 3	
Competing sugar	[Sugar]...	2 mM	10 mM	2 mM	10 mM
L-Glucose		100	100	100	100
3-Deoxyglucose		102 ± 4	100 ± 5	101 ± 7	85 ± 7
3-Fluoroglucose		96 ± 5	85 ± 5*	60 ± 6*	18 ± 3*

Table 2 shows that D-allose, the C-3 epimer, is an effective inhibitor of glucose transport by GLUT 2, but not by GLUT 1, 3 or 4. Thus these data may be cautiously interpreted to imply that hydrogen-bonding at the C-3 position of GLUT 2 may contribute to the overall binding of sugar to this isoform differently to that observed in the other isoforms, since the displacement of the C-3 hydroxy group in space has not reduced the affinity to the same extent as, for example, observed with 3-deoxyglucose. We would therefore speculate that these observations could be rationalized if the C-3 hydroxy group were still able to weakly participate in hydrogen-bonding, even in the *allo* configuration (Figure 3).

3-Deoxyglucose was found to be a poor inhibitor of transport mediated by GLUT 3 and 4 (Table 2), indicating that the C-3 position is involved in hydrogen-bonding in both these isoforms. Replacement of the hydrogen with a fluorine restored the affinity of the analogue for the transporter, and thus suggests the presence of a hydrogen bond from the transporters to the sugar at this position, with the hydrogen being donated from the transporter. This result was further strengthened by the comparison of 2 mM and 10 mM 3-deoxy and 3-fluoro analogues (Table 5), which demonstrates that the fluoro analogue at this position inhibits the rate of 2-deoxyglucose transport to a greater extent than the deoxy analogue. In the case of GLUT 2, the inhibition induced by 10 mM 3-deoxyglucose was comparable

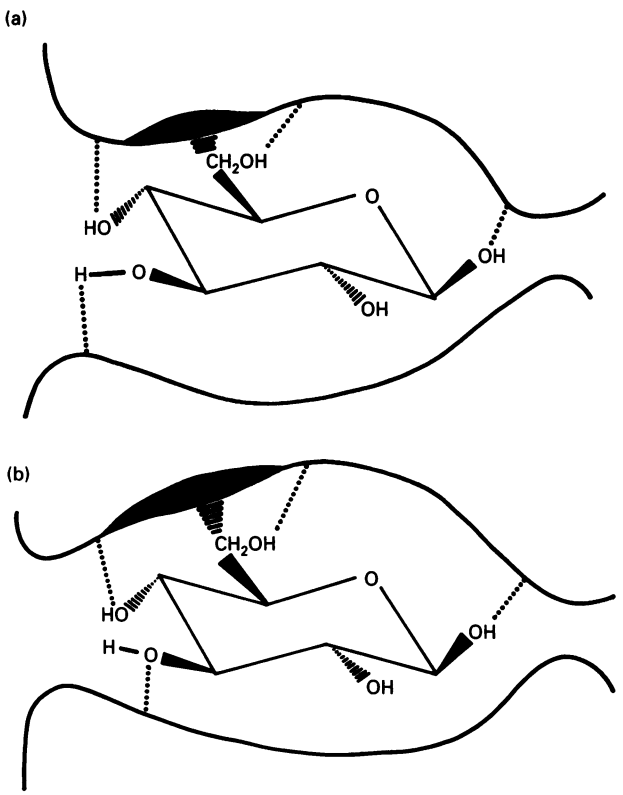

Figure 3 Models of the interaction of glucose with GLUT 2, 3 and 4

(**a**) A model for the binding of β-D-glucose to GLUT 2 is illustrated. Note the unusual nature of the hydrogen bond to the C-3-position hydroxy group compared with GLUTs 3 and 4 (**b**). Hydrogen bonds are shown as dotted lines, and the hydrophobic interaction between the transporter and the C-6 position methylene group indicated by dashed lines. (**b**) Generalized model for β-D-glucose binding to GLUT 3 and 4. Note, however, that in the case of GLUT 3, the hydrogen bond at the C-4 position is likely to play a relatively less significant role in substrate binding, since this transporter efficiently binds D-galactose, the C-4 epimer of glucose.

with that by 10 mM 3-fluoroglucose; similar results were obtained at 20 mM analogue and provide further support for the different nature of the direction of the hydrogen bond in this isoform (Table 5 and Figure 3).

3-*O*-Methyl-D-glucose and 3-*O*-propylglucose which can also form a hydrogen bond were also found to inhibit transport by these isoforms. D-Allose (the 3-epimer) was a poor inhibitor of transport. Thus GLUT 3 and 4 would appear to be similar to GLUT 1 in that a hydrogen bond from the C-3 position hydroxy group in the *gluco* configuration to the protein is indicated by these results. 3-*O*-Propylglucose appears to have a lower affinity for GLUT 3 and 4 than 3-*O*-methyl-D-glucose, but the ability of the relatively large propyl-substituted sugar analogue to inhibit transport by these isoforms indicates that the C-3 position is able to accommodate relatively large substitutions before steric effects significantly effect binding.

Binding at the C-4 position

4-Deoxyglucose was not available for this analysis. However, D-galactose, the C-4 epimer, which is transported by all transporter isoforms studied here, was observed to have a lower affinity for both GLUT 2 and GLUT 4 than D-glucose, as assessed by lack of inhibition observed in the presence of 10 mM D-galactose compared with 10 mM D-glucose (Table 3). In contrast, GLUT 3, which has been shown to have a higher affinity for D-galactose than either GLUT 1, 2 or 4, was effectively inhibited by D-galactose, but to a smaller extent than that measured in the presence of an equivalent concentration of D-glucose. These results may be interpreted to imply the presence of a hydrogen bond to the hydroxy group in the *gluco* configuration of the C-4 position in both GLUT 2 and GLUT 4, similar to the results proposed by Barnett et al. (1973) for GLUT 1. However, GLUT 3 would appear to interact with the C-4 position hydroxy group to a much less significant extent than GLUT 1, 2 or 4, since D-galactose is an effective inhibitor of transport. This result is consistent with the observed decreasing order of affinities of galactose reported by us and others, GLUT 3 > GLUT 1 > GLUT 2 (Burant and Bell, 1992; Colville et al., 1993), and thus we propose that hydrogen-bonding to the C-4 hydroxy group is of minor importance when compared with other factors for the substrate binding and/or recognition by this isoform.

Maltose, cellobiose and ethylideneglucose all inhibit GLUT 3 at concentration of 20 mM. In addition, in a previous study, we have demonstrated that maltose is a competitive inhibitor of transport by GLUT 2 and 3, but with a relatively high K_i (Colville et al., 1993). Since all of these sugars can be regarded as C-4 analogues of glucose, it is likely that the C-4 position of the glucose is surrounded by a relatively large 'space' which is able to accommodate bulky derivatives. One interpretation would therefore be that the sugar enters the binding site C-1 position first, and thus leaves C-4 oriented such that it faces out of the sugar-binding site (see below). This has already been proposed for GLUT 1 and GLUT 4 (Barnett et al., 1973; Holman and Rees, 1981).

Binding at the C-6 position

6-Deoxyglucose is a good inhibitor of all transporter isoforms. Thus we would argue that a simple hydrogen bond to the C-6 position is unlikely to be a factor in sugar recognition at this site. The potential for hydrophobic interactions between the transported sugar and the protein around the C-6 position has been suggested for GLUT 1 (Barnett et al., 1973). The observation that D-xylose (which lacks the C-5 hyroxymethyl group of D-glucose) was less effective an inhibitor than 6-deoxyglucose supports a similar interaction between the transported substrate and all of the isoforms studied here.

This proposal is further strengthened, in particular for GLUT 3, by the use of a series of 6-substituted galactose analogues, specifically L-arabinose (D-galactose without the C-5 hydroxymethyl group), D-fucose (6-deoxy-D-galactose), 6-*O*-methylgalactose and 6-*O*-propylgalactose. Each of these analogues retains the same stereochemistry as D-galactose. Replacement of the C-6 hydroxymethyl group with a hydrogen atom (L-arabinose) results in a dramatic loss of affinity for the transporter. In contrast, removal of the C-6 hydroxy group (D-fucose) results in only a small loss of affinity of the analogue, since this analogue is almost as good an inhibitor as D-galactose itself. These data suggest that a C-5 substituent is important for binding, and that a methylene group is the minimum requirement. On the basis of the data in Table 4 we propose that hydrophobic interaction at C-6 may be more important for the substrate binding than the presence of the C-6 hydroxy group.

Similarly, for GLUT 2 and 4, hydrophobic interactions are likely to be important for substrate binding. In this case, D-galactose is a poor inhibitor of transport (consistent with the more significant role of the *gluco* C-4 in hydrogen-bonding in

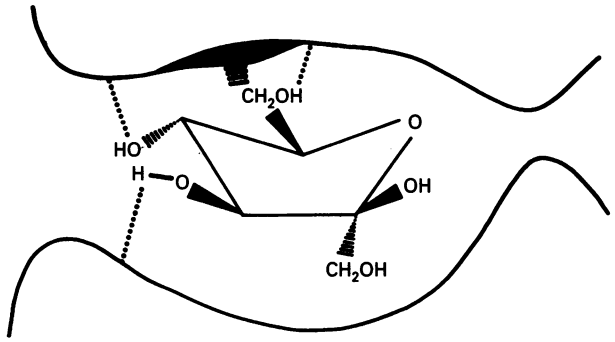

Figure 4 Model for the interaction of fructose with GLUT 2

Shown is a proposed model of the interaction of β-D-fructofuranose with GLUT 2. The sugar is proposed to bind in the furanose-ring form.

these isoforms suggested above). However, D-fucose is as effective as 6-deoxyglucose in inhibiting transport, indicating that hydrophobic interaction around the C-6 position is also important for GLUT 2 and 4 and may, in fact, contribute more significantly to substrate binding than the C-4 hydrogen bond (particularly when the C-4 hydroxy group is in the *galacto* configuration).

One notable difference amongst the isoforms studied here is that 6-*O*-methylgalactose is an effective inhibitor of GLUT 2 and 4, but not GLUT 3. We interpret this result as implying that, for GLUT 2 and 4, further hydrophobic interactions are important factors in the recognition of the C-6 position, since the 6-*O*-methyl analogue of galactose is more effective an inhibitor of transport than D-galactose itself; however, it must be pointed out that this result contrasts with those of Holman et al. (1981), who found that 6-*O*-methyl-D-galactose was a poor inhibitor of GLUT 4. We at present have no explanation of this result. Presumably, the alkoxy analogues are not effective inhibitors of GLUT 3 due to steric considerations.

The ability of D-fucose (6-deoxygalactose) to inhibit transport to a greater extent than D-galactose for all isoforms implies that a hydrophobic interaction between the transporter and sugar at the C-6 position is important for substrate recognition.

Interaction of β-D-glucose with the transporters: models of the binding sites for GLUT 2, 3 and 4

Models for the interaction of glucopyranose with each of the transporters are presented in Figure 3. For all isoforms, some degree of hydrogen-bonding between the C-1, C-3 and C-4 positions is indicated by the results presented above. Some differences are evident, however, the most significant being the difference in the hydrogen bond to the C-3 position of GLUT 2 compared with GLUT 1, 3 or 4. In addition, we propose that, for GLUT 3, hydrogen-bonding at the C-4 position plays a relatively minor role in sugar binding by this isoform, consistent with this transporter being able to bind (and transport) D-galactose, the C-4 epimer of D-glucose, with high affinity. Hydrogen-bonding to the C-1 position is also an important contributor to binding.

Hydrophobic interaction at the C-6 position is also implicated in sugar binding. The ability of 6-deoxyglucose to inhibit all three of the transporter isoforms studied here suggests that hydrogen-bonding is not crucial for substrate binding; nonetheless, the lower affinity of 6-deoxyglucose than glucose itself does suggest the presence of a hydrogen bond at this position. The role of hydrophobic interactions has been best established in studies of GLUT 3, using a series of C-6 galactose analogues. In this case, D-fucose is as effective as D-galactose in inhibiting glucose transport, and suggests that hydrophobic interactions at this position are more important than hydrogen bonds, at least when the C-4 hydroxy group is in the *galacto* conformation. Similar results have also been obtained for GLUT 2 and 4 (Table 4).

Interaction of D-fructose with GLUT 2

A recent study of the hexose transporter of *Trypanosoma brucei* has revealed that, like GLUT 2, the hexose transporter present in this organism is capable of mediating the transport of both glucose and fructose with equal efficiency (Fry et al., 1993). Studies using a variety of pyranose and furanose sugars have indicated that, although glucose is transported in the pyranose form, fructose binds to, and is transported in, the furanose form. This surprising result prompted us to evaluate the conformation in which D-fructose binds to GLUT 2.

We have examined the ability of L-sorbose, a fructose analogue predominantly in the pyranose-ring form (see Figure 1) and 2,5-anhydromannitol, a fructose analogue locked in the furanose-ring form (see Figure 1) to inhibit 2-deoxyglucose transport by GLUT 2. Note that both these fructose analogues retain the same stereochemistry at C-3, C-4 and C-6 as D-fructose itself. Analysis of the results (Figure 2) shows that the K_i for anhydromannitol ($\sim$ 26 mM) is substantially less than that for either L-sorbose ($\sim$ 170 mM) or D-frustose ($\sim$ 200 mM; Colville et al., 1993). We interpret this result to indicate that D-fructose binds to GLUT 2 preferentially in the furanose form, and this interaction is schematically represented in Figure 4. Thus, for fructose binding, hydrogen-bonding at C-3 and C-4 is important, but not at C-2. Compared with the binding of D-glucose, the most significant difference is likely to be the absence of a hydrogen bond between the transporter and the C-1 position of D-glucose (Figure 4). This may account in part for the lower affinity of D-fructose for the transporter.

Interaction of D-galactose with GLUT 3

D-Galactose is transported by GLUT 3 with a relatively high affinity ($K_m \sim 8$ mM; Colville et al., 1993) compared with other isoforms (e.g. K_m for transport by GLUT 2 $\sim$ 90 mM; Colville et al., 1993). It is therefore likely that the region around C-4 would constitute less to the binding of sugars to GLUT 3 than the other isoforms, since D-galactose is the C-4 epimer of D-glucose. The data in Table 3 are consistent with this, since D-galactose is a relatively poorer inhibitor of GLUT 2 and 4 compared with GLUT 3.

Conclusions

We have analysed the patterns of hydrogen-bonding of glucose to three members of the facilitative glucose-transporter family. Our results indicate that hydrogen-bonding at the C-1, C-3 and C-4 positions are important determinants of glucose binding, and hydrophobic interactions between the transporter and the sugar in the C-6 position also play a significant role. Subtle differences between the isoforms are detectable, and account for the different substrate selectivities of these isoforms. It is, however, important to note that, although hydrogen bonds are, at some stage in the transport of the substrate, involved at several positions on the sugar molecule, they need not all hydrogen-bond at the same point during the transport cycle. The

relative importance of each potential hydrogen bond may change as the sugar is passed through the transport protein.

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