Facilitated Hexose Transporters: New Perspectives on Form and Function

The recent sequencing of the human genome has resulted in the addition of nine new hGLUT isoforms to the SLC2A family, many of which have widely varying substrate specificity, kinetic behavior, and tissue distribution. This review examines some new hypotheses related to the structure and function of these proteins.

Hexoses are a major metabolic fuel for numerous cell types, and the original studies of their movement across cell membranes and epithelia led to the development of the whole field of membrane transport proteins. Hexose transporters belong to one of two protein super families: the sodium/solute symporter family (SSSF; Ref. 41) and the major facilitative superfamily (MFS; Ref. 33), which are expressed in numerous phyla. This review will focus on the MFS hexose transporters, of which most are facilitated carriers, i.e., they use the energy of the transported solute concentration gradient to drive net movement across the membrane. In humans, the facilitated transport of hexoses is mediated by the GLUT proteins (gene family SLC2A), which can move hexoses into or out of cells and were among the very first facilitated transporters to be studied systematically (22). Their detailed kinetic analysis led to the development of a number of key concepts as to how these proteins might work, yet it is the recent application of molecular techniques that has significantly contributed to our understanding of these processes. The recent sequencing of new genomes and the cloning of numerous orthologs from other species has allowed for a widely expanded search for conserved residues and motifs. In this review, we will attempt to bring together the current evidence as to what a GLUT protein may look like and, most intriguingly, how these “simple” facilitators might actually recognize and then translocate their substrates. Recent evidence suggests that substrate recognition and binding may not be the same process.

Kinetic Predictions of Transporter Mechanisms

The application of Michaelis-Menten kinetics to hexose transport into red cells (52), across the epithelia of sheep placenta (53) and intestine (17), led to development of the “simple carrier” theory, which predicted that two related, but separate, processes must occur during transport. The first step is binding or adsorption of the substrate to a recognition site on the protein. This interaction is considered to be much like that between an enzyme and its substrate, but unlike an enzyme no chemical change occurs to the substrate during the second step: translocation. This involves some kind of conformational change within the protein that leads to the presentation of the binding site to the other side of the membrane and the subsequent release of the substrate. To complete the cycle, the binding site then needs to reorient to face the side of the membrane where the initial binding process started. In the case of facilitated hexose transporters, net movement of substrate results from the solute concentration gradient across the membrane, although hexoses can flow in either direction. It also became quickly appreciated that the affinity for the substrate may not be the same on opposite sides of the membrane, but to fulfill thermodynamic laws this must mean that the maximal rate of transport in one direction would also have to be greater. This could be satisfied if the ratio of the half-maximal saturation constant (Km) to the maximal rate of transport (Vmax) was the same for movement in both directions (Km/Vmax = Km/Vmax) (48). Moreover, it was postulated that the rate-limiting step for transport is the reorientation of the empty carrier, a concept supported by trans-acceleration experiments (29). If transport of solute in one direction is measured in the presence or absence of transportable solute on the opposite (trans) side, it will be faster when solute is present on both sides (47). This is interpreted to result from the acceleration of the reorientation of the occupied binding site. Similarly, if kinetic constants for transport are measured when the solute concentration is the same on both sides of the membrane, the Vmax is greater than when measured under zero trans conditions.

The determination of how hexoses might interact with the binding site of the protein largely resulted from a series of extremely elegant experiments measuring the transport of different hexose analogs into and out of red cells or cell lines transfected with other hexose transporter isoforms (3). This work suggested that hydrogen bonds are formed between specific hydroxyl groups on the hexoses and the side chains of certain hydrophilic amino acids lining an aqueous cleft or vestibule. Also, it appeared that these bonds were modified as the protein went through its conformational change so that the binding site facing the exofacial side was partially or completely different from that facing the inside (4). However, it also reinforced the notion that the specificity of the red cell hexose transporter would be defined by which sugars could interact with the binding sites as they faced either the exofacial or endofacial environment.
Finally, it needs to be appreciated that a structural difference between the two binding sites may not be reflected by a kinetic asymmetry since, at least for GLUT1, influx and efflux appear to be the same when measured at 37°C (6). Similar analyses have not been made for other GLUT isoforms.

Basic GLUT Structure

In 1985, the first GLUT protein was cloned and found to be a 492-amino acid single protein constitutively expressed in almost all cell types (36). However, differing kinetic parameters and substrate specificities in some tissues suggested the existence of several additional discreet entities responsible for moving hexoses. Subsequent expression cloning confirmed this hypothesis with the rapid expansion of the hGLUT family to five members (see Table 1) (7-9). This small number of hexose transporters appeared to satisfy most physiological metabolic processes, with only two members showing any ability to promote fructose movement. However, the recent sequencing of the human and other genomes has transformed this landscape. There are now 14 members of the human SLC2A gene family, and many of the newly described members have discreet tissue expression and function (FIGURE 1). The original hydropathy analysis of GLUT1 indicated that the protein folded into 12 transmembrane α-helices that combined to form a central aqueous pore or channel through which the substrate crossed the lipid bilayer. A comprehensive series of studies using cysteine scanning mutagenesis supports such a model with up to 8 of the 12 helices contributing to parts of the surface lining of the pore (Ref. 39; FIGURE 2). Recent X-ray crystal structure analysis of two related bacterial proteins, which are also members of the MFS superfamily (the proton coupled lactose transporter LacY and the glycerol-3-phosphate transporter GlpT), indicates a barrel structure in which two clusters of six transmembrane helices surround the aqueous pore in the center (1, 20). The alternating tilting of these two clusters appears to close the channel on one side and simultaneously open it on the other, i.e., form the outward or inward facing clefts. The substrate binding site for both proteins was identified as being at the center of the aqueous pore, such that the alternating tilting sequentially exposed bound substrate to one side of the membrane or the other. Computer analysis of the structure of GLUT1, using the coordinates for GlpT, has generated a very similar model (44). Most of the helices predicted by this model to form the walls of the pore correspond with those identified by the cysteine scanning studies, although, not surprisingly, there are some discrepan-

Table 1. Class I-III GLUT properties

<table>
<thead>
<tr>
<th>GLUT</th>
<th>Km (Oocyte)</th>
<th>Tissue</th>
<th>Special Feature</th>
<th>Substrate Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLUT1</td>
<td>5 mM</td>
<td>Ubiquitous; red cells</td>
<td></td>
<td>Glucose/galactose</td>
</tr>
<tr>
<td>GLUT3</td>
<td>1 mM (high affinity)</td>
<td>Neurons</td>
<td></td>
<td>Glucose/galactose</td>
</tr>
<tr>
<td>GLUT4</td>
<td>5 mM</td>
<td>Fat, muscle including heart</td>
<td>Trafficking regulated by insulin (LL motif)</td>
<td>Glucose, not galactose</td>
</tr>
<tr>
<td>GLUT2</td>
<td>11 mM (low affinity)</td>
<td>Intestine, kidney, liver, beta-cell</td>
<td>Mediates both uptake and efflux</td>
<td>Glucose/galactose/fructose</td>
</tr>
<tr>
<td>GLUT5</td>
<td>6 mM</td>
<td>Intestine, sperm</td>
<td>Found primarily in fructose-metabolizing tissues</td>
<td>Fructose (glucose)</td>
</tr>
<tr>
<td>GLUT7</td>
<td>0.3 mM</td>
<td>Intestine</td>
<td>Apical membrane targeting</td>
<td>Glucose/fructose, not galactose</td>
</tr>
<tr>
<td>GLUT9a and 9b</td>
<td>0.3 mM</td>
<td>Kidney BLM</td>
<td>Liver expression co-localizes with GLUT2 but not kidney</td>
<td>Glucose/fructose, not galactose</td>
</tr>
<tr>
<td>GLUT11</td>
<td>0.2 mM</td>
<td>Muscle, heart, fat, placenta, kidney, and pancreas</td>
<td>Three isoforms (A, B, and C) with differing tissue distribution</td>
<td>Glucose/fructose, not galactose</td>
</tr>
<tr>
<td>GLUT6</td>
<td>High Km</td>
<td>Brain and spleen</td>
<td>Has LL targeting motif</td>
<td>Glucose/?</td>
</tr>
<tr>
<td>GLUT8</td>
<td>2.4 mM (2DG)</td>
<td>Testis, brain, fat, liver, and spleen</td>
<td>Has LL targeting motif</td>
<td>Glucose/(fructose)</td>
</tr>
<tr>
<td>GLUT10</td>
<td>0.3 mM (2DG)</td>
<td>Heart and lung</td>
<td></td>
<td>Glucose/galactose, not fructose</td>
</tr>
<tr>
<td>GLUT12</td>
<td>4.5 mM</td>
<td>Insulin-sensitive tissues</td>
<td>Has LL targeting motif</td>
<td>Glucose, galactose, fructose</td>
</tr>
<tr>
<td>HMIT (GLUT13)</td>
<td>0.1 mM</td>
<td>Brain</td>
<td>Proton-coupled substrate movement</td>
<td>Myoinositol</td>
</tr>
</tbody>
</table>
high affinity for both hexoses with $K_m$ in the micromolar range (32). However, none of these transporters are able to recognize 2DG as a substrate, which likely explains the failure to identify these proteins in the earlier expression cloning strategies that employed 2DG. Several studies of both naturally occurring and engineered point mutations in GLUT1 suggested that, within helix 7, there are a number of residues that are very important for functional activity of the transporter. In particular, analysis of GLUT1–5 indicated that the motif “QLS” was a site involved in substrate recognition. GLUT1, 3, and 4, which transport glucose but not fructose, have the QLS sequence in helix 7, whereas GLUT2 and 5, which both transport fructose, have HVA or MGG, respectively (FIGURE 4). Chimeras constructed from GLUT2 and 3, in which the amino acid sequence for GLUT2 from the beginning of helix 7 to the COOH terminus was inserted into GLUT3, converted the protein to a glucose/fructose transporter with GLUT2 kinetics, i.e., high capacity/low affinity (2). A subsequent mutation experiment in which GLUT2 had HVA replaced with QLS and GLUT3 had the HVA inserted resulted in a partial reversal of their respective kinetics and substrate selectivities (46). This demonstration for the role of a series of residues in the center of helix 7 combined with the structural modeling reinforced the concept of a single central substrate binding site. The comparison of a number of hexose analogs as substrates or inhibitors of transport using GLUT1–5 also led to the conclusion that substrate binding involved the formation of a number of hydrogen bonds between protein and hexose. In GLUT1, 3, and 4, the hydroxyls on C1 and C2 of the hexose ring are critical for binding and transport, whereas binding to C4 is possible but not essential (8). The differing affinities between isoforms for D-galactose (GLUT3 > GLUT1 > GLUT2) supports the view that C4 binding is of less significance. The situation regarding the C6 position is less clear, and it has been suggested by analysis of GLUT1–4 that a hydrophobic interaction between the methylene group and part of the pore lining may occur when the hexose enters the outer vestibule (3, 8). We will return to the possible role of hydrophobic interactions later.

But what of fructose binding and transport? Within the original limited panel of human isoforms, both GLUT2 and GLUT5 are able to handle this hexose, which exists in either the furanose or pyranose structure when in solution, 30 vs. 70% (8), respectively. Competition experiments suggest that GLUT2 recognizes fructose in the furanose form, allowing alignment with the same residues within the binding pocket as for the pyranose structures. Thus C2 and C3 of the furanose ring would form the hydrogen bonds, whereas C6 may still provide the possible hydrophobic interaction. It is also likely that, for both glucose and fructose, GLUT2 does not form the conventional hydrogen bond where the protein is the proton donor to the hydroxyl on C3.

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Substrate Selectivity or Specificity

Functional characterization of the five original GLUTs indicated that only GLUT2 and 5 were able to transport fructose (7, 9) and that, although GLUT2 could also readily mediate glucose fluxes, GLUT5 appears to have a very limited capacity for glucose (26). In addition, GLUT2 has been shown to be able to handle glucosamine as a substrate as well as 2-deoxy-D-glucose (2DG), which suggest that either the C2 position is not important for hydrogen bonding or that there is the possibility for additional hydrophobic interactions given the higher affinity for glucosamine (51). Recently, characterization of the class II GLUTs (7, 26, 28, 31) show that apparently they can all transport glucose as well as fructose, and the latter three all have
(glucose) or C2 (fructose), but instead the hexose appears to donate the proton (8). Another series of studies used a whole series of specially synthesised hexose analogs to investigate their interaction with GLUT5. These data indicate that this isoform can recognize fructose in both the furanose and with a lower affinity for the pyranose conformations and that binding involves the C1, C2, C3, and C4 positions of the hexose (24). Although all of these results indicate subtle differences in how hexoses bind to each GLUT isoform, in all cases it appears that the hexose needs to enter the exofacial vestibule with C1 at the front. However, with the exception of GLUT1 in human red cells, this type of analysis has only been possible for the exofacial binding. Technical obstacles have largely prevented a full analysis to be performed for the endofacial binding site. However, efflux and trans-acceleration experiments do indicate that the endofacial binding is somewhat different, as would be expected. Primarily, it appears that binding to C6 is more important, and the C1 position of the hexose now faces the opening of the inner vestibule. Thus, during the transport process, the orientation of the hexose appears to remain unaltered.

Finally, there is additional evidence from both plant and plasmodial orthologs that indicates significant differences between the handling of glucose and fructose. The point mutation of Q298N (equivalent to the glutamine of the QLS motif in GLUT1, helix 7) or N436Q in helix 11 of HUP1 expressed in the algae Chlorella kessleri, both produce an altered affinity for glucose and galactose (54). However, these mutations have little effect on the kinetics of mannose or fructose transport. Conversely, the mutation of Q189N in the hexose transporter pHIT1 expressed in Plasmodium falciparum (the malarial parasite) abolished fructose transport but had no effect on glucose kinetics (55).

Expansion of the GLUT Family

Expansion of the SLC2A gene family to 14 members necessitated the adoption of a uniform nomenclature for these proteins. Consequently, some GLUTs have been renamed or removed because of the presence of a pseudo gene and one cloning artifact (22). In addition, numerous orthologs from other species over a wide range of phyla have now been reported. The power of bioinformatics allows for quite sophisticated comparison of these sequences and the identification of a number of conserved motifs and key sequences in several of the predicted helices. Sequence alignments indicate that the 14 human proteins can be clustered into three families or subclasses I, II, and III (FIGURE 1; Ref. 22). The majority of the originally identified members belong to class I, along with the new GLUT14, which appears to have resulted from a gene duplication of GLUT3 (56), with which it shares 93.5% identity. GLUT5 has been placed in class II along with GLUTs 7, 9, and 11, all of which transport fructose as well as glucose (24, 30, 31, 45) and have $K_m$ of <1 mM (excepting GLUT5). They also have quite specific and limited tissue expression, suggesting that they play very distinct metabolic roles. As yet, none have been functionally characterized to the level of detail obtained with GLUT1–5; however, one key observation is that none of the class IIs can transport galactose or 2DG. This would indicate that these four GLUTs do form additional hydrogen bonds with the hydroxyls at positions C2 and C4, as had originally been proposed for GLUT5 (24).

The structure of the class III GLUTs still preserves the 12 helix arrangement, but there is a longer extracellular loop between H9 and H10 on which the glycosylation tag appears to reside rather than between H1 and H2 as in the classes I and II. There is less information available on the functional activity of the class III GLUTs, but most do transport hexoses. GLUT8 (initially called X1) has been identified in three species (human, rat, and mouse), but only the rat ortholog has been functionally characterized and can transport glucose, 2DG, and fructose (21). GLUT10 appears to transport glucose, galactose, and 2DG with a $K_m$ of about 0.3 mM but does not transport fructose (12). GLUT12, found in adult human muscle, heart, and prostate (42), appears to transport both glucose and fructose (43). GLUT6 (formally GLUT9) can transport glucose with a high $K_m$, which has not been defined (15). There is no reported data on other possible substrates for this isoform. GLUT13 (HMIT) is a proton coupled myo-inositol transporter, making it the only GLUT that has been demonstrated to use a proton gradient to energize the movement of substrate (5, 50). It is tempting to speculate that GLUT13 represents an ancient hexose transporter for two reasons. First, it...
couples proton movement to that of its organic substrate, a mechanism that is employed throughout phyla. Secondly, the sequence of this ortholog is highly conserved across species. Also, given the different substrate specificity of HMIT (GLUT13) and the ability of GLUT2 to transport glucosamine, it must be kept in mind that hexoses may not be the only physiological substrates for some GLUTs.

**Possible Hydrophobic Interactions During the Transport Process**

Hydrophobic residues manifest their structural role through the so called “hydrophobic effect” or “hydrophobic interaction” exerted between the side chains of the nonpolar residues that are responsible for compact folding. It has also been proposed that they interact with water during protein folding and that these interactions play a crucial role in achieving stable three-dimensional structures (13, 25). This theory assumes that nonpolar residues, through side chain-side chain hydrophobic interactions, get “buried” in the three-dimensional structure of the protein and the water molecules are cooperatively squeezed out from the hydrophobic core (18, 27). Also, the ordering of water molecules in the exterior makes them available for future hydrogen bonds at the expense of a loss of randomness in the system (25). Water molecules buried within proteins have much longer mean resistance times than water in the first hydration shell, and thus they constitute an integral part of the protein structure (13, 18, 40). This energetic state is more prone to subsequent conformational changes, i.e., helps the protein to exist in a meta-stable condition where small energy changes, such as substrate binding, could result in large changes in conformation. Therefore, mutations of hydrophobic residues could possibly affect the number of structural water molecules within the core of a protein and disrupt essential interactions mediated by ordered water contacts, resulting in functional changes (10).

Also, it is likely that the hydrophobic residues play important roles in influencing the permeation of the substrate through the aqueous pore. It has been postulated that the length of side chains of hydrophobic residues lining the pore of hexose transporters could influence the substrate selectivity of these proteins by exerting hydrophobic interactions with the water shell surrounding the substrate (30, 31). This interaction could create a funneling action on the substrate orienting it to properly interact with the ligand binding site (FIGURE 3).

The concept of hydrophobic substrate selectivity filters in membrane proteins is not novel. The family of opioid receptors appears to have two components to their ligand recognition termed the “address” and the “message.” The address has been proposed to provide steric hindrance determining which ligands can actually gain access to the highly conserved binding pocket (the message). The interaction between the ligand and residues in the extracellular loops or at the end of

**FIGURE 3.** Three-dimensional reconstruction of hGLUT7 showing some of the helices proposed to form the aqueous channel through the bilayer

The high-affinity docking of fructose is shown at the core of the channel with three hydrogen bonded interactions with Arg150 from helix 2, and Ser309 and Gln306 from helix 7. Also, note the pairing of hydrophobic residues Trp89 and Ile34 at the opening of the exofacial vestibule and Val93 with Trp38 at the opening of the endofacial vestibule. These pairs of residues could form hydrophobic interactions with each other or provide sites for substrate-specific hydrophobic binding at the entrance to the translocation pore. Taken from Ref. 30 with permission.

**FIGURE 4.** Sequence alignments of the putative helix 7

Sequence alignments of the putative helix 7 for human GLUT isoforms and other facilitative hexose transporter orthologs. Red highlights the proposed NXI/V motif, and gray highlights the QLS motif.
some helices is thought to restrict access to the binding pocket through possible hydrophobic interactions. Notably, two hydrophobic residues have been identified as being involved in this process: valine and tryptophan (16, 34, 35).

There are a number of point mutations of hydrophobic residues that appear to have a very significant effect on the function of GLUTs and some orthologs from other species. A mutation from a diabetic patient of a valine to an isoleucine in the center of helix 5 in GLUT2 (V165I) abolished 2DG transport, but unfortunately fructose transport was not investigated (37). Introduction of the same point mutation in hGLUT1 also resulted in a comparable reduction of 2DG transport. Intriguingly, the authors then went on to show that this residue lies between the exofacial vestibule and the central binding site(s) and that it was in contact with the lumen of the pore. Furthermore, the addition of bulky side chains at this site interfered with substrate permeation, suggesting that hydrophobic residues projecting into the pore can influence solute permeation (38).

Recently, two very different types of analysis have raised the possibility that hydrophobic residues lining the aqueous pore of GLUTs may indeed play a key role is substrate selectivity quite distinct from that of the central “substrate binding site(s).” First, a structurally conservative substitution of a valine for an isoleucine at the exofacial end of helix 7 in hGLUT7 abolished fructose transport (30). However, this mutation had no effect on the glucose transport kinetics, indicating that the overall structure and functionality of the protein had not been significantly affected. Comparison of sequence alignments for the class I and II GLUTs shows that, in almost all of these isoforms at the homologous position, this residue is either an isoleucine or a valine. Interestingly, those that cannot transport fructose have a valine and those that can have an isoleucine. Similar mutational experiments confirmed that for GLUT2, 5, and 9 the replacement of the isoleucine with valine also significantly reduced fructose fluxes with no effect on glucose transport. The one exception appears to be GLUT11, which can also transport both hexoses but has a valine at the equivalent position. However, this residue is expressed within a different environment as part of a DSV motif, whereas the others all have NXV or NXI (FIGURE 4; Ref. 31).

Indeed, this motif is highly conserved between almost all of the GLUTs and many of their orthologs. This then raises the issue of how a hydrophobic region lining the exofacial vestibule could influence substrate specificity. The first clue is the original suggestion that there may be a hydrophobic interaction with the C6 region of the hexoses during transport. The second element is the recent application of a docking algorithm to study how hexoses interact with the previously generated three-dimensional model of GLUT1 (11). The results indicated the possible existence of a series of sites for hexose binding/docking in addition to the original high-affinity one at the core. Of interest was the finding that, although glucose could bind at the entrance of the exofacial vestibule, two hexoses that are not substrates of GLUT1, l-glucose, and 2-fructose could not dock at this site. However, apparently both glucose and fructose could bind to the central high-affinity site. These observations suggest that substrate selectivity for the GLUT proteins is determined in part at the entrance to the exofacial vestibule, possibly through hydrophobic interactions between the pore wall and the hexoses. There is also other evidence that residues close to the opening of the exofacial vestibule in HUP1; a plant hexose transporter may form part of a substrate selectivity filter. Mutation of asparagine 45 located at the extracellular end of helix 1 in HUP1 has been suggested to exclude galactose entry into the pore, with no effect on glucose transport (54).

Summary

There appears to be evidence that GLUT proteins may have, in addition to their core substrate binding/translocation site, some form of selectivity filter at the entrance to the exofacial vestibule. The interaction between residues lining the entrance to the pore and substrate could be the result of steric hindrance or possibly some form of hydrophobic docking. This hypothesis still needs much more testing, but it does raise some interesting possibilities as to how a simple facilitated transporter may function. As shown in FIGURE 3, hexoses could approach the outer vestibule, and if they are unable to gain entry to the vestibule then they would fail to gain access to the central high-affinity binding site. Conversely, those that move past the entrance could then progress through the pore to the center, where binding induces the conformational change resulting in translocation. Such a model raises some very interesting questions with regard to which other residues in the outer vestibule might contribute to such a selectivity filter and also whether there is a similar structure in the inner (endofacial) vestibule controlling which hexoses can access the binding site from the cytoplasm. In the absence of crystal structures for both the outward- and inward-facing conformations of the GLUT proteins, it is going to be difficult to resolve these questions, and our understanding of how these transporters achieve substrate translocation has in reality progressed only to a small degree from the models proposed 50 years ago.

References
