Substitution of Tyrosine 293 of GLUT1 Locks the Transporter into an Outward Facing Conformation

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Tyrosines 292 and 293 in the mammalian glucose transporter GLUT1 have been substituted by either iso-leucine or phenylalanine. Chinese hamster ovary clones that were transfected with Tyr-292→Ile, Tyr-292→Phe, Tyr-293→Ile, and Tyr-293→Phe constructs of GLUT1 were shown, by Western blotting and cell surface carbohydrate labeling, to have expression levels that were comparable with the wild-type. The V_m, for 2-deoxy-2-18O-D-glucose transport was markedly reduced only as a result of the Tyr-293→Ile mutation. The ability of the Tyr-293→Ile mutated GLUT1 to bind the exofacial ligand 2-azido-2,2,2-trifluoroethylbenzoyl-1,3-bis(o-mannos-4-yloxy)-2-propylamine (ATB-BMPA) and the endofacial ligand cytochalasin B were assessed by photo labeling procedures. The ability to bind the bis-mannose compound was unimpaired, whereas the ability to bind cytochalasin B was totally abolished, and the level of labeling was lower than in the nontransfected clone.

Affinities of the wild-type and Tyr-293→Ile GLUT1 for 2-deoxy-2-18O-D-glucose, the exofacial ligands (ATB-BMPA and the endofacial ligand cytochalasin B) were assessed by the ability of these agents to displace the radioactive ATB-BMPA photolabel. These data indicated that the Tyr-293→Ile substitution produced no change in the affinity for 2-deoxyglucose, a relatively small enhancement in the affinity for exofacial ligands, but a large ~600-fold reduction in affinity for cytochalasin B, suggesting that the mutated GLUT1 is locked in an outward facing conformation. The observation that the Tyr-293→Ile mutant transporter can bind nontransported C4 and C6 substituted hexose analogues but cannot catalyze transport is interpreted as indicating that Tyr-293 is involved in closing the exofacial site around C4 and C6 of 2-deoxy-D-glucose in the transport catalysis process.

The mechanism by which glucose transporters catalyze glucose transport is thought to involve alternating conformational changes that sequentially expose the hexose binding site to the external and internal surfaces of the protein. A combination of ligand binding and site-directed mutagenesis studies have been used to investigate the protein domains responsible for this mechanism. Photo labeling studies involving active site ligands suggest that the bis-mannose compound, ATB-BMPA (an exofacial ligand), cytochalasin B (an endofacial ligand), and 3-iodo-4-azido-phenethylamino-7-O-succinyl-deacetyl-forskolin interact only with the C-terminal half of GLUT1 (in transmembrane helices (TM) 7–11) (Holman and Rees, 1987; Cairns et al., 1987; Wadzinski et al., 1988). Domain assembly experiments suggest that the N- and C-terminal halves of sugar transporters are both necessary for function (Kaback, 1992). The N-terminal half of the GLUT1 may be responsible for providing a packing surface for stabilizing the C-terminal half in a ligand binding conformation. A reduction in exofacial ATB-BMPA binding and poor cell surface expression (Asano et al., 1991, 1993) have been observed in mutants in which the glycosylated amino acid Asn-45, in the C-terminal half of GLUT1, is substituted. Experiments in which a C-terminally truncated GLUT1 is expressed in CHO cells suggest that the C-terminal region is required for exposing the exofacial ATB-BMPA binding site. C-terminal truncation produces a locking of GLUT1 into a stable inwardly directed conformation (Oka et al., 1990).

Mutagenesis has been carried out on Pro-385 in TM 10 (Tamori et al., 1994). Reduction in transport and exofacial ATB-BMPA photolabeling occur as the result of substitution with iso-leucine but not with glycine at this position. These results suggest that retention of flexibility in this region is required for the transport catalysis process. This study (Tamori et al., 1994) and molecular dynamic simulations of a model of the three-dimensional structure of GLUT1 (Gould and Holman (1993) have suggested that the proline- and glycine-rich region in TM 10 provides a pivotal point for a conformational change, which allows TM 11 and TM 12 to either pack against the outside (ATB-BMPA binding) site in TM 7–9 or against the inner (cytochalasin B binding) site at the base of TM 10.

Mutagenesis studies have been used for examining amino acids that may be important in binding at the endofacial (cytochalasin B/forskolin binding) site. Tryptophan fluorescence changes occur on the binding of these ligands (Appleman and Lienhard, 1985; Carruthers, 1986) and tryptophan residues have been postulated to be involved in the photochemical labeling mechanism (Deziel et al., 1984; Cairns et al., 1987). Therefore, tryptophan 412 and 388 have been mutated. Mutation of Trp-412 in TM 11 of GLUT1 results in reduced transport.

1 The abbreviations used are: ATB-BMPA, 2-azido-2,2,2-trifluoroethylbenzoyl-1,3-bis(o-mannos-4-yloxy)-2-propylamine; TM, transmembrane segment; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary.


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activity but with either no change in affinity for cytochalasin B (Garcia et al., 1992) or only a 40% reduction in cytochalasin B labeling (Katagiri et al., 1992) and no alteration in 3-iodo-4-azidophenethylamino-7-O-succinyldeacetyl-forskolin binding (Schurmann et al., 1993). Mutation of Cys-421 to Arg in TM 11 of GLUT1 reduced the affinity for cytochalasin B by ~50%, and this effect was attributed to a perturbation of the neighboring tryptophan 412, also in TM 11 (Wellner et al., 1992). Mutation of Trp-388 of GLUT1 expressed in COS (Schurmann et al., 1993) and CHO (Katagiri et al., 1993) cells resulted in reduced transport but either a 70% decrease (Schurmann et al., 1993) or no decrease (Katagiri et al., 1992) in labeling with 3-iodo-4-azidophenethylamino-7-O-succinyldeacetyl-forskolin. The Trp-388 mutant expressed in oocytes showed a marked reduction in the affinity for cytochalasin B as measured by its ability to inhibit glucose transport activity (Garcia et al., 1992).

We have identified that a region toward the exofacial end in TM 7, Q^{283}XSGGN^{285}XXFY^{287}, which is present in all the mammalian transporters and is highly conserved in all members of the wider glucose transporter superfamily (Henderson, 1990), is an important domain responsible for binding exofacial ligands. We have shown that a Q282L mutant has extremely low affinity for exofacial ligands such as ABT-BMPA and 4,6-O-ethylidene-3-glucose (Hashiramoto et al., 1992). Surprisingly, and by contrast, mutagenesis of the highly conserved ABT-284, which is an exofacial ligand binding and transport (Hashiramoto et al., 1992). In the study described here, we examine the effects of mutagenesis of residues in the hydrophobic patch at the boundary of this sequence. Tyrrosines 292 and 293 are mutated to either isoleucine or phenylalanine. The Tyr-293 residue is of particular interest, as this residue is completely conserved in all members of the glucose transporter superfamily. Substitution of this residue by isoleucine produces a transporter with full retention of ABT-BMPA binding but with total loss of cytochalasin B binding. The transporter is therefore locked in an outward facing conformation with exactly the opposite properties of C-terminally truncated transporters (Oka et al., 1990), which are locked in an inward facing conformation.

**EXPERIMENTAL PROCEDURES**

**Materials—**Phloretin, cytochalasin E, galactose oxidase, neuraminidase, Protein A-Sepharose, and molecular weight markers were from Sigma. 2-Deoxy-[2,6,6-3H]2-glucose, 14-H-[3H]cytochalasin B, and 1125-I-labeled Protein A-Sepharose from Amersham. The detergent nonaethylene glycol dodecyl ether (C12E8) was from Boehringer Mannheim. ABT-282 (1-butylmaleimide (specific activity 10 Ci/mmol)) was synthesized as described (Clark and Holman, 1990). An affinity-purified antibody against the C-terminal portion of human GLUT1 was used for Western blot analysis, while crude antisera was used in immunoprecipitation experiments (Hashiramoto et al., 1992).

**Site-directed Mutagenesis and Construction of Human GLUT1 cDNA and Expression in CHO Cells—**A full-length human glucose transporter (GLUT1) cDNA was kindly provided by Dr. G. I. Bell (University of Chicago). Point mutations were introduced according to the method of Kunkel et al. (1985). The template for mutagenesis was prepared in Escherichia coli RZ1032, and mutagenesis was carried out by using the mutagenic primer 5'-GGCTGTTCTTTCCTTACCCAG'3' for Y292F, 5'-GGCTGTTCTTTCCTTACCCAG'3' for Y292L, 5'-GGCTGTTCTTTCCTTACCCAG'3' for Y293F, and 5'-GGCTGTTCTTTCCTTACCCAG'3' for Y293C, respectively, with each containing 1- or 2-base pair changes from the sequence determined by Mueckler et al. (1985). Suitable mutant clones were selected, and their sequences were confirmed by dideoxy-nucleotide sequencing in M13. The final construct encoded the amino acid sequences Ile-Ala-Ala-Val-Phe-Phe-Tyr-Ser-Thr-Ser-Ile (Y292F), Ile-Ala-Ala-Val-Phe-Phe-Tyr-Ser-Thr-Ser-Ile (Y292L), Ile-Ala-Ala-Val-Phe-Phe-Tyr-Ile-Ser-Thr-Thr-Ile (Y293F), and Ile-Ala-Ala-Val-Phe-Phe-Tyr-Ile-Ser-Thr-Thr-Ile (Y293C), which span the residues 287-297 of the GLUT1 protein. The mutated fragments were reinserted into the BS1214XbaI site of pRC-CMV(GT1)WT, previously constructed in the vector pRC-CMV, which has the human cytomegalovirus IE1 promoter and the bacterial neomycin resistance gene fused to the SV40 promoter (Hashiramoto et al., 1992). Mutant GLUT1 cDNAs were transfected by the calcium phosphate method into CHO-K1 cell lines, which were maintained in Ham's F-12 medium containing 10% fetal calf serum. The clones that obtained neomycin resistance were selected with 600 μg/ml of the neomycin derivative G418 (Life Technologies, Inc.), and the cells were subsequently subjected to Western blotting analysis using anti-peptide antibody against the C-terminal domain of human GLUT1, as described (Hashiramoto et al., 1992), to identify clones that overexpress these glucose transporters. Mutant clones expressing similar amounts of protein to the wild-type clone were selected for further study.

**Assays of Glucose Transport Activity in CHO-K1 Cells—**Cells in 24-well (16 mm) plates were grown to confluence for 2-3 days and then were washed three times in Krebs-Ringer phosphate buffer, containing 130 mM NaCl, 5 mM KCl, 1.3 mM CaCl2, 1.3 mM MgSO4, and 10 mM Na2HPO4, pH 7.4. 2-Deoxy-[2,6,6-3H]2-glucose was then added to give a final assay concentration of 0.1-10 μM in 0.5 ml of Krebs-Ringer phosphate buffer. Uptake was terminated at 1 min by an addition of 1 ml/well of ice-cold phosphate-buffered saline (PBS) containing 0.3 mM phlorizin, followed by three rapid washes in ice-cold PBS/phlorizin. Following the arrest of transport, cells were solubilized with 0.4 ml of 0.1% SDS, and the extract was added to scintillant for estimation of radioactivity. Zero time uptake was determined by adding phlorizin before the transported substrate.

**Labeling of Transporter Cell Surface Carbohydrate with Tritiated Sodium Borohydride—**The CHO-K1 clones transfected with either wild-type or mutated GLUT1 were grown to confluence in 35-mm culture dishes. The cells were then simultaneously treated with 10 units of galactose oxidase and 1 unit of neuraminidase in 1 ml of PBS for 30 min at 18°C. The dishes were then washed three times with 2 ml of PBS, 1 μCi of tritiated sodium borohydride (specific activity 13.4 Ci/mmol) in 40 μl of 50 mM NaOH was rapidly mixed with 20 μl of 45 mM HCl and added immediately to the cells in 1 ml of PBS. After maintaining the cells at 18°C for 10 min, the dishes were washed four times in PBS, and then the cells were solubilized in 1 ml of detergent buffer containing 2% C12E8 in PBS, pH 7.2, with the proteinase inhibitors antipain, aprotinin, pepstatin A, and leupeptin, each at 1 μg/ml. Following centrifugation at 20,000 × g for 10 min, the supernatant was subjected to immunoprecipitation with 20 μl of protein A-Sepharose coupled to 100 μl of anti-GLUT1 antisera. The immunoprecipitates were washed four times in 0.2% C12E8, detergent buffer and then directly solubilized in electrophoresis sample buffer containing 10% SDS, 6 M urea, and 10% mercaptoethanol. The supernatant was subjected to electrophoresis on 10% acrylamide gels. Gel slices, in scintillation vials, were dried at 80°C for 2 h and were then dissolved in 0.5 ml of alkaline hydrogen peroxide (2% v/v ammonium hydroxide in 30% hydrogen peroxide) at 80°C for an additional 2 h. Scintillant was added and the radioactivity counted. The positions of the photobleached peaks were compared with the positions of molecular weight markers in adjacent lanes. The levels of radioactivity associated with each peak were obtained by summing the radioactivity in all slices under the peak and subtracting a background based on the average radioactivity of the slices on either side of the peak.

**ATB-BMPA and Cytochalasin B Photolabeling of GLUT1 Transfected CHO-K1 Cells—**Confluent cells in 35-mm dishes were washed four times in PBS buffer and were then incubated at 18°C with 100 μCi of ATB-282 (1-butylmaleimide) or 1.4 μCi of cytochalasin B and 100 μCi cytochalasin E in 250 μl of PBS for 2 min and then irradiated for 1 min in a Rayonet.

**FIG. 1. Western blot analysis of mutated GLUT1 transporters.** CHO-K1 cells were transfected with cDNA coding for wild type (lane 2), Y292F (lane 3), Y292L (lane 4), Y293F (lane 5), and Y293C (lane 6) and compared with nontransfected cells (lane 1). A crude membrane fraction was obtained from stably transfected cells and subjected to Western blotting with GLUT1 C-terminal peptide antibody.
Y293F mutant GLUT1 were efficiently labeled with ATB-BMPA. In comparison with the wild-type GLUT1, the using this technique, the Tyr-293 mutant transporters were confirmed to be expressed at the cell surface to levels of 112.7 ± 29.7% (n = 3) for Y292F and 137.3 ± 18.5% (n = 4) for Y293F, respectively, of that occurring in the wild-type clone. By contrast, the CHO-K1 clone was labeled to a level that was only 14.8 ± 3.4% (n = 3) of the wild-type clone.

2-Deoxy-D-glucose uptake measurements in the Y293I clone showed that a marked reduction in the V

 where L

 and L are the levels of labeling observed without and with the competing ligand (I). The K values in the wild-type and Y293I mutant GLUT1 are 8.5 ± 0.5 mm and 3.0 ± 0.2 mm for 4,6-O-ethyldene-D-glucose and 118.8 ± 3.8 μM and 80.0 ± 4.3 μM for ATB-BMPA, respectively, indicating an ~2-fold higher affinity of the mutated GLUT1 for these exofacial ligands.

| Results are the mean ± S.E. of the indicated number of experiments except where the results are from two separate experiments, as noted. |

**Table I**

<table>
<thead>
<tr>
<th>Western blotting (% of wild type; n = 3)</th>
<th>CHO-K1</th>
<th>Wild type</th>
<th>Y292F</th>
<th>Y292I</th>
<th>Y293F</th>
<th>Y293I</th>
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<tbody>
<tr>
<td>14.4 ± 2.2</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>129.0 ± 35.3</td>
<td>137.3 ± 18.5</td>
<td>134.8 ± 3.4</td>
</tr>
<tr>
<td>Borohydride labeling (% of wild type; n = 4)</td>
<td>14.8 ± 3.4</td>
<td>100</td>
<td>136.5 ± 5.7</td>
<td>131.9 ± 31.0</td>
<td>137.3 ± 18.5</td>
<td>112.7 ± 29.7</td>
</tr>
<tr>
<td>Transport K_{m} (µM; n = 3)</td>
<td>2.6 (3.4, 1.8)</td>
<td>1.98 ± 0.1</td>
<td>2.46 ± 0.32</td>
<td>2.17 ± 0.33</td>
<td>2.0 ± 0.12</td>
<td>1.38 ± 0.15</td>
</tr>
<tr>
<td>Transport V_{max} (nmol/min/mg of protein; n = 3)</td>
<td>7.1 (7.0, 7.2)</td>
<td>74.9 ± 18.2</td>
<td>72.7 ± 23.6</td>
<td>75.1 ± 19.2</td>
<td>83.5 ± 11.7</td>
<td>10.3 ± 2.8</td>
</tr>
<tr>
<td>ATB-BMPA labeling (% of wild type; n = 3)</td>
<td>16.2 ± 5.3</td>
<td>100</td>
<td>147.6 ± 60.7</td>
<td>118.7 ± 15.8</td>
<td>105.8 ± 27.1</td>
<td>126.2 ± 34.9</td>
</tr>
<tr>
<td>Cytochalasin B labeling (% of wild type; n = 4)</td>
<td>18.2 ± 2.3</td>
<td>100</td>
<td>105.5 ± 8.6</td>
<td>53.8 ± 7.3</td>
<td>107.3 ± 8.1</td>
<td>4.5 ± 2.1</td>
</tr>
</tbody>
</table>

* Results are from two separate experiments.

**Results**

The four mutant GLUT1 cDNAs encoding Tyr-292 → Phe (Y292F), Tyr-293 → Ile (Y292I), Tyr-293 → Phe (Y293F), and Tyr-293 → Ile (Y293I) were transfected into CHO-K1 cell lines by the calcium phosphate method, and the stable transformants were selected by their resistance to neomycin. Western blot analysis of crude membranes isolated from the neomycin-resistant clones using an anti-peptide antibody directed against residues 478-492 of the C-terminus of GLUT1 enabled clones that had levels of expression comparable with the wild-type clone previously isolated to be selected (Hashiramoto et al., 1992) (Fig. 1). Using Western blot analysis, the expression levels in these clones were shown to be ~7-fold higher than that observed with a nontransfected CHO-K1 clone. The transfection efficiency was calculated as 100.3 ± 29.7% (n = 3) for Y292F, 129.0 ± 15.3% (n = 3) for Y292I, 97.4 ± 8.9% (n = 3) for Y293F, and 134.8 ± 3.4% (n = 3) for Y293I of that occurring in the wild-type clone.

The substitution of tyrosine 292 by neither isoleucine nor phenylalanine altered cell surface labeling using sodium borohydride, 2-deoxy-D-glucose transport activity, or the labeling by ATB-BMPA. In comparison with the wild-type GLUT1, the Y292I GLUT1 showed slightly reduced cytochalasin B labeling. (Table I).

The results of cell surface labeling the Y293I and Y293F clones using sodium borohydride are compared with the labeling of the wild-type and the nontransfected clones in Fig. 2. Using this technique, the Tyr-293 mutant transporters were confirmed to be expressed at the cell surface to levels of 112.7 ± 29.7% (n = 4) for Y293I and 157.5 ± 18.5% (n = 4) for Y293F, respectively, of that occurring in the wild-type clone. By contrast, the CHO-K1 clone was labeled to a level that was only 14.8 ± 3.4% (n = 3) of the wild-type clone.

2-Deoxy-D-glucose uptake measurements in the Y293I clone showed that a marked reduction in the V_{max} for transport that was only 14% of that observed in the wild type (Fig. 3). By contrast, the Y293F clone retained a relatively high level of transport activity. The V_{max} was similar to that of the wild-type clone (Table I).

We initially assessed the ability of the Tyr-293 mutated clones to bind exofacial and endofacial ligands using photolabeling procedures. As shown in Fig. 4A, both the Y293I and Y293F' mutant GLUT1s were efficiently labeled with ATB-BMPA.
Fig. 6, A and B, compares the displacement of ATB[2-^3]H]BMPA by \( \sigma \)-glucose and cytochalasin B in the wild-type and Y293I mutated GLUT1. The competition between 10 mM \( \sigma \)-glucose and ATB[2-^3]H]BMPA is similar in the wild-type and Y293I mutated GLUT1. By contrast, a range of concentrations of cytochalasin B that markedly inhibit ATB[2-^3]H]BMPA photolabeling in the wild-type GLUT1 has virtually no inhibitory effect on the extent of labeling in the Y293I mutated GLUT1. The approximate \( K_a \) for cytochalasin B in the wild-type and Y293I mutated GLUT1 are 0.5 \( \mu \)M and 144 \( \mu \)M, respectively. These values are only approximations, because in neither case was the range of concentrations of cytochalasin B ideal for determining the affinity constant. Nevertheless, the \( K_a \) for cytochalasin B is at least 250-fold higher in the Y293I mutated GLUT1.

A simple analysis (Stein, 1986; Clark and Holman, 1990) suggests that the \( K_a \) for binding exofacial and endofacial ligands are determined according to Eq. 2,

\[
\frac{K_a}{K_{d1}} = \frac{K_{d2}}{K_a + A}
\]

where \( K_a \) are the dissociation constants, and \( A \) is the ratio of distribution of alternately exposed sites at inside compared with outside surfaces. If, in the wild-type clone, the value of \( A \) is approximately unity, i.e. if the alternately exposed binding sites are equally available at inside and outside surfaces, then it follows that the effects of mutagenesis of Tyr-293 could be entirely due to a redistribution of available sites from inside to outside so that \( A \) is very low. This explanation would account for the small ~2-fold decrease in \( K_a \) (the 4,6-O-ethylidene-\( \sigma \)-glucose and ATB-BMPA affinity constants) and a large increase in \( K_a \) (the cytochalasin B affinity constant).

**DISCUSSION**

\( \sigma \)-Glucose analogues in which bulky alkyl groups have been substituted have provided information on the spatial requirements for hexose binding at the outer and inner sites of the hexose transporters, GLUT1 and GLUT4 (Barnett et al., 1973a, 1973b, 1975; Holman and Rees, 1982). Substitutions into the C-1 position of \( \sigma \)-glucose markedly reduce interaction with the exofacial site, suggesting that there is a close approach to the front of the glucose molecule as it interacts at the outer binding site. By contrast, analogues with hydrophobic alkyl substitutions into the C-4 and C-6 positions of \( \sigma \)-glucose were found to have high affinity for the transporter, in some cases exceeding that of \( \sigma \)-glucose. The high affinity of the hydrophobic C-6 substituted analogues led to the suggestion that there is a hydrophobic patch on the transporter, which is close to the C-6 of the sugar as it locates in the exofacial site. Although binding well to the exofacial site, the C4 and C6 substituted analogues were not transported substrates. Therefore, it was suggested that on initial binding of the sugar, there was not a close approach to the C4 and C6 positions but that in the absence of a bulky substitution at C-6, the transporter could close the hydrophobic patch around the back of the sugar and so facilitate the trans-
Functional Role of Tyr-293 in GLUT1

Fig. 5. Exofacial displacement of ATB[2-3H]BMPA photolabel in wild-type and Tyr-293 → Ile GLUT1. Cells of the wild-type (●) and Y293I (▲) clones were grown to confluence in 35-mm dishes and then labeled with 100 μCi of ATB[2-3H]BMPA in the presence of increasing concentrations of either nonlabeled ATB-BMPA (A) or 4,6-O-ethylidene-α-glucose (B). Following irradiation, the cells were washed five times in PBS and directly solubilized in SDS-electrophoresis sample buffer and subjected to electrophoresis on 10% acrylamide gels. The total radioactivity associated with the labeled transporter peak either in the presence (L) or absence (Lₒ) of competing ligand were calculated. Results are the mean of two similar experiments. Kᵢ values were calculated by least squares regression of the equation \( L/Lₒ = 1 + I/K_i \), where I is the concentration of displacing ligand.

Fig. 6. Glucose and cytochalasin B displacement of ATB[2-3H]BMPA photolabel in wild-type and Tyr-293 → Ile GLUT1. Cells of the wild-type (●) and Y293I (▲) clones were grown to confluence in 35-mm dishes and then labeled with 100 μCi of ATB[2-3H]BMPA in the presence of increasing concentrations of either D-glucose or cytochalasin B. Following irradiation, the cells were washed five times in PBS and directly solubilized in SDS-electrophoresis sample buffer and subjected to electrophoresis on 10% acrylamide gels. The total radioactivity associated with the labeled transporter peak either in the presence (L) or absence (Lₒ) of competing ligand were calculated. Results are the mean of two similar experiments, except the D-glucose displacement from the wild-type GLUT1 are the mean ± S.E. of three experiments. Kᵢ values were calculated by least squares regression of the equation \( L/Lₒ = 1 + I/K_i \), where I is the concentration of displacing ligand.

There is a relatively minor perturbation of cytochalasin B labeling in the Y293I mutated GLUT1, but the cytochalasin B labeling is totally abolished as a result of the Y293I substitution. We interpret these data as indicating that the residues F291, Y292, and Y293 constitute part of the hydrophobic patch, previously postulated from studies involving hexose analogues (Fig. 7). Size and hydrophobicity of the tyrosine residue are both important properties, as the relatively small but hydrophobic isoleucine group is insufficient to allow the normal conformational change that accompanies the transport catalysis process. The bulky phenylalanine substitution does, however, allow this change. If the residues of this hydrophobic patch move around the back of the sugar to separate it from the external solution, then this process may occlude the sugar within the center of the protein and strengthen the interaction with hydrophobic amino acid side chains in this region. The sequence Q²⁸²QLSGINAFFYY²⁹³ in TM 7 of GLUT1 is therefore suggested to have a role in both hydrogen bonding to D-glucose and closing the exofacial site during the transport catalysis process (Fig. 7). This interpretation is consistent with the observation that substitution of Q282 by leucine abolishes ATB-BMPA binding. The glutamines in this region may be responsible for providing some of the hydrogen bonding to the C1, C3, and ring-oxygen positions that previous studies on fluoro- and deoxy-hexose analogue have suggested are the hydrogen bonding positions on the sugar (Barnett et al., 1973a).

The Y293I mutant GLUT1 shows a decrease in glucose transport activity that is associated with a complete loss of affinity for the inside specific ligand cytochalasin B but a retention of high affinity for the outside specific ligand ATB-BMPA. These results are in marked contrast with those observed in C-terminally truncated GLUT1, where the decreased glucose transport activity is associated with corresponding loss of labeling by ATB-BMPA but with retention of high affinity for cytochalasin B (Oka et al., 1990). Oka et al. (1990) interpreted these results as indicating that the truncated GLUT1 is locked into an in-
Fig. 7. Proposed role of Tyr-293 in closing the exofacial hexose binding site. The sequence Q293QLSGINAVY in TM 7 of GLUT1 is suggested to have a role both in hydrogen bonding to o-glucose and closing the exofacial site necessary for facilitating the occlusion of this transported sugar. Only the hydrogen bonding is necessary for interaction with the exofacial ligands ATB-BMPA and 4,6-O-ethylidene-glucose, which are substituted here suggest that the Y293I transporter is locked in an outward facing conformation. By contrast, the results presented here suggest that the Y293I transporter is locked in an outward-facing conformation.

From a combination of ligand binding and mutagenesis studies, it is now apparent that conformational flexibility is necessary for the transport catalysis process. The glycine- and proline-rich region of TM 10 may act as a pivotal point in this process that alternately allows opening and closing of the outer and inner sites (Tamori et al., 1994). Nontransported ligands may stabilize the outward and inward directed conformations (Barnett et al., 1973b; Holman and Rees, 1982, 1987), but mutagenesis and proteolytic enzymes can also lock transporters in these stable conformations (Oka et al., 1990; Clark and Holman, 1990). It is particularly striking that a completely stable and outward directed conformation can be induced by just a single amino acid substitution, Tyr → Ile at position 293.

REFERENCES