Structure−Function Studies of the Brain-Type Glucose Transporter, GLUT3: Alanine-Scanning Mutagenesis of Putative Transmembrane Helix VIII and an Investigation of the Role of Proline Residues in Transport Catalysis†

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ABSTRACT: The brain-type glucose transporter (GLUT3) is a high-affinity transporter for D-glucose and D-galactose and is a member of a family of mammalian sugar transporters, each of which are proposed to adopt a secondary structure containing 12 transmembrane helices. In an effort to understand structure−function relationships within such transporters, we have employed alanine-scanning mutagenesis to examine the functional importance of each residue within putative transmembrane helix VIII of the human GLUT3 isoform. Each residue in this helix was replaced individually with alanine, and the functional properties of the mutants were examined by microinjection of *in vitro* transcribed mRNA into *Xenopus* oocytes. We show that substitution of residues 305, 306, 308−314, and 316−325 with alanine had minimal effect on the functional activity of the transporter, as determined by measurement of the K_m for deoxyglucose transport and the K_i for maltose. In contrast, Asn-315 > Ala-315 exhibited a significant increase in the K_m for deoxyglucose independently of any effect on the K_i for maltose. This data suggests that, despite the strong sequence conservation in this helix among the GLUT family, no individual residue is absolutely required for transport catalysis by this isoform. We have also examined the role of proline residues in transport catalysis mediated by GLUT3. Substitution of Pro-203 (helix VI), Pro-206, Pro-209 (cytoplasmic loop between helices VI and VII), Pro-381, Pro-383 and Pro-385 (helix X), Pro-399 (intracellular loop between helices X and XI), or Pro-451 (in the carboxy terminus, close to the end of helix XII) with alanine did not change the K_m for deoxyglucose transport for any mutant. However, both Pro-381 and Pro-385 when mutated to alanine exhibited a reduction in the K_i for cytochalasin B. In addition, the K_i for maltose inhibition of deoxyglucose transport was increased for mutants Pro206Ala, Pro381Ala, Pro383Ala, and Pro451Ala. These results will be discussed in terms of proposed structural models for the transporters.

The transport of glucose across the plasma membrane of animal cells is mediated by a family of transporters of the facilitative diffusion type. These transporters are expressed in a tissue-specific fashion resulting in the highly coordinated control of blood glucose [for review, see Bell et al. (1993) and Gould and Holman (1993)]. The ectopic expression of different members of the transporter family in a variety of cellular systems has resulted in a detailed analysis of the kinetic properties and substrate selectivities of these proteins (Burant & Bell, 1992; Colville et al., 1993b; Gould et al., 1991; Keller et al., 1989; Nishimura, 1993), and issues of structure−function relationships within this important class of membrane transporters are the subject of intense investigation in many laboratories (Hashiramoto et al., 1992; Hresko et al., 1994; Mori et al., 1994; Mueckler, 1994; Tamori et al., 1994). Upon the basis of computer analysis of the primary amino acid sequence, two distinct models of the glucose transporters have been proposed (Fishbarg & Vera, 1995; Gould & Bell, 1990; Saravolac & Holman, 1997; Zeng et al., 1996). The most widely accepted proposes that the transporters contain 12 putative membrane-spanning helices, and this model is consistent with most of the experimental data available to date [reviewed in Saravolac and Holman (1997)].

Glucose transporters have been proposed to exist in either of two conformations, with the substrate binding site exposed to either the extracellular side (exofacial conformation) or the intracellular space (endofacial conformation) (Baldwin, 1993). Structural separation of these sites has been proposed on the basis of both kinetics and inhibitor selectivity (Appleman & Lienhard, 1989; Baldwin, 1993; Baldwin et al., 1979; Bloch, 1973; Hebert & Carruthers, 1991, 1992). Studies by Holman and colleagues using baculovirus expressed protein have shown that the C-terminal half of the erythrocyte-type (GLUT1) protein contains sufficient information to recognize and bind both the endofacial ligand cytochalasin B (CB)[1] and the exofacial ligand ATB-BMPA. Such studies suggest a key role for the C-terminal cluster of six putative transmembrane helices in the binding of glucose (Cope et al., 1994). The identification of functionally important residues within the transporter has been the subject of intense investigation [recently reviewed in Saravolac and Holman (1997)]. Such studies have employed both chimeric

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[1] Abbreviations: DeGlc, 2-deoxy-D-glucose; CB, cytochalasin B.

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transporter species (Arbuckle et al., 1996; Buchs et al., 1995; Katagiri et al., 1992) and individual point mutations within the transporters [for example Garcia et al. (1992), Hashiramoto et al. (1992), Katagiri et al. (1991), Mueckler (1994), and Wellner (1995)], and subsequent expression and analysis of the mutant proteins in oocytes or cultured cells. These studies have provided important insight into the regions of the protein which are important in both substrate selection and transport activity.

In this study, we set out to determine the role of individual residues within one of the helices in the carboxy-terminal half of the protein, helix VIII. This helix was chosen because of its high degree of conservation between different GLUT isoforms. We have sequentially replaced each residue within this helix with alanine and examined the functional properties of these mutants by heterologous expression in *Xenopus* oocytes. Our data show that no individual residue within this helix is crucial for transport activity, but mutation of Asn-315 > alanine resulted in a small but significant increase in the K_m for 2-deoxy-D-glucose (deGlc). These data are discussed in terms of the published models of the structure of the GLUT family.

We have also examined the role of several proline residues in GLUT3 function. Proline has been implicated as an important amino acid in membrane proteins as the isomerisation of this amino acid has been suggested to endow regions of protein with conformational flexibility which may be crucial for transport function (Milner-White et al., 1992; Wellner et al., 1995; Williams & Deber, 1991). Studies on GLUT1 have shown that the proline residues in putative transmembrane helices VI and X are individually not crucial for transporter function, but kinetic analysis of these mutants was limited (Wellner et al., 1995). Here, we have examined the role of these residues in GLUT3 function with a view to determining the generality of the results obtained with GLUT1 and also examined the effect of mutation of other proline residues predicted to lie close to the endofacial side of the membrane or in the intracellular loop(s) of the GLUT3 protein. Subtle alterations in the kinetic properties of these mutants were observed which are discussed in relation to models of GLUT function.

MATERIALS AND METHODS

Materials. Wild caught *Xenopus laevis* were purchased from the African Xenopus Facility (Noordhoek, Republic of South Africa). All isotopes were from DuPont/NEN (U.K.), and sugars purchased from Sigma (Poole, U.K.). Reagents for *in vitro* transcription, DNA ligations/digestions, *Taq* polymerase, and dNTPs were from Promega (Southampton, U.K.). Pfu polymerase was from New England Biolabs. All other reagents were as described (Arbuckle et al., 1996; Colville et al.; 1993a, Gould et al., 1991).

Isolation of Oocytes and Microinjection. Female *X. laevis* were maintained at 18 °C on a 12 h light/dark cycle. Individual oocytes were dissected and stored in DNOM buffer (Arbuckle et al., 1996); this buffer is a modification of the more commonly used Barths buffer which we have used previously (Colville et al., 1993a; Gould et al., 1991). DNOM buffer was prepared by supplementing 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) with nonessential and essential amino acids (Gibco, Paisley, Scotland), MEM-vitamins (Gibco, Paisley, Scotland), all at 1× the manufacturers recommended concentrations, 1 mM oxaloacetate, and 1 mM pyruvate. All subsequent procedures were performed in DNOM buffer, except for the assays of hexose transport, which were performed in Barths buffer alone. Oocytes were injected with water or 50 nL of cRNA (usually ∼50 ng), prepared and purified as described in (Colville et al., 1993b), and incubated in DNOM buffer at 18 °C for 48 to 72 h prior to assay; the medium was replaced every 12 h.

Hexose Transport in Oocytes. Transport of deGlc. Groups of 8 oocytes were washed three times in Barths buffer and incubated in 0.45 mL of Barths buffer at pH 7.4 in 13.5 mL centrifuge tubes at room temperature (∼18 °C). Transport measurements were initiated by the addition of a 50 μL aliquot of [2,6-^{3}H]deGlc to the concentration indicated in the figures and tables. The reaction was stopped after the requisite time interval (30 min unless otherwise stated) by quickly aspirating the media and washing the oocytes with 5 mL of ice-cold phosphate buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate, pH 7.4) containing 0.1 mM phloretin, a potent transport inhibitor. The oocytes were washed in this fashion two more times and dispensed to scintillation vials, 1 oocyte per vial. These three washes were completed within 30 s. Sodium dodecyl sulfate (1 mL of 1%) was added to each scintillation vial and incubated at room temperature for 1 h with agitation, prior to the addition of scintillant and measurement of radioactivity. Under these conditions, we have demonstrated that transport of deGlc, and not its subsequent phosphorylation, are rate limiting in oocytes (Colville et al., 1993a,b; Gould et al., 1991). Parallel assays were undertaken using oocytes microinjected with water as a control. These transport rates were subtracted from those obtained in oocytes expressing a functional transporter, and here we present only the value of transport rates obtained after such subtraction. Such control assays were performed for every condition in every experiment. In general, the water-injected oocytes exhibit a deGlc transport rate of between 3 and 15% of that measured in an identical oocyte population microinjected with a functional transporter. K_i values were obtained at a range of deGlc (substrate) and CB or maltose (inhibitor) concentrations as previously described (Colville et al., 1993a; Gould et al., 1991).

Generation of Mutant Transporter Species. Recombinant mutant transporter cDNAs were constructed either with the Amersham site-directed mutagenesis kit (Amersham Ltd., U.K.), or using a PCR-based approach (Arbuckle et al., 1996; Katagiri et al., 1992). For the latter, complementary oligonucleotide primers and PCR were used to generate two fragments with overlapping ends containing the mutation of interest. These primary PCR fragments were combined in a subsequent secondary PCR reaction in which the overlapping ends anneal allowing the 3′ overlap of each strand to serve as a primer for the extension of the complementary strand. The sequences of the oligonucleotides used are presented in Table 1. PCR reactions were carried out using either Taq polymerase or *Pfu* polymerase. The PCR generated primary and secondary products were purified by gel electrophoresis followed by passage through a DEAE-sephacel column (EluTip, Schliecher and Scheull). Primary products were purified in this fashion before the subsequent overlap extension PCR reaction.

Table 1: Details of Oligonucleotides Used in Mutagenesis[a]

	sequence	mutation
helix VIII mutants		
antisense strand	GGTGGCATATGCGGGCTCTG	Ile^{305}→Ala
sense strand	CAAGAGCCCGCATATGCCACC	Ile^{305}→Ala
antisense strand	ATGGTGGCGGCGATGGGTC	Tyr^{306}→Ala
sense strand	GAGCCCATCGCCGCCACCATC	Tyr^{306}→Ala
antisense strand	GCGCCGATTGCGGCATAAT	Thr^{308}→Ala
sense strand	ATCTATGCCGCAATCGGCGCG	Thr^{308}→Ala
antisense strand	CGCGCCTGCGGTGGCATA	**Ile^{309}→Ala**
antisense strand	CACACCCGCTGCGATGGTGGC	**Gly^{310}→Ala**
antisense strand	ATTAACCACTGCCGCGCCGAT	Gly^{312}→Ala
sense strand	TTCACTGTAGCATCTCTATTT	Gly^{312}→Ala
antisense strand	AGTATTAACTGCACCCGCGCC	**Val^{313}→Ala**
antisense strand	GATAGTATTTGCCACACCCG	Val^{314}→Ala
sense strand	GCGGGTGTGGCAAATACTATC	Val^{314}→Ala
antisense strand	AAGATAGTTGCAACCACACC	Asn^{315}→Ala
sense strand	TTTCTCTATTTGCAGTGGAAAGGG	Asn^{315}→Ala
antisense strand	GTGAAGATTGCATTAACCAC	Thr^{316}→Ala
sense strand	GGTGTGGTTGCAACTATCTT	Thr^{316}→Ala
antisense strand	TACAGTGAATGCAGTATTAAC	**Ile^{317}→Ala**
antisense strand	AACTACAGTTGCGATAGTATT	Phe^{318}→Ala
sense strand	AATACTATCGCAACTGTAGTT	Phe^{318}→Ala
antisense strand	AGAAACTACTGCGAAGATAGT	**Thr^{319}→Ala**
antisense strand	TAGAGAAACTGCAGTGAA	**Val^{320}→Ala**
antisense strand	AAATAGAGATGCTACAGTGAA	**Val^{321}→Ala**
antisense strand	CAGAAATAGTGCAACTACAGT	**Ser^{322}→Ala**
antisense strand	CACCAGAAATGCAGAAACTAC	Leu^{323}→Ala
sense strand	GTAGTTTCTGCATTTCTGGTG	Leu^{323}>Ala3
antisense strand	CCTTTCCACCAGTGCTAGAGAAAC	Phe^{324}→Ala
sense strand	GTTTCTCTAGCACTGGTGGAAAG	Phe^{324}→Ala
antisense strand	CCCTTTCCACTGCAAATAGAGAAA	Leu^{325}→Ala
sense strand	GTGGTTAATGCAAATCTTCAC	Leu^{325}→Ala
pro mutants		
antisense strand	ACGGGTGTCTGCGACTTTGAAGAA	Pro^{451}→Ala
sense strand	TTCTTCAAAGTCGCTGAGACCCGT	Pro^{451}→Ala
antisense strand	CATCGCAGCTGCGCGGGGGCCCTG	Pro^{399}→Ala
sense strand	CAGGGCCCCGGCGCAGCTGCGATG	Pro^{399}→Ala
antisense strand	AATAAACCAGGCAATGGGGCCTGG	Pro^{385}→Ala
sense strand	CCAGGCCCCATTGCCTGGTTTATT	Pro^{385}→Ala
antisense strand	CCAGGGAATGGCGCCTGGTCCAAT	Pro^{383}→Ala
sense strand	ATTGGACCAGGCGCCATTCCCTGG	Pro^{383}→Ala
antisense strand	AATGGGGCCTGCTCCAATTTCAAA	Pro^{381}→Ala
sense strand	TTTGAAATTGGAGCAGGCCCCATT	Pro^{381}→Ala
antisense strand	CAAAAATCTGGCACTTTCAGGGCA	Pro^{209}→Ala
sense strand	TGCCCTGAAAGTGCCAGATTTTTG	Pro^{209}→Ala
antisense strand	GGGACTTTCAGCGCAAAATGGAAG	Pro^{206}→Ala
sense strand	CTTCCATTTTGCGCTGAAAGTCCC	Pro^{206}→Ala
antisense strand	AGGGCAAAATGCAAGGGCTGCACT	Pro^{203}→Ala
sense strand	AGTGCAGCCCTTGCATTTTGCCCT	Pro^{203}→Ala
external oligos		
G3 end	*gtcgacgtcgac*GAGGGAGAGGTGGCTTTCCCATGCC	
G3 start	*gtcgacgtcgac*TCACCCCTAGATCTTTCTTGAAGAC	

[a] This table lists the oligonucleotides used in the site-directed mutagenesis and PCR protocols; note that the latter employed two oligonucleotides (see Materials and Methods). The sequences are shown reading from 5′ to 3′, and the codon which encodes the substituting alanine residue is underlined. Each pair of oligonucleotides (sense and antisense) were used to generate primary PCR products with overlapping complimentary ends containing the mutation; for these reactions, the external oligos G3 start or G3 end were also used. These were then combined as templates for a secondary PCR reaction employing G3 start and G3 end to generate a full-length cDNA containing the mutation of interest. Mutations prepared using the Amersham kit required only a single oligonucleotide, and these are indicated by the amino acid being written in bold type. G3 start and G3 end are designed to introduce specific restriction sites (lower case italics) into the amplified products.

The PCR fragments were engineered such that restriction sites at the 5′ and 3′ ends allowed subcloning into plasmid pSP64T (Kayano et al., 1990). Large scale plasmid preparations of all positive clones were then made for subsequent analysis. DNA sequencing was performed using DyeTermination reactions on an ABI automated DNA sequence analysis unit. All mutants were fully sequenced on both strands twice prior to functional analysis.

RESULTS

Alanine-Scanning Mutagenesis of Helix VIII. We have employed *Xenopus* oocytes as an expression system to study the effects of mutations on GLUT3 function. Helix VIII was chosen for this analysis because of its high degree of conservation among the members of the GLUT family which are capable of mediating glucose transport (i.e., GLUTs 1, 2, 3, and 4. See Table 2). This high degree of conservation may be interpreted to imply that this region is of particular functional importance (Baldwin, 1993). To that end, we prepared a range of mutants in which each residue in helix VIII of GLUT3 was individually changed to alanine.

Each of the mutants reproducibly exhibited high levels of 2-deoxy-D-glucose (deGlc) transport when expressed in *Xenopus* oocytes by microinjection of *in vitro* transcribed

Table 2: Sequence Alignment of Putative Helix VIII of the Human Glucose Transporters

GLUT1	...VYATIGSGIVNTAFTVVSLFV...
GLUT2	...VYATIGVGAVNMVFTAVSVFL...
GLUT3	...IYATIGAGVVNTIFTVVSLFL...
GLUT4	...AYATIGAGVVNTVFTLVSVLL...
consensus	...x**YATIG**x**G**x**VN**$^{T}_{M}$x**FT**x**VS**$^{VFL}_{LLV}$...

[a] The predicted amino acid sequence of putative helix VIII of GLUTs 1, 2, 3, and 4 is shown, together with the consensus sequence of this region. All sequences are written from the amino terminus to the carboxy terminus, left to right. For GLUT3, the amino acids shown are residues 305−325. Note that the sequence of the murine homologue of GLUT3 is completely identical to the human isoform in this region.

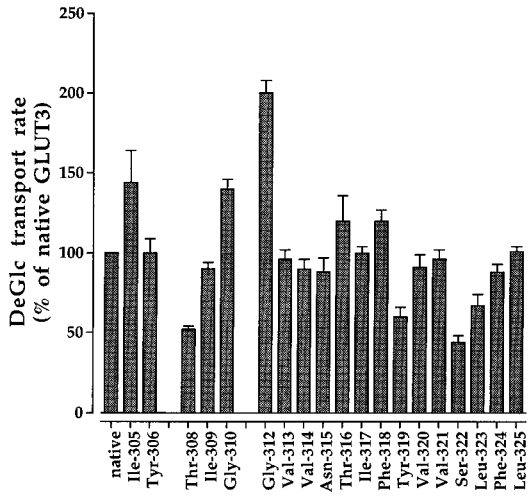

FIGURE 1: Expression of the helix VIII mutant GLUT3 species in *Xenopus* oocytes. Each of the mutants was expressed in *Xenopus* oocytes by microinjection of ~50 ng of *in vitro* transcribed mRNA. After 48 h, the rate of deGlc transport was determined at a concentration of 50 μM deGlc as described in Materials and Methods. In all experiments, a similar amount of mRNA encoding native GLUT3 was also injected and the oocytes were assayed. The observed transport rates were corrected by performing duplicate assays in water-injected oocytes. Transport rates for the mutants were expressed as a percentage of the native GLUT3 rate measured in the same oocyte preparation, and the data above represent data from three experimental measurements for each of the mutant species.

mRNA (Figure 1). Although it is not possible to compare these mutants directly from such a simple analysis, this experiment demonstrated that none of the mutations completely abolished transport activity. Such data argues that none of the residues mutated are absolutely required for function of the protein. We therefore undertook a more rigorous analysis of the kinetic properties of these mutants, and measured the K_m for deGlc transport. The results of this analysis, together with the simultaneous measurement of the K_i for maltose is presented in Table 3. Maltose was chosen for this analysis as it is a disaccharide (and therefore not transported by the GLUT isoforms) and has been previously established to act as a competitive inhibitor of deGlc transport by binding at the exofacial substrate binding site (Arbuckle et al., 1996; Baldwin, 1993; Barnett et al., 1975). Inspection of the data in Table 3 indicates that alteration of any of the residues in helix VIII of GLUT3 (with the exception of Asn-315) did not markedly change the K_m for deGlc transport or the K_i for maltose inhibition of transport.

Asn315Ala exhibited a markedly elevated K_m for deGlc compared to native GLUT3 (12.2 ± 4.9 *vs* 1.2 ± 0.03 mM, respectively); this increase in K_m was observed in the absence of any significant change in the K_i for maltose. The K_m value measured using deGlc is a function of both substrate binding and release from the exo- and endofacial substrate binding sites and also the rate of interconversion of the transporter between these two conformation states. In contrast, the K_i for maltose is chiefly a function of an event at the exofacial substrate binding site and thus is independent of the endofacial to exofacial conformational change. Hence, one interpretation of the increased K_m for deGlc of the Asn315Ala mutant independent of any change in the K_i for maltose is that this mutation has altered the rate of the transporter conformational change. However, definitive proof of such a contention would require accurate determination of transporter turnover numbers, a measurement which is difficult in oocytes (Arbuckle et al., 1996). Small increases in the K_i for maltose inhibition of deGlc transport were observed for mutants Ser322Ala and Leu325Ala (see Table 3).

Table 3: Kinetic Analysis of the Helix VIII Mutants[a]

mutation	K_m 2-deoxy-D-glucose (mM)	K_i maltose (mM)
native GLUT3	1.2 ± 0.03	33 ± 2.1
Ile305Ala	1.2 ± 0.36	39 ± 12
Tyr306Ala	2.1 ± 0.6	30 ± 15
Ala307Ala		
Thr308Ala	2.8 ± 0.9	nd
Ile309Ala	1.1 ± 0.3	20 ± 2
Gly310Ala	1.5 ± 0.1	37 ± 2
Ala311Ala		
Gly312Ala	1.5 ± 0.02	37 ± 8
Val313Ala	1.16 ± 0.1	nd
Val314Ala	1.3 ± 0.5	nd
Asn315Ala	12.2 ± 4.9[b]	28 ± 3
Thr316Ala	2.9 ± 1.0	53 ± 12
Ile317Ala	1.53 ± 0.3	45 ± 17
Phe318Ala	1.7 ± 0.3	40 ± 9
Thr319Ala	2.7 ± 0.4	34 ± 3
Val320Ala	1.1 ± 0.5	50 ± 16
Val321Ala	2.4 ± 0.8	nd
Ser322Ala	1.3 ± 0.3	55 ± 9[b]
Leu323Ala	1.9 ± 0.6	31 ± 2
Phe324Ala	2.6 ± 0.3	33 ± 7
Leu325Ala	1.3 ± 0.5	62.5 ± 16[b]

[a] Shown are the values for the K_m for deGlc and the K_i for maltose inhibition of deGlc transport, measured for each of the indicated GLUT3 mutations when expressed in oocytes. The values are the mean ± sd of at least three determinations for each mutant. Values for the native GLUT3 were measured in parallel in roughly half of the experiments presented above, and these values, together with published data (Colville et al., 1993b) were used to determine the native GLUT3 values presented. nd, not determined. [b] Statistically significant difference from native GLUT3 ($p < 0.05$).

Role of Proline Residues in GLUT3 Function. We also studied the effect of substitution of proline residues with alanine within the GLUT3 isoform with the view to examining the role of these residues in transporter function. Table 4 shows the result of mutation of Pro203Ala in helix VI, Pro206Ala, Pro209Ala (both predicted to lie in the large cytoplasmic loop), Pro381Ala, Pro383Ala and Pro385Ala (helix X), Pro399Ala (intracellular loop between helices X and XI) or Pro451Ala (in the carboxy terminus, close to the end of helix XII) on the K_m for deGlc transport, and the respective K_i values for cytochalasin B and maltose inhibition of deGlc transport. All of the mutants exhibited broadly comparable rates of deGlc transport into oocytes (not shown). Although we have not directly measured the GLUT3 protein content in oocytes expressing different mutants, this observa-

Table 4. Kinetic Analysis of Proline Mutants[a]

mutant	K_m deGlc (mM)	K_i maltose (mM)	K_i cytochalasin B (μM)
native GLUT3	1.2 ± 0.03	33 ± 2	2.8 ± 0.6
Pro206Ala	1.7 ± 0.2	76 ± 17[b]	2.1 ± 0.9
Pro209Ala	0.95 ± 0.5	56 ± 13	2.1 ± 1.2
Pro381Ala	0.96 ± 0.1	80 ± 14[b]	0.5 ± 0.4[d]
Pro383Ala	1.46 ± 0.6	71 ± 2[c]	nd
Pro385Ala	0.62 ± 0.3	43 ± 9	0.97 ± 0.7[d]
Pro451Ala	1.23 ± 0.2	67 ± 9[c]	1.76 ± 0.5

[a] Shown are the values for the K_m for deGlc, and the K_i for cytochalasin B (endofacial ligand) and maltose (exofacial ligand) inhibition of deGlc transport, measured for each of the indicated mutations in oocytes. The values are the mean ± sd of at least three determinations for each mutant. Values for the native GLUT3 were determined in parallel in roughly half of the experiments presented above, and these values, together with published data (Colville et al., 1993b) were used to calculate the native GLUT3 kinetic parameters presented above. nd, not determined. [b] Statistically significant changes from wild-type ($p = 0.05$). [c] Statistically significant changes from wild-type ($p < 0.05$). [d] Statistically significant changes from wild-type ($p < 0.02$).

tion suggests that none of the proline residues are absolutely required for transport catalysis, a result in general agreement with the data of Wellner et al. (1995). However, some subtle differences in kinetic properties were noted compared to wild-type GLUT3 (Table 4). These are discussed below.

DISCUSSION

Information Content of Helix VIII of GLUT3. Several models have been proposed for transporter structure. Zeng et al. (1996) have proposed that the transmembrane channel through which glucose moves across the membrane is composed of either helices III, IV, VII, VIII, and XI, or alternatively, helices II, V, VII, VIII, and XI. The amphipathic nature of helix VIII was an important consideration in the construction of these models, and this helix is strikingly well conserved among the GLUT isoforms which mediate glucose transport (Table 2). If the amphipathic nature of helix VIII is important in lining a water-filled channel through the transporter, then it may be predicted that mutation of the charged residues within this helix should have a significant functional impact, reflected by alterations in either the K_m for deGlc transport or the K_i value of an exofacial binding site inhibitor. Helix VIII of GLUT3 contains two Thr residues, a Ser, and an Asn residue. Of these, only substitution of Asn-315 with alanine exhibited any measurable change in transport kinetics (Table 3). Hydrophobic interaction between the transported sugar and the transporter proteins has also been proposed (Barnett et al., 1975; Colville et al., 1993a). However, transport function was not affected by substitution of the highly conserved Tyr and Phe residues within this helix. In fact, substitution of any residue in helix VIII by alanine (with the exception of Asn-315) produced no significant effect on the K_m for deGlc and in most cases no alteration in the K_i for maltose (Table 3). We argue that this implies that this helix does not play a major role in the recognition of glucose at the exofacial site or in the mechanism of transport catalysis. Although we have measured an increased K_m for deGlc for Asn315Ala, this is independent of any change in the K_i for maltose (Table 3), so it is unlikely that this residue is involved in substrate recognition. Rather, we would suggest that perhaps this residue is crucial for the maintenance of structural integrity of the transporter (see below).

In contrast to the models of Zeng et al., (1996), others have proposed that helix VIII may not be intimately involved in either the endofacial or the exofacial binding of substrate in the GLUTs (Baldwin, 1993; Saravolac & Holman, 1997). The data presented here are consistent with this proposal. It is tempting to speculate that the role of helix VIII is mainly in the establishment of structural integrity of the transporter protein as a whole.

The information content of helix VIII has been studied in a distinct sugar transporter, the lac permease of *Escherichia coli* (Hinkle et al., 1990). This transporter is also predicted to contain 12 membrane-spanning helices (Kaback, 1996) and has been subjected to intense mutagenesis studies [reviewed in Kaback 1996)]. It has been suggested that there is a stripe of low information content on one face of helix VIII which interacts with the membrane lipid and is tolerant of a range of amino acid substitutions which do not significantly affect function. The ability of helix VIII of GLUT3 to tolerate amino acid substitutions with little alteration in function may by analogy suggest a similar structural role for this helix. The ability to tolerate the presence of alanine (with its smaller side chain volume) at so many positions may imply that a much larger proportion of this helix is in contact with the membrane lipid or engaged in hydrophobic interactions with other helices than is the case for the lac permease.

Clearly, definitive proof of the arguments presented here will require the solution of the structure of the GLUTs by biophysical approaches. Nevertheless, the data presented in Table 3 clearly show that individual mutation of the residues within helix VIII to alanine does not ablate function of the transporter. Hence, we argue that this helix is not intimately involved in substrate recognition or transport catalysis; rather we would argue for a mainly structural role for helix VIII.

Proline Residues and GLUT3 Function. Proline residues have been implicated as factors in the conformational changes within membrane proteins accompanying transport (Williams & Deber, 1991). To address this, several studies have examined the role of membrane buried proline residues within membrane transport proteins, including GLUT1 (Baldwin 1993; Tamori et al., 1994). Pro-385 (in helix X of GLUT1)[2] is conserved in all mammalian glucose transporters and is located in a proline-glycine-rich region thought to confer high flexibility to this helix. Replacement of Pro-385 with isoleucine resulted in a loss of GLUT1 transport activity and labeling by ATB-BMPA, while cytochalasin B labeling was retained, suggesting that this mutation locks the transporter into the endofacial conformation (Tamori et al., 1994). There are 23 proline residues located in GLUT1, of which eight reside in either helix VI or helix X. When these residues were replaced with alanine (Tamori et al., 1994; Wellner et al., 1995), wild-type deGlc transport rates were observed. Therefore the presence of proline in any of these positions (Pro-187, Pro-196, Pro-383, Pro-385, Pro-387)[2] may not be absolutely required for transport. Such data have questioned the role of membrane buried proline residues within GLUT1. However, it should be noted that these studies contained no information on the kinetics of the transporter and so may have overlooked subtle alterations in function (Tamori et al., 1994; Wellner et al., 1995).

[2] Amino acid numbers refer to GLUT1 isoform.

Here, we have generated a range of proline mutants [including the proline residues in GLUT3 equivalent to those in GLUT1 studied by Tamori et al. (1994) and Wellner et al. (1995)] and studied the kinetic properties of these proteins when expressed in oocytes. Mutation of Pro-203, Pro-206, Pro-209, Pro-381, Pro-383, Pro-385, Pro-399, or Pro-451 to alanine did not produce any significant changes in the K_m for deGlc (Table 4), and all of the mutants exhibited broadly comparable rates of deGlc transport into oocytes (not shown), indicating that none of the proline residues are absolutely required for transport catalysis.

More detailed kinetic analysis has revealed interesting changes in the phenotype of some of these mutants. Pro381Ala and Pro385Ala were found to exhibit a ~3−4-fold lower K_i for CB than native GLUT3 (Table 4). Interestingly, the residue corresponding to Pro-385 in GLUT1 when mutated to isoleucine was found to lock the transporter in an endofacial conformation (Tamori et al., 1994). These authors conclude that this proline residue is important for the endofacial to exofacial conformational change during GLUT1 transport catalysis. Our data provides further proof of this hypothesis and extends the model to propose that Pro-381 is also important in this regard as this mutant exhibited an increased K_i for maltose (Table 4). We did not observe a similar increase in the K_i for maltose in mutant Pro385Ala. One possible explanation for this apparent anomaly may be that Pro-385 is somewhat less important in this context than Pro-381; consistent with this, an elevated K_i value for maltose in mutant Pro385Ala is observed, but the value is not statistically significant. Pro-381 and Pro-385 are located in the well-conserved glycine-proline-rich region at the base of putative transmembrane helix X; hence, we suggest that the region encompassing these residues is important for the exofacial to endofacial conformational change, and may act as a conformational switch during transport catalysis. These observations prompt the question of why no effect of these mutations on the K_m for deGlc was observed, as such changes might be expected if the rate of transporter reorientation is altered by these mutations. In order to fully investigate the effects of these mutations on transporter function, measurement of the V_{max} of the transporter is required (discussed below). However, such measurements are technically difficult in oocytes and had not been attempted [discussed in Arbuckle et al. (1996)]. Hence, it is possible that changes in V_{max} have occurred which mask changes in the K_m values for deGlc. Alternatively, it is possible that the effects measured using maltose to probe the exofacial substrate binding site are minimal within the context of the complete catalytic cycle of the transporter protein.

Inspection of the data of Table 4 also shows that two other proline substitutions (Pro206Ala and Pro451Ala) also exhibited significant increases in the K_i for maltose, but these changes were not accompanied by changes in the K_i for CB. It is proposed that the loops connecting the putative membrane-spanning helices of the GLUTs on the endofacial side of the membrane are shorter than those on the exofacial surface. Thus, the base of the helices have been proposed to be conformationally restricted (Baldwin, 1993; Gould & Holman, 1993). Interestingly, Pro-206 and Pro-451 reside close to the base of the membrane-spanning helices VI and XII, respectively. Hence, it may be speculated that mutation of these residues has resulted in global changes to the protein which have an effect on the exofacial binding site, reducing the affinity of exofacial ligands. Although such effects are not manifest in alterations in deGlc K_m values, it should be remembered that this term is a reflection of substrate binding the exofacial binding site, the reorientation of the loaded transporter to the endofacial conformation, the release of substrate, and the subsequent conformational change of the transporter back to the exofacial conformer. It is possible that the effects of proline mutations on the exofacial binding site are small compared to other aspects of the turnover of the protein.

We have interpreted our data within the framework of the alternating conformation model for transporter function. It should be clearly stated that other models for GLUT1 transporter function have been proposed [see, for example, Carruthers (1991) and Baldwin (1993)]. At present, there is insufficient data to distinguish between these possible models for GLUT3; hence, in this paper, we have assumed that this isoform exhibits transport properties of an alternating conformational model.

Taken together, the data outlined here provides several conclusions. Firstly, mutation of any individual proline residue does not significantly alter the K_m for deGlc, suggesting that the replacement of proline residues individually does not *grossly* perturb transporter function. Pro381Ala and Pro385Ala exhibit decreased values for the K_i for CB; in the case of Pro381Ala, this is accompanied by an increased K_i for maltose. We suggest that these residues are involved in the conformational change which accompanies transport catalysis. Our extensive kinetic analysis has, however, highlighted a significant change in the K_i for maltose in several of the proline mutants examined (Table 4). Such changes would not have been evident from the previous studies of GLUT1 containing proline mutations as detailed kinetic analysis was not performed (Tamori et al. 1994; Wellner et al., 1995).

Summary. We show that helix VIII of the human GLUT3 isoform, when investigated by alanine-scanning mutagenesis, does not contain any amino acids which are absolutely essential for transport catalysis. Mutation of Asn-315 to alanine does result in an increase in the K_m for deGlc transport independently of an effect on the K_i for maltose, suggesting that this residue may be involved in either the exofacial to endofacial conformational change, or (perhaps more likely) in the maintenance of structural integrity of the transporter. The absence of other measurable effects within this helix may imply that the role of helix VIII is mainly to provide a framework for structural packing within the transporter molecule. We suggest that proline residues are important in transporter function on the basis of small but significant changes in the kinetic properties observed in some of the proline to alanine substitutions. We suggest that the regions encompassing Pro-381 and Pro-385 is important in the conformational change of the protein. None of the proline mutants exhibited significantly decreased rates of glucose transport, indicating that no specific proline residue is absolutely required for transport catalysis. However the collective role of proline residues in transporter function remains unknown, particularly when one considers that substitutions at single prolines may be compensated for by the flexibility of neighboring proline residues.

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