Identification by Comprehensive Chimeric Analysis of a Key Residue Responsible for High Affinity Glucose Transport by Yeast HXT2*

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Hxt2 and Hxt1 are, respectively, high affinity and low affinity facilitative glucose transporter paralogs of Saccharomyces cerevisiae. We have previously investigated which amino acid residues of Hxt2 are important for high affinity transport activity. Studies with all the possible combinations of 12 transmembrane segments (TMs) of Hxt2 and Hxt1 revealed that TMs 1, 5, 7, and 8 of Hxt2 are necessary for high affinity transport. Systematic shuffling of the 20 amino acid residues that differ between Hxt2 and Hxt1 in these TMs subsequently identified 5 residues as important for such activity: Leu59 and Leu61 (TM1), Leu201 (TM5), Asn331 (TM7), and Phe366 (TM8). We have now studied the relative importance of these 5 residues by individually replacing them with each of the other 19 residues. Replacement of Asn331 yielded transporters with various affinities, with those of the Ile331, Val331, and Cys331 mutants being higher than that of the wild type. Replacement of the Hxt2 residues at the other four sites yielded transporters with affinities similar to that of the wild type but with various capacities. A working homology model of the chimeric transporters containing Asn331 or its 19 replacement residues indicated that those residues at this site that yield high affinity transporters (Ile331, Val331, Cys331) face the central cavity and are within van der Waals distances of Phe208 (TM5), Leu357 (TM8), and Tyr427 (TM10). Interactions via these residues of the four TMs, which compose a half of the central pore, may thus play a pivotal role in formation of a core structure for high affinity transport.

Monosaccharides are an important carbon and energy source for most living cells. In the yeast Saccharomyces cerevisiae, the movement of hexoses across the plasma membrane and into the cell occurs by facilitated diffusion via hexose transporters. The yeast cells must be able to adapt to the broad range of sugar concentrations to which they are exposed under natural conditions. A diversity of hexose transporter genes has evolved in S. cerevisiae, with these genes encoding 17 different transporters (Hxt1–11, Hxt13–17, and Gal2) (1, 2) that belong to the major facilitator superfamily (MFS) (3). Among these 17 hexose transporters, Hxt2 and Hxt1 are high affinity ($K_m = 3.3$ mM) and low affinity ($K_m = 46$ mM) glucose transporters that share $\approx 70\%$ amino acid sequence identity in corresponding putative transmembrane segments (TMs) and inter-TM loops. There are 252 amino acid residues in the 12 putative TMs of both Hxt2 and Hxt1 (4), with 75 of these residues differing between the two transporters. We have been attempting to identify which of these 75 sites of Hxt2 are the key determinants of high affinity transport.

We have adopted a comprehensive chimeric approach in studies with two closely related paralogs that differ in substrate specificity (5–7) or affinity (8–10) to overcome drawbacks of analyses based on site-directed mutagenesis. This approach greatly reduces the number of mutants necessary for saturation mutagenesis. For example, with this approach, the simultaneous random mutation of 12 sites yields $2^{12} = 4,096$ independent clones, whereas ordinary random mutagenesis at even four sites yields $2^4 = 16,000$ clones, a number so high as to render screening impractical. We previously studied which TMs of Hxt2 are important for high affinity glucose transport with the use of a new procedure designated TM shuffling (8). Each of the 12 TMs of Hxt2 was randomly replaced with the corresponding region of Hxt1 at the DNA level, and clones encoding transporters with a high affinity for glucose were selected by plating transformants on glucose-limited agar plates. Our results demonstrated that a minimal combination of TMs 1, 5, 7, and 8 of Hxt2 is necessary for high affinity glucose transport. The chimeric transporter C1578 (supplemental Fig. S1), in which all TMs but 1, 5, 7, and 8 of Hxt2 are replaced with the corresponding TMs of Hxt1, thus showed high affinity transport activity similar to that of Hxt2. Twenty amino acid residues in these four TMs differ between Hxt2 and Hxt1. We further examined which residues in C1578 are important for high affinity glucose transport by systematically shuffling these 20 residues with the corresponding residues of Hxt1. Analysis of transporters with high affinity and high capacity revealed that 5 residues of Hxt2 are important for such activity: Leu59 and Leu61 in TM1, Leu201 in TM5, Asn331 in TM7, and Phe366 in TM8 (9, 10) (supplemental Fig. S1). A working homology model indicated that Asn331 is located in the middle of TM7 and faces the central substrate permeation pathway, whereas the other residues are situated in the hydrophobic periphery (10).

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2 The abbreviations used are: MFS, major facilitator superfamily; TM, transmembrane segment.
As a final step in our analysis, we have now investigated which of these 5 residues of Hxt2 are most important for high affinity transport by replacing each of them with the other 19 amino acid residues and analyzing the transport activity of the resulting transporters. Our results indicate that Asn^{331} in TM7 is the key determinant of high affinity glucose transport. We thus found that replacement of Asn^{331} with each of the other 19 residues generated transporters with various affinities, with three of them possessing an affinity higher than that of the wild type.

Structural insight into MFS transporters was provided by determination of the crystal structures of two bacterial transporters, the lactose transporter LacY and the glycerol 3-phosphate transporter GlpT (11, 12). The configurations of the TMs in these two transporters are highly similar. The structures of two additional bacterial MFS transporters also proved to be similar to those of LacY and GlpT (13, 14). These results suggest that the MFS transporters share a similar conformation regardless of their substantial sequence differences. With the use of a previous homology model based on GlpT (10), we have now obtained results consistent with the notion that Asn^{331} of Hxt2, or its corresponding residue in high affinity mutants, interacts hydrophobically with residues in TMs 5, 8, and 10, thereby generating at least a part of a core TM structure required for high affinity glucose transport.

**EXPERIMENTAL PROCEDURES**

**Construction of Vectors**—The plasmid Hxt2mnx-pVT, which comprises HXT2 under the control of the ADH1 promoter in the multicopy vector pVT102-U (YEp URA3 blia), was constructed as described previously (8). The expression vector C1578-pVT, which encodes the chimeric transporter C1578 (in which all of the TMs of Hxt2, with the exception of TMs 1, 5, 7, and 8, are replaced with those of Hxt1), was also described previously (8).

**Mutagenesis**—Replacement of Leu^{59}, Leu^{61}, Leu^{201}, Asn^{331}, or Phe^{366} in C1578 with the other 19 residues was performed with the use of a polymerase chain reaction-based approach as described (10). In brief, mutation of the 5 target residues was achieved by replacing each target codon with NNK for all mutants with the exception of Leu^{201} mutants, for which NNN was used. DNA sequences for the mutated transporters were determined with a DNA sequencer (model 310, Applied Biosystems) and supplemented with adenine, amino acids, and sucrose concentration of 0.1 or 20 mM were expressed as picomoles of glucose/1 x 10^7 cells/5 s and were corrected for the background activity determined either in the presence of 0.5 mM HgCl_2 or with 0.1 or 20 mM L-[^14C]glucose as substrate. In some experiments, transport activity was calculated as a percentage of that obtained with the cells expressing C1578.

**Construction of a Three-dimensional Model of Hxt2**—The crystal structure of GlpT (Protein Data Bank accession number 1PW4) formed the basis for construction of a structural model of Hxt2. A working homology model of C1578 and Hxt2 was generated with the Biopolymer module of Insight II (version 2000; Accelrys, San Diego, CA) as described (10).

**RESULTS AND DISCUSSION**

We previously identified 5 residues of Hxt2 (Leu^{59} and Leu^{61} in TM1, Leu^{201} in TM5, Asn^{331} in TM7, and Phe^{366} in TM8) that are required for the high affinity and high capacity glucose transport activity of C1578 (10) (supplemental Fig. S1). To investigate the relative roles of these 5 residues, we individually replaced each one with each of the other 19 residues. The resulting mutant proteins were expressed in KY73 cells in which genes for the eight major glucose transporters (Hxt1–7, Gal2) are disrupted and which therefore do not exhibit substantial glucose transport activity. The transport activities of the mutant proteins were measured for 5 s at 30 °C at two substrate concentrations, 0.1 and 20 mM D-glucose, and they were normalized by cell number and expressed as a percentage of the activity of C1578 at each substrate concentration (Fig. 1 and supplemental Tables S1–S5). The transport activity of almost all Leu^{59}, Leu^{61}, Leu^{201}, or Phe^{366} mutants was reduced when compared with that of C1578. A few mutants, however, including 59F, 59T, 59N, and 61Q, maintained >90% of the activity of C1578 at both 0.1 and 20 mM glucose (supplemental Tables S1 and S2). The mutants were distributed more or less along the diagonal line connecting the origin to C1578 in Fig. 1, suggestive of no substantial change in K_m and various changes in V_max. In contrast, the Asn^{331} mutants showed a pattern markedly different from that of the others (Fig. 1 and supplemental Table S4). The transport activity of the mutants 331I, 331V, and 331C with 0.1 mM D-glucose was substantially higher than that of C1578, indicative of an increase in affinity.

We performed kinetic analysis for the Asn^{331} mutants (supplemental Table S6). A wide range of K_m (0.87–54 mM) and V_max (430–1320 pmol/5 s/10^7 cells) values was obtained. Only the Asn^{331} mutants showed such a diversity of K_m values (Fig. 2 and supplemental Table S7). The 331I, 331V, and 331C mutants exhibited affinities (K_m values of 0.87, 1.3, and 2.2 mM, respectively) substantially higher than that of C1578 (5.3 mM).
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FIGURE 1. Glucose transport activities of Leu59, Leu61, Leu201, Asn331, and Phe366 mutants of C1578. Each residue at positions 59, 61, 201, 331, and 366 of C1578 was replaced with each of the other 19 residues. KY73 cells expressing each of the resulting mutant transporters were grown to log phase at 30 °C in S2Mal synthetic medium, after which glucose transport activity was measured for 5 s at 30 °C with D-glucose at 0.1 or 20 mM as substrate. Transport activities were normalized by cell number and expressed as a percentage of the value for C1578. Each circle represents an individual mutant according to the indicated color code. Closed circles indicate mutants for which kinetic analysis was performed, with the results being shown in supplemental Tables S1–S5.

FIGURE 2. Affinities of mutant transporters for D-glucose. KY73 cells expressing the indicated Leu59, Leu61, Leu201, Asn331, or Phe366 mutant transporters were cultured to log phase at 30 °C in S2Mal synthetic medium, after which the kinetic parameters of D-glucose transport were measured for 5 s at 30 °C with 0.1–100 mM D-glucose as substrate as described (17). Affinity is expressed as 1/Km and data are means ± S.E. (n = 3).

In contrast, the affinities of 331I, 331P, and 331E (Km values of 20, 21, and 54 mM, respectively) were markedly reduced when compared with that of C1578. The Km values for transporters mutated at the other sites were 80–190% of that of C1578, with the exception of 201H, whose Km was 280% (Fig. 2 and supplemental Table S7).

We also studied whether the role of Asn331 in wild-type Hxt2 is the same as that in C1578. We thus replaced Asn331 of Hxt2 with Ile, Val, or Cys to generate 331I/Hxt2, 331V/Hxt2, and 331C/Hxt2, respectively. The Km and Vmax values (means ± S.E.) were 0.76 ± 0.03 mM and 160 ± 10 pmol/5 s/107 cells for 331I/Hxt2 (n = 3), 0.51 ± 0.02 mM and 320 ± 10 pmol/5 s/107 cells for 331V/Hxt2 (n = 3), and 0.98 ± 0.05 mM and 900 ± 60 pmol/5 s/107 cells for 331C/Hxt2 (n = 3), whereas those for wild-type Hxt2 were 3.3 ± 0.3 mM and 1170 ± 160 pmol/5 s/107 cells (n = 8). The 331I, 331V, and 331C mutations thus also markedly increased the affinity of Hxt2 for glucose according to the rank order 331V > 331I > 331C when compared with 331I > 331V > 331C for C1578. The replacement of Asn331 with each of these 3 residues therefore had similar effects in C1578 and Hxt2 with the slight differences in the relative magnitude of these effects possibly reflecting small structural differences between the two transporters.

We prepared a crude membrane fraction from cells and examined the expression level of the various mutant transporters by immunoblot analysis with antibodies to the COOH-terminal region of Hxt2. All mutants yielded a major immunoreactive band at a position corresponding to that of Hxt2 (47 kDa); representative data for the Asn331 mutants are shown in supplemental Fig. S2. Quantitative analysis of these bands revealed that the expression level for the mutants was ~60–140% (n = 3–5) relative to the value for C1578, with the exception of 201T (170%), 331H (27%), 366K (41%), and 366R (26%) (supplemental Tables S1–S5).

The Vmax/Km value provides a measure of transport efficiency (9) and was calculated for mutant transporters (supplemental Tables S6 and S7). Normalization by expression level changed individual values but did not affect the general profile (see expression levels in supplemental Tables S1–S5). Most of the Leu59, Leu61, and Phe366 mutants showed Vmax/Km values higher than that of C1578, with the values for 59V, 59I, 61N, and 61Q being similar to that of wild-type Hxt2. The Vmax/Km value for all of the Leu201 mutants examined was reduced when compared with that of C1578, confirming the essential role of Leu201 (9). The Asn331 mutants showed a wide range of Vmax/Km values (20–210%) of that of C1578. Transport efficiencies of the 331I, 331V, and 331C mutants were greater than that of C1578, with that of 331I also being higher than that of wild-type Hxt2.

Substrate specificities were examined for 331I, 331V, and 331C (supplemental Fig. S3). No substantial difference in the effects of the addition of nonradioactive D-galactose or L-glucose on D-glucose transport activity was apparent between the mutants and C1578. The addition of D-fructose inhibited the glucose transport activity of the mutants and C1578 by similar extents. 2-Deoxy-D-glucose, D-mannose, D-xylose, and 6-deoxy-D-glucose each inhibited the glucose transport activity of the mutants to a greater extent than they did that of C1578. 3-O-Methyl-D-glucose is not transported by Hxt2, Hxt1, or C1578 (7, 8), but this hexose inhibited the glucose transport activity of the mutants. We measured the inhibition constant (Ki) for 3-O-methyl-D-glucose with 0.05 mM [14C]-D-glucose as the substrate, obtaining values (n = 3) of >50 mM for C1578 when compared with 8.7 ± 0.7 mM for 331I, 14 ± 0 mM for 331V, and 13 ± 0 mM for 331C. These results indicate that the recognition of glucose was less selective in the Asn331 mutants, suggesting that the observed changes in substrate specificity are not due to a change in the recognition of a specific position of glucose.
The role of TM7 has been intensively studied in members of the mammalian GLUT family of glucose transporters. Exposure of cells expressing a single Cys-substituted form of GLUT1 to a water-soluble sulfhydryl reagent revealed that the exofacial portion of TM7 is accessible to the external solvent (19). A QLS motif present in TM7 of GLUT family proteins has been proposed to interact with the C-1 position of D-glucose (20), and Ile314 situated at the outer edge of TM7 of GLUT7 was shown to be required for fructose transport (21). Studies of other MFS transporters have also shown the importance of TM7. TM7 contributes to the substrate permeation pathway in the crystal structures of LacY and GlpT (11, 12), and one of the expected substrate binding sites (Arg269) in TM7 of GlpT corresponds to Gln327 of C1578 in our current homology model. TM7 of human Na+/H+ exchanger, another MFS transporter, was also shown to play an important role as an interrupted helix (22). These various observations thus indicate that TM7 as a whole plays a key role in substrate recognition by MFS transporters.

Construction of a homology model of C1578 based on the structure of GlpT revealed Asn331 to be situated in the middle of TM7 facing the central pore (10). A close examination of Asn331 and its environment shows that this residue is located on the edge of a hydrophobic pocket consisting of Phe208 (TM5), Leu357 (TM8), and Tyr427 (TM10) and that these 4 residues are positioned within van der Waals distances of each other (Fig. 3A). The model indicated that TMs 5, 7, 8, and 10 comprised a half of the central pore. Replacement of Asn331 with each of the other 19 residues revealed that Ile, Val, and Cys fit into the pocket and increase the amount of space in the central pore. Replacement of Asn331 with smaller residues such as Gly, Ala, and Ser increases the amount of space in the pocket as a result of weakening of the interactions with the other residues, but the predicted effects on $K_m$ are minimal. Breakage of the $\alpha$ helix by replacement of Asn331 with Gly or Pro is also possible. The remaining 12 residues substituted for Asn331 have protruding hydrophilic or hydrophobic side chains that resulted in steric hindrance. These observations are in good agreement with our experimental results showing that Asn, Ile, Val, and Cys at position 331 each support high affinity and high capacity glucose transport, whereas transporters containing the other residues at this position exhibit either lower affinity or impaired activity. They are also consistent with the observation that the recognition of glucose became less strict in the 331I, 331V, and 331C mutants. The unexpected inhibition of the glucose transport activity of these three mutants by 3-O-methyl-D-glucose is thus likely attributable to the extra space conferred by each mutation, which allows recognition of the methyl group of this sugar. The model also reveals that TMs 5, 7, 8, and 10 are closest to this pocket region and form an hourglass-like structure (Fig. 3B). It is thus

![FIGURE 3](image_url)
likely that van der Waals interactions among Phe208, Asn331, Leu357, and Tyr427 pull their respective TMs toward each other, resulting in bending of each TM.

With comprehensive chimeric analysis, we had previously identified 5 amino acid residues necessary for high affinity and high capacity glucose transport activity of Hxt2. We have now shown that one of these 5 residues, Asn331, is a critical determinant of transporter affinity for glucose.

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