Eight Amino Acid Residues in Transmembrane Segments of Yeast Glucose Transporter Hxt2 Are Required for High Affinity Transport*

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Hxt2 and Hxt1 are high affinity and low affinity facilitative glucose transporter paralogs of Saccharomyces cerevisiae, respectively, that differ at 75 amino acid positions in their 12 transmembrane segments (TMs). Comprehensive analysis of chimeras of these two proteins has previously revealed that TMs 1, 5, 7, and 8 of Hxt2 are required for high affinity glucose transport activity and that leucine 201 in TM5 is the most important in this regard of the 20 amino acid residues in these regions that differ between Hxt2 and Hxt1. To evaluate the importance of the remaining residues, we systematically shuffled the amino acids at these positions and screened the resulting proteins for high affinity and high capacity glucose transport activity. In addition to leucine 201 (TM5), four residues of Hxt2 (leucine 59 and leucine 61 in TM1, asparagine 331 in TM7, and phenylalanine 366 in TM8) were found to be important for such activity. Furthermore, phenylalanine 198 (TM5), alanine 363 (TM8), and either valine 316 (TM7) or alanine 368 (TM8) were found to be important for high capacity glucose transport activity. Construction of a homology model suggested that asparagine 331 interacts directly with the substrate and that the other identified residues may contribute to maintenance of protein conformation.

Facilitated diffusion of glucose across the plasma membrane of the yeast Saccharomyces cerevisiae is mediated by a variety of hexose transporters (Hxt1–Hxt17, Gal2) (1, 2) that belong to the major facilitator superfamily (MFS) (3). A common structural feature of members of this superfamily is the presence of 12 putative transmembrane segments (TMs), with both the NH2- and COOH-terminal domains being present on the cytoplasmic side of the membrane. Among the 18 hexose transporters of S. cerevisiae, Hxt2 is a major high affinity glucose transporter (Km = 3.6 mM), and Hxt1 is a low affinity glucose transporter (Km = 44 mM). The numbers of amino acid residues in each putative TM and inter-TM loop of Hxt2 are identical to those in the corresponding regions of Hxt1, and the two proteins share ~70% sequence identity in these regions, with only 75 of the ~250 residues in TMs differing between Hxt2 and Hxt1.

Site-specific mutagenesis has been used extensively to determine the contribution of individual amino acid residues to the structure and function of transporters. In some instances, however, single point mutations have indirect effects on protein function through an extensive, rather than localized, distortion of protein structure. To circumvent this problem and to identify positively the contributing amino acid residues, we have adopted a comprehensive chimeric approach with two closely related paralogs that differ in substrate specificities (4–6) or affinities (7, 8). We previously investigated which TMs of Hxt2 are important for high affinity glucose transport with the use of a new procedure designated TM shuffling (7). We thus randomly replaced each of the 12 TMs of Hxt2 with the corresponding segments of Hxt1 at the DNA level. Clones encoding transporters with a high affinity for glucose were selected by plating the 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 CI578 (Fig. 1), in which all TMs but 1, 5, 7, and 8 of Hxt2 are replaced with the corresponding TMs of Hxt1, excluding a contribution of 55 of the 75 TM residues that differ between Hxt2 and Hxt1, thus exhibited high affinity and high capacity glucose transport activity similar to that of Hxt2. We subsequently showed that, among the 20 residues that differ between Hxt2 and Hxt1 in these four TMs, Leu201 in TM5 of Hxt2 is the most important for high affinity glucose transport (8).

The crystal structures of the bacterial MFS transporters LacY (9) and GlpT (10) have been determined at a resolution of <4 Å, revealing that the configurations of the TMs in these two transporters are highly similar and that TMs 1, 5, 7, and 8 contribute to the central pore. Although alignment of the amino acid sequences of MFS transporters has revealed substantial sequence variability, these findings together with a low resolution crystal structure of the MFS transporter OxlT (11) and molecular modeling (12) suggest that MFS transporters share a similar conformation.

We have now examined which amino acids among the 19 remaining residues in TMs 1, 5, 7, and 8 of Hxt2 that differ from those of Hxt1 are important for high affinity and high capacity glucose transport activity. Our results show that, in addition to Leu201, four of these residues are important for and three residues are supportive of such activity. Molecular modeling suggests that most of these residues do not directly interact with the substrate but rather have a structural role.

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 (YPEP URA3 bla), was constructed as described previously (7). In brief, HXT2 was modified to be divisible into four regions by the introduction of MroI, Nhel, XhoI, and ClaI sites into the nucleotide sequences corresponding to the NH2-terminal end of TM4, the loop between TM6 and TM7, and the loop between TM9 and TM10, as well...
as immediately after the sequence corresponding to the COOH terminus, respectively. The expression vector C1578-pVT, which encodes the chimeric transporter C1578 (in which all of the TMs of Hxt2, with the exception of 1, 5, 7, and 8, have been replaced with those of Hxt1), was also described previously (7) (Fig. 1).

Mutagenesis—Site-directed mutants were prepared by replacing each target codon with modified sequences with the use of a PCR-based approach. Saturation mutagenesis of residues in TMs 1, 5, 7, and 8 was performed as described (8). In brief, mutation of 14 target residues in these TMs was achieved by PCR with each of the four regions of the modified HXT2 sequence divided by EcoRI, MroI, Nhel, and Xhol sites and with degenerate primers. The PCR products for the EcoRI-MroI, MroI-Nhel, and Nhel-Xhol regions were connected first with the use of MroI and then with the use of Nhel. The final product was used to replace the corresponding region of a modified version of C1578-pVT (C1578K-pVT) as described (16) and immunoblot analysis of this fraction was performed with rabbit polyclonal antibodies specific for the COOH-terminal region of Hxt2 (4) and with 125I-labeled protein A (GE Healthcare). The intensity of bands corresponding to immune complexes was measured with imaging plates (BAS 1800II, Fuji Film) (16) within the range proportional to the amount of protein. Cell number was determined with a particle counter (Z2, Beckman Coulter). Protein concentration was measured with bicinchoninic acid (Pierce).

RESULTS AND DISCUSSION

Elimination of Five Residues and Construction of C1578K—We started our analysis with C1578, in which 19 amino acid residues were potential contributors to high affinity glucose transport. By saturation mutagenesis of TMs 1, 5, 7, and 8 in C1578, we previously found that five residues of Hxt2 (Ile57, Val69, Tyr215, Ile317, and Ile359) were present in 50% of the 60 clones isolated that encoded high affinity transporters (8). These five residues were thus considered not to be required for high affinity glucose transport and were excluded from the present study. We therefore constructed a modified form of C1578 (C1578K) in which these five residues were replaced with the corresponding residues of Hxt1. Yeast cells expressing C1578K grew on selection plates (S0.1D) at 30 °C in S2Mal plates (malto, 20 mg/ml). Modified portions of all clones selected in the present study were verified by DNA sequencing with an automated sequencer (model 310, Applied Biosystems).

Transport Assay—Cells harboring plasmids were grown to log phase (optical density at 650 nm, 0.3–0.6) at 30 °C in S2Mal synthetic liquid medium. Glucose transport by the cells was measured at 30 °C for 5 s as described (4, 14). Transport activities measured at a D-[^14C]glucose concentration of 0.1 mM were expressed as picomoles of glucose/1 × 10^7 cells/5 s and were corrected for the background activity determined either in the presence of 0.5 mM HgCl2 or with 0.1 mM 1-[^14C]glucose.

Construction of a Three-dimensional Model of Hxt2—The crystal structure of GlpT (Protein Data Bank 1PW4) formed the basis for construction of a structural model of Hxt2. CLUSTAL W (15) was used to align residues in the putative TMs of Hxt1 to Hxt7, Gal2, GlpT, and LacY, and the alignment was modified manually. A working homology model of Hxt2 was generated with the Biopolymer module of Insight II (version 2000; Accelrys, San Diego, CA) as described (8).

Other Assays—A crude membrane fraction was prepared from cells as described (16), and immunoblot analysis of this fraction was performed with rabbit polyclonal antibodies specific for the COOH-terminal region of Hxt2 (4) and with 125I-labeled protein A (GE Healthcare). The intensity of bands corresponding to immune complexes was measured with imaging plates (BAS 1800II, Fuji Film) (16) within the range proportional to the amount of protein. Cell number was determined with a particle counter (Z2, Beckman Coulter). Protein concentration was measured with bicinchoninic acid (Pierce).

Hxt2 Residues Required for High Affinity Glucose Transport

![Schematic representation of the C1578 transporter. C1578 is a chimera in which all TMs but 1, 5, 7, and 8 of Hxt2 are replaced with those of Hxt1. The structure of C1578 is based on a previous model (20). Each circle represents one amino acid, with gray shading indicating residues common to Hxt2 and Hxt1, black shading indicating residues of Hxt1, and open circles indicating residues of Hxt2. The numbers within the circles indicate residue positions for C1578, which are identical to those for Hxt2.](image)
residues of Hxt1, generating 120,000 transformants corresponding to the 2^{14} = 16,384 possible combinations of Hxt1 and Hxt2 residues at these sites. Selection on S0.1D plates yielded 1300 transformants, 40 of which were subjected to plasmid extraction and DNA sequencing. The 40 clones encoded 17 distinct proteins, all of which contained four residues of Hxt2 (Leu59 and Leu61 in TM1, Asn331 in TM7, and Phe366 in TM8) on the C1578K background (Table 1). We therefore considered these four residues to be important for high affinity and high capacity glucose transport activity. Five additional residues of Hxt2 (Cys58 and Ile63 in TM1, Cys195 in TM5, Leu357, and Val367 in TM8) were present in 59% of the 17 transporters and were therefore considered unimportant. We replaced these five residues of C1578K with the corresponding residues of Hxt1, thereby generating a transporter (H1) that contained a total of 10 Hxt2-derived residues in TMs 1, 5, 7, and 8, and this transporter showed high affinity and high capacity glucose transport activity similar to that of C1578 (Table 2). The remaining five of the 14 residues examined (Phe198 in TM5, Val316 in TM7, and Gln352, Ala363, and Ala368 in TM8) were present in 71–94% of the 17 transporters and were subjected to further examination.

**TABLE 1**

| Amino acid residues at 14 sites of C1578K-based chimeric transporters exhibiting high affinity and high capacity glucose transport activity
| --- |
| Amino acid residues at 14 sites of TMs 1, 5, 7, and 8 of C1578K were shuffled. Transformants were selected on S0.1D plates and subjected to DNA sequencing, resulting in the identification of 17 distinct transporters, Hxt2-derived residues are shown as uppercase letters, and Hxt1-derived residues as lowercase letters. The percentage of Hxt2-derived amino acids at each position among the 17 transporters is shown in the bottom row. The corresponding residues of Hxt1 and residue 201, which was not subjected to random mutagenesis, are also shown.

| TM1 | TM5 | TM7 | TM8 |
| --- | --- | --- | --- |
| Clone | 58 | 59 | 61 | 63 | 195 | 198 | 201 | 316 | 331 | 352 | 357 | 363 | 366 | 367 | 368 |
| Hxt1 | i | c | v | v | l | c | v | t | d | e | f | f | c | c | s |
| C1578K | C | L | L | I | C | F | L | V | N | Q | L | A | F | V | A |
| IF15 | i | L | L | I | I | F | L | V | N | e | L | A | F | V | A |
| IF16 | i | L | L | I | I | F | L | V | N | Q | L | f | F | c | A |
| IF17 | i | L | L | v | I | F | L | V | N | Q | L | f | F | c | A |
| IF4b | i | L | L | v | I | F | L | V | N | Q | L | F | F | V | A |
| CA20 | C | L | L | v | C | F | L | V | N | e | f | A | F | c | A |
| CA1b | C | L | L | I | C | F | L | V | N | Q | L | A | F | V | s |
| CA3b | C | L | L | I | C | F | L | V | N | Q | f | A | F | V | s |
| CA6b | C | L | L | I | C | F | L | V | N | Q | f | A | F | V | A |
| CA7b | C | L | L | I | I | F | L | V | N | Q | L | A | F | V | s |
| CA8b | C | L | L | v | C | F | L | V | N | Q | L | A | F | V | A |
| CA14b | C | L | L | I | C | F | L | V | N | Q | f | A | F | V | A |
| IA9b | i | L | L | I | I | F | L | t | N | Q | L | A | F | c | A |
| IAA23b | i | L | L | I | c | C | L | V | N | Q | L | A | F | V | A |
| IA16b | i | L | L | I | C | F | L | V | N | Q | L | A | F | V | s |
| C1578H | i | L | L | v | C | F | L | V | N | e | f | A | F | c | A |
| C1578N | i | L | L | v | I | F | L | V | N | e | f | A | F | c | s |
| C1578P | i | L | L | v | I | F | L | V | N | e | f | A | F | c | A |
| Hxt2(%) | 41 | 100 | 59 | 47 | 94 | 94 | 100 | 71 | 59 | 82 | 100 | 59 | 71 |

Comprehensive Mutagenesis of the Remaining Five Residues—All 32 possible combinations of these five residues in the H1 background were generated (Table 2). We prepared a crude membrane fraction from cells expressing each of these 32 chimeric transporters and examined the extent of transporter expression by immunoblot analysis with antibodies to the COOH-terminal region of Hxt2 (Fig. 2). All 32 chimeras yielded a predominant immunoreactive band at a position corresponding to that of Hxt2 (47 kDa). Quantitative analysis of these bands revealed an expression level for the chimeras of 68–118% (n = 3 to 5) relative to the value for C1578 (Table 2). Eight (H1, H2, H3, H5, H6, H9, H13, and H17) of the 32 clones conferred the ability to grow on S0.1D plates, although the colony size varied. Whereas H1 possessed all five Hxt2-derived residues, H2, H3, H5, H6, and H17 possessed four and H6 and H13 possessed three Hxt2 residues at the five targeted positions. An assay of glucose transport activity revealed that the \( K_m \) values of all eight mutants were similar to that of C1578, whereas the \( V_{max} \) values varied (Table 2). No marked differences in substrate specificity were apparent among these eight transporters (Fig. 3).
TABLE 2
Characterization of the 32 chimeras containing all possible combinations of Hxt2 and Hxt1 residues at positions 198, 316, 352, 363, and 368

KY73 cells expressing the chimeric transporters were subjected to plate assays with S2D and S0.1D media. Cell growth or no growth after incubation for 3–4 days at 30°C is indicated by + or − signs, respectively; + (s) indicates that the size of the colonies was smaller than that for cells expressing C1578. The level of transporter expression was determined by quantitative analysis of immunoblots. For assay of glucose transport activity, cells were grown to log phase at 30 °C in S2Mal synthetic liquid medium, after which activity was measured for 5 s at 30 °C with 0.1 mM D-glucose as the substrate. The $K_m$ and $V_{max}$ values were determined with 1–100 mM D-glucose and are expressed as mM and pmol/107 cells/5 s, respectively; the $V_{max}/K_m$ ratio is expressed as pmol/107 cells/5 s/mM. All values are the means ± S.E. from at least three experiments. Clones encoding high affinity and high capacity glucose transporters are indicated in bold type.

| Clone | TM5 | TM7 | TM8 | Plate | Immunoblot (%) | 0.1 mM | $K_m$ (mM) | $V_{max}$ (pmol/107 cells/5 s) | $V_{max}/K_m$ (pmol/107 cells/5 s/mM) |
|-------|-----|-----|-----|-------|---------------|-------|-----------|-------------------------------|----------------------------------|
| H1    | F   | V   | Q   | A    | + (+)        | 94±4  | 23.0±0.2  | 3.9±0.3                      | 1010±50                          |
| H2    | F   | V   | Q   | A    | + (s) +     | 88±10 | 19.5±1.2  | 4.9±0.7                      | 1280±160                         |
| H3    | F   | V   | Q   | f    | + (s) +     | 113±9 | 16.9±1.3  | 5.2±0.3                      | 1060±70                          |
| H4    | F   | V   | Q   | f    | − +         | 111±4 | 10.8±0.4  |                             |                                  |
| H5    | F   | V   | e   | A    | + +         | 94±8  | 21.4±0.7  | 3.6±0.2                      | 830±50                           |
| H6    | F   | V   | e   | A    | + (s) +     | 94±7  | 20.0±1.8  | 3.9±0.4                      | 790±80                           |
| H7    | F   | V   | e   | f    | − +         | 85±6  | 10.9±0.5  |                             |                                  |
| H8    | F   | V   | e   | f    | − +         | 78±8  | 10.0±0.4  |                             |                                  |
| H9    | F   | t   | Q   | A    | + (s) +     | 68±6  | 16.4±0.9  | 5.0±0.2                      | 920±70                           |
| H10   | F   | t   | Q   | A    | − +         | 92±7  | 13.9±0.4  |                             |                                  |
| H11   | F   | t   | Q   | f   | − +         | 96±7  | 8.7±0.5   |                             |                                  |
| H12   | F   | t   | Q   | f   | − +         | 98±3  | 3.7±0.3   |                             |                                  |
| H13   | F   | t   | e   | A    | + (s) +     | 77±8  | 19.3±0.7  | 5.6±0.4                      | 950±70                           |
| H14   | F   | t   | e   | A    | − +         | 85±5  | 8.5±0.5   |                             |                                  |
| H15   | F   | t   | e   | f   | − + (s)    | 92±9  | 6.1±0.3   |                             |                                  |
| H16   | F   | t   | e   | f   | − −         | 75±2  | 3.7±0.5   |                             |                                  |
| H17   | c   | V   | Q   | A    | + (s) +     | 82±3  | 20.3±1.8  | 3.6±0.2                      | 810±60                           |
| H18   | c   | V   | Q   | A    | − +         | 90±3  | 9.6±0.6   |                             |                                  |
| H19   | c   | V   | Q   | f   | − +         | 108±17| 8.3±0.3   |                             |                                  |
| H20   | c   | V   | Q   | f   | − + (s)    | 95±7  | 5.4±0.2   |                             |                                  |
| H21   | c   | V   | e   | A    | − −         | 107±12| 13.7±0.4  |                             |                                  |
| H22   | c   | V   | e   | A    | − + (s)    | 94±6  | 7.8±0.2   |                             |                                  |
| H23   | c   | V   | e   | f   | − −         | 104±11| 5.0±0.3   |                             |                                  |
| H24   | c   | V   | e   | f   | − −         | 95±9  | 3.6±0.2   |                             |                                  |
| H25   | c   | t   | Q   | A    | − −         | 95±11 | 8.3±0.6   |                             |                                  |
| H26   | c   | t   | Q   | A    | − −         | 77±7  | 6.8±0.2   |                             |                                  |
| H27   | c   | t   | Q   | f   | − −         | 88±4  | 4.4±0.1   |                             |                                  |
| H28   | c   | t   | Q   | f   | − +         | 118±8 | 4.5±0.2   |                             |                                  |
| H29   | c   | t   | e   | A    | − −         | 98±9  | 7.4±0.7   |                             |                                  |
| H30   | c   | t   | e   | A    | − −         | 117±8 | 3.4±0.3   |                             |                                  |
| H31   | c   | t   | e   | f   | − −         | 110±14| 2.8±0.2   |                             |                                  |
| H32   | c   | t   | e   | f   |             | 87±6  | 1.6±0.2   |                             |                                  |
| C1578 | F   | V   | Q   | A    | + +         | 100   | 21.3±0.9  | 5.2±0.1                      | 1180±70                          |
| Hxt2  | F   | V   | Q   | A    | + +         | 35.4±1.0| 3.6±0.3   | 1280±50                      |
| Hxt1  | c   | t   | e   | f   | − −         | 9.4±0.5| 44±3      | 2800±270                     | 64                              |
Complementary Approach to Construct Chimeras That Mediate Low Affinity Transport—The reverse approach, determining which residues of Hxt1 are important for low affinity glucose transport, was problematic. A chimera in which all the TMs are derived from Hxt1 and the remainder of the molecule is derived from Hxt2, which was named C0 in a previous study (7), was found to be expressed but was virtually inactive, indicating the importance of structures adjacent to TMs. Efforts were continued with the chimera H5 generated in the present study. After replacement of the essential Leu201 of Hxt2 with Hxt1-derived Val, we performed saturation mutagenesis of eight residues (Leu59, Leu61, Phe198, Val316, Asn331, Ala363, Phe366, and Ala368). The clone that exhibited the lowest $K_m$ of 17 mM showed a reduced $V_{\text{max}}$ of 870 pmol/10^7 cells/5 s; the corresponding values for C1578 were 5.2 mM and 1180 pmol/10^7 cells/5 s, and those for Hxt1 were 44 mM and 2800 pmol/10^7 cells/5 s. The phenotype of this clone thus appears not to reflect that of Hxt1 but rather that of an impaired Hxt2. Given that Hxt2 and Hxt1 show similar substrate specificities and are both inhibited only by sulfhydryl reagents, further studies aimed at identifying residues necessary for low affinity transport will likely require the development of a new strategy.

Location of Important Residues Inferred from a Homology Model—To evaluate the functional roles of the identified residues of Hxt2, we modified our previous homology model (8) on the basis of the crystal structure of GlpT, a glycerol 3-phosphate transporter of *E. coli* (10). The alignment of GlpT, Hxt2, and other transporters was performed with CLUSTAL W and then modified manually. Important and supportive amino acid residues with regard to high affinity and high capacity glucose transport activity of Hxt2 are indicated by asterisks, and residues found in the hydrophobic environment are shown in bold type. Phenylalanine 431 in TM10 was previously identified as critical for the differential recognition of glucose and galactose (6).
Conclusions—Of the ~250 amino acid residues in the 12 TMs of Hxt2, five have been found to be important for high affinity and high capacity glucose transport and three to be supportive of maximal activity. A working homology model suggests that among these eight residues, Asn^331 interacts directly with the substrate and the others contribute to the protein conformation required for high affinity transport. Our present results suggest the possibility that fine structural tuning is important for construction of a high affinity and high capacity transporter. A subtle modification in structure may bring about a substantial change in transport characteristics. It also seems likely that most residues responsible for the direct recognition of glucose are common to both Hxt2 and Hxt1 and were not isolated in the present study. Previous results (6) and our present data suggest that Asn^331 (TM7) and Phe^431 (TM10) are two candidates for substrate-interacting residues. The functional role of Asn^331 should be the subject of further study.

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