Alteration of Substrate Affinities and Specificities of the Chlorella Hexose/H+ Symporters by Mutations and Construction of Chimeras*

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The dDNAs HUP1 and HUP2 of Chlorella kessleri code for monosaccharide/H+ symporters that can be functionally expressed in Schizosaccharomyces pombe. By random mutagenesis three HUP1 mutants with an increased $K_m$ value for $d$-glucose were isolated. The 40-fold increase in $K_m$ of the first mutant is due to the amino acid exchange N436I in putative transmembrane helix XI. Two substitutions were found in a second (G97C/I303N) and third mutant (G120D/F292L), which show a 270-fold and 50-fold increase in $K_m$ for $d$-glucose, respectively. An investigation of the individual mutations revealed that the substitutions I303N and F292L (both in helix VII) cause the $K_m$ shifts seen in the corresponding double mutants. These mutations together with those previously found support the hypothesis that helices V, VII, and XI participate in the transmembrane sugar pathway.

Whereas for most mutants obtained so far the $K_m$ change for $d$-glucose is paralleled by a corresponding change for other hexoses tested, the exchange D44E exclusively alters the $K_m$ for $d$-glucose. Moreover the pH profile of this mutant is shifted by more than 2 $p$H units to alkaline values, indicating that the activity of the transporter may require deprotonation of the corresponding carboxyl group.

Chimeric transporters were constructed to study the 100-fold lower affinity for $d$-galactose of the HUP1 symporter as compared with that of the HUP2 protein. A crucial determinant for the differential $d$-galactose recognition was shown to be associated with the first external loop. The effect could be pinpointed to a single amino acid change: replacement of Asn-45 of HUP1 with isoleucine, the corresponding amino acid of HUP2, yields a transporter with a 20 times higher affinity for $d$-galactose. The reverse substitution (I47N) decreases the affinity of HUP2 for $d$-galactose 20-fold.

The green alga Chlorella kessleri possesses an inducible transport system, capable of accumulative uptake of a variety of monosaccharides using an electrochemical proton gradient as driving force (1–4). Three dDNAs coding for highly homologous Chlorella monosaccharide/H+ symporters were cloned by differential screening (5) and named HUP1–3 (hexose uptake protein). Their identities have been confirmed by heterologous expression in Schizosaccharomyces pombe (6, 7). Furthermore, the HUP1 transporter retains its uptake activity after solubilization from the membrane of transgenic fission yeast, purification to homogeneity, and reconstitution into proteoliposomes (8, 9).

The HUP symporters belong to a large family of substrate transporters, called the “major facilitator superfamily” (10). Members of this major facilitator superfamily are thought to consist of 12 $a$-helical transmembrane segments connected by internal and external loops. Support for this topological model comes from alkaline phosphatase fusion protein analysis of the Escherichia coli lactose permease lacY (11) and N-glycosylation scanning mutagenesis studies on the human glucose facilitator GLUT1 (12). However, hard structural data on the nature of the binding sites and translocation pathways of substrates and cosubstrates have not been obtained. Since no three-dimensional structure of a transporter is in sight, one has to be content with indirect evidence, deduced for example from mutagenesis studies.

Structure-function analysis of the HUP1 transporter (13, 14) was carried out in a sugar uptake deficient S. pombe strain (15). Several mutants with an increased $K_m$ value for $d$-glucose uptake were found by site-directed mutagenesis (13) and by polymerase chain reaction random mutagenesis with subsequent selection for decreased sensitivity toward the toxic sugar 2-deoxyglucose (14). The affected amino acids cluster in the middle of the transmembrane helices V (Gln-179), VII (Gln-298 and Gln-299), and XI (Val-433 and Asn-436), with the exception of Asp-44 putatively located at the beginning of the first external loop (Fig. 1). The fact that predominantly acidic amino acids and their amides were identified correlates well with the finding that binding sites of periplasmic sugar-binding proteins are built up by such residues (16, 17).

The symporters HUP1 and HUP2 differ significantly in their substrate specificity (6, 18). Especially, the affinity for $d$-galactose is more than 100 times higher for the HUP2 protein. The amino acids of the HUP1 protein probably involved in substrate recognition (see above) are also present in the HUP2 transporter. The different substrate specificities of the two transporters must, therefore, be determined by differing residues at still unidentified positions. Recently, a study using chimeric proteins revealed that the exchange of a 30-amino acid span at the beginning of the first extracellular loop of HUP1 for that of HUP2 increases the affinity for $d$-galactose by about 15-fold (18).

The present work tries to find answers to the following questions. 1) Do additional residues exist in the HUP1 symporter, which give rise to an increased $K_m$ value for $d$-glucose uptake upon replacement? 2) Do all these HUP1 mutants also exhibit decreased affinities for other sugars, or do some of them show substrate specific effects? 3) Is it possible to narrow down the segment of the first external loop of HUP2 participating in $d$-galactose recognition?
EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—All cloning steps were carried out in E. coli DH5α with the plasmid vector pUC18. E. coli TGI served as host for the phagemid pUC18 and the helper virus M13K07 in site-directed mutagenesis. The Leu- and sugar uptake deficient strain S. pombe YGS-B25 (15) used for heterologous expression of the various transporter cDNAs was grown in 2% glucose, 2% yeast extract. Transformed S. pombe cells were cultivated in minimal medium containing 2% glucose and 0.67% yeast nitrogen base without amino acids.

Transformation of S. pombe YGS-B25—Wild-type, point-mutated, and chimeric transporter cDNAs were cloned via SacI/BamHI into the shuttle vector pEVP11 (19) or pART3 (20), the latter allowing significant higher expression. S. pombe YGS-B25 was transformed as described in Ref. 7.

Random Mutagenesis by Polymerase Chain Reaction—The full-length cDNA of HUP1 was amplified by polymerase chain reaction under suboptimal conditions as described previously (14) in order to achieve one error per cDNA fragment on average. The pool of randomly mutagenized cDNAs was ligated into pEVP11 or pART3 and introduced in S. pombe cells leading to the RMY and RGY transformants, respectively.

Recovery and Sequencing of the Mutated HUP1 cDNAs—Plasmid reisolation from RGY52 was performed by the phenol/chloroform/isoamyl alcohol procedure (21) with the following addition. The aqueous phase containing the recovered plasmids was purified and precipitated by successive treatment with phenol, diethyl ether, and ethanol prior to transformation of E. coli DH5α. However, this procedure for plasmid isolation from yeast cells as well is not described in the case of RMY126 and RMY254. Therefore, one big colony of each of these transformants was picked and directly applied to a standard polymerase chain reaction. Plasmids were released from the cells due to preincubation at 94 °C for 10 min. The mutated HUP1 cDNAs were amplified afterward using flanking primers that bind in the promoter and the polylinker region of pEVP11. Then they were subcloned via SacI/BamHI into pUC18 and their nucleotide changes were determined by sequencing using the T7Sequencing™ kit (Pharmacia Biotech) and synthetic oligonucleotides.

Separation of the Mutations of the Double Mutants RMY126 and RGY52—The transformants RMY126 and RGY52 both exhibited two point mutations in the HUP1 gene (see “Results”). These mutations could be separated using a unique KpnI restriction site lying in between (Fig. 1). The SacI/KpnI fragment and the KpnI/BamHI fragment coding for the N- and C-terminal part were ligated to the respective missing sequences from the wild-type clone. This resulted in HUP1 cDNA regions carrying either the one or the other mutation. Those originating from RMY126 were resubcloned into pEVP11, those originating from RGY52 into pART3. S. pombe YGS-B25 was transformed as described above.

Site-directed Mutagenesis—Preparation of the single-stranded HUP1 and HUP2 template DNA was performed as described previously (13, 18). Site-specific mutagenesis was carried out with the Sculptor™ in vitro mutagenesis system (Amersham) according to the instructions of the manufacturer. The sequences of the synthetic oligonucleotides used were as follows (changes beneath are underlined): (a) K59K/R60M, 5′-C-TGGAGAAGAACCTCTGGCTAAGGTCCTC-3′; (b) R144L, 5′-ACCAATGCGCTACAGGAAATCTCG-3′; (c) R204L, 5′-GACCACCGGAGCAGACCCGTGGTGTTG-3′; (d) N45L, 5′-CCAGTCAACCAGTACTATATAGGA-3′; (e) V52T, 5′-CCTGCCAGGGAGGTGACGCCACCAG-3′; (f) 147N, 5′-CCAGTCAACCCAGTCTGGTGTTG-3′; (g) 292 (helix VII) is changed to leucine. As a consequence the Asp120 (helix III) is replaced by an aspartic acid and phenylalanine. (h) 270. The point mutations in the HUP1 protein. The substitution of the asparagine residue at position 436 in helix XI for isoleucine decreases the affinity of the HUP1 transporter for its sugar substrate(s). Therefore Km mutants should lead to the identification of amino acid residues most probably involved in substrate binding. Recently we reported an unbiased functional screening for such Km mutants (14). It is based on a 1000-fold increase in 2-deoxyglucose sensitivity upon transformation of a sugar uptake deficient S. pombe strain with the HUP1 cDNA. A pool of randomly mutated HUP1 cDNAs was generated by polymerase chain reaction and used for transformation. Transformants with intermediate 2-deoxy-D-glucose sensitivity were selected and tested for decreased affinity for D-glucose. Four Km mutants had been obtained in this way (14). In the meantime further use of this strategy has been made and three additional mutants have been isolated (Table I).

The HUP1 cDNA isolated from RMY254 exhibits three nucleotide changes, but only one of them affects the primary structure of the HUP1 protein. The substitution of the asparagine residue at position 436 in helix XI for isoleucine decreases the affinity for D-glucose 40-fold. The cDNAs of the other Km mutants only contain two point mutations, which both result in amino acid changes. Thus, the HUP1 symporter of RMY26 contains cysteine and asparagine instead of glycine 97 (helix II) and isoleucine 303 (helix VII), respectively. The Km value for D-glucose is dramatically increased by a factor of about 270. The point mutations in the HUP1 cDNA of RGY52 alter the transport protein in the following way: Glycine 120 (helix III) is replaced by an asparagine residue, as well as isoleucine 292 (helix VII) is changed to leucine. As a consequence the Km value for D-glucose rises approximately 50-fold as compared with that of the wild-type. These Km mutants also show a dramatic decrease in Vmax value, which correlates well, however, with the poor level of expression (Table I). A reduced amount of transport protein is not unusual for clones originating from the random mutagenesis approach, since a lower uptake rate also contributes to higher 2-deoxy-D-glucose resistance (14). It is not really under-

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stood, however, why these mutations lead to such a low expression of transport protein. The possibility that mutations affect protein secondary structure and/or protein stability, leading to indirect effects on $K_m$ values, unfortunately cannot be ruled out. Although, in principle this is true for all mutations described here or elsewhere, particular caution is required for those reducing the expression level.

Separation of the Mutations in the HUP1 Double Mutants—In order to elucidate whether the $K_m$ change found in RMY126 and RGY52 is brought about by both substitutions acting additively or whether it is simply caused by one of them, the mutations were separated from each other as described under “Experimental Procedures.” Table II lists the $K_m$ values of the single mutants. Obviously, the effect of substituting Gly-97 for Cys on the $d$-glucose affinity of HUP1 is negligible. The nearly identical $K_m$ values of mutant and wild-type transporter manifest that glycine 97 is not important for the interaction with the substrate. On the other hand mutant I303N exhibits a $K_m$ value very similar to that of the double mutant RMY126. Therefore, it is suggested that the isoleucine residue in helix VII is involved in $d$-glucose binding.

In the case of RGY52 the results are more complex. Both substitutions in question generate effects on $d$-glucose affinity, but they are not additive. The replacement F292L causes a 50-fold increase of the $K_m$ value equal to that of the double mutant. In the G120D mutant the $K_m$ value is raised somewhat less but still significantly (about 30-fold). It is not clear why the effects are not additive to some extent. It seems that the structural aberration caused by F292L includes the one induced by G120D. Since G120D is a much more drastic change when compared with F292L, the results are taken as indication that the phenylalanine residue in helix VII is important for correct substrate binding. Finally, it should be emphasized that for all single mutants the $V_{max}$ value correlates very well with the amount of transport protein detected by immunoblotting (Table II).

Substrate Specificity of Several Mutated HUP1 Transporters—The Chlorella HUP1 symporter enables transformed S. pombe cells to take up a great number of monosaccharides. The affinities for the particular sugar substrates differ widely, however. Thus, the $K_m$ values for the uptake of $d$-glucose, $d$-mannose, $d$-fructose, $d$-xylose, and $d$-galactose turned out to be in the range of $1.5 \times 10^{-5}, 1.5 \times 10^{-4}, 3 \times 10^{-4}, 1.5 \times 10^{-3}$, and $3 \times 10^{-3}$ M, respectively (Table III). In previous publications (13, 14), as well as in this paper, several HUP1 mutants with an increased $K_m$ value for $d$-glucose were described. It was of interest to find out whether these mutants show less efficient binding also of other substrates, i.e. whether sugar specific effects or more general ones are produced by the mutations. For this purpose the mutated HUP1 transporters listed in Table III were chosen for a detailed analysis of their substrate specificities and compared with that of the wild-type protein.

Three mutations, Q179E, I303N, and N436Y, equally diminish the affinities for all sugars tested (d-glucose, d-mannose, and d-fructose). In the case of Q298N and N436Q the $K_m$ values for $d$-glucose, d-xylose, and d-galactose transport are significantly increased while those for d-mannose and d-fructose uptake are only mildly affected. The conservative exchange D44E, however, influences the substrate specificity in an extraordinary way. As previously pointed out, this substitution increases the $K_m$ for $d$-glucose uptake by more than 10 times (13). Surprisingly, none of the $K_m$ values for other sugar substrates is altered in the D44E mutant significantly, i.e. by more than a factor of 2. These results suggest a special role of aspartate 44 in the glucose transport process. Therefore, this amino acid position has been investigated more thoroughly.

Characterization of Position Asp-44—In the transporter model of Fig. 1 aspartate 44 is situated at the very beginning of transmembrane helix VII. It both are neutralized by forming salt bridges with lysine residues in helix X and XI, respectively (24). It is a characteristic feature of these charge pairs that simultaneous replacement of both partners by neutral amino acids does not impair the translocation process, whereas the exchange of only one partner, leaving the other one unpaired, inactivates the permease completely. Since the mutation D44N leads to a total inactivation of the HUP1 transporter (13), it might be suspected that the aspartic acid residue likewise is involved in a salt bridge. The primary structure of the Chlorella transporter provides several basic amino acids that might act as the positively charged counterpart. Some of them were replaced individually or in combination by

### Table I

$d$-Glucose transport characteristics of three additional S. pombe strains transformed with randomly mutated Chlorella HUP1 cDNAs

| S. pombe strain | Nucleotide exchange | Amino acid exchange |
|-----------------|---------------------|---------------------|
| wt-HUP1         | G97C                 | V81Y                |
| RMY254          | AGG→CGG             | R197R               |
| RMY126          | AAC→ATC             | N436I               |
| RGY52           | GGT→GAT             | G97C                |
|                 | I303N               |                     |
|                 | GGT→GAT             | I303N               |
|                 | TTT→TAT             | F292L               |

| Location         | $K_m$ ($M$) | $V_{max}$ (%) | Transport protein |
|------------------|-------------|---------------|-------------------|

| S. pombe strain | $K_m$ ($M$) | $V_{max}$ (%) | Transport protein |
|-----------------|-------------|---------------|-------------------|
| wt-HUP1         | 1.5 $\times$ 10^{-5} | 100 | 100 |
| RMY254          | 6 $\times$ 10^{-4} | 2 | 6 $\times$ 10^{-4} |
| RMY126          | 4 $\times$ 10^{-3} | 7 | 4 $\times$ 10^{-3} |
| RGY52           | 7 $\times$ 10^{-4} | 2 | 7 $\times$ 10^{-4} |

### Table II

$d$-Glucose transport characteristics of S. pombe strains expressing HUP1 transporter with the single mutations G97C, I303N, G120D, or F292L

$K_m$, $V_{max}$, and expression values were determined as described in Table I.

| Mutant | $K_m$ ($M$) | $V_{max}$ (%) | Transport protein |
|--------|-------------|---------------|-------------------|
| wt-HUP1 | 1.5 $\times$ 10^{-3} | 100 | 100 |
| G97C   | 5 $\times$ 10^{-5} | 3 | 5 $\times$ 10^{-5} |
| I303N  | 6 $\times$ 10^{-3} | 120 | ND* |
| G120D  | 3 $\times$ 10^{-4} | 9 | 15 |
| F292L  | 7 $\times$ 10^{-4} | 13 | 15 |

* ND, not determined.
neutral residues (Table IV). The ability to take up D-glucose is maintained in the mutants R144L, R204L, and K59Q/K60M, albeit with dramatically reduced overall activities. These reductions, however, primarily reflect the low expression levels. There is no indication that the catalytic activity per se is impaired. Moreover D-glucose transport tests of all three mutants yield Km values nearly identical to that of wild-type HUP1. These results make it unlikely that one of the basic amino acids at position 59, 60, 144, or 204 in the transport protein interacts with aspartate 44 via a salt bridge.

Previous studies not only demonstrated the absolute necessity of a carboxyl group at position 44 but also the importance of its precise location for the transporter to be active. Thus, increasing the side chain length by changing aspartic into glutamic acid (D44E) decreases the Vmax of D-glucose uptake by 90% and raises the Km by 15-fold as compared with the wild-type, although similar amounts of transport protein are present in the cells (13). In order to elucidate whether the potential for protonation/deprotonation of Glu-44 as compared with that of Asp-44 is affected, the influence of extracellular pH on the translocation reaction catalyzed by the two symporters has...
Experimental Procedures. The uptake rate at pH 6.0 corresponds to

\[ K_m \] 3. As to the D-glucose uptake, C7 and C8 show

exchanging the posterior and anterior halves, respectively (Fig.

Additional HUP1/2/1 chimeras, C7 and C8, were generated by

Despite the high homology (74%), the

HUP2—Chlorella optimum at about pH 7.0.

ificity (6, 18). Whereas both carriers efficiently transport D-

porters HUP1 and HUP2 differ significantly in substrate spec-

Km values of C7 and C8 coincide well with those

The initial rates of D-[U-14C]glucose transport were measured

B25 expressing the Chlorella wild-type HUP1 or the D44E sym-

been examined. The D-glucose uptake activity of HUP1 is opti-

mal at about pH 4.5 and declines steeply toward lower and

higher values (Fig. 2). The D44E mutant shows a completely
different pH dependence. As ambient pH is increased from 3.0
to 9.0, D-glucose uptake is accelerated gradually, reaching an

optimum at about pH 7.0.

Determination of Substrate Specificities of HUP1 and

HUP2—Despite the high homology (74%), the Chlorella sym-

porters HUP1 and HUP2 differ significantly in substrate specific-

ity (6, 18). Whereas both carriers efficiently transport D-

with comparable affinities, there exists a large
difference concerning D-galactose. This hexose is only poorly

accepted by HUP1 and favored by HUP2. The \( K_m \) values for

D-galactose uptake differ from one another by 2 orders of mag-
nitude (3 \( \times 10^{-5} \) m versus 2.5 \( \times 10^{-7} \) m). In a study using

chimeric proteins it was recently shown that exchanging only

the front part of the first extracellular loop of HUP1 for that of

HUP2 (chimera C6), gives rise to a 15-fold higher affinity for

d-galactose as compared with that of the wild-type HUP1 (18).

A sequence alignment of the interchanged segments demon-

strates that HUP1 and HUP2 differ in 16 out of 29 positions in

the corresponding HUP2 sequence. In the alignment shown above only

those amino acids of HUP2 are listed that differ from the HUP1 se-

quence. The exchanged loop segment can be bisected by

Asp700I restriction as indicated by the dotted line. Replacing the posterior and

anterior section independently leads to chimeras C7 and C8, respectively.

Finally it should be mentioned that the \( V_{max} \) values of the chimeras and the HUP1 and HUP2 mutants correlate without exception well with the levels of expressed transport protein (data not shown). Although a specific antibody for HUP2 is lacking, the amounts of wild-type HUP2 and I47N transporter could be estimated, because the polyclonal anti-HUP1-B antiserum (13) shows weak cross-reactivity.

DISCUSSION

The HUP proteins of Chlorella catalyze the uptake of several

monosaccharides in co-transport with protons. How transport function is accomplished by the Chlorella symporters or, for that matter by transporters of any other organism, is an open and intriguing question. Regarding the nature of the sugar-binding site and the translocation pathway, it is assumed that residues directly interacting with the substrate should not be replaceable without causing a significant change in the affinity of the transporter for the substrate. Several HUP1 mutants with an increased \( K_m \) value for D-glucose were found previously (13, 14). Apart from Asp-44, which will be discussed later, the following amino acids were affected: Gln-179 (in helix V), Gln-282 and Gln-299 (both in helix VII), Val-433 and Asn-436 (both in helix XI). The HUP1 symporter of mutant RMV126 revealed two amino acid exchanges, G97C (in helix II) and I303N (in helix VII), but

![Fig. 2. pH dependence of D-glucose uptake by S. pombe YGS-B25 expressing the Chlorella wild-type HUP1 or the D44E symporter. The initial rates of D-[U-14C]glucose transport were measured for the wild-type (■) and the D44E mutant (○) as described under “Experimental Procedures.” The uptake rate at pH 6.0 corresponds to 131 and 2.8 μmol/h × g fresh weight for the wild-type HUP1 and the D44E mutant, respectively.](http://www.jbc.org/)

![Fig. 3. Construction of chimeras C6–C8. The chimeric transporter C6 consists mainly of HUP1 sequence. Only the front part of the first external loop, represented by the hatched box, is replaced by the corresponding HUP2 sequence. In the alignment shown above only those amino acids of HUP2 are listed that differ from the HUP1 sequence. The exchanged loop segment can be bisected by Asp700I restriction as indicated by the dotted line. Replacing the posterior and anterior section independently leads to chimeras C7 and C8, respectively.](http://www.jbc.org/)
Comparison of the $K_m$ values for D-glucose and D-galactose uptake of wild-type, chimeric, and point mutated transporters

The initial uptake of D-[U-14C]glucose, and D-[U-14C]galactose was measured at different substrate concentrations as described under "Experimental Procedures." $K_m$ values were calculated from Lineweaver-Burk plots. The $V_{max}$ values of the single mutants for both sugar substrates amount to more than 50%, but at least 20% of the respective wild-type values (data not shown).

| Substrate | Wild-types | HUP1/2 chimeras | HUP1 mutants | HUP2 mutants |
|-----------|------------|-----------------|--------------|--------------|
| Glucose   | HUP1       | HUP2            | C6           | C7           | C8           | V52T         | N45I         | I47N         |
| $K_m$ (mM) | 1.5 x 10^{-5} | 4.5 x 10^{-6}  | 2.5 x 10^{-5} | 2.5 x 10^{-5} | 2.5 x 10^{-5} | 7.5 x 10^{-6} | 4.5 x 10^{-5} | 1 x 10^{-5} |
| Galactose | 3 x 10^{-3}  | 2.5 x 10^{-5}  | 2 x 10^{-4}  | 3.5 x 10^{-3} | 1.5 x 10^{-4} | 3 x 10^{-3}  | 1.5 x 10^{-4} | 5.5 x 10^{-4} |

Fig. 4. Lineweaver-Burk plot for D-galactose uptake. The initial uptake velocity of *S. pombe* YGS-B25 expressing the *Chlorella* wild-type HUP1 (□) or the N45I symporter (○) was measured at different D-galactose concentrations. The $K_m$ value is decreased from 3 to 0.15 mM due to the exchange.

Fig. 5. Helical wheel plot of putative helix VII of HUP1, including positions 289–306. The helix is viewed from the cytoplasmic surface of the membrane with the N terminus at the top. Numbers inside the wheel indicate the succession of the amino acids in the helix. Residues found to be exchanged in HUP1 $K_m$ mutants are shown as enlarged bold face letters.

only the latter proved to be responsible for the detected $K_m$ effect (Table II). A parallel situation exists in mutant RGY52, where only F292L (in helix VII) induces a $K_m$ shift identical to that in the double mutant. However, the efficiency of D-glucose binding is also influenced by the second exchange, G120D in N45I (30, 31), corresponding evidence for HUP transporters is missing.

Taken together, the new $K_m$ mutants strengthen the importance of residues within helices VII and XI, which have previously been suggested to play a role in D-glucose binding. Remarkably, residues Phe-292, Gln-298, Gln-299, and Ile-303 cluster on one face of putative helix VII when viewed on a helical wheel plot (Fig. 5). One might speculate therefore that Phe-292, Gln-298, and Ile-303, which are located in closest vertical proximity, are probably guiding the sugar substrate along the translocation path. Stacking of hydrophobic patches of the glucopyranose ring with aromatic residues is clearly visible in the binding pocket of the D-glucose-binding protein of *E. coli* (17). Considering phenylalanine 292, the same could hold for D-glucose binding of HUP1.

By x-ray crystallography it was shown that sugar binding in periplasmic binding proteins is mediated mainly by charged amino acids and their amides via hydrogen bonds (16, 17). It is striking that most of the HUP1 residues identified as good candidates for sugar interaction are amides (Gln-179, Gln-298, Gln-299, and Asn-436). In addition, there is a charged residue, Asp-44, in HUP1 that cannot be changed without affecting D-glucose binding. The topological model of HUP1 (Fig. 1) puts Asp-44 outside the hydrophobic membrane, but a location within helix I is imaginable as well. Supposing it were so, then helices I, V, VII, and XI would participate in sugar binding. Although it might be a coincidence that the first and fifth transmembrane spanning domains of the N- and C-terminal half of HUP1 would then have been identified, it is nevertheless remarkable, since the 12-helix arrangement of the major facilitator superfamiy transporters most likely has arisen by a gene duplication event of an ancestral gene encoding a protein with six transmembrane spans (28). Helices with functional importance may very well be corresponding ones in each half of the protein.

When the proposed helix packing of HUP1 is compared with that of the intensively studied lactose permease of *E. coli* a number of parallels like the postulated neighborhood of helices V, VII, and XI can be seen (29). The importance of helices I and VII of lactose permease is, furthermore, underpinned by sugar specificity mutants (Refs. 30 and references therein). Only for helix X, potentially playing a major role in lactose transport (30, 31), corresponding evidence for HUP transporters is missing.

Oppositely charged residues in transmembrane segments sometimes neutralize each other by forming a salt bridge (24). Provided that Asp-44 is located in helix I, it might also be paired with a basic amino acid. Candidates that could possibly act as positive counterions for Asp-44 were selected and replaced by neutral residues (Table IV). Evidence that Asp-44 is linked to a basic amino acid via a salt bridge has not been obtained, however. The shift in the pH optimum from pH 4.5 of the wild-type HUP1 protein to pH 7.0 of the D44E transporter indicates that the carboxyl group of the mutant is located in a drastically changed environment (increased hydrophobicity), leading to such a large increase in its $pK_a$. The shift also suggests that a negative charge is required at this position for transport activity; in the case of the mutant one would have to assume that the proton dissociates only under the more alkaline condition. Of course, this is in accordance with the obser-
Substrate Affinity and Specificity of Two Sugar Transporters

A 20-fold increased HUP2 mutant carrying the reverse substitution, I47N, reveals affinity for D-galactose, implying that there are at least two separate determinants in the N-terminal half of HUP2 (Fig. 3). This chimera still has a 15-fold increased affinity for D-galactose, indicating that the critical positions lie within the N-terminal half (18). In the chimera C6 only the first 29 amino acids of putative loop 1 were removed and replaced by the corresponding residues of HUP2 (Fig. 3). This chimera still has a 15-fold increased affinity for D-galactose, implying that there are at least two separate determinants in the N-terminal half of HUP2, one inside the interchanged loop segment and one outside.

By use of additional chimeras and site-directed mutants it has been proven that one single exchange within loop 1, N45I, causes the improved D-galactose affinity seen in C6 (Table V). A HUP2 mutant carrying the reverse substitution, I47N, reveals a 20-fold increased Km value for D-galactose uptake indistinguishable from that of the high affinity transporter HUP2, indicating that the critical positions lie within the N-terminal half (18). In the chimera C6 only the first 29 amino acids of putative loop 1 were removed from HUP1 and replaced by the corresponding residues of HUP2 (Fig. 3). This chimera still has a 15-fold increased affinity for D-galactose, implying that there are at least two separate determinants in the N-terminal half of HUP2, one inside the interchanged loop segment and one outside.

Recently the construction of various chimeras between two closely related facilitators of S. cerevisiae have been reported, i.e. the Gal2 protein, which transports D-galactose and D-glucose, and the Hxt2 protein, which is specific for D-glucose. Various chimeras between these two transporters were previously constructed in order to identify crucial site(s) for the differential D-galactose recognition (18). A chimera consisting of the N-terminal half of HUP2 and the C-terminal half of HUP1 shows a Km value for D-galactose uptake indistinguishable from that of the high affinity transporter HUP2, indicating that the critical positions lie within the N-terminal half (18). In the chimera C6 only the first 29 amino acids of putative loop 1 were removed from HUP1 and replaced by the corresponding residues of HUP2 (Fig. 3). This chimera still has a 15-fold increased affinity for D-galactose, implying that there are at least two separate determinants in the N-terminal half of HUP2, one inside the interchanged loop segment and one outside.

By use of additional chimeras and site-directed mutants it has been proven that one single exchange within loop 1, N45I, causes the improved D-galactose affinity seen in C6 (Table V). A HUP2 mutant carrying the reverse substitution, I47N, reveals a 20-fold increased Km value for D-galactose uptake and therefore strongly supports the notion that the exchanged position is a crucial determinant.

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