Kₘ mutants of the Chlorella monosaccharide/H⁺ cotransporter randomly generated by PCR

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ABSTRACT The HUP1 gene codes for the monosaccharide/H⁺ cotransporter protein of Chlorella kessleri. The gene is functionally expressed in Schizosaccharomyces pombe. This heterologous system has been used to screen for Kₘ mutants of the Chlorella symporter. Since S. pombe transformed with HUP1 cDNA showed a 1000-fold increase in sensitivity toward the toxic sugar analogue 2-deoxyglucose, we screened for transformants with a decreased 2-deoxyglucose sensitivity. The transformants were produced with HUP1 cDNA randomly mutagenized by PCR. From 73 transformants with decreased 2-deoxyglucose sensitivity, four mutants with increased Kₘ values for D-glucose were obtained. The amino acid exchanges responsible for the increased Kₘ values are located in the center of the putative transmembrane helices V (Q179E), VII (Q298R), and XI (V433L/N436Y). Q179N and Q299N had previously been shown by directed mutagenesis to affect the Kₘ value of the transporter for D-glucose. The drastic mutational changes Q298R and N436Y gave rise to very high Kₘ values; however, the corresponding conservative amino acid changes Q298N or N436Q obtained by directed mutagenesis also result in Kₘ values increased by a factor of 10 or 20, respectively. The data therefore support the proposal that at least helices V, VII, and XI may line the sugar translocation path and determine its specificity. These results are discussed in relation to other sugar transporters and to the interaction of the yeast hexokinase B with D-glucose as known from published crystal structures.

The hexose transporter of Chlorella kessleri (corrected name; previously designated Chlorella vulgaris), an inducible plasma membrane protein, is an intensively studied plant protein catalyzing transmembrane transport (1–3). It is capable of accumulative transport of hexose analogues using H⁺ gradients for electrogenic secondary active transport (4–7). The cDNA of this transporter has been cloned (HUP1; hexose uptake protein 1) and its identity has been proven by heterologous expression in Schizosaccharomyces pombe (8, 9). As such it was the first plant gene cloned that codes for a transporter responsible for cellular uptake processes.

The Chlorella hexose H⁺ symporter shows high homology to the human glucose transporter (10) and belongs to a large family of substrate transporters (11), the most well known of which certainly is the Escherichia coli lac permease (12, 13). From a large quantity of primary sequences, therefore, a well substantiated topological model suggesting 12 membrane-spanning helices has been postulated (13, 14). Nevertheless, transmembrane translocation of sugars—and for that matter of any other small molecule—has remained a black box due to the lack of hard structural data of the corresponding membrane proteins. Thus, in spite of an increasing number of site-directed mutagenesis studies (13, 15, 16), the evidence for substrate and cosubstrate (here H⁺) binding sites and translocation paths is necessarily circumstantial (13). However, as long as crystals of transport proteins or NMR images are not available, these indirect pieces of information are the only possible contributions to try understanding an important biological phenomenon: the catalysis of transmembrane transport.

Structure/function analysis of the Chlorella hexose transporter can be carried out, since the HUP1 cDNA as well as corresponding mutants are functionally expressed in S. pombe (9, 17). Recently, a S. pombe mutant (YGS-B25), not able to grow on glucose (36), has been used for this purpose (17). When these cells were transformed with Chlorella HUP1 cDNA, they not only grew on glucose again, but their glucose uptake and accumulation behavior was indistinguishable from that of Chlorella (17). Mutated HUP1 cDNA produced by site-directed mutagenesis and tested in S. pombe gave the following results (17): transmembrane helices V and VII are candidates for substrate binding, since Q179 N (helix V) and Q299 N (helix VII) resulted in a significant increase in Kₘ. All these as well as a number of additional site-specific changes were based on sequence comparisons with other eukaryotic glucose transporters. It is obvious, however, that an unbiased, completely independent functional screening for Kₘ mutants would be preferable. Such a new screen and the first four mutants obtained in this way are described here. The screen is based on the fact that S. pombe mutant 25, not able to grow on glucose, is also highly resistant to 2-deoxyglucose, an analogue of D-glucose and D-mannose, which most likely is toxic because of its incorporation into glycoproteins instead of D-mannose (18). YGS-B25 transformed with HUP1 wild-type cDNA, on the other hand, is highly sensitive to 2-deoxyglucose. When HUP1 cDNA was mutagenized randomly with PCR and this mutated gene pool was used for S. pombe transformation, we could select for cells with an intermediate 2-deoxyglucose sensitivity. Among these, four Kₘ mutants were obtained, which allow us to postulate the sugar binding site in the Chlorella monosaccharide/H⁺ symporter protein.

MATERIALS AND METHODS

Chemicals. D-[¹⁴C]Glucose, deoxyadenosine 5’-[(α-[³⁵S]thio)triphosphate, the oligonucleotide-directed mutagenesis system, and the Western blot detection system were purchased from Amersham Buchler (Braunschweig, F.R.G.). 4-Aminobenzenemidinehydrochloride was from Merck (Darmstadt, F.R.G.); phenylmethylsulfonyl fluoride and 2-deoxy-D-glucose were from Sigma (Deisenhofen, F.R.G.).

Strains. The S. pombe wild-type strain leu1-32 (19) and, for heterologous expression of the HUP1 cDNA, a sugar transport-deficient mutant (YGS-B25; leu¹) were used; the latter was obtained from B. Milbradt and M. Höfer (Bonn).

Plasmids. The HUP1 cDNA was cloned into pEVP11 (20) or pUC18 (21) for heterologous expression or PCR random mutagenesis, respectively.

Growth Conditions. Transformed S. pombe cells were grown in minimal medium containing 2% gluconate and 0.67% yeast nitrogen base without amino acids. For culti-
vating *S. pombe* YGS-B25, 4.4 × 10⁻⁴ M 1-leucine was added to the medium. Growth behavior in the presence of 2-deoxyglucose was tested on plates containing various concentrations of the cytotoxic reagent.

**Random Mutagenesis by PCR.** The 1770-bp Sac I/BamHI fragment (9), containing the full-length cDNA of the *Chlorella* hexose carrier HUPI, was ligated into the Sac I/BamHI-digested plasmid pUC18. According to Eckert and Kunkel (22) the standard protocol of PCR as indicated in the Perkin-Elmer/Cetus GeneAmp DNA amplification kit was modified to achieve an error rate of 1/1800 bp after 25 cycles. Therefore, the MgCl₂ concentration was increased from 1.5 to 5.0 mM, the concentration of each dNTP was raised from 200 to 500 μM, and the pH was lowered from 8.3 to 8.0. Twenty nanograms of DNA and 1 μM universal and reversed primer were used per 100-μl reaction mixture. Each of the 25 PCR cycles consisted of the following steps: 1 min at 94°C, 1.5 min at 60°C, and 2 min at 72°C.

**Transformation of *S. pombe* Mutant.** The pool of randomly mutagenized HUPI cDNA was digested with Sac I YGS-B25/BamHI and cloned into the expression vector pEVP11. *S. pombe* YGS-B25 was transformed as described (9).

**Transport Tests.** Sugar uptake was measured as described elsewhere (9), except for the following change: to work with optimally energized *S. pombe* cells, ethanol was added to a final concentration of 120 mM 2 min before the test was started.

**Isolation of Plasmids.** The reisolation of plasmids from transformed *S. pombe* cells was carried out according to the instructions of Robzyk and Kassir (23).

**Sequencing.** To determine the nucleotide changes, the mutated HUPI cDNAs were resubcloned into pUC18 and sequenced by the dyeodeoxyxynucleotide chain-termination method (24) using the T7 polymerase kit (United States Biochemical).

**Isolation of Whole Membranes, SDS/PAGE, and Immunoblotting.** From the same cultures that had been used for transport tests, 30 ml with an OD₅₇₈ (Eppendorf 1101 M photometer) of 1 were pelleted by centrifugation. Their membranes were isolated as described (17). The protein content was assayed by the method of Bradford (25). SDS/PAGE and immunoblotting with anti-HUPI-A antibody were carried out as described (17).

**Oligonucleotide-Directed Site-Specific Mutagenesis.** Preparation of single-stranded template DNA and site-specific mutagenesis were performed as described (17). The sequences of the oligonucleotides used were as follows (asterisks mark changed bases):

Q298N 5'-CGGTGAACTCGTGGAGGACTGG-3'  *
N436Q 5'-GAACAGGACTGGCCGACCACCG-3'  *

To exclude the possibility of additional mutations, the whole length of the cDNA fragment introduced into the mutagenesis system was sequenced as described above.

**RESULTS**

**Rationale for Selecting *Kₘ* Mutants.** *S. pombe* YGS-B25, which can be grown on gluconate as carbon source, was isolated by selecting for 2-deoxyglucose insensitivity (36). Whereas *S. pombe* wild type does not grow on gluconate anymore in the presence of 10⁻⁶ M 2-deoxyglucose, the mutant is unaffected by the same concentration (Fig. 1) and even grows slowly at 10⁻² M (data not shown). YGS-B25 transformed with *Chlorella* HUPI cDNA (17), however, is highly sensitive toward 2-deoxyglucose and does not grow on gluconate when the toxic sugar analogue is present at 10⁻⁵ M (Fig. 1). The drastically increased sensitivity toward 2-deoxyglucose as compared to *S. pombe* wild type is due to a

**Fig. 1.** 2-Deoxyglucose toxicity. α-Glucose uptake mutant of *S. pombe* (YGS-B25; Upper), *S. pombe* wild type (leu1-32; Lower Right), and *S. pombe* YGS-B25 transformed with HUPI cDNA (TCY96; Lower Left). 2-Deoxyglucose concentrations are given next to each plate. Cells were grown on gluconate medium.

100-fold higher affinity of the *Chlorella* hexose transporter as compared to the *S. pombe* one (9). The existing difference of 1000-fold in 2-deoxyglucose sensitivity between *S. pombe* YGS-B25 and the corresponding transformants expressing *Chlorella* HUPI cDNA has been used for selecting HUPI cDNA mutants expressing a glucose transporter with increased *Kₘ* values. From *Chlorella* HUPI cDNA random mutations were generated by PCR. With the pool of mutated HUPI cDNAs, *S. pombe* YGS-B25 cells were transformed by using the LEU2 expression vector pEVPII (20).

**Mutant Classes Obtained.** In a first experiment, 814 transformants were picked and subsequently plated to grow on gluconate in the presence of 10⁻⁶ to 10⁻³ M 2-deoxyglucose. Table 1 shows that 673 (82.5%) transformants grew only at 10⁻⁶ M 2-deoxyglucose, indicating that they were as sensitive as cells containing untreated HUPI cDNA. This must be due to transformation with fully active HUPI cDNA, which either was not mutated during PCR or contained neutral mutations. Twenty-six transformants (3.5%) grew at 10⁻³ M 2-deoxyglucose and four transformants (0.5%) grew at 10⁻⁴ M, indicating a 10- and 100-fold increased resistance to 2-deoxyglucose, respectively. The residual 111 transformants (13.5%) were as insensitive as *S. pombe* YGS-B25, indicating that an inactive transporter was expressed or that incomplete HUPI cDNA may have been transferred in the transformation.

Of mutant class I (growth in the presence of 10⁻³ M 2-deoxyglucose but not higher) all 26 transformants were individually tested for changes in *Kₘ* values for glucose

<table>
<thead>
<tr>
<th>Table 1. <em>S. pombe</em> transformants partially or fully resistant to 2-deoxyglucose</th>
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<tr>
<td><strong>No. of transformants in class</strong></td>
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<tr>
<td>Screening 1</td>
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<td>Screening 2</td>
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Carbon source for growth was gluconate (see Fig. 1). Number of transformants in different classes of sensitivity: class I, growth up to 10⁻² M 2-deoxyglucose; class II, growth up to 10⁻⁴ M 2-deoxyglucose; class III, growth at 10⁻³ M 2-deoxyglucose and higher.

*Growth up to 10⁻³ M 2-deoxyglucose; *S. pombe* YGS-B25 transformed with the original, nonmutated *Chlorella* HUPI gene also grows up to this concentration.

*Not determined; transformants were grown right away in the presence of 10⁻³ M 2-deoxyglucose and 303 such transformants were obtained.
uptake. Only one transformant showed a clear increase in \( K_m \) value by a factor of 10; this was mutant TDY63. Of class II (growth in the presence of \( 10^{-4} \) M 2-deoxyglucose but not higher) all four transformants have been checked; two showed a 10-fold increase in \( K_m \) (mutants TDY55 and TDY134). Finally, of class III (growth in the presence of \( 10^{-3} \) M 2-deoxyglucose) only 10 transformants have been tested; none showed any uptake activity. In a second screening experiment only transformants able to grow in the presence of \( 10^{-5} \) M 2-deoxyglucose were further characterized. Of 303 such transformants, 18 also grew in the presence of \( 10^{-4} \) M 2-deoxyglucose but not higher and 26 grew in the presence of \( 10^{-3} \) M 2-deoxyglucose or higher (Table 1). One of the 18 mutants in class II, 2 showed an increase in \( K_m \) of \( \approx 1000 \)-fold (RMY95 and RMY644). Because of the results of the first screening the class III transformants were not further investigated.

The altogether low yield of \( K_m \) mutants is not surprising, since all the other transformants within the corresponding classes showed a dramatically decreased \( V_{\text{max}} \) value. Resistance against toxic 2-deoxyglucose can be caused by a decreased amount of 2-deoxyglucose entering the cell, independent of whether this is due to a less active transporter or to one with decreased substrate affinity. Obviously mutations affecting the overall activity of the transporter (its catalytic activity, its correct intracellular targeting, or the total amount produced) are more common.

### Determination of the DNA Changes in the \( K_m \) Mutants.

The five mutants with clear-cut changes in \( K_m \) values for D-glucose uptake were sequenced. The changed nucleotides and the deduced amino acid changes are summarized in Table 2. One mutation, V433L (helix XI), has been obtained twice (TDY55 and TDY134), which may be due to the PCR method used and therefore is not evidence for an independent mutational event. Three of the five \( K_m \) mutants obtained possessed single mutations, indicating that the random PCR method applied and aimed at one mutation per 1800 bp worked very well. The double mutation in TDY63 (Q179E/F497S) showed a \( K_m \) value that was increased by a factor of \( \approx 10 \). Since we had already reported (17) that the less dramatic change Q179N showed an identical change in \( K_m \), it seems obvious that the glutamine in the putative transmembrane helix V is responsible for the wild-type \( K_m \) of 1-2 \times 10^{-5} \) M. The other double mutation in RMY644 resulted in one amino acid exchange, Q298R, in helix VII, whereas the change of codon GGA at V6 to GGT did not result in an amino acid replacement. Finally, N436 in helix XI in mutant RMY95 was replaced by Y. The dramatically increased \( K_m \) values of RMY644 and RMY95 may be due at least in part to the fact that the two amides were exchanged by vastly differing amino acids, Q298R and N436Y, which are likely to change the overall structure of the protein. When Q298 was exchanged by N and N436 was exchanged by Q by site-directed mutagenesis, thus by two amino acids differing only in length (i.e., by one CH2 unit), these conservative changes indeed led to a much smaller but still pronounced increase of the \( K_m \) value by a factor of 10 or 20, respectively (1-4 \times 10^{-4} \) M (data not shown).

Two of the \( K_m \) mutants (Q298R and V433L) also show a dramatically decreased \( V_{\text{max}} \) value (Table 2). In the case of Q298R, this can at least be explained to the extent that the corresponding transport protein also is not expressed very well (Fig. 2); thus, a \( V_{\text{max}} \) value 1/9th of normal exactly matches the 11% signal on the Western blot for the expressed protein. Mutant V433L, on the other hand, shows only 6.6% of wild-type \( V_{\text{max}} \), although >50% of the corresponding mutant protein is expressed (Table 2 and Fig. 2). Thus, this mutation either affects the overall catalytic activity of the transporter as well or the mutated protein may be partly mistargeted and not reach the \( S. \) pombe plasmalemma efficiently.

### DISCUSSION

Independent of what may be causing changes in \( V_{\text{max}} \) values, one possible interpretation of clear-cut changes in \( K_m \) values—being independent of the actual amount of transporter expressed and its correct intracellular translocation—may be a straightforward one: the amino acids mutated could possibly form contacts with the sugar substrate. The various amino acids exchanged in the \( K_m \) mutants are summarized in Fig. 3. All four seem to be localized approximately in the middle of the corresponding transmembrane helices as these have been postulated, admittedly without any further evidence except the hydrophathy plots. Since previously the change Q299N also led to a 10-fold increase in \( K_m \) for glucose (17), this additional observation leads to a clustering of mutations affecting \( K_m \) values in putative transmembrane helices VII and XI. Together with Q179E (or Q179N) in the middle of helix V, a model with at least helices V, VII, and XI lining the sugar transport channel can be postulated (Fig. 3 Inset).

From crystal studies, it is known that proteins always interact with carbohydrates via hydrocarbon bonds or van der Waals forces (26). In yeast hexokinase B crystallized with a derivatized glucose, the hydrocarbon bond-forming residues are an aspartic acid residue and four amides (27), the latter ideally suited to serve as hydrogen donor or/and acceptor (26). Four of the five amino acid substitutions resulting in \( K_m \) changes of the \( Chlorella \) glucose transporter also affect amides (Fig. 3).

Considering all conserved amino acids in nine D-glucose transporters cloned and unequivocally identified so far, facilitating transporters and \( H^+ \) symporters as well (i.e., human GLUT1 and -4; yeast SNF3; Arabidopsis STP1, -2, and -4; tobacco MST1; bluegreen algae Gtr; and \( Chlorella \) HUPI1),

### Table 2. D-Glucose transport properties of \( S. \) pombe mutant 25 transformed with mutated \( Chlorella \) HUPI1 cDNA

<table>
<thead>
<tr>
<th>HUPI1 mutation</th>
<th>( K_m ) M</th>
<th>( V_{\text{max}} )</th>
<th>Transport protein expressed, %</th>
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<tbody>
<tr>
<td>TCY96 (HUPI control)</td>
<td>—</td>
<td>—</td>
<td>1-2 \times 10^{-5}</td>
</tr>
<tr>
<td>TDY63</td>
<td>CAG \rightarrow GAG</td>
<td>Q179E</td>
<td>Helix V</td>
</tr>
<tr>
<td></td>
<td>TTC \rightarrow TCC</td>
<td>F497S</td>
<td>C terminus</td>
</tr>
<tr>
<td></td>
<td>CAG \rightarrow CGG</td>
<td>Q298R</td>
<td>Helix VII</td>
</tr>
<tr>
<td></td>
<td>GTA \rightarrow GGT</td>
<td>V6V</td>
<td>N terminus</td>
</tr>
<tr>
<td></td>
<td>GTG \rightarrow TTG</td>
<td>V433L</td>
<td>Helix XI</td>
</tr>
<tr>
<td></td>
<td>AAC \rightarrow TAC</td>
<td>N436Y</td>
<td>Helix XI</td>
</tr>
</tbody>
</table>

\( *V_{\text{max}} \) is given as \( \mu \)mol of D-glucose taken up per g of fresh weight per hr.
only 36 amino acid positions or 7% based on an average protein length of 500 amino acids are identical in all these proteins (28). Six of these are found in helix VII including Q298 and Q299, thus showing the highest degree of identity (28%) within the whole glucose transporter family. Three amino acids including Q179 are conserved in helix V, whereas helix XI contains only one such amino acid. These conservations have to be compared with those helices of glucose transporters where not one amino acid position has been evolutionarily conserved—i.e., helices II, III, VI, VIII, and XII (28).

For lac permease, only three amino acids are discussed as possibly located close to the substrate binding site. Y236 in helix VII and A177 in helix VI have been found to alter the substrate specificity (29-31). C148 in helix V is not essential, like all cysteines in the lac permease (16); its bulky modification with N-ethylmaleimide, however, most likely interferes with substrate binding. Helices IX and X, previously discussed as part of a charge/relay system for the proton (13), are still considered to be an essential element for active lactose translocation, but not necessarily for lactose interaction (32).

From inhibitory studies with the bismannose compound 2-N-4-(1-azi-2,2,2-trifluoroethyl)benzoyl-1,3-(D-mannose-4-loyx)-2-propylamine (ATB-BMPA), which binds at or near the postulated outward-facing sugar binding site, and cytochalasin B, which binds at or near the postulated inward-facing site, helices VIII, X, and XI are considered to interact with D-glucose in human glucose transporters (33). In addition a replacement of W412 in helix XI of GLUT1 impaired glucose transport activity (34). The corresponding position in helix XI of the Chlorella transporter is F437, the neighbor of N436, which is discussed in this paper. The replacement of Q282 with L in GLUT1 (35), the homologue of Q298 in helix VII of the Chlorella transporter, had little effect on sugar transport but decreased the affinity of the human transporter at the postulated outward binding site. Altogether no strong evidence concerning the site of substrate interaction for any transporter has been obtained so far. Therefore, the data reported here, partly favoring different helices as possibly lining a sugar transfer channel (V, VII, and XI in Chlorella HUP1 as compared to V, VI, and VII in E. coli lac permease and VII, VIII, and XI in human glucose transporter GLUT1), are not in serious disagreement with published results.

The mutation D44E previously obtained by site-directed mutagenesis (17) also affected the $K_m$ value for D-glucose.

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**Fig. 2.** Western blot of *S. pombe* crude membranes with anti-HUP1-A antibody. Band at 43 kDa represents the HUP1 protein. Mut25, glucose uptake negative mutant of *S. pombe*; TCY96, transformant containing HUP1 control cDNA; other transformants are described in text and in Table 2.

**Fig. 3.** Sequence and putative topology of the *Chlorella* HUP1 transporter protein. Boldface print indicates amino acids replaced in the four $K_m$ mutants described in this paper. Q299 (Q in black circle) was tested by site-directed mutagenesis; Q299N resulted in an increased $K_m$ value (17). (Inset) Model of the three transmembrane helices forming the putative D-glucose recognition site.
According to the location of D44 at the external edge of helix I, this aspartic acid residue so far is the only mutation not fitting the model (Fig. 3 Inset). The method described here to select for $K_m$ mutants may be extended to saturate the transporter with such mutations. If it substantiated that only this small number of amino acids are mutated, the argument that they constitute the D-glucose binding site would, of course, become stronger.

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