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K_m 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 K_m 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 K_m values for D-glucose were obtained. The amino acid exchanges responsible for the increased K_m 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 K_m value of the transporter for D-glucose. The drastic mutational changes Q298R and N436Y gave rise to very high K_m values; however, the corresponding conservative amino acid changes Q298N or N436Q obtained by directed mutagenesis also result in K_m 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 to understand 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 K_m. 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 K_m 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 K_m mutants were obtained, which allow us to postulate the sugar binding site in the *Chlorella* monosaccharide/H^+ symporter protein.

MATERIALS AND METHODS

Chemicals. D-[^{14}C]Glucose, deoxyadenosine 5′-[α-[^{35}S]thio]triphosphate, the oligonucleotide-directed mutagenesis system, and the Western blot detection system were purchased from Amersham Buchler (Braunschweig, F.R.G.). 4-Aminobenzamidinehydrochloride 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*; leu$^-$) 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-

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vating *S. pombe YGS-B25*, 4.4×10^{-4} M L-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/*Bam*HI fragment (9), containing the full-length cDNA of the *Chlorella* hexose carrier HUP1, was ligated into the *Sac* I/*Bam*HI-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 $MgCl_2$ 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 *HUP1* cDNA was digested with *Sac* I *YGS-B25*/*Bam*HI 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 *HUP1* cDNAs were resubcloned into pUC18 and sequenced by the dideoxynucleotide 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 OD_{578} (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-HUP1-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′-CGGTGAACT*G*GT*T*GAAGAACTGG-3′

N436Q 5′-GAACAGGAA*C*T*G*GCCGACCACGG-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 K_m 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^{-3} 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 HUP1* 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

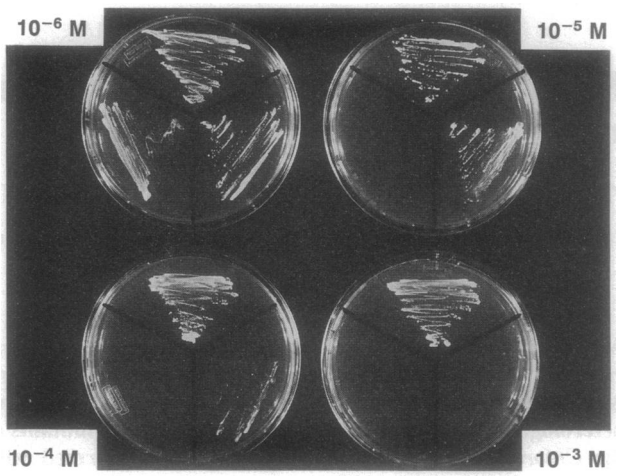

FIG. 1. 2-Deoxyglucose toxicity. D-Glucose uptake mutant of *S. pombe* (*YGS-B25*; *Upper*), *S. pombe* wild type (leu1-32; *Lower Right*), and *S. pombe YGS-B25* transformed with *HUP1* cDNA (TCY96; *Lower Left*). 2-Deoxyglucose concentrations are given next to each plate. Cells were grown on gluconate 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 HUP1* cDNA has been used for selecting *HUP1* cDNA mutants expressing a glucose transporter with increased K_m values. From *Chlorella HUP1* cDNA random mutations were generated by PCR. With the pool of mutated *HUP1* cDNA, *S. pombe YGS-B25* cells were transformed by using the LEU2 expression vector pEVP11 (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 *HUP1* cDNA. This must be due to transformation with fully active *HUP1* cDNA, which either was not mutated during PCR or contained neutral mutations. Twenty-six transformants (3.5%) grew at 10^{-5} 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 *HUP1* cDNA may have been transferred in the transformation.

Of mutant class I (growth in the presence of 10^{-5} M 2-deoxyglucose but not higher) all 26 transformants were individually tested for changes in K_m values for glucose

Table 1. *S. pombe* transformants partially or fully resistant to 2-deoxyglucose

	No. of transformants in class			
	0*	I	II	III
Screening 1	673	26	4	111
Screening 2	ND†	25	18	260

Carbon source for growth was gluconate (see Fig. 1). Number of transformants in different classes of sensitivity: class I, growth up to 10^{-5} 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^{-6} M 2-deoxyglucose; *S. pombe YGS-B25* transformed with the original, nonmutated *Chlorella HUP1* gene also grows up to this concentration.

†Not determined; transformants were grown right away in the presence of 10^{-5} 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 260 grew in the presence of 10^{-3} M 2-deoxyglucose or higher (Table 1). Of the 18 mutants in class II, 2 showed an increase in K_m of ≈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_{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 ≈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 × 10^{-5} M. The other double mutation in RMY644 resulted in one amino acid exchange, Q298R, in helix VII, whereas the change of codon GTA at V6 to GTT 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 CH_2 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 × 10^{-4} M) (data not shown).

Two of the K_m mutants (Q298R and V433L) also show a dramatically decreased V_{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_{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_{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_{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 hydropathy 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 helixes 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 hydrogen bonds or van der Waals forces (26). In yeast hexokinase B crystallized with a derivatized glucose, the hydrogen 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* HUP1),

Table 2. D-Glucose transport properties of *S. pombe* mutant 25 transformed with mutated *Chlorella HUP1* cDNA

Transformant of *S. pombe* mutant 25	HUP1 mutation					
	bp exchange	Amino acid exchange	Localization	K_m, M	V_{max}*	Transport protein expressed, %
TCY96 (HUP1 control)	—	—	—	1–2 × 10^{-5}	90	100
TDY63	CAG → GAG TTC → TCC	Q179E F497S	Helix V C terminus	1–2 × 10^{-4}	52	27
RMY644	CAG → CGG GTA → GTT	Q298R V6V	Helix VII N terminus	≥5 × 10^{-2}	10	11
TDY134 TDY55	GTG → TTG	V433L	Helix XI	2–3 × 10^{-4}	7	59
RMY95	AAC → TAC	N436Y	Helix XI	≥5 × 10^{-2}	87	122

*V_{max} is given as μmol of D-glucose taken up per g of fresh weight per hr.

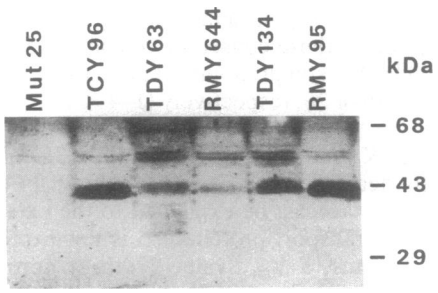

FIG. 2. Western blot of *S. pombe* crude membranes with anti-HUP1-A antibody. Band at 43 kDa represents the HUP1 protein. Mut25, glucose uptake negative mutant of *S. pombe*; TCY96, transformant containing *HUP1* control cDNA; other transformants are described in text and in Table 2.

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-yloxy)-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, X, 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.

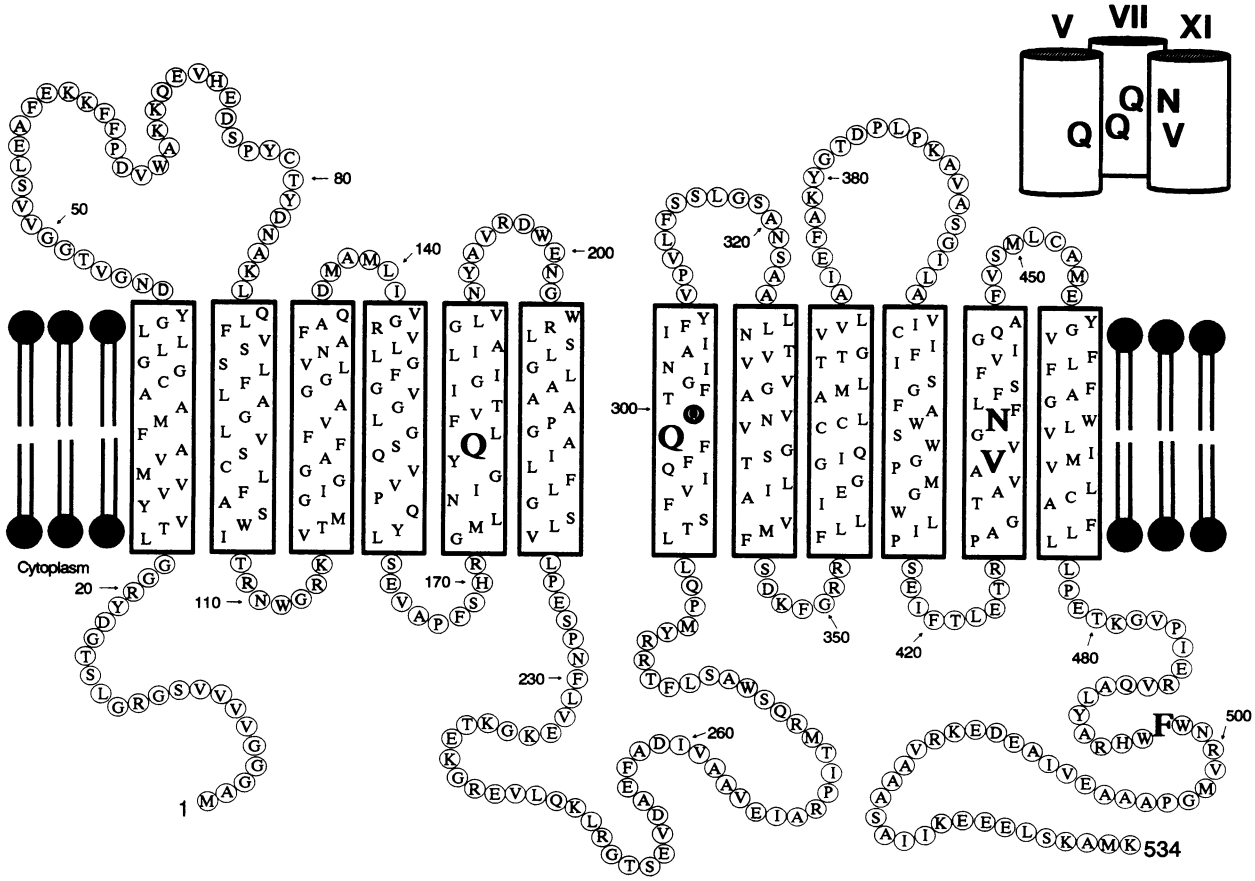

FIG. 3. Sequence and putative topology of the *Chlorella* HUP1 transporter protein. Boldface print indicates amino acids replaced in the four K_m mutants described in this paper. Q299 (Q in black circle) was tested by site-directed mutagenesis; Q299N resulted in an increased K_m value (17). (*Inset*) 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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