Structure/Function Relationship of the Chlorella Glucose/H⁺ Symporter*

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The Chlorella kessleri HUP I gene coding for a hexose/H⁺ symporter has been expressed in a glucose uptake-deficient mutant of Schizosaccharomyces pombe. The transformants are able to grow on glucose and to accumulate 3-O-methylglucose 100-fold. This system has been used to test the activity of specifically mutated HUP I cDNAs. All three histidyl residues were exchanged with arginine (H73R, H170R, and H495R) without a major effect on transport activity. When Asp-44 within the first transmembrane helix was replaced by Asn, the transporter was inactive; replacement by Glu (D44E) resulted in a loss of activity by 90% and a 15-fold increased Kₘ value. Glutamine residues conserved in all glucose transporters sequenced so far were exchanged: Q179N (in helix 5), Q298G and Q299N (both in helix 7). Whereas Q298G only resulted in a small Kₘ change, both Q179N and Q299N showed an increase in Kₘ by a factor of 10. Inserting 4 additional amino acids each into the two largest loops (1 and 6) reduced the activity dramatically; only in the latter case this was due to decreased protein synthesis or stability. Two COOH-terminal deletions (-27 and -43 amino acids) were also tested. The 27 COOH-terminal amino acids, but not the 43 COOH-terminal amino acids, could be removed without affecting transporter activity.

Active hexose transport in plant cells is catalyzed by proton symporters. Genes of such symporters, one from Chlorella and one from Arabidopsis have been cloned and functionally expressed in Schizosaccharomyces pombe (1-3). Wild type S. pombe cells are not able to accumulate glucose analogues like 3-O-methylglucose (4), whereas transformants exhibit accumulation ratios of 10-15-fold (2, 3). With this heterologous expression system, which has been improved in the meantime (3), it is possible now to study the function of various defined mutations of the H⁺ symporter gene.

The plant hexose/H⁺ symporters belong to the family of glucose transporters with 12 transmembrane helices originally described from mammalian cells (5, 6); they are not related to the Escherichia coli H⁺/lactose and the mammalian Na⁺/glucose cotransporter (7-9). So far extensive mutational studies have only been carried out with the E. coli Lac permease (8). A number of single amino acid exchanges and COOH-terminal deletions have recently also been carried out with the human GLUT1 gene (10-12).

By mutational analysis of the Chlorella glucose/H⁺ symporter coded for by the HUP1 gene answers to the following questions were sought. 1) Do any of the histidyl residues have an essential function for sugar and/or H⁺ transport? 2) What happens if most highly conserved amino acids present in all glucose transporters sequenced so far are exchanged? 3) Are the loops connecting the transmembrane helices highly fixed structures, or are they just simple links between the transmembrane helices? 4) What are the effects of COOH-terminal deletions? Results related to these four questions are reported in this study.

MATERIALS AND METHODS

Chemicals—All radioactive compounds, the site-directed mutagenesis system, and the Western blot detection system were purchased from Amersham Buchler (Braunschweig, Germany (FRG)). 4-Aminobenzamidine hydrochloride was from Merck (Darmstadt, FRG), and phenylmethylsulfonylfluoride was from Sigma (Deisenhofen, FRG). All oligonucleotides were synthesized by TIB MOLBIOL (Berlin, FRG).

Strains—The E. coli strain TG1 (32) was used as host for the phagemid pUC118 and the helper virus M13KO7 (13). For heterologous expression we used S. pombe leu1-32 and a glucose transport-deficient mutant thereof (mutant 25), obtained from Dr. M. Höfer, Bonn, FRG.

Cloning and Preparation of Single-stranded DNA—Starting with the 1770-bp SacI-BamHI fragment (1) containing the full-length cDNA of the Chlorella hexose carrier, we used an internal KpnI site to clone a 566-bp NH₂-terminal SacI-KpnI fragment and a 1204-bp COOH-terminal KpnI-BamHI fragment into the phagemid pUC118 and the helper virus M13KO7 and preparation of the single-stranded DNA was performed according to Vieira and Messing (13).

Oligonucleotide Directed Site-specific Mutagenesis—For site-specific mutagenesis the Amersham Buchler system was used, which is based on the method of Sagers et al. (14). Sequences of all oligonucleotides used are summarized as follows.

| Oligonucleotide | Sequence |
|----------------|----------|
| H73R | 5'-TGTTCCTGCGCAGCTTC-3' |
| H170R | 5'-ATGCCCGGCAGGGGAGA-3' |
| H495R | 5'-CAGAACACCGGCCGG-3' |
| Q179N | 5'-GACAAAGAGATTGTAGCCGAT-3' |
| Q298G | 5'-GACAAAGAGATTGTAGCCGG-3' |
| Q299N | 5'-GACAAAGAGATTGTAGCCAGAT-3' |
| Q298N | 5'-TGAACTGCCCGAAGAAACT-3' |
| Q299N | 5'-TGAACTGCCCGAAGAAACT-3' |
| H73R | 5'-CCGGTGAAGTTGCAGAAGA-3' |
| D44E | 5'-CCCGTGGTTCTCATAGCC-3' |
| D44N | 5'-CCCGTGGTTCTCATAGCC-3' |
| -27: | 5'-CAATGACTTACGGCG-3' |
| -43: | 5'-GGGCGGTTAGTGGC-3' |
| EcoRV | 5'-CGCGCCACGATATCTGCAACC-3' |

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The abbreviations used are: bp, base pair(s); PAGE, polyacrylamide gel electrophoresis.
Insertion of Additional Amino Acids and COOH-terminal Deletions—To introduce additional codons, a 12-bp EcoRI linker fragment (5'-CCGGAATTCGCCG-3') was ligated in frame into the HUP1 cDNA. Between helix 1 and 2 a single restriction site for Asp7001 was used, starting at the third base in the glutamate codon number 58 and in the central loop an EcoRV site was introduced by site-directed mutagenesis without changing the amino acid sequence, starting at the first base in the aspartate codon number 259.

The COOH-terminal deletions were achieved by introducing a new stop codon.

Sequencing and Cloning in S. pombe—The point mutations introduced were sequenced using the dye-ode chain-termination method (15). To locate the complete coding sequence, the corresponding wild type fragments were ligated into the phagemid pUC118, which carries the mutated fragment, using the common KpnI site. The resulting SacI-BamHI cDNA fragment was ligated into the SacI-BamHI-digested plasmid pEVP11. This plasmid and all transformation steps have been described previously (2).

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

Isolation of Membranes—30 ml of S. pombe cells with A580 of 1–1.5 were pelleted and resuspended in 10 mM Tris, pH 7.5, 5 mM EDTA, pH 7.5, 2.5 mM 4-aminobenzamidinhydrochloride, 1 mM phenylmethylysulfonyl fluoride to a final volume of 200 µl. For homogenization, cells were vortexed four times for 15 s with 0.5-g glass beads (inner diameter 0.45 mm, Braun, Melsungen, FRG). Beads were washed three times with 200 µl of homogenization buffer. Cell walls were pelleted for 5 min at 1500 × g and membranes for 20 min at 40,000 × g. The membrane pellets were solubilized in 100 µl of 20% (w/v) glycerol/homogenization buffer. The protein content was assayed by the method of Peterson (16).

Preparation of Antibodies—Polyclonal antibodies were raised against two different fusion proteins of β-galactosidase and fragments of the HUP1 protein.

Anti-HUPl-A Antibody—A fragment encoding the 186 amino acids of incomplete cDNA clone pTF15 as a BamHI/PstI fragment of 1029 base pairs (1). It was subcloned as an in-frame fusion into the BamHI/PstI sites of the expression vector pTRBO (17). This antibody did not recognize the transporter corresponding to the 27 deletion although the transformant expressing it showed 76% of the wild type activity (Table I). Therefore, a second antibody against a considerably larger part of the transporter protein was prepared (see below).

Anti-HUPl-B Antibody—The PstI fragment of 1791 base pairs of the complete HUP1 cDNA clone pTF201 (1) was subcloned into the PstI site of pTRBI. The antigen antibody contained the 448 carboxy-terminal amino acids of HUP1 as an in-frame fusion with β-galactosidase. The two different fusion proteins were isolated and injected in rabbits as described before (18).

SDS-PAGE and Immunoblotting—SDS-PAGE was carried out according to Laemmli (19); proteins were transferred electrophoretically to nitrocellulose (20) and immunodecorated with the ECL detection kit according to the Amersham manual. The intensity of immunostaining was determined with a densitometer (Hirschmann Elscript, Unterhaching, FRG).

RESULTS AND DISCUSSION

Improvement of the Heterologous Expression System—The S. pombe strain used previously for the expression of hexose transporters (2, 3) possesses an endogenous glucose transporter, which facilitates the equilibrium of sugar analogues (4). Accordingly this heterologous system has two major drawbacks for studying the structure/function relationship of an H+ symporter molecule in detail. First, the accumulation ratios of 3-O-methylglucose achieved with such cells are about 10-fold lower than those obtained with Chlorella; this is mainly due to the fact that the endogenous facilitator acts like a hole countereacting the actively transporting heterologous protein (2). Second, any mutation within the heterologous transporter giving rise to a low rate of uptake or its potential transformation to a facilitator could not be picked up against the endogenous background in transport. It has been fortunate, therefore, that in the laboratory of Höfer (Bonn), an S. pombe mutant had been obtained recently which does not transport glucose and, therefore, is not able to grow on it. This S. pombe mutant (mutant 25) when transformed with the Chlorella hexose/H+ symporter gives rise to strain TCY96, which grows again on glucose and accumulates 3-O-methylglucose more than 100-fold (Fig. 1). Thus, an ideal system for the mutational analysis of the Chlorella hexose/H+ symporter had been established.

Single Amino Acid Changes in the Chlorella H+ Hexose Symporter—Site-directed mutagenesis of single amino acids has been led by the following considerations. 1) Previous in vivo results had indicated that histidyl residues may participate in the overall transport of hexoses in Chlorella (21, 22). 2) It had been suggested (23) that the aspartyl residue in helix 1 (Fig. 2) may participate in the proton translocation. 3) When all sugar transporters cloned and sequenced so far from E. coli to man, belonging to the same homologous family of transporters, are compared, a fairly small number of amino acids is found to be identically present in all transporters specific for glucose; these are marked in Fig. 2. When each of the 3 histidyl residues was replaced by arginine (H73R, H170R, and H495R), transport was little affected (Table I). Although the uptake activities differ considerably from that of the wild type, they correlate well with the amount of protein expressed as detected by immunoblot (Table I and Fig. 3A). Changes of Km values by a factor < 2 have not been statistically supported. Thus none of the histidyl residues seems to be of special importance for the activity of the transporter. Since the amount of transporter protein in the plasma membrane may vary for a number of reasons like the number of plasmids per cell, the amount and stability of the protein synthesized, and the correctness and efficiency of its targeting, changes in Vmax values may not at all be related to the catalytic activity of a mutated protein.

In the following two mutations, however, the amount of

2 B. Milbrand and M. Höfer, unpublished results.
TABLE I
Transport characteristics of S. pombe cells transformed specifically mutated Chlorella HUP1 transporters

Kₘ and Vₘₐₓ values have been determined for D-glucose. The amount of transport protein present was estimated from the Western blot of Fig. 3 by densitometry. TCY 96 corresponds to the control transformant containing the vector only; its Vₘₐₓ value amounts to 110 μmol/g fresh weight·h⁻¹. All Kₘ and Vₘₐₓ data given are average values of three experiments.

| TCY  | Kₘ  | Vₘₐₓ | Protein |
|------|------|------|---------|
| 96   | 1.5 x 10⁻⁵ | 100  | 100     |
| H73R | 2.0 x 10⁻⁵ | 27   | 30      |
| H170R| 1.5 x 10⁻⁵ | 51   | 59      |
| H495R| 2.5 x 10⁻⁶ | 186  | 179     |
| Q179N| 1.5 x 10⁻⁴ | 61   | 37      |
| Q298G| 3.0 x 10⁻⁴ | 49   | 112     |
| Q299N| 1.5 x 10⁻⁴ | 85   | 52      |
| D44N | Inactive | 78   |         |
| D44E | 3.0 x 10⁻⁴ | 9    | 88      |
| K60TGIPE| 2.5 x 10⁻⁵ | 2   | 90      |
| D290DPEFR| Inactive | <3  |         |
| −27  | 3.0 x 10⁻⁶ | 76   | 14      |
| −43  | 2.0 x 10⁻⁵ | 2   | <5      |

the negative charge but also its precise location is important for activity. A change in Kₘ for glucose by a factor of 15 was observed in this mutant as well (Fig. 4). Concerning the activity, however, the possibility cannot be excluded that the mutated transport protein is not correctly translocated to the cell surface and integrated into the plasma membrane.

Of the amino acids conserved in all glucose transporters (Fig. 2), the 3 glutamines at positions 179, 298, and 299 were exchanged. Glu-298 was changed to a small amino acid (Q298G) since transmembrane helix 7 of the Klyveromyces maltose carrier (24) contains a GQ instead of a QQ pair. Glucose uptake decreased by a factor of 2 in Q298G, and the Kₘ value increased somewhat (Table I); maltose uptake could not be observed, however. Mutants Q179N and Q299N showed a big change in Kₘ for glucose (Fig. 4). Whereas Vₘₐₓ was only slightly affected by these mutations, the amount of protein expressed, the Kₘ value increased by a factor of 10. The Vₘₐₓ change of Q298G could be caused by a problem of correct and efficient targeting, since the amount of protein found to be present in total membranes had not decreased.

To test the amount of transport protein synthesized, all mutants were checked on Western blots for the amount of transporter present. As can be seen in Fig. 3, the amount of immunopositive material in all but two point mutants (H73R and Q179N) differs by less than a factor of 2, as compared to the wild type (see also Table I).

Amino Acid Insertions into Extramembranous Loops—to test how flexible or rigid the various postulated loops between
the potential transmembrane helices are, 4 additional amino acids were inserted into the two largest loops, i.e. into the one between helix 1 and 2 and into the one between helix 6 and 7. In the first case amino acid lysine 60 was omitted, and instead the 5 amino acids TGPIPE were inserted. The rate of uptake of this mutant decreased to about 2% of that of the wild type; a change in kinetic parameters was not observed. The immunoblot (Fig. 3B) showed that the carrier protein of the mutant K60TGPIPE was synthesized. When 4 additional amino acids were inserted into the central loop past amino acid D259 (D259DPEFR), uptake was almost completely gone and these cells reached only concentration equilibrium (not shown). The immunoblot indicated, however, that less than 3% of the amount of protein found in control cells was expressed in this insertion mutant (Fig. 3B). It is difficult therefore to draw any additional conclusions concerning this mutation. The insertion into loop 1 suggests that this structure is fairly rigid and not only a link between membrane helices. This result is clearly different from results with the Lac permease of E. coli. There it was shown that the transporter remains active even when up to 6 contiguous His residues are inserted into most of the loops (25) or when the biotin acceptor domain from the Klebsiella pneumoniae oxalacetate decarboxylase (26), cytochrome bo, or the HisP protein* are inserted into the middle cytoplasmic domain of the permease. Furthermore, functional Lac permease molecules can be formed by expressing the lacY gene in fragments encoding NH2- and COOH-terminal portions of the polypeptide (8, 27, 28).

C-terminal Deletions—According to the model of the HUP1 transporter (Fig. 2), approximately 50 amino acids at the COOH-terminal end should be facing the cytoplasmic side of the plasmalemma. Within this peptide the sequence around histidine 495 is highly conserved in all plant sugar transporters cloned so far in our laboratory (Fig. 5). Thus this sequence is present in two other highly homologous carriers of Chlorella (HUP2 and HUP3),4 the STP1 (3) and the STP2, 3, and 4 gene products of Arabidopsis thaliana,5 and the MST1 gene product of tobacco (18), but not in non-plant genes coding for monosaccharide or disaccharide transporters (5, 6). Deleting the last 27 amino acids resulted in an active gene product,

3 H. R. Kaback, unpublished results.

4 N. Sauer, unpublished results.

5 K. Baier and N. Sauer, unpublished results.
whereas the deletion of 43 COOH-terminal amino acids, including the conserved region, decreased the activity to 2% of the wild type gene product (Table 1). Although immunoblots indicate that in both cases the transporter is synthesized, the possibility cannot be excluded that the protein with the large COOH-terminal deletion may be handicapped in reaching the plasmalemma. The immunoblots of the COOH-terminal deletions give a weak signal (Fig. 3B); this is to a large extent due to the fact that the COOH terminus is mainly responsible for immunogenicity. Thus, the transformant with the –27 deletion shows 76% of wild type activity but only 14% of immunoblot intensity (Table 1, Fig. 3B) with an antibody prepared against 448 out of 534 amino acids of the transporter protein (anti-HUP1-B, see “Materials and Methods”). The anti-HUP1-A antibody, directed against the carboxyl-terminal 166 amino acids of the transporter, did not recognize the –27 and –43 deletions at all (data not shown), although all the strong signals shown in Fig. 3A and in the first three lanes of Fig. 3B, were obtained with exactly this antibody.

**CONCLUSIONS**

The system that consists of S. pombe cells transformed with plant sugar transporters is well suited for studying structure/function relationship of these membrane transporters. Whereas it has become clear from the results reported herein that the last 27 amino acids of the Chlorella transporter do not play any role in the uphill sugar translocation, it is more difficult, based on the other results described, to support any detailed model concerning the transporter topology of the HUP1 gene product. The observation, however, that three minor and fairly conservative amino acid exchanges (D44E, Q179N, and Q299N) all lead to a significant $K_m$ change without a major effect on $V_{max}$ in the two latter cases (once corrected with the amount of protein), at least supports a model in which the corresponding helices 1, 5, and 7 might take part in the substrate binding site and possibly form part of a potential glucose channel. Since an independent search for $K_m$ mutants gave rise so far to two mutants, Q179E and V433L, the latter being located in the 11th helix, it is suggestive that also helix 11 may line the postulated glucose channel. Helix 11 from the human GLUT1 carrier has also been proposed to be involved in $\alpha$-glucose interaction (6).

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