The C-terminal tetrapeptide HWFW of the *Chlorella* HUP1 hexose/H^+-symporter is essential for full activity and an α-helical structure of the C-terminus

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Received 29 November 1999

Edited by Richard Cogdell

Abstract C-terminal tails of plant hexose/H^+-symporters of the major facilitator superfamily contain a highly conserved motif of four amino acids: HWFW. A deletion of these four amino acids in the *Chlorella* HUP1 protein leads to a decrease in transport activity by a factor of 3–4. The mutated tail is highly sensitive to trypsin; it does not show α-helical conformation in contrast to the wild type C-terminal peptide with an α-helical content of at least 15%. The production of monoclonal antibody 416B8 recognizing an epitope within the central loop of HUP1 protein has been a prerequisite for the experiments described.

Key words: Sugar transport; HUP1; C-terminus structure; Tryptic digestion; Monoclonal antibody; *Chlorella kessleri*

1. Introduction

In the unicellular green alga *Chlorella kessleri* D-glucose induces a well characterized hexose transport system, capable of accumulative sugar uptake using a proton gradient as driving force [1,2]. The cDNAs for the corresponding genes (hexose uptake protein, HUP1–3) were cloned by differential screening [3,4] and their identities were proven by heterologous expression in *Schizosaccharomyces pombe* [4,5]. Structure/function relationship of the HUP1 transporter has been obtained by defining amino acids participating in substrate binding and translocation by either site directed mutagenesis of conserved amino acids [6] or random mutagenesis and selection for decreased sensitivity to a toxic sugar 2-deoxyglucose [7,8].

The HUP1 protein belongs to a large family of substrate cotransporters, called the 'major facilitator superfamily' (MFS) [9]. A high degree of homology between HUP1 and other proton monosaccharide symporters is found both within hydrophobic domains and cytoplasmic C-terminal regions. A highly conserved motif of four amino acids HWFW is present exclusively in the C-terminal tails of plant hexose/H^+-symporters (see Fig. 1).

It was shown previously that a HUP1 mutant lacking the last 43 C-terminal amino acids (including the highly conserved box) is nearly inactive whereas the deletion of 27 amino acids did not influence the symporter activity [6]. The question therefore arises what significance for substrate translocation can be attributed to the conserved amino acids HWFW.

In this study we show that a deletion of the conserved box in the C-terminal region of HUP1 results in a considerable decrease in glucose transport activity. Additionally, the relevance of the four amino acids for a native α-helical conformation of the C-terminus is demonstrated. The generation of a monoclonal anti-HUP1 antibody detecting the middle loop domain of the symporter was a prerequisite for investigating the significance of the C-terminal tail of HUP1.

2. Materials and methods

2.1. Strains, vectors and growth conditions

All cloning steps were carried out in *E. coli* DH5α with vector pUC18. *E. coli* BL21(DE3) was used for expression of the middle loop and the C-terminal tail peptides of HUP1 in vector pET24a.

The leu$^-$ and glucose uptake deficient strain *Schizosaccharomyces pombe* YGS-B25 [10] was used for heterologous expression of HUP1 cDNAs as described in [6].

2.2. Transformation of S. pombe YGS-B25 and transport tests

Wild-type and mutated HUP1 cDNAs were cloned into shuttle vector pEVP11 [11] via *Sac*I/*Bam*HI and *S. pombe* cells were transformed as described elsewhere [5]. Transport measurements in transgenic yeast cells were carried out essentially as described in [8] but in 100 mM sodium phosphate buffer at pH 5.5.

2.3. Production of antibodies

2.3.1. Polyclonal antiserum anti-HUP1 C. A peptide of 58 amino acids of HUP1 C-terminus expressed in *E. coli* and purified to homogeneity (see below), was used for rising an antiserum in rabbit.

2.3.2. Monoclonal antibodies. Female Balb/c mice (Charles River, Germany) were intraperitoneally injected with 30 μg purified HUP1-BioHis6 protein [12] in ABM2 (PAN Systems, Germany) and subsequently three times in ABM1 in intervals of 3 weeks. For production of monoclonal antibodies, P3X63Ag8.653 myeloma cells were grown in RPMI 1640 Dutch modification medium (GIBCO) supplemented with 10% heat inactivated fetal calf serum (Boehringer-Mannheim), 2 mM glutamine, 10 μM 2-mercaptoethanol and 100 μg/ml kanamycin (GIBCO). Spleen cells were fused with myeloma cells using 50% polyethylene glycol 1500 (Boehringer-Mannheim). Hybridoma cells were selected by RPMI 1640 Dutch modification medium containing hypoxanthine, aminopterin and thymidine. The cells were cultivated and cloned according to standard protocols [13,14].

Screening for cells producing monoclonal antibodies against HUP1 was performed by ELISA. Wells of ELISA plates (Falcon) were coated over night at 4°C with *S. pombe* plasma membranes (10 μg protein/ml in TBS) containing either wild type HUP1 or HUP1BioHis6. Non-specific sites were blocked with 0.2% BSA in Tris-buffered saline (TBS) containing 0.1% Tween 20. Wells were incubated for 1 h at room temperature with hybridoma cell culture supernatant (see above). Plates were washed three times with TBS and further incubated with secondary antibody anti-mouse IgG conjugated with alka-

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PII: S0014-5793(00)01230-8

line phosphatase (whole molecule, Sigma) at a dilution of 1:5000 in TBS. The color reaction was developed with *p*-nitrophenylphosphate (1 mg/ml) in buffer containing 10% (w/v) diethanolamine, pH 9.8 and 0.5 mM $MgCl_2$.

2.4. Deletion of four amino acids in the C-terminal domain of the HUP1 protein

Four conserved amino acids HWFW in the C-terminal domain of the HUP1 transporter were deleted applying a PCR method of Ho and coworkers [15]. As a template the 1770 bp *Sac*I/*Bam*HI HUP1 cDNA fragment in pUC18 was utilized. The PCR product corresponded to a 420 bp *Bgl*II/*Bam*HI fragment and was used to replace the wild-type C-terminus of HUP1 by taking advantage of a single *Bgl*II site in HUP1 cDNA.

2.5. Isolation of membranes, SDS–PAGE, immunoblotting and Coomassie staining

Total and plasma membranes of transgenic *S. pombe* cells were isolated essentially as described in [16]. The protein content was assayed by the method of Bradford [17]. After SDS–PAGE [18], proteins were electroblotted onto nitrocellulose and immunodetected either with monoclonal anti-HUP1 antibody 416B8 or polyclonal anti-HUP1 antiserum B [6] and C (see above). Proteins were detected by secondary antibodies conjugated with horseradish peroxidase (Sigma) and blots were developed using the ECL kit (Amersham).

For staining proteins with Coomassie Blue the protocol of Fairbanks [19] was followed.

2.6. Limited tryptic proteolysis

Plasma membranes of *S. pombe* cells expressing wild type HUP1 (20 μg of plasma membrane protein) or ΔHWFW HUP1 (60 μg of plasma membrane protein) were proteolytically digested on ice in 50 mM Tris–HCl pH 8.0 with a trypsin/protein ratio of 1/100 in a final volume of 500 μl. Aliquots of 50 μl were taken at 0, 0.25, 0.5, 1, 2, 5, 10, and 20 min and added to 10 μl of 20% SDS to stop the reaction. All samples were analyzed by SDS–PAGE and immunoblotting as described.

2.7. Construction of middle loop and C-terminus cDNAs

cDNAs of the HUP1 middle loop, wild type and ΔHWFW C-terminus were constructed by PCR starting from wild type or ΔHWFW HUP1 cDNAs in pUC18. With the selected 5′-primers a start ATG was introduced within a *Nde*I site used for cloning into pET24a. For subsequent affinity purification a 6× histidine-tag was added to each construct by 3′-oligonucleotides. The constructed DNAs were transformed into *E. coli* BL21(DE3).

2.8. Purification of the middle loop and tail peptides

The induction of *E. coli* BL21(DE3) with 1 mM IPTG was carried out at 37°C for 1 or 3 h for wild type tail and ΔHWFW tail expressing cells, respectively. Middle loop expressing cells were induced at 20°C over night to avoid the formation of inclusion bodies. Cells were broken by sonifying in lysis buffer (50 mM sodium phosphate pH 6.3, 1 M NaCl, 20% glycerol, 20 mM imidazole). Cell debris was pelleted by centrifugation (10 000 × *g*, 20 min) and the supernatant was merged with Ni^{2+}–NTA agarose (Quiagen) and loaded into a column. Before eluting, the bound peptides were washed in two subsequent steps (washing buffer 1 and 2: 50 mM sodium phosphate pH 6.3, 50 mM imidazole, and 1 M or 300 mM NaCl, respectively). Pure peptides were eluted using 250 mM imidazole in washing buffer 2.

2.9. Spectral analysis

2.9.1. Circular dichroism. Far-UV CD-spectra were recorded with an AVIV 62 DS spectropolarimeter at 0.46 mg/ml (HUP1-tail) and 1.8 mg/ml (ΔHWFW-tail) protein concentration in 50 mM sodium phosphate buffer pH 5.5 containing 300 mM NaCl and 1 mM EDTA at 20°C using a 0.01 cm path length cell. The bandwidth was 1 nm and the averaging time 5 s/nm. Five scans have been accumulated. The values were corrected for contributions of the buffer. The mean residue ellipticity [Θ] was calculated from the measured ellipticity according to Schmid [20].

2.9.2. UV absorption. UV absorption measurements were performed in an Uvikon 931 spectrophotometer. The molar extinction coefficients of 12660 M^{-1} cm^{-1} and 1280 M^{-1} cm^{-1} for wild type HUP1 tail and ΔHWFW tail, respectively, were determined from the amino acid sequence according to Gill and von Hippel [21].

YAR**HWFW**NR	HUP1	Chlorella kessleri
YAR**HWFW**KK	HUP3	Chlorella kessleri
FAR**HWLW**GR	HUP2	Chlorella kessleri
WRS**HWYW**SR	STP1	Arabidopsis thaliana
WEK**HWFW**RR	STP3	Arabidopsis thaliana
WRD**HWFW**KK	HEX6	Ricinus communis
WRK**HWFW**KK	SGT2	Saccharum sp.
WRK**HWFW**KK	STA1	Ricinus communis
WKS**HWFW**SR	VvHT1	Vitis vinifera
WAK**HWYW**KR	PaMST1	Picea abies
WKE**HWFW**SK	MST1	Nicotiana tabacum
WKS**HPFW**SR	MtST1	Medicago truncatula
WKS**HPYW**SR	VfSTP1	Vicia faba

Fig. 1. Multiple sequence alignment. A stretch of nine C-terminal amino acids from hexose symporters of several plant species has been chosen. The gray box shows four amino acids that are highly conserved throughout a large number of plant monosaccharide/H^+-symporters.

3. Results

3.1. Homologies within the C-terminal domain of plant sugar/H^+-symporters

As revealed by an alignment of C-terminal domains of homologous cotransporters, a conserved box of four amino acids (Fig. 1) corresponding to HWFW 495–498 in HUP1, is found exclusively in plant monosaccharide/H^+-symporters. The histidine and the second tryptophan are fully conserved among all plant hexose symporters known so far. Besides the *Chlorella* proteins HUP1-3, the alignment in Fig. 1 includes STP1 [22] and STP3 (accession no.: 4127417) from *Arabidopsis thaliana*, HEX6 and STA1 (= Hex1) from *Ricinus communis* [23], SGT2 from *Saccharum sp.* [24], VvHT1 from *Vitis vinifera* [25], PaMST1 from *Picea abies* (accession no.: 2258137), MST1 from *Nicotiana tabacum* [26], MtST1 from *Medicago truncatula* [27], and VfSTP1 from *Vicia faba* [28].

3.2. Deletion of the conserved box in HUP1 and characterization of the mutant

The highly conserved amino acids $HWFW_{495–498}$ of HUP1 were deleted and the mutant protein was expressed in *S. pombe* YGS-B25.

Fig. 2A shows glucose uptake into *S. pombe* cells expressing either the wild type or the mutated protein. The ΔHWFW mutant exhibits a significantly reduced rate of glucose uptake of 14 μmol/h/g fresh weight as compared to that of the wild type (240 μmol/h/g fresh weight). This significant reduction of glucose uptake activity to about 5–10% is only partly caused by a lower amount of the ΔHWFW transporter since at least 30% of the mutated protein is present in the plasma membrane (Fig. 2B). One therefore has to assume that the decreased transport activity is mostly due to the deletion of the conserved box. Substrate affinity was not affected since a K_m-value of 30 μM was determined for both the wild type and mutant protein.

3.3. Generation and characterization of a monoclonal anti-HUP1 antibody

To understand why the HUP1 ΔHWFW mutant exhibited this significantly impaired activity, we tried to obtain information on the conformation of the C-terminal part of HUP1. For this kind of studies, another specific antibody recognizing HUP1 protein at an epitope distinct from the C-terminus was indispensable.

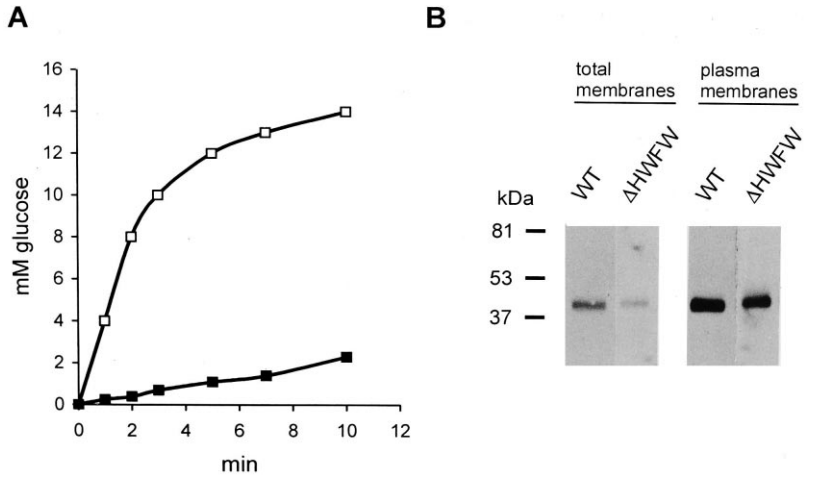

Fig. 2. A: Uptake of D-glucose by *S. pombe* cells transformed with either wild type (□) or ΔHWFW (■) HUP1 cDNA. B: Western blot determining the amount of protein present in crude and plasma membranes of transformed cells. Equal amounts of membrane extracts (30 μg of crude and 10 μg of plasma membranes) were separated on SDS–PAGE, blotted and immunodetected with the monoclonal anti-HUP1 antibody 416B8.

Monoclonal antibodies were raised against the purified HUP1BioHis6 protein as described in Section 2. One of the positive antibodies recognizing the HUP1 protein in Western blots and not crossreacting with the HUP2 protein (data not shown) was chosen for further studies and named 416B8. To detect the HUP1 epitope that is recognized by this monoclonal antibody, membranes of different *S. pombe* strains expressing chimeric proteins of HUP2/HUP1 (C4 to C1) [29] (see Fig. 3A) were analyzed. The chimeric HUP2/HUP1 symporters in the strains C4 and C3 were recognized by the antibody 416B8 while no signal was obtained in the strains C2 and C1, although all chimeric proteins were present in the membrane as revealed by polyclonal antiserum B (Fig. 3B). These results led to a conclusion that the epitope of HUP1 recognized by the antibody is located between the fourth transmembrane helix and a part of the big cytoplasmic loop (Fig. 3A). To narrow down the binding site, the cytoplasmic loop itself was expressed in *E. coli*. As shown in Fig. 3C, the monoclonal antibody recognized the recombinant peptide with an apparent molecular weight of 6 kDa. Thus, the antibody binding site could be limited to 35 amino acids from amino acid 224 to 259 (Fig. 3A).

3.4. Limited tryptic proteolysis

In the next step we focused on putative conformational changes within the C-terminal domain of the ΔHWFW mu-

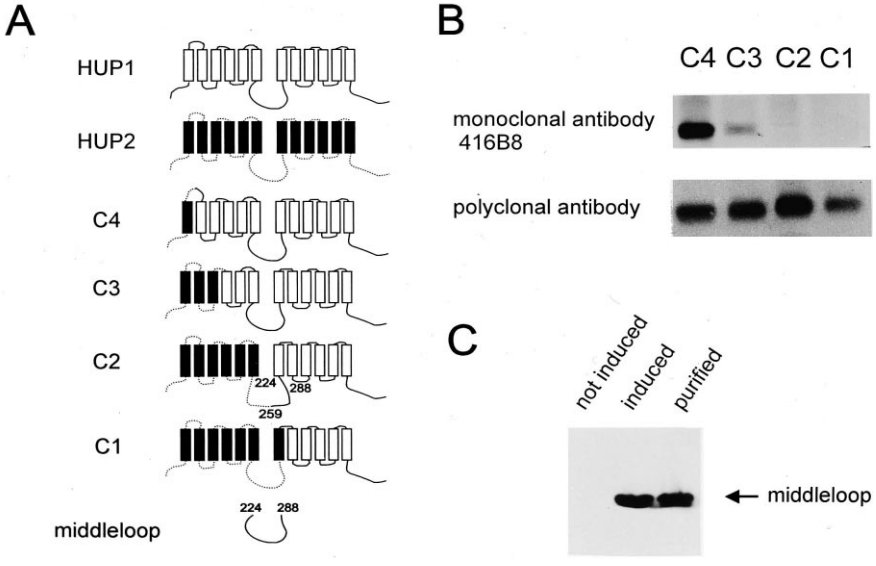

Fig. 3. A: Schematic drawing of the wild type HUP2/HUP1 and chimeric transporters. The HUP2 portion (black rectangles) of the chimeric proteins increases from C4 to C1 [29]. At the bottom the middle loop domain apart from the whole HUP1 protein is shown with indicated amino acid positions. B: Western blot of *S. pombe* crude membranes (30 μg/lane) containing chimeric HUP2/HUP1 transporters (C4–C1). The blot was incubated either with monoclonal anti-HUP1 antibody 416B8 or with polyclonal antiserum anti-HUP1 B [6]. C: Western blot of *E. coli* crude extracts expressing the middle loop before and 2 h after induction with 1 mM IPTG, and the affinity purified middle loop peptide (2 μg). The blot was incubated with monoclonal anti-HUP1 antibody 416B8.

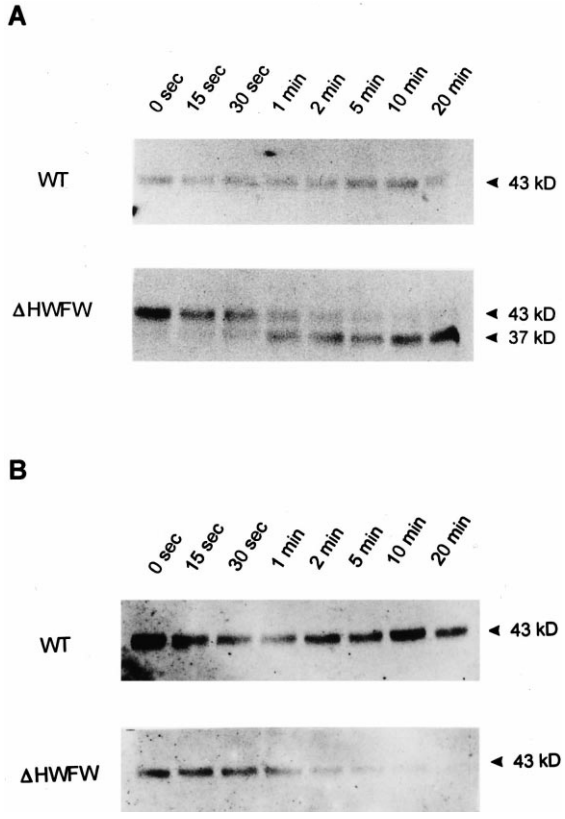

Fig. 4. Limited tryptic digestion of wild type and mutant HUP1 protein. Purified plasma membranes of the *S. pombe* strains expressing the ΔHWFW or the wild type HUP1 were subjected to trypsin proteolysis for the indicated times. A: HUP1 was immunodetected with the monoclonal anti-HUP1 antibody 416B8. B: HUP1 was detected with the polyclonal antiserum anti-HUP1 C.

tant using limited tryptic digestion. The rational behind this classical method is that proteases preferentially cleave within solvent-exposed, unstructured regions rather than within folded domains. Plasma membranes purified from *S. pombe* cells expressing the ΔHWFW or the wild type HUP1 protein were digested with trypsin and HUP1 integrity was analyzed with the middle loop detecting antibody 416B8. At room temperature, the ΔHWFW protein of 43 kDa was shortened by 6 kDa immediately after the addition of trypsin (data not shown). Under the same conditions, the wild type HUP1 protein exhibited a shift in molecular mass of 1–2 kDa only after 20 min (data not shown). By performing the digestion on ice, after 1 min about half of the mutant protein, and after 20 min the total mutant protein showed a mobility consistent with a molecular mass of 37 kDa (Fig. 4A), whereas wild type HUP1 did not show any shift when incubated on ice for the same time period (Fig. 4A). To distinguish whether the mobility shift is a consequence of cleavage of the C-terminal domain rather than digestion from the N-terminus or in some of the small loops connecting the α-helices, the polyclonal antiserum C against the C-terminus (see Section 2) was used. The immunoblot revealed that only the 43 kDa band carries an intact C-terminus, as the 37 kDa band could not be recognized by the antibody (Fig. 4B). Evidently, the disappearance of the 43 kDa signal correlates well with the appearance of the 37 kDa signal.

3.5. *Expression and purification of mutant and wild-type C-terminal tail peptides and structural investigations*

The results above indicate that the mutated form of the HUP1 C-terminal tail lost its folded conformation. To obtain information on the structure of the HUP1 C-terminus, peptides of both the wild type and the mutated protein consisting of 58 and 54 C-terminal amino acids, respectively, have been expressed in *E. coli*.

Both the wild type and the ΔHWFW tail peptides were expressed in significant amounts, appearing as a prominent band of 6 kDa in crude *E. coli* extracts on Coomassie stained

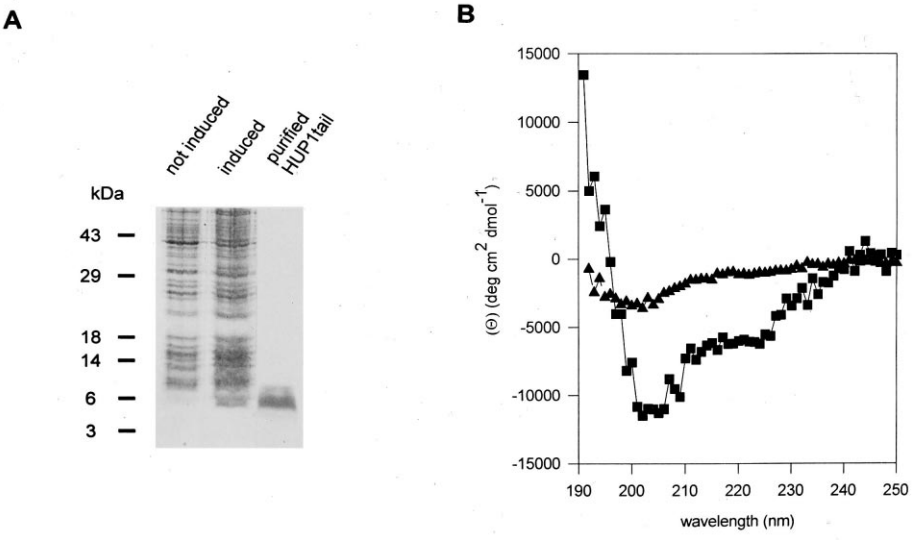

Fig. 5. A: Purification of the C-terminal tail of wild type HUP1. Crude extracts of HUP1 tail expressing *E. coli* cells, before and after induction, were separated on SDS–PAGE and analyzed by Coomassie staining. The third lane shows HUP1-tail purified by Ni^{2+}–NTA affinity chromatography. B: Far-UV circular dichroism spectra of wild type (■) and ΔHWFW (▲) HUP1-tail peptides.

SDS-gels (Fig. 5A). We succeeded in purifying both peptides to homogeneity in a one step affinity chromatography (Fig. 5A) and we obtained sufficient amounts of highly pure peptides. To investigate the secondary structure of wild type and ΔHWFW tail peptides, circular dichroism was measured. As shown in Fig. 5B, the far-UV CD-spectrum of the wild-type C-terminus exhibits two minima at 209 and 222 nm thus indicating an α-helical structure. According to Chen et al. [30], the spectrum corresponds to approximately 15% α-helix. In contrast, the recorded spectrum for the ΔHWFW C-terminus shows a very weak single minimum at 200 nm (Fig. 5B) and is typical for random coiled conformation. The results obtained both from the limited tryptic digestion and CD studies suggest that the deletion of the four amino acids in the C-terminus of HUP1 results in a complete loss of its structure.

4. Discussion

There exists a very high degree of homology between a variety of transport proteins within the sugar porter family (Family 1) of the MFS [9]. In the group of plant monosaccharide/proton-symporters a strict conservation of one histidine and one tryptophan can be observed in the C-terminal regions. The multiple sequence alignment in Fig. 1 provides only a brief overview since there are further candidates in the given plant species and there are further plant species with representatives all showing this conservation [22–28].

For the HUP1 protein, the fully conserved histidine H495 has been considered as potential intracellular pH-sensor, as in *Chlorella* the sugar uptake is inhibited at acidic intracellular pH-values. The amino acid exchange H495R, however, did not show any effect on the glucose transport activity or the K_m-value [6]. But on the other hand the deletion of H495 together with the three other highly conserved amino acids WFW496–498 leads to a considerable decrease in transport activity indicating that the conserved box is very important for sugar/H^+-translocation. The demonstrated sensitivity of the mutant protein to trypsin indicates a possible role of the amino acids HWFW in preserving a specific 'active' conformation of the HUP1 C-terminus. This conformation is lost upon deletion of the conserved box.

It might be assumed that similarly to plant plasma membrane ATPases, the symporter C-terminus exhibits regulatory properties. In plant plasma membrane ATPases, it is known that a large C-terminal region has inhibitory effects on the protein [31]. Mutations in the C-terminal domain of ATPases lead to a conformational change, as shown by tryptic digestion, resulting in the displacement of the inhibitory C-terminus and in the activation of the protein [32]. In contrast, in the case of HUP1, the conformational change within the C-terminus obviously leads to an inhibition of the protein activity.

Our data demonstrate that the soluble C-terminus of wild type HUP1 possesses an α-helical conformation which is important for maximal activity of the symporter. One possible explanation for the necessity of an α-helix in the tail region is that the C-terminus might regulate the protein activity by integrating into the membrane or contacting the membrane/water interface. It has been shown with synthetic α-helical peptides that tryptophan side chains within the peptides have a specific affinity for a well defined site near the lipid carbonyl region at the interface [33]. This kind of interaction might be also considered for the tryptophan residues in the HUP1 C-terminus.

Another explanation might consist in a specific role of the C-terminus in ensuring a protein–protein interaction. For instance, it is known for the plasma membrane ATPases from plants that their C-terminus interacts directly with a protein of the 14-3-3 protein family [34].

A direct interaction of the C-terminal domain of a transporter with a soluble protein was also demonstrated for the glucose transporter GLUT1 in mammalian cells. It was shown that the glucose transporter binding protein GLUT1CBP specifically interacts with the functionally important GLUT1 C-terminus [35].

The specific function of the C-terminal domain of HUP1 protein as a plant hexose/H^+-symporter can only be speculative at this moment. But the data presented in this article clearly demonstrate that an intact cytoplasmic C-terminus containing an α-helical structure is required to guarantee full transport activity.

Acknowledgements: We are grateful to Dr. Stephan Wenzl for help with the generation of monoclonal antibodies and to Dr. Doris Wassenberg for assistance in CD-measurements. We thank Claudia Strohmeier for her skillful technical help. This work was supported by Deutsche Forschungsgemeinschaft (SFB521), by Grant Agency of Czech Republic (204/99/0492) and by a NATO Collaborative Research Grant (LST.CLG 974804).

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