Three Surface Subdomains Form the Vestibule of the Na+/Glucose Cotransporter SGLT1*

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Theeraporn Puntheeranurak15*, Myriam Kasch5, Xiaobing Xia5, Peter Hinterdorfer5, and Rolf K. H. Kinne11

From the 1Department of Biology, Faculty of Science, Mahidol University, and Center of Excellence, National Nanotechnology Center at Mahidol University, Bangkok, 10400, Thailand, the 2Max Planck Institute of Molecular Physiology, Otto-Hahn Strasse 11, 44227, Dortmund, Germany, and the 3Institute for Biophysics, Johannes Kepler University of Linz, Altenbergerstrasse 69, A-4040, Linz, Austria

A combination of biophysical and biochemical approaches was employed to probe the topology, arrangement, and function of the large surface subdomains of SGLT1 in living cells. Using atomic force microscopy on the single molecule level, Chinese hamster ovary cells overexpressing SGLT1 were probed with atomic force microscopy tips carrying antibodies against epitopes of different subdomains. Specific single molecule recognition events were observed with antibodies against loop 6–7, loop 8–9, and loop 13–14, demonstrating the extracellular orientation of these subdomains. The addition of D-glucose in Na+ containing medium decreased the binding probability of the loop 8–9 antibody, suggesting a transport-related conformational change in the region between amino acids 339 and 356. Transport studies with mutants C345A, C351A, C355A, or C361S supported a role for these amino acids in determining the affinity of SGLT1 for D-glucose. MTSET, [2-(Trimethylammonium)ethyl] methanethiosulfonate and dithiothreitol inhibition patterns on α-methyl-glycoside uptake by COS-7 cells expressing C255A, C560A, or C608A suggested the presence of a disulfide bridge between Cys255 and Cys608. This assumption was corroborated by matrix-assisted laser desorption ionization time-of-flight mass spectrometry showing mass differences in peptides derived from transporters biotinylated in the absence and presence of dithiothreitol. These results indicate that loop 6–7 and loop 13–14 are connected by a disulfide bridge. This bridge brings also loop 8–9 into close vicinity with the former subdomains to create a vestibule for sugar binding.

Several molecular biological approaches have been employed to determine the membrane topology of SGLT1, indicating that it contains 14 transmembrane α-helices and 13 surface domains or loops connecting the transmembrane segments (4–6). There is still controversy, especially regarding the orientation of the large C-terminal loop connecting transmembrane helices 13 and 14 and the C-terminal end of SGLT1. Recently, single-molecule force spectroscopy using AFM2 has been utilized as an alternative method for probing the surface topology of loop 13–14 of the rabbit SGLT1 on living cells (7). This study confirmed an extracellular orientation of the disputed loop 13–14 of SGLT1, which is also supported by previous studies by Gagnon et al. (8) using the substituted cysteine accessibility method (SCAM) and fluorescent labeling methods. Both groups have proposed that this loop possibly acts as a reentrant loop during substrate translocation.

The N-terminal half of SGLT1 contains the Na+ binding sites, whereas the sugar pathway is located in the C-terminal domain, particularly in helices 10–13 of the protein (9–11). It is known that SGLT1 strongly discriminates among its natural substrates, D-glucose, D-galactose, and other hexoses (12). Many researchers have attempted to localize a substrate translocation pathway in SGLT1. Studies on chimera proteins of SGLT1/SGLT2 and a truncated protein (residues 407–648) showed that residues from 381–662 of SGLT1 are important for sugar transport, however, with less sugar specificity and lower sensitivity to the competitive inhibitor phlorizin (10, 13). Also residues 457, 468, and 499 have been shown to play an important role in controlling the sugar binding of SGLT1 (14, 15). Additional studies have demonstrated that loop 13–14 is involved in phlorizin binding and possibly contains a glucose-binding site (7, 16–18). Nevertheless, the precise substrate-binding domains have not yet been defined. In this study, we have speculated that a region before residue 380 might play an important role in high affinity glucose binding, because the mutations at residues 292 and 355 in human SGLT1 lead to the hereditary disease glucose-galactose malabsorption (19, 20). SGLT1 possesses 14 conserved cysteine residues, and there is evidence indicating that the existence of (at least) two disulfide bonds is important for functioning of this transporter (21, 22).

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To whom correspondence should be addressed. Tel.: 49-0231-9742-6491; Fax: 49-0231-9742-6479; E-mail: rolf.kinne@mpi-dortmund.mpg.de.

2 The abbreviations used are: AFM, atomic force microscopy; aa, amino acid(s); DTT, dithiothreitol; MTSET, ---; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; AMG, α-[14C]methyl glucoside; KRH, Krebs-Ringer-HEPES; PEG, polyethylene glycol; MTSET, [2-(Trimethylammonium)ethyl] methanethiosulfonate.
Disulfide groups play a key role in stabilizing the three-dimensional structure of a protein by forming intra- and interchain disulfide bonds. In addition, free sulfhydryl groups often have important catalytic or regulatory roles. So far, there is evidence showing one disulfide bridge between residues Cys355 and Cys511 in human SGLT1 (23). In rabbit SGLT1 there are three large extramembranous loops or surface subdomains (loop 6–7, loop 8–9, and loop 13–14) that contain cysteine residues and therefore are potential sites for bringing the transmembrane helices 4 and 5 close to the transmembrane helices 10–13. Thereby, a vestibule containing a glucose recognition site might be formed similar to the results from the γ-aminobutyric acid transporters, showing that three surface loops form a pocket in which the substrate initially binds to the transporters (24).

To prove this assumption, the investigations using single-molecule AFM were further expanded to study the binding of epitope-specific antibodies to surface domains of SGLT1 during D-glucose translocation. In addition, site-directed mutagenesis, uptake assays, and MALDI-TOF mass spectrometry were employed to strengthen this study.

The results clearly show that the region between aa 339 and 361 in loop 8–9, especially residues Cys351 and Cys361, are involved in glucose binding. There is also evidence for a disulfide bridge between Cys355 and Cys608 in rabbit SGLT1. Taken together, these studies demonstrate the potential of AFM techniques in combination with biochemical and genetic approaches to investigate the structure and function of subdomains of SGLT1. Thereby, considerable insight is provided into the arrangement of transmembrane helices and the substrate-binding pocket of the rabbit SGLT1. This is important information for understanding the mechanism of action of this carrier protein.

**EXPERIMENTAL PROCEDURES**

**Materials**—All of the chemicals were of the highest purity available and were purchased from Sigma unless other sources are indicated. Immunopurified polyclonal antibodies QIS30 (subdomain I), PAN2-2 (subdomain II), and PAN3-2 (subdomain III) were raised against oligopeptides from the amino acid sequences aa 243–272 (loop 6–7, i.e. the loop between the transmembrane helices 6 and 7), aa 339–356 (loop 8–9), and aa 606–630 (loop 13–14) of rbSGLT1 (rabbit isoform), respectively, as described before (6, 25, 26). The specificity of the interaction of the antibodies with the supposed epitopes has been shown in independent previous immunohistochemistry studies where surface labeling of the cells could be prevented in the presence of the peptides used for their generation.

**Mutagenesis of SGLT1 cDNA and Plasmid Construction**—All of the mutants were prepared by site-directed mutagenesis using a Chameleon™ double-stranded site-directed mutagenesis kit (Strategene, La Jolla, CA). The following primers with mutated nucleotides (underlined) were used for mutagenesis: C252A, 5′-CATCCATCCAGAGGTGGCCCGTGTCAGCCAAAAGGGAGGCCGCG—3′; C345A, 5′-GCCTGCTAGCTGGACAGAAGATAGGCAGCGATGACGCTGGAGCCTACGCCG—3′; C351A, 5′-GCCTGCTAGCTGGACAGAAGATAGGCAGCGATGACGCTGGAGCCTACGCCG—3′; C355A, 5′-GCCTGCTAGCTGGACAGAAGATAGGCAGCGATGACGCTGGAGCCTACGCCG—3′; C356A, 5′-GCCTGCTAGCTGGACAGAAGATAGGCAGCGATGACGCTGGAGCCTACGCCG—3′; C560A, 5′-GCCTGCTAGCTGGACAGAAGATAGGCAGCGATGACGCTGGAGCCTACGCCG—3′; and C608A, 5′-GCCTGCTAGCTGGACAGAAGATAGGCAGCGATGACGCTGGAGCCTACGCCG—3′.

For preparation of mutants, plasmid pSGLT1 (2207 bp of rabbit DNA) was used as a template. After site-directed mutagenesis all of the mutants were confirmed by DNA sequencing and cloned from plasmid pSGLT1 into expression plasmid pHook™-2 (Invitrogen) with appropriate enzymes. Final plasmids were purified with a Qiagen endotoxin-free plasmid maxi kit.

**Cell Cultures and Transient Transfection**—G6D3 cells or cell line stably transfected with the rabbit SGLT1 (25) and COS-7 cells were grown in 25-cm² flasks (Falcon, Heidelberg, Germany) under 5% CO₂ at 37 °C. The G6D3 cells were cultured in Dulbecco’s modified Eagle’s medium, containing high glucose (25 mM) supplemented with 5% fetal calf serum, 1 mM pyruvate, 2 mM L-glutamine, 1 × minimal essential medium, 25 μM β-mercaptoethanol, and 400 μg/ml paneticin G420 (PAN Biotech GmbH, Aidcnbach, Germany). The COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1% L-glutamine. The culture media were renewed three times a week, and the cells were subcultured at 80% confluence. Cell passages below 15 were used for all experiments. For AFM investigation, the G6D3 cells were seeded on 22-mm² poly-L-lysine-coated glass coverslips, and the experiments were performed within 1–4 days of seeding the cells.

COS-7 cells were grown in 6-well culture plates to 70–80% confluence before transfection. Transient transfection was performed by using the activated dendrimer transfection reagent SuperFect™ (Qiagen) as described earlier (16). The efficiency of transfection was tested in COS-7 cells that were simultaneously transfected with the pHook™-2lacZ control plasmid expressing β-galactosidase under identical conditions (Invitrogen). The post-transfection period for an optimal expression of β-galactosidase was 48 h. The transfection was performed as described in the manufacturer’s manual, and ~35–40% efficiency was found for the wild type and all mutants.

**Transport Studies**—Na⁺/D-glucose cotransport activity of transiently transfected COS-7 cells (wild type and mutants) was assessed by examining α-[14C]methyl glucoside (AMG) uptake at 37 °C in Na⁺-containing Krebs-Ringer-HEPES (KRH) medium as described previously for Chinese hamster ovary cells (25). Prior to the transport assay, COS-7 cells cultured in 6-well plates were incubated in a D-glucose-free medium for 1 h at 37 °C to reduce the intracellular glucose concentration to a nonsignificant level. Uptakes of AMG were carried out at a concentration of 0.02 mM AMG (containing 2 μCi of 14C-labeled AMG). The maximum uptake rate of wild type was 2.8 ± 0.3 nmol (mg protein)⁻¹ (15 min)⁻¹. The results were expressed as percentages of AMG uptake of the wild type (mean values ± S.D., n = 6). In kinetic measurements for the determination of the apparent Km, AMG (mmol/liter), two concentrations of substrate, i.e. 0.1 mm (S₁) and 3 mm (S₂) were used. The results were expressed as mean values ± S.D. (n = 3). The calculations were performed as described earlier (16).

For determination of the free thiol groups on the surface of SGLT1, MTSET (a thiol-reactive compound) was used. The
transiently transfected wild type COS-7 cells and mutants were incubated with 1 mM MTSET for 12 min before performing AMG uptake assays in Na\(^+\)-KRH medium containing 1 mM MTSET. The results are presented as a percentage of inhibition compared with the uptake of the respective cells in the buffer without DTT and were obtained from measurement repeated in triplicate (mean values ± S.D., n = 4).

To demolish possible intramolecular disulfide bonds of SGLT1, 20 mM dithiothreitol (DTT) were added in the AMG uptake assays of the transiently transfected COS-7 cells (wild type and mutants). The results were expressed as percentages of inhibition compared with the uptake of the respective cells in the buffer without DTT and were obtained from measurement performed in duplicate (mean values ± S.D., n = 3).

**Conjugation of Antibodies to AFM Tips**—Conjugation of immunopurified subdomain antibodies (i.e. QIS30, PAN2-2, and PAN3-2) to AFM tips via a flexible PEG-cross-linker, generating subdomain I tip, subdomain II tip, and subdomain III tip, respectively, were done similarly as described previously (27). In brief, AFM tips were first functionalized with ethanamine by an overnight incubation with ethanolamine hydrochloride solubilized in Me₃SO. Then the heterobifunctional PEG linker (aldehyde-PEG-N-hydroxysuccinimide) was covalently bound to the amino groups on the tip surface via the N-hydroxysuccinimide ester function. Next, the specific antibodies were coupled via the aldehyde function to the PEG-conjugated AFM tips (28). The tips were finally washed in the AFM working buffer and stored in the cold room. This method provides tips to which only a few antibodies, functionally separated from each other, are attached. These tips have been used successfully in single-molecule recognition studies (7, 27).

**Atomic Force Microscopy**—All AFM experiments were performed using a magnetically driven dynamic force microscope (Agilent 5500 AFM; Agilent Technologies, Tempe, AZ) in the Na\(^+\)-containing KRH medium except where Na\(^+\)-free medium was stated. For the detection of antibody-SGLT1 recognition, force distance cycles were performed at room temperature using ligand-coated cantilevers (rectangular cantilever; Veeco Instruments, Mannheim, Germany) with 0.02 N/m nominal spring constants in the conventional contact force spectroscopy mode as described previously (7). Spring constants were determined according to the thermal noise method (29). Force distance cycles were recorded at the cell surfaces with the assistance of a CCD camera for positioning the AFM cantilever. An AFM tip carrying antibody was first approached (trace) and then retracted (retrace) from a cell surface, and the deflection angle of the cantilever was measured as a function of the z position. The deflection is proportional to the interaction force according to \( f = k \Delta z \) (Hook’s law, \( k \) is the spring constant of the cantilever, \( \Delta z \) is the deflection up (+) or down (−) of the cantilever). The sweep-amplitude of the force-distance cycles was 1000 nm at 1 Hz sweep rate. Up to 500–1000 force-distance cycles were performed for each area on the surface of cells, in the average four different cells for each condition, i.e. initial condition, ligand addition, and washout condition. For ligand addition, 0.3 μM free antibodies (QIS30, PAN2-2, and PAN3-2) and 10 mM D-glucose were separately applied in different conditions. The binding probability for each condition was derived and expressed as the mean value ± S.E. (n = 1500–4000). The statistical significance was tested using a Student’s t test. Several experiments were performed, and one typical experiment for each condition is shown. Analysis of force distance cycles was performed using Matlab version 6.5 (Math Works, Natick, MA) as previously described (27, 30).

**Sample Preparation for MALDI-TOF Mass Spectrometry**—The rbSGLT1 was purified by immunoprecipitation from G6D3 cells as previously described (25). The extracted protein was then incubated with 10 milliunits of peptide-N-glycosidase F (Roche Diagnostics) at 37 °C for 1 h and subsequently precipitated with CHCl₃/MeOH (31). The protein pellets were solubilized in 10 mM Tris-HCl, pH 6.8, containing 0.5% SDS at 90 °C for 5 min in two conditions, i.e. in the absence or presence of 5 mM DTT. Cysteine residues were then biotinylated using 10 mM maleimide-PEO₂-biotin (molecular weight, 525.6; Pierce) at room temperature in the dark for 1 h. The reaction was stopped by adding excess l-cysteine HCl and followed by SDS-PAGE. The SGLT1 protein was digested with modified trypsin (20 μg/ml) in 25 mM ammonium bicarbonate overnight at 37 °C. The tryptic peptides were extracted twice with acetonitrile/water/trifluoroacetic acid (66:33:0.1, v/v/v) solution and subsequently dried and resuspended in phosphate-buffered saline buffer, pH 6.5. The detrimental effects of proteases were avoided by treating the protein with 4-(2-aminoethyl)-benzenesulfonyl fluoride (Roche Diagnostics). The peptides were then purified with avidine coupled agarose, and the biotinylated peptides were then eluted with acetonitrile/water (60:45, v/v) containing 5% formic acid.

**MALDI-TOF Mass Spectrometry**—The sample was spotted onto the MALDI plate and air-dried at room temperature. MALDI-TOF mass spectra were acquired on a Voyager MALDI system (PE-Biosystems). MALDI peptide spectra were collected by using the linear delayed mode (100 ns) with 94% grid voltage (guide wire voltage) and 0.05% low mass gate, 500. The spectra were externally calibrated with insulin, thioredoxin, and apomyoglobin (PE-Biosystems).

**RESULTS**

**Orientation of Three Subdomains Determined by Antibodies Coupled to AFM Cantilevers**—AFM force spectroscopy was employed to verify the surface topology of subdomain I (loop 6–7), subdomain II (loop 8–9), and subdomain III (loop 13–14) (Fig. 1). rbSGLT1-transfected G6D3 cells, which overexpress SGLT1 on the cell surface (25), were used as a cell model for this investigation. Using three different AFM tips primed with epitope-specific antibodies, i.e. subdomain I tip, subdomain II tip, and subdomain III tip (see “Experimental Procedures”), force-distance cycles were performed at a fixed lateral position above the cells in Na\(^+\)-containing KRH medium. The principle of single-molecule recognition force detection of the ligand-receptor complex on living G6D3 cells by using AFM has been described previously (7). Specific recognition events were observed with a unique characteristic from the force signal, as illustrated by a typical force curve using the subdomain I tip (Fig. 2A). The recognitions diminished in the presence of specific subdomain I antibodies in solution (Fig. 2B), suggesting that the antibody coupled to the cantilever only reacts with
SGLT1 and no other proteins. Specific unbinding events that can be prevented by free antibodies were also observed when epitope-specific antibodies against subdomain II and subdomain III were coupled to the AFM tips. The binding probabilities (probability to find an unbinding event in force distance cycles) from several experiments were derived. From typical experiments (n = 3 for subdomain I and II, n = 9 for subdomain III), a binding probability of subdomain I, II, and III antibodies on G6D3 cells of 9.5 ± 0.7, 8.0 ± 1.9, and 10.6 ± 0.7%, respectively, were observed, (Fig. 2, C, D, and E, first bars). For confirmation of the specific recognition of SGLT1 by the antibodies, blocking experiments by injecting free antibodies were performed. Binding probability decreased to 2.1 ± 0.2% when free subdomain I antibodies were present in the medium and subsequently recovered to 6.9 ± 0.7% after wash-out of the antibodies (Fig. 2C). Likewise, the binding probabilities decreased to 1.7 ± 0.5 or 3.6 ± 0.9% when free subdomain II and III antibodies were injected. After subdomain II and III antibodies were subsequently removed the binding probabilities increased again to 4.9 ± 1.5 and 6.6 ± 2.3%, respectively (Fig. 2, D and E).

From these results it can be concluded that the epitopic regions of the subdomain I, II, and III of SGLT1 are localized extracellularly in living cells.

By determining $f_u$, the maximum of the probability density function of the unbinding forces, the most probable unbinding force for the interaction strength of the different antibodies with SGLT1 was quantified. The results are shown in Table 1. The interaction force between subdomain III and SGLT1 was 95.2 ± 9.9 pN, which is in agreement with previous investigations (7). However, the unbinding forces between the subdomain I antibody and SGLT1 and between the subdomain II antibody and SGLT1 were slightly lower (61.9 ± 2.2 and 60.7 ± 6.2 pN, respectively) (n = 3).

Effect of D-Glucose and Na\textsuperscript{+} on the Subdomain Antibody Recognition Observed by AFM—To determine whether the three subdomains are involved in glucose-sodium cotransport, the effect of D-glucose and sodium on the recognition of the three subdomains by the antibodies was investigated. As illustrated in Fig. 3, before the addition of D-glucose, SGLT1 could be recognized by the three antibodies on the AFM tips in both Na\textsuperscript{+}-containing (KRH-NaCl) and Na\textsuperscript{+}-free (KRH-NMG) buffers with 8.7 ± 0.4% binding probability for both conditions (n = 15). Upon the addition of 10 mM D-glucose, the binding probability was significantly reduced only with the subdomain II antibody tip and only in the presence of Na\textsuperscript{+} (from 9.6 ± 1.1 to 4.3 ±...
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TABLE 1

| Antibodies         | Binding probability | Maximum unbinding force $f_{u}^{*}$ | $p$ Value |
|--------------------|---------------------|-------------------------------------|-----------|
| Subdomain I (aa 243–272) | 9.5 ± 0.7           | 61.9 ± 2.2                          | 0.0013    |
| Subdomain II (aa 339–356) | 8.0 ± 1.9           | 60.7 ± 6.2                          | 0.0013    |
| Subdomain III (aa 606–630) | 10.6 ± 0.7          | 95.2 ± 9.9                          | 0.0013    |

$^{*}$ Binding probability and maximum unbinding force from several independent experiments with four locations on four cells for each experiment. The values are the means ± S.E. ($n = 3$ except for subdomain III, where $n = 9$).

TABLE 2

| Mutant   | Apparent $K_{m}$ of AMG $^{a}$ | AMG uptake rate of wild type $^{b}$ |
|----------|---------------------------------|------------------------------------|
| Wild type| 1.0 ± 0.2                        | 100.0                              |
| C345A    | 3.3 ± 1.5                        | 27.4 ± 4.8                         |
| C351A    | > 20.0                           | 3.9 ± 1.7                          |
| C355A    | 5.1 ± 1.6                        | 27.5 ± 4.5                         |
| C361S    | > 20.0                           | 37.3 ± 5.7                         |

$^{a}$ Kinetics constants ($K_{m}$) were obtained from measurements repeated in triplicate. The values are the means ± S.D. ($n = 3$).

$^{b}$ Maximum AMG uptake rate of wild type was 2.8 ± 0.3 nmol (mg protein)$^{-1}$ (15 min)$^{-1}$ at 0.02 mM AMG. The values are the means ± S.D. ($n = 6$).

A–F  | ![Diagram A](image1.png) | ![Diagram B](image2.png) | ![Diagram C](image3.png) | ![Diagram D](image4.png) | ![Diagram E](image5.png) | ![Diagram F](image6.png)

FIGURE 3. Effect of d-glucose and Na$^+$ on the recognition of SGLT1 by antibodies. Binding probabilities of subdomain I (A and B), subdomain II (C and D), and subdomain III (E and F) tips to the surface of G603 cells under various conditions in the presence of Na$^+$ (KRH-NaCl buffer) and in the absence of Na$^+$ (KRH-NMG buffer), respectively. The values are the means ± S.E. ($n = 1500–4000$; **, $p < 0.01$ and ***, $p < 0.005$) compared with levels in the relevant controls (absence of d-glucose or antibodies, value from the first bar).

0.7%, $p = 0.0013$) (Fig. 3C, second bar). Again, injection of the respective antibody resulted in a drastic significant reduction of binding (Fig. 3, A–F, third bars). After the free antibodies were removed from the medium, the binding probabilities recovered (Fig. 3, A–F, fourth bars). These results show that in the presence of d-glucose and Na$^+$, the region of aa 339–356 on subdomain II, where the antigenic sites are located, becomes either less accessible to the antibody or that its conformation changes dramatically. Interestingly, the antigenic sites on subdomain I and III were still reachable from the outside by the antibodies under the same conditions.

Effect of Mutation of Cys$^{345}$, Cys$^{351}$, Cys$^{355}$, or Cys$^{361}$ in Subdomain II on Transport Properties of SGLT1—In the previous section, single-molecule recognition investigations have demonstrated a sodium-dependent effect of d-glucose on the region between aa 339 and 356 on subdomain II of SGLT1. Hence, it was interesting to investigate whether this region is indeed involved in sugar translocation. Therefore, transport studies were performed with wild type and mutants of the subdomain II region. Four mutants were generated by site-directed mutagenesis to replace cysteine by alanine or serine, i.e. C345A, C351A, C355A, and C361S. Subsequently, the wild type and mutant SGLT1 gene were transiently transfected into COS-7 cells for functional characterization. The uptake rate of wild type was 2.8 ± 0.3 nmol (mg protein)$^{-1}$ (15 min)$^{-1}$ at a concentration of 0.02 mM AMG. All of the replacements showed clearly a reduction of the transport activity. The most prominent effect was observed for C351A, i.e. ~4% of the maximum wild type activity, whereas the other mutants were ~27–37% of the wild type one (Table 2). The kinetic properties of wild type and mutants were also investigated. As depicted in Table 2, the replacements of cysteine residues in the subdomain II strongly affected the affinity of the transporter for AMG. The most striking effects were observed for C351A and C361S with the apparent $K_{m}$ values for AMG at least 20-fold higher than the apparent $K_{m}$ of the wild type. These results are in agreement with the observed effect of d-glucose on the recognition of the epitopic region of aa 339–356 in loop 8–9 by the subdomain II antibody, suggesting a possible glucose-binding pocket in this region (see “Discussion”).

Proximity of the Three Subdomains—Several studies have shown that segments in subdomain III of rabbit SGLT1 are involved in phlorizin binding (7, 16–18). It also has been demonstrated that phlorizin obstructs the initial glucose-binding site of rbSGLT1 (12). Hence, we hypothesized that subdomain III of rbSGLT1 is in close vicinity to subdomain II, which we found above to be involved in glucose binding. Such proximity can be created by an intramolecular disulfide bridge. To determine whether this assumption is true, the cysteine residues in subdomain III were investigated as possible candidate acceptors of forming a disulfide bond. To this end, two mutants, i.e.
C560A and C608A were generated, and the inhibitory effect of MTSET (which blocks free SH groups on the membrane surface) on AMG uptake by COS-7 cells transiently transfected with SGLT1 wild type and the mutants was measured. As illustrated in Fig. 4, MTSET inhibited uptake both in cells expressing wild type SGLT1 and in cells expressing C608A, where the cysteine at position 608 is replaced by alanine. The percentages of inhibition of wild type and C608A were not significantly different. However, almost no inhibition by MTSET was observed when uptake in cells expressing C560A was determined. These results imply that a free SH group exists at position 560 on the subdomain III; binding of MTSET to this cysteine inhibits sugar transport by SGLT1. They also suggest that the cysteine at position 608 might be forming a disulfide bond with another part of SGLT1.

To identify the donor and acceptor residues for such a disulfide bridge, we determined the effect of DTT on AMG uptake by COS-7 cells transiently transfected with SGLT1 wild type and mutants in subdomain I (C255A), subdomain II (C345A, C351A, C355A, and C361S), and subdomain III (C608A). As depicted in Fig. 5, cells expressing the wild type SGLT1 showed ~40% inhibition of sugar uptake by DTT. All of the mutants in subdomain II showed high DTT inhibition (i.e. ~50–70%), whereas the mutants in subdomain I (C255A) and III (C608A) showed no or low inhibition by DTT (~6 ± 12.7% and 20 ± 9.3%, respectively). These results suggest that the residues Cys$^{255}$ and Cys$^{608}$ possibly form an intramolecular disulfide bond in rbSGLT1 (see “Discussion”).

Finally, surface biotinylation and MALDI-TOF mass spectrometry was used to locate the regions containing the candidate disulfide acceptor and donor groups. Cysteine residues were labeled in the immunopurified rbSGLT1 with PEO-maleimide-activated biotin (PMAB) in the absence or presence of 5 mM DTT. Then the protein was digested with trypsin as described under “Experimental Procedures.” MALDI-TOF mass spectra of the biotinylated tryptic peptides from purified SGLT1 without and with reduction are shown in Fig. 6. Upon treatment with DTT, two new biotinylated peaks with molecular mass (mass/charge, m/z ratios) of 1165.2 and 1914.7 were observed. In Table 3, the theoretical and measured masses of biotinylated tryptic peptides of SGLT1 are compared. This comparison shows that two new peptides were generated under reducing conditions with the amino acid sequences CYTRP (residues 255–259) and AYDLFCGLDQDK (residues 603–614) conjugated with PMAB. Thus, in the presence of DTT, but not in its absence, PMAB could react with the cysteine residues Cys$^{255}$ and Cys$^{608}$, respectively. These results confirm our assumption that residues Cys$^{255}$ and Cys$^{608}$ form a disulfide bridge in rbSGLT1.

**DISCUSSION**

**Surface Topology of the Three Subdomains—**The structural topology of SGLT1 has been investigated in several studies (4–8, 32). However, contradictory results have been obtained that might be due to various factors, such as the methods employed and/or the expression systems used. The orientation of loop 13–14 of SGLT1 is the most ambiguous. The current study used a recently developed technique as an alternative way to verify the surface topology of subdomains I, II, and III of the rabbit SGLT1 in the nearly native structure in the living cells. We demonstrated that antibodies against these regions coupled separately to AFM tips could interact specifically with the antigenic-binding epitopes of SGLT1. These results confirm the extracellular orientations of the regions of the subdomain I (aa 243–272), II (aa 339–356), and III (aa 606–630), which support the assumptions derived from previous studies (5–8, 33) and
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![Image](49x579 to 408x734)

FIGURE 6. MALDI-TOF mass spectra of the biotinylated tryptic peptides from purified SGLT1. The purified protein was labeled with maleimide-PEO₂-biotin in the absence or presence of 5 mM DTT for 1 h in the dark at room temperature and digested with trypsin. The tryptic peptides were purified and prepared for MALDI-TOF. A, peptides obtained after labeling without DTT. B, peptides obtained after labeling in the presence of DTT.

### TABLE 3

| Tryptic peptide                          | Expected mass | Measured mass |
|----------------------------------------|---------------|---------------|
| Without reduction                       |               |               |
| YCGRT-PMAB (residues 354–358)           | 1125.3        | 1125.7        |
| LCWSLR-PMAB (residues 559–564)          | 1303.6        | 1303.2        |
| With reduction                          |               |               |
| YCGRT-PMAB (residues 354–358)           | 1125.3        | 1125.3        |
| LCWSLR-PMAB (residues 559–564)          | 1303.6        | 1303.1        |
| CYTRP-PMAB (residues 255–259)           | 1165.4        | 1165.2        |
| AYDLFCLGDLQDK-PMAB (residues 603–614)   | 1914.1        | 1914.7        |

The receptor-ligand forces found in this work are in the range of the ones observed for other biological systems, i.e. in the range of 50–250 pN (27, 36, 37). The unbinding force required to disrupt the association of either subdomain I or subdomain II antibodies from SGLT1 was, however, detected to be lower than the interaction of subdomain III antibody to SGLT1. The difference in forces might be due to differences in accessibility and/or interaction strength of the different antibodies and their specific epitopes. According to topology models the epitopic region in subdomain III is located in the late part of loop 13–14, which is also the longest loop in the SGLT1, whereas the subdomain II epitope in loop 8–9 lies close to the membrane plane, and this loop is much shorter than the one of subdomain III. Moreover, antigenicity plots (JaMBW) for each antigenic peptide showed that the peptide from subdomain III exhibits the highest antigenic index, i.e. the number of probable interactive sites between the antigen and the antibody is the highest.

### Critical Residues for D-Glucose Binding

We further confirmed our statement by using site-directed mutagenesis to generate the mutants in subdomain II of SGLT1, which were then transiently transfected into COS-7 cells. These cells have been shown in previous studies to lack endogenous Na⁺-dependent glucose transport (6, 16). All of the mutant clones showed lower AMG uptake rates, which might be due to insufficient sorting into the plasma membrane, inappropriate conformational structure of the expressed SGLT1 in the plasma membrane and/or a global reorganization of the disulfide bonds. Such reorientation has also been assumed recently for the rat isoform of SGLT1 expressed in HEK-293 cells (38).

The change in the kinetic properties indicates an important role for the subdomain II in glucose binding. All of the mutants in this loop clearly showed a significantly lower affinity for AMG, especially those with mutations at residues 351 and 361. Moreover, mutation in this region, especially residues 351 and 361, also showed very low inhibition affinity (high Kᵢ) for phlorizin.

3 These results suggest that the two cysteines either interact directly with the glucose molecule or help to stabilize this region of the SGLT1. Replacement of these residues possibly

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3 M. Kasch and R. Kinne, unpublished data.
causes a structural collapse of the glucose-binding pocket, which in turn affects the affinity for substrate translocation.

**Proximity of the Three Subdomains and the Proposed Topology of SGLT1**—In SGLT1 there are three distinct subdomains that we have speculated may be involved in sugar translocation. By using single-molecule AFM, we have proposed that in the presence of Na\(^+\) subdomain III of rbSGLT1 acts as a reentrant loop containing a substrate- and/or phlorizin-binding site, which subsequently transfers a substrate close to the translocation pathway (7). In the current study, the region in subdomain II was found to be involved with either glucose or phlorizin binding, so we hypothesized that subdomains II and III (and possibly with subdomain I) may lie close together. There is evidence from other transporters showing that exposed surface loops can act as a substrate binding region (24) or can be a reentrant pore-loop-like structure with the accessibility depending on the conformation of the transporter (39–41). For this reason we investigated the proximity of these three exposed loops of SGLT1 by specifically delineating intramolecular disulfide bonds. Site-directed mutagenesis in subdomain III (C560A and C608A) and chemical modification of free cysteines by methanethiosulfonate derivatives indicated the possibility that MTSET binds to the residue Cys\(^{560}\) because we could observe the inhibitory effect in wild type and the Cys\(^{608}\) mutant and not for the Cys\(^{560}\) mutant. These results also imply that residue Cys\(^{560}\) is not available for a reaction and thus might act as an acceptor for a disulfide bond. The presence of a free SH group at the \(\alpha\)-glucose-binding site is in accordance with previous studies on the protection of this site by \(\alpha\)-glucose in \(N\)-ethylmaleimide labeling experiments (42). In other investigations it was reported that SGLT1 is not sensitive to alkylation by \(N\)-ethylmaleimide or methanethiosulfonate. The reason for this discrepancy is unclear. It might be related to species differences and/or the incubation conditions used. Furthermore, depending on the conformation of the carrier (see for example Ref. 43), different disulfide linkages may be formed.

Our results from the effect of DTT on AMG transport and labeling of peptides were in agreement and support the existence of an intramolecular disulfide bond between Cys\(^{255}\) and Cys\(^{608}\) in rabbit SGLT1. These results differ from the recent work by Gagnon et al. (23) proposing a bond between Cys\(^{255}\) and Cys\(^{511}\) in human SGLT1. It is noteworthy in this context that different isoforms of SGLT1 exhibit individual properties in term of kinetics, substrate specificity, and inhibitor affinity (44). Therefore, minor differences in the functional structure of different SGLT1 isoforms, particularly in the surface loops, might be expected. Such diversity is also evident in recent studies concerning the evolution of SGLT (1). Irrespective what the exact partners are, such an intramolecular disulfide bridge would bring loop 6–7, loop 8–9, and loop 13–14 closer together and create the critical conditions for phlorizin and \(\alpha\)-glucose binding. We thus suggest that the three subdomains of SGLT1 form a vestibule that acts as a \(\alpha\)-glucose-binding pocket.

Finally, this study nicely demonstrates the advantage of using different techniques, i.e. AFM with the potential for nearly native environmental investigation and molecular biochemical approaches, in cooperation to investigate the structure and dynamics of the cotransporter SGLT1 which currently has become an important target in oral rehydration therapy (45).

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