C-terminal Loop 13 of Na\(^+\)/Glucose Cotransporter 1 Contains Both Stereospecific and Non-stereospecific Sugar Interaction Sites*§

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To investigate whether the C-terminal loop 13 of rabbit sodium/glucose cotransporter SGLT1 is involved in the recognition of the substrate D-glucose, isolated loop 13 (amino acids (aa) 541–638) was immobilized to a lipid bilayer. Interactions were investigated by surface plasmon resonance spectroscopy using an antibody directed against the late part of the loop (aa 606–631) or the glucoside transport inhibitor phlorizin. Specific binding of the antibody to the loop could be detected. The number of bound antibodies decreased upon the addition of D-glucose but not upon the addition of L-glucose. Phlorizin also significantly lowered the number of bound antibodies. Binding of phlorizin to the loop could also be demonstrated directly. Binding of phlorizin was, however, reduced to a similar extent upon the addition of either D-glucose or L-glucose, indicating their unspecific competition with the inhibitor’s sugar moiety. Thus, the presence of a stereospecific glucose interaction site in the late part of the loop and a second, but non-stereospecific, sugar binding site on the same loop was assumed. To investigate whether the early part of loop 13 contains this non-stereospecific sugar binding site, peptides containing aa 541–598 were expressed in Escherichia coli and purified. Both D-glucose and L-glucose quenched the peptides tryptophan fluorescence and reduced the Trp accessibility to acrylamide to a similar degree. In view of the recently proposed transmembrane orientation of loop 13, the two binding sites may be part of the extracellular (stereospecific) and intracellular (non-stereospecific) sugar interaction sites of SGLT1.

In the human kidney two transporters accomplish proximal-tubular glucose resorption on the luminal side of tubule cells; whereas the low affinity sodium/glucose cotransporter SGLT2 is located in the early proximal kidney tubule, the high affinity sodium/glucose cotransporter SGLT1 can be found in the late proximal tubule (1). On the basal side of the epithelial cells sugar transport to the blood is mediated by the sugar uniporter GLUT2. SGLT1 and SGLT2 are secondary active sodium/glucose symporters, i.e. they use the electrochemical sodium gradient across the membrane, which is maintained by the basolateral Na\(^+\)/K\(^+\)-ATPase, to accumulate their substrates within the cell.

The sodium/glucose cotransporters SGLT2 in brush border membrane vesicle preparations from human renal outer cortex, representing segments of the early proximal tubule, have a low affinity for D-glucose (K\(_m\) ≈ 6 mM) and a transporter stoichiometry of 1 Na\(^+\) to 1 glucose (2). In contrast, SGLT1 in brush border membrane vesicle from the rabbit outer medulla (K\(_m\) ≈ 0.35 mM) and skate kidney (K\(_m\) ≈ 0.12 mM) have a significantly higher affinity for D-glucose and a transporter stoichiometry of 2 Na\(^+\) to 1 glucose (3).

Several models for sodium/glucose cotransport have been developed by correlating structure-determining and functional studies of SGLT1 (e.g. Refs. 4 and 5). Expressing only parts of SGLT1 has further increased the knowledge about this transporter (e.g. Refs. 6 and 7). Substrate binding and translocation by SGLT1 is thought to be a multistep process showing strong stereospecificity at the extracellular face of the transporter (D-glucose is favored over L-glucose) (e.g. Refs. 4 and 8) and functional asymmetry (sugar selectivity seems to be different between the extracellular and the cytoplasmic face) (9). Recently we have shown that there are at least two sugar interaction sites on rabbit SGLT1, one representing the initial binding site and one related to the translocation pathway (10).

SGLT1 can be inhibited by several glucoses with either aromatic or aliphatic aglucone residues (11, 12). Phlorizin, a β-glucoside of the aromatic compound phloretin, is the most potent inhibitor with an apparent K\(_i\) value of 1 μM (11). In previous studies we have shown that phlorizin binds to loop 13 of rabbit SGLT1 (aa 541–638) via the aromatic aglucone moiety inducing conformational changes of the loop, predominantly in the region between aa 600 and aa 621 (4, 6) of the C terminus. Mutagenesis experiments provided also data on individual amino acids critically important for the interaction with the inhibitors (6, 13, 14).

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3 The abbreviations used are: SGLT, sodium-glucose cotransporter; rbSGLT1, sodium-glucose cotransporter 1 (rabbit isoform); FC, fluid cell; RU, resonance units; MALDI, matrix-assisted laser desorption ionization; GST, glutathione S-transferase; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-
phosphatidylcholine; NTA, N-nitrilotriacetic acid; DOGS-Ni\(^{2+}\)-NTA, nickel salt of 1,2-dioleyl-sn-glycero-3-{[N-(5-amino-1-caboxypentyl)]liminodiacetacacid]succinyl]; aa, amino acid(s).
In addition, the role of loop 13 in the interaction with phlorizin has been investigated by examining binding of PAN3-2 antibody. PAN3-2, raised against a peptide representing aa 606–630 on the C-terminal ("late") part of loop 13, was shown to interact with the extracellular side of whole SGLT1 in brush border membrane vesicles and intact cells, and this interaction could be blocked by phlorizin (e.g. Ref. 4, 8, and 15).

Although the interaction of the transporter with phlorizin and its aglucone phloretin is fairly well understood, there are only few studies that investigate the interaction of the transporter with its substrate D-glucose. Panayotova-Heiermann et al. (16, 17) have shown that the C-terminal half of the protein is responsible for D-glucose binding and translocation. Several mutagenesis studies and tryptophan scanning analysis of the isolated transporter have identified a variety of amino acids in the transmembrane helix between aa 454 and aa 460 (18, 19). There is also evidence from single molecule recognition studies on brush border membrane vesicles using PAN3-2-poised cantilevers that D-glucose interacts with loop 13 of the transporter. These studies face, however, the problem that no distinction between binding sites and translocation pathways of the transporter can be made. In the current study, we therefore employed isolated loop 13 to directly study the interaction of sugars with this domain of the transporter. The complete loop was immobilized on a phospholipid bilayer, and the interactions were analyzed by surface plasmon resonance spectroscopy. Specific binding of the PAN3-2 antibody and of phlorizin to the complete loop 13 could be detected which was significantly but differently reduced in the presence of D-glucose and L-glucose, suggesting the involvement of loop 13 in glucose binding. Its role in sugar binding was also confirmed by tryptophan fluorescence and acrylamide quenching studies on isolated peptides representing the early parts of loop 13.

The data suggest that loop 13 contains a non-stereospecific interaction site for sugars in its early part (aa 548–571), probably located intracellularly, and a stereospecific sugar binding site in its late part (aa 620–631), definitely located extracellularly. To what extent these sites are indeed involved in the sugar binding and translocation process of SGLT1 remains to be determined.

EXPERIMENTAL PROCEDURES

Preparation of Lipid Vesicles and Solutions—For preparation of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC) vesicles, 2 mg of POPC was dissolved in 2 ml of CHCl₃. For preparation of POPC-Ni²⁺-NTA vesicles, 5 mol % of DOGS-Ni²⁺-NTA, the nickel salt of 1,2-dioleoyl-sn-glycero-3-[N-{[(S)-1-2-oxypropyl]liminomethyl}acrylic acid]succinyl was included. CHCl₃ was evaporated by a stream of nitrogen, and the resulting lipid film was further dried at 1–10 pascals for at least 10 min. Vesicles were generated by ultrasonication in 2 ml of buffer (10 mM HEPES, 150 mM NaCl, pH 7.4) under argon atmosphere until the solution became clear. All solutions were filtered through 0.22-μm pore size filters (Nalgene) before usage. HEPES buffer containing 5 mM oxidized glutathione was used to prevent a breaking open of the disulfide bridge between Cys-560 and Cys-608. Glutathione-containing buffer was mixed, degassed, and held oxygen-free during experiments by continuous purging with helium. Although buffers were held at room temperature during BIACORE experiments, vesicles and samples were kept on ice and light-protected. Stock solutions of sugars and inhibitors were freshly prepared before experiments. Dilution of these components with glutathione-containing HEPES buffer to the desired final concentrations (10 mM sugar, 0.5 mM phlorizin, 0.3 mM phloretin, 0.3 mM arbutin) was done immediately before injection into the BIACORE.

Running BIACORE Sensograms—All experiments were done on a BIACORE X setup at a temperature of 25 °C using sensor chip L1 (produced by Biacore AB, a GE Healthcare company). cDNAs coding for whole loop 13 of rabbit SGLT1 (aa 541–638) were amplified by PCR (13), His₉-tagged, expressed, and purified as described earlier (20). The antibody PAN3-2 and the short peptide A606 representing the amino acid sequence of the PAN3-2 epitope were synthesized and purified as described before (15). The serial order of flow cells had to be kept in mind to avoid contaminations of flow cell 2 (FC2) with material coming from flow cell 1 (FC1), whereas a multichannel flow path (FC1-2) was used (for a detailed description, see Ref. 21).

Injections had a volume of 100 μl each. If dissociation of analytes was taken into consideration (to reach a stable value after injection), then the automatic wash cycle of the injection port normally after an injection was delayed for 120 s.

Sensor chips were treated with 40 mM n-octyl-β-D-glucopyranoside solution for 2 min first, then vesicle solution was injected into both FCs with a flow rate of 5 μl/min. To remove multilayers and entire vesicles, both FCs were flooded with 100 mM NaOH (at 50 μl/min for 2 min) afterward. It has been shown that a dense lipid layer causes a stable increase in the resonance signal of about 1000 resonance units (short term, RU) (22). Hence, the signal level after the end of the NaOH injection had to remain higher than this critical mark; otherwise, vesicle and NaOH injections were repeated (see Fig. 1A). Freshly diluted loop 13 (in a final concentration of 1 μg/ml) was injected using multimode flow path FC1-2 next (5 μl/min) till saturation. Again, the peptide was required to cause a signal increase of more than 100 RU, and the signal level after the end of the peptide injection had to remain higher than this critical mark for at least 2 min; otherwise, the injection was repeated.

Blocking experiments were characterized by three consecutive injections with 10 μl/min for minutes 10 each after the peptide immobilization. First, 3.5 μg/ml PAN3-2 or 0.5 mM phlorizin was injected into FC2 to get a reference value for unblocked interaction between immobilized loop 13 and the antibody or the inhibitor, respectively. Second, sugar or inhibitor (acting as blocking reagent of PAN3-2 or phlorizin binding, respectively, during experiments) was injected in FC1-2 to interact with unaffected loop 13 in FC1 and to show if it can remove already bound antibody/phlorizin from peptides in FC2. And third, a mixture of this blocking reagent and antibody or the inhibitor, respectively, was injected in FC1-2 to interact with unaffected loop 13 in FC1 and to show if it can remove already bound antibody/phlorizin from peptides in FC2. As the last step of a sensogram, SDS was used for removal any immobilized material from the sensor chip. A whole sensogram is given in Fig. 1A. The injection schemes of some control experiments (Figs. 1, B and C, and 2) differed from the scheme.
given above and could not be generalized, but normally FC1 was used as “investigation cell” and FC2 as “control cell.”

Evaluation of Sensograms—Sensograms were evaluated using Biacore evaluation, Version 3. To test the stability of signal levels after injections of vesicles, loop 13, PAN3-2, or phlorizin, mean signal values from the end of the respective injection to 90 s afterward were calculated and S.D. were determined (Table 1).

In general, the percentage of “residual binding” represents the signal due to antibody or phlorizin binding in the presence of a blocking reagent (FC1), related to that one caused by binding in pure buffer (FC2). First, the starting point of peptide injection was set to time point zero ($x$ transformation), and both signal curves were set to a common baseline ($y$ transformation). Subsequently, the sensogram representing FC2 was equalized to its counterpart of FC1 using RU values at the end of peptide injection as reference values, and mean values of curve parts preceding antibody/phlorizin injections were set to $RU_{FC1}/RU_{FC2}$ = 0. To get the mean percentage of residual antibody/phlorizin binding over the time span of 90 s after the end of the respective injections, $100 \times RU_{FC1}/RU_{FC2}$ was calculated for every single time point. Examples for modified curves are shown in Figs. 3 and 4.

Expression and Purification of the Early Parts of Loop 13—Primers, designed to amplify a 171-bp-long fragment of the early part of loop 13 (aa 541–598; see Fig. 6), were 5'-GGA TCC GTT GTG GTT TCC CTC TCC ACC AAG-3' and 5'-GAA TTC TCA TCC TTT CTT CTT AGG AAC TTC-3'. PCR conditions were optimized, and the product was purified by phenol/chloroform extraction and alcohol precipitation. DNA was digested with BamHI and EcoRI sites introduced in forward and reverse primers, respectively, are indicated in bold letters. PCR conditions were optimized, and the product was purified by phenol/chloroform extraction and alcohol precipitation. DNA was digested with BamHI and EcoRI, separated on 1.5% agarose gel, and extracted using QIAquick gel extraction kit (Qiagen). After the DNA fragment had been subcloned into the pGEX-4T-1 vector, the recombinant plasmid was sequenced and then transformed into BL21 (DE3) cells. The GST-tagged part of loop 13 was expressed using 1 mM isopropyl-$\beta$-D-thiogalactopyranoside induction for 3.5 h at 37 °C in a 2-liter culture. Cells were pelleted by centrifugation at 8000 × g for 10 min at 4 °C and resuspended in 20 ml of phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$ adjusted to pH 7.3) supplemented with 5 mM dithiothreitol, 0.1% Triton X-100, 1 mg/ml lysozyme, and 1 tablet of protease inhibitor mixture. Afterward, they were effectively lysed using a SONOPULS ultrasonic homogenization system under mild conditions. The lysate was centrifuged at 17,000 g for 45 min at 4 °C, and supernatants containing GST-tagged peptides were applied to glutathione-Sepharose columns (Amersham Biosciences) after passage through a 0.2-μm filter. Soluble fractions of early loop 13 were generated by treating the glutathione-Sepharose columns with 5 mM free thiol.
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one-Sepharose-immobilized fusion peptide, derived from pGEX-4T-1, with 2 units of thrombin per ml of column (in phosphate-buffered saline) for 16 h at 4 °C after removing protease inhibitor.

Protein purity was assessed by SDS-PAGE gel electrophoresis (using a precasted Bis-Tris 4–12% gel in Tris-glycine running buffer) and staining with Coomassie Blue (a picture of the gel is shown in the supplemental material). Finally, protein concentration was determined by the method according to Lowry using bovine serum albumin as a standard (23).

A short fusion protein of GST (representing aa 548–590 of SGLT1) was a gift from Jutta Luig and Hendrike Schütz from the Max Planck Institute of Molecular Physiology, Dortmund, Germany. Soluble fractions of this peptide were generated by treating the glutathione-Sepharose-immobilized fusion peptide with 2 units of factor Xa per ml of column (in phosphate-buffered saline) for 16 h at 4 °C after removing protease inhibitor. The purity of the GST-cleaved peptide (~5 kDa) was assessed by SDS-PAGE (data not shown).

MALDI Mass Spectrometry—Mass spectra were acquired in the positive ion linear mode on a Voyager DE-PRO MALDI system (PE Biosystems) as described previously (6). Briefly, after mixing the solubilized protein with 2.5-dihydroxybenzoic acid matrix (saturated 2.5-dihydroxybenzoic acid solution in 0.1% trifluoroacetic acid and 50% acetonitrile), 0.5-μl aliquots of the mixture were dispensed onto the sample support followed by 0.5 μl of α-cyano-4-hydroxycinnamic acid matrix solution (solution of α-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid and 50% acetonitrile). Samples were deposited on a MALDI plate and dried at room temperature before collecting the spectrum (the spectrum can be seen in the supplemental material).

Tryptophan Fluorescence and Acrylamide Quenching—All fluorescence experiments were performed using a PerkinElmer Life Sciences LS 50B Luminescence spectrometer fitted with 450-watt xenon arc lamp at room temperature. The samples were excited at 295 nm, and emission spectra were collected between 300 and 400 nm. The bandwidths for both excitation and emission monochromators were 5 nm.

Acrylamide quenching of tryptophan fluorescence was performed to test the accessibility of Trp-561 as a function of D/L-glucose (see Fig. 5, A and C). Acrylamide was added in aliquots from a 5 mM stock solution to the peptide solution up to a concentration of about 18 mM. The Stern-Volmer equation was used to analyze the quenching data (24): $F_0/F = 1 + K_{SV}[Q]$, where $F_0$ is the Trp fluorescence in the absence of quencher, and $F$ is the observed fluorescence at the concentration [Q] of the quencher. $K_{SV}$ is the collisional quenching constant, which was determined from the slope of Stern-Volmer plots (see Fig. 5, B and D, Table 3). The inner filter effects of the ligands for the titration of an L-Trp solution (5 μM) at an excitation of 295 nm and emission at 355 nm were found to be very weak; hence, they were not considered further in our measurements.

Circular Dichroism Analysis—A Far-UV CD spectrum was recorded between 190 and 250 nm (350 μl sample volume) on a Jasco J-715 spectropolarimeter (equipped with a temperature-controlled incubator) at 20 °C using a 1-mm optical path length quartz cells. The step size was 0.5 nm with a 1.0-nm bandwidth at a scan speed of 50 nm/min. An average of 10 scans was obtained for blank and peptide spectrum, and data were corrected for buffer contributions. All measurements were performed under nitrogen flow. Predicted percentages for different kinds of secondary structure were calculated using the K2d computer modeling program, and the results were named as mean residue ellipticity in units of degrees/cm²/dmol (see supplemental material). The spectrum was recorded at 10 μM peptide concentration in 10 mM phosphate buffer, pH 6.8.

RESULTS

Immobilization of Isolated Whole Loop 13 to a Lipid Bilayer—To investigate the optimum conditions under which loop 13 could be immobilized on bilayers, the interaction of the peptide with lipid bilayer chips was examined by real-time surface plasmon resonance spectroscopy (BIACORE). Initially, chip surfaces of flow cells were covered by POPC vesicles containing 5% of DOGS-Ni²⁺-NTA, the nickel salt of 1,2-dioleoyl-sn-glycero-3-[N-[(5-amino-1-carboxypentyl)iminodiacetic-acid][succinyl]] to fix the peptides via their His-tags, a method used to purify the peptides during their isolation. Indeed, immobilization of loop 13 to POPC-Ni²⁺-NTA vesicles gave a value of 566 ± 36 RU (mean values ± S.D., n = 3, Table 1), suggesting interaction of the peptide with POPC-Ni²⁺-NTA bilayer. The Ru values were obtained from signal levels representing the 90 s after either the start of the NaOH injection, which removed POPC-Ni²⁺-NTA or POPC multilayers and led to a stable signal due to the presence of a dense bilayer, or the start of the buffer wash after peptide, PAN3-2, or phlorizin injection (see Fig. 1A). Mean values and S.D. are given. n = number of experiments.

| Injected compound          | x ± S.D. | n |
|----------------------------|---------|---|
| POPC-Ni²⁺-NTA vesicles     | 5083 ± 423 | 3 |
| POPC vesicles              | 4480 ± 676 | 10 |
| Loop 13, injected over POPC-Ni²⁺-NTA bilayer | 566 ± 36 | 3 |
| Loop 13, injected over POPC bilayer | 468 ± 216 | 10 |
| PAN3-2, injected over POPC-fixed loop 13 | 202 ± 37 | 5 |
| Phlorizin, injected over POPC-fixed loop 13 | 156 ± 8 | 5 |

Changes in BIACORE signals by phospholipids, loop 13, PAN3-2 antibody, and phlorizin

The RU values were obtained from signal levels representing the 90 s after either the start of the NaOH injection, which removed POPC-Ni²⁺-NTA or POPC multilayers and led to a stable signal due to the presence of a dense bilayer, or the start of the buffer wash after peptide, PAN3-2, or phlorizin injection (see Fig. 1A). Mean values and S.D. are given. n = number of experiments.
groups. Therefore, in all further studies the peptides were immobilized to pure POPC bilayers to guarantee maximum flexibility and optimum functionality of the loop.

Interaction of Immobilized Loop 13 with PAN3-2—To investigate whether the POPC-immobilized whole loop 13 showed functions demonstrated for loop 13 when studied as part of the membrane-embedded intact carrier, its interactions with PAN3-2 antibody and with phlorizin were analyzed. As shown in Fig. 2A, PAN3-2 injection caused a ∼4-fold higher signal increase with loop 13-containing POPC membranes than on “bare” POPC membranes, indicating that PAN3-2 interacts with loop 13 also after immobilization. Furthermore, the short peptide A606, which represents the sequence of the PAN3-2 epitope on loop 13, was used to study the nature of PAN3-2 interaction. As demonstrated in Fig. 2B, PAN3-2 could not bind to immobilized loop 13 in the presence of A606, whereas in the absence it did. Thus, the antigenic epitope of the immobilized loop 13 to which PAN3-2 binds (see Fig. 6) appears to be freely accessible.

Effect of Transport Inhibitors and Sugars on PAN3-2 Interactions with the Whole Loop 13—As in the intact carrier (4, 8), PAN3-2 binding (original values of 202 ± 37 RU, mean values ± S.D., n = 5, Table 1) could be blocked by the SGLT1 inhibitors phlorizin, phloretin, and arbutin to 48, 79, and 62% residual binding, respectively (Fig. 3, A, B, and C, Table 2; for molecular structures see the supplemental material). Thus, apparently the sites of phlorizin, phloretin, and arbutin binding, which partly overlap with the PAN3-2 epitope, are also functional.

To check whether sugars affect PAN3-2 binding to loop 13, the influence of β-D-glucose (the sugar natively transported by SGLT1) (25) and l-glucose (a non-transported sugar) on the interactions was investigated. The binding of PAN3-2 to POPC immobilized whole loop 13 was significantly reduced in the presence of β-D-glucose to 65% residual binding, suggesting interactions between β-D-glucose and loop 13 (Fig. 3D). Besides β-D-glucose, l-glucose was used to test if the whole transporter stereospecificity could be detected. Only a small inhibition resulting in 92% residual binding was found in the presence of l-glucose (Fig. 3E). Hence, the existence of at least one stereospecific sugar binding site on immobilized whole loop 13 of rbSGLT1 could be demonstrated at this point. Because in the intact carrier loop 13 has been shown to play an essential role in phlorizin binding, we checked whether it was possible to demonstrate this interaction directly using surface plasmon resonance spectroscopy. Indeed, as shown in Fig. 4, signals related to phlorizin binding could be well detected (156 ± 8 RU, mean value ± S.D., n = 5, Table 1).
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To examine the molecular basis of this interaction, we asked which part of the glucoside, the aglucone or the sugar residue, is responsible for the interaction. As shown in Fig. 4, A and B, the phlorizin aglucone phloretin inhibited binding by 35%, whereas the glucoside arbutin inhibited binding by 53%. This result suggested that both the aglucone and the sugar moiety of phlorizin might contribute to the binding. Indeed, as shown in Fig. 4C, β-D-glucose reduced phlorizin binding by 54%

Importantly, unlike in the experiments with PAN3-2, both D-glucose and L-glucose inhibited phlorizin binding (Fig. 4, C and D). The extent of inhibition was almost identical for both sugars (46% and 47% residual binding, respectively; all percentages are given in Table 2). Thus, a second, presumably non-stereospecific, sugar interaction site was postulated to exist on whole loop 13 of rabbit SGLT1.

Because PAN3-2 antibody was raised against the sequence aa 606–630, it reports mainly on interactions at the antibody binding site located on the C-terminal (late) part of loop 13. Furthermore, as shown above, the PAN3-2 epitope overlaps with a stereospecific sugar binding site as the antibody interaction with the whole loop 13 could be blocked by D-glucose but not by L-glucose. Hence, it was speculated that the stereospecific and the non-stereospecific sugar binding sites are located on different parts of the peptide.

Purification of the Early Part of Loop 13 and Identification by MALDI Mass Spectrometry—We, therefore, produced a peptide in which the late part of loop 13 was absent. The early part of loop 13 (aa 541–598) of rabbit SGLT1 was expressed as a GST fusion protein in Escherichia coli and purified with a yield of about 200 µg/liter culture. The purity of the peptide was assessed by SDS-PAGE to be higher than 95%. To identify whether the purified peptide was indeed the desired product, MALDI-mass spectrometry was performed. As expected for the early loop 13, a mass peak at m/z 6795.20 was found (data shown in the supplemental material). Because attempts to immobilize this peptide on phospholipids failed and the early part of loop 13 contains an intrinsic Trp at position 561, we turned to studies in solution.

Determination of Secondary Structure of the Early Part of Loop 13 by CD Spectroscopy—To determine whether the early part of loop 13 contains structural elements that might provide a scaffold for sugar binding, the far UV CD spectrum was recorded between 190 and 250 nm. There were characteristic minima at 225 and 206 nm, from which the secondary structure was estimated as 14% α-helix, 40% β-sheet, and 46% random coil. The spectrum is given in the supplemental material.

Effects of D-Glucose and L-Glucose on Trp Fluorescence of the Early Part of Loop 13—To detect changes in the conformation of loop 13 in the presence of D-glucose or L-glucose, Trp fluorescence emission spectra were recorded. The corrected emission spectrum (λ<sub>ex</sub> = 295 nm) of the early part of loop 13 is shown in Fig. 5A. The emission maximum of Trp-561 was located at 345 nm, which is typical for a slightly apolar environment of the Trp. As depicted also in Fig. 5A and in Table 3, upon the addition of 100 µM concentrations of either D-glucose or L-glucose, the fluorescence intensity decreased by 28 and 33%, respectively.

Effects of D-Glucose and L-Glucose on Trp Fluorescence of a Truncated Early Part of Loop 13 (aa 548–590)—To even better localize the non-stereospecific sugar interaction site, Trp-561 fluorescence emission spectra (λ<sub>ex</sub> = 295 nm) of the even shorter truncated early part of loop 13 available in our laboratory were recorded as well, showing maximal fluorescence emission at 345 nm due to a slightly apolar environment of the Trp again (Fig. 5C). Quenching of Trp fluorescence was observed for both sugars (D-glucose decreased the fluorescence intensity by 20%, L-glucose by 14%).

Effects of D-Glucose and L-Glucose on Acrylamide Quenching of the Trp Fluorescence of the Early Part of Loop 13 and of a Truncated Early Part of Loop 13—Trp fluorescence quenching and shifts in maxima

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**TABLE 2**

**Effect of D-glucose, L-glucose, and glucosides on PAN3-2 antibody and phlorizin binding to immobilized whole loop 13 of rbSGLT1 (aa 541–638)**

Values of two experimental series are given separated by a slash. Rounded average percentages are mentioned in the text. Molecular structures of the sugars and glucoside inhibitors are shown in the supplemental material; sensograms representing the experiments are shown in Figs. 3 and 4.

| Compound          | Residual binding of PAN3-2 (3.5 µg/ml) % of control | Residual binding of phlorizin (0.5 mM) % of control |
|-------------------|----------------------------------------------------|----------------------------------------------------|
| 10 mM β-D-glucose | 64.9/67.9                                          | 44.2/48.4                                          |
| 10 mM L-glucose   | 91.7/92.9                                          | 44.8/49.1                                          |
| 0.5 mM phlorizin  | 46.1/50.1                                          | 30.3/35.4                                          |
| 0.3 mM phloretin  | 78.6/79.0                                          | 64.2/65.4                                          |
| 0.3 mM arbutin    | 61.1/63.6                                          | 46.3/47.9                                          |

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**FIGURE 4.** Phlorizin binding to loop 13 immobilized on POPC-bilayers and the influence of glucosides and sugars in BIACORE experiments. Effect of 0.3 mM phloretin (A), 0.3 mM arbutin (B), 10 mM β-D-glucose (C), and 10 mM L-glucose (D), respectively. The experimental approach and data evaluation described in the legend to Fig. 3 were used. One representative experiment is shown for each compound. Mean values are summarized in Table 2.
It is obvious that the addition of either D-glucose or L-glucose resulted in a comparable decreased accessibility of Trp-561 in both peptides. Thus, both sugars interact with the early part of loop 13 and the truncated early part, and they induce similar conformational changes.

**DISCUSSION**

A key prerequisite in structural and functional studies of isolated loop 13 of rabbit SGLT1 (aa 541–638) by surface plasmon resonance spectroscopy was the stable and oriented immobilization of the peptide on the phospholipid bilayer with the preservation of native conformation and reactivity. For this purpose, end-tags with six histidines were added at both sides of the loop. The initial immobilization experiments done with a mixture of POPC and DOGS-Ni²⁺-NTA gave a strong indication for the expected interactions between His₆-tagged loop 13 and Ni²⁺-NTA via the Ni²⁺-NTA/His₆ system, as EDTA could prevent immobilization. Removal of the loop by competition with imidazole, which usually disrupts such interactions, failed, however; hence, additional interactions between the phospholipids and other parts of the peptide had to be assumed. Indeed, the whole loop showed interactions with pure POPC bilayers. The layer's hydrophobic part as well as the space between the bottom sheet of the bilayer and the covalently bound dextran-matrix of the hydrophilic sensor chip, appeared to facilitate incorporation of whole loop 13 into the membrane (also see Ref. 26). Although the exact nature of binding between loop 13 and the lipid bilayers has not been determined, hydrophobic interactions seem most plausible. The point of attachment is probably the hydrophobic part between the phospholipids and other parts of the loop. The layer's hydrophobic part as well as the space between the bottom sheet of the bilayer and the hydrophobic sensor chip, appeared to facilitate incorporation of whole loop 13 into the membrane (also see Ref. 26). Although the exact nature of binding between loop 13 and the lipid bilayers has not been determined, hydrophobic interactions seem most plausible. The point of attachment is probably the hydrophobic part between the phospholipids and other parts of the loop.

**Structure/Function Relationship of SGLT1 Loop 13**

**FIGURE 5. Effect of D- and L-glucose on Trp-561 fluorescence of early parts of loop 13 in solution.** Corrected fluorescence emission spectra of 5 μM of the early part of loop 13 (aa 541–598) in the absence of sugars (solid line) and in the presence of 100 μM D-glucose (○) or L-glucose (■) are shown in A. The effect of increasing concentrations of acrylamide on the Trp fluorescence is shown in B. Quenching in the absence (○) and presence of 50 μM D-glucose (○) and L-glucose (■) was investigated. Fig. S. C and D, show identical experiments with the truncated early part of loop 13 (aa 548–590). The slopes of the best fit linear regression lines of the Stern-Volmer plots for each data set (KSV values) are given in Table 3. a.u., arbitrary units.

**TABLE 3**

Effect of D-glucose and L-glucose on the Trp fluorescence of the early part of loop 13 (aa 541–598) and of the truncated early part of loop 13 (aa 548–590)

| Peptide                  | Sugars | Quenching | KSV a | b     |
|-------------------------|--------|-----------|-------|-------|
| Early part of loop 13   | D-glucose | 28 | - 6.3 ± 0.8 |
| (aa 541–598)            | L-glucose | 33 | - 6.3 ± 0.8 |
| Truncated early part    | D-glucose | 20 | 5.8 ± 0.5  |
| of loop 13 (aa 548–590) | L-glucose | 14 | 5.4 ± 0.8  |

a The parameters were derived from the fluorescence quenching data shown in Figs. S. A and C. Values represent fluorescence quenching and blue shifts in the presence of 100 μM D-glucose/L-glucose.

b Acrylamide quenching experiments were conducted in the absence (−) and presence (+) of 50 μM D-glucose/L-glucose.

*The Stern-Volmer quenching constants were determined from the slopes of the lines of (F0/F) = 1 + KSV [Q] shown in Fig. 5, B and D. Values are the mean values ± S.D. of two or three experiments.

Suggested conformational changes in both peptides upon interaction with D-glucose and L-glucose. This was assessed in a more distinct manner by using the collisional quencher acrylamide to detect changes in the accessibility of Trp from the aqueous environment.

The Stern-Volmer quenching plots of both the early part of loop 13 and the truncated early part of the loop, in which F0/F (fluorescence in the absence and fluorescence in the presence of both sugars) are plotted against acrylamide concentrations, were linear and are shown in Fig. 5, B and D. The Stern-Volmer constants representing acrylamide accessibility are compiled in Table 3.
In the current studies these results could only be confirmed indirectly as an interaction between the sugars and the immobilized loop could not be detected by surface plasmon resonance spectroscopy, probably because of the smaller size of the sugars compared with the antibody and the phlorizin molecule. However, D-glucose but not L-glucose was able to reduce the binding of PAN3-2 antibody to the whole cotransporter could be blocked by D-glucose but not by L-glucose during atomic force microscopy force distance cycles (4, 8). PAN3-2 is directed against aa 606–630 and, thus, reports mainly on changes in the late part of loop 13. For structural features of whole loop 13, see Fig. 6.

In the current studies these results could only be confirmed indirectly as an interaction between the sugars and the immobilized loop could not be detected by surface plasmon resonance spectroscopy, probably because of the smaller size of the sugars compared with the antibody and the phlorizin molecule. However, D-glucose but not L-glucose was able to reduce the binding of PAN3-2 antibody to whole loop 13 (Fig. 3, D and E).

Besides the immobilized loop’s interaction with PAN3-2, the interaction of loop 13 with phlorizin was also clearly detectable by the BIACORE. As observed previously, phloretin and arbutin decreased phlorizin binding (Fig. 4, A and B, Table 2). Regarding sugar competition of phlorizin binding to immobilized loop 13 (Fig. 4, C and D), the results obtained with D-glucose agree well with the studies available thus far on isolated brush border membrane vesicles (e.g. Refs. 4, 28, and 29). In contrast, most authors did not find any interactions between SGLT and L-glucose (e.g. Refs. 4, 28, and 29), but it has to be pointed out that in these studies mainly interactions with the extracellular side of the cotransporter were investigated. In transport studies, however, there have been strong indications for an interaction of L-glucose with the intracellular side of the SGLT, as SGLT-mediated secretion of L-glucose was observed in the proximal tubule of rat kidney (30).

Sugar binding to early parts of loop 13 could also be detected via changes in Trp fluorescence (Fig. 5, A and C). Our investigations showed that this truncated peptide provides at least one non-stereospecific glucose interaction site as the peptide intrinsic fluorescence decreased upon the addition of both D-glucose and L-glucose. This reduction was not due to a direct effect of sugars on the fluorescence yield of Trp, as no quenching was observed when sugars were added to free L-Trp in solution. The quenching, thus, indicates an internal re-arrangement of the peptide, increasing the internal quenching of the tryptophan. A close association between the sugars and the peptide is also evident from the fact that the accessibility for the water-soluble collisional quencher acrylamide was decreased, perhaps via an interaction with the backbone of the sugars (see Ref. 31) by each of the stereoisomers (Fig. 5, B and D).

If we accept the hypothesis that two sugar binding sites are present on loop 13, the question arises where these are located. The postulated domains are indicated in Fig. 6.

The stereospecific sugar interaction site is probably located around aa 620–631. In previous studies binding of the aglucone moiety of phlorizin could be shown to occur around aa 602 (ring B) and between aa 606 and aa 611 (ring A) (6). These interactions would bring the phlorizin molecule into a position where the glucose moiety points to a region downstream to the C terminus. Indeed, the region around aa 621 showed changes in conformation when phlorizin was added to loop 13. Thus, the C-terminal α-helix of loop 13 containing aa 621 is now proposed to represent the stereospecific sugar binding site in the late part of SGLT1 loop 13 (see Fig. 6).

The secondary structure of the early part of loop 13 (aa 541–598) was estimated from the CD spectrum to consist of 14% α-helix, 40% β-sheet, and 46% random coil. Effects of D-glucose and L-glucose on Trp-561 fluorescence emission spectra of early loop 13 were similar to the effects on Trp-561 fluorescence emission spectra of the shorter truncated early part of loop 13 (aa 548–590). The addition of either D-glucose or L-glucose resulted in a comparable decreased accessibility of Trp-561 for the collisional quencher acrylamide in both peptides as well. Therefore, Trp fluorescence, acrylamide quenching, and CD spectroscopy data indicate that the early part of loop 13 contains some secondary structures, particularly hydrophobic areas around tryptophan 561 (Fig. 6) from aa 548 to 571, which might be important for the formation of the newly detected non-stereospecific sugar interaction site. Hydrophobic interaction sites with sugars have also been postulated for the porin family (32).

This region probably also provides an additional binding site of the peptide for phlorizin. In fluorescence studies with the early part of loop 13, indeed quenching of Trp-561 fluorescence by phlorizin and phloretin was observed (data not shown).

In the intact sodium/glucose cotransporter loop 13 seems to cross the membrane, with the early part located intracellularly, and the late part located extracellularly. Evidence for such an orientation is provided by the presence of a phosphorylation consensus site, spanning aa 561–564 on the early loop (33), and the detection of the late part of loop 13 on the outer surface of SGLT1 expressing cells in single molecule recognition atomic force microscopy experiments (4, 8).

As a result of the current studies, the following sequence of events can be imagined for the transport of D-glucose across the sodium/glucose cotransporter SGLT1. The initial stereose-
cific interaction, discriminating between D- and L-glucose, occurs on the extracellular surface in the late part of loop 13. Then the sugar enters the recently demonstrated hydrophilic pocket within the membrane (19) that leads to the translocation pathway, where an additional screening of the sugar occurs. Before the sugar leaves the carrier it is attached to the early part of loop 13 which is located intracellularly.

It should be kept in mind, however, that the current experiments have been performed under rather artificial conditions using parts of the transporter separated from the carrier and the membrane lipids. Therefore, other techniques such as time-resolved affinity labeling and crystallization of the transporter in various states of translocation have to be used to show whether the assumptions presented above are right or wrong.

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