Phlorizin Recognition in a C-terminal Fragment of SGLT1 Studied by Tryptophan Scanning and Affinity Labeling*

Received for publication, June 27, 2003, and in revised form, September 3, 2003
Published, JBC Papers in Press, September 3, 2003, DOI 10.1074/jbc.M306881200

M. Mobeen Raja, Navneet K. Tyagi, and Rolf K. H. Kinne‡
From the Department of Epithelial Cell Physiology, Max Planck Institute for Molecular Physiology, Otto-Hahn Strasse 11, 44227 Dortmund, Germany

SGLT1 as a sodium/glucose cotransporter is strongly inhibited by phlorizin, a phloretin 2'-glucoside that has strong interactions with the C-terminal loop 13. We have examined phlorizin recognition by the protein by site-directed single Trp scanning mutagenesis experiments. Six mutants (Q581W, E591W, R601W, D611W, E621W, and L630W) of truncated loop 13 (amino acids 564–638) were expressed in Escherichia coli and purified to homogeneity. Changes in Trp quenching and positions of the emission maxima were determined after addition of phlorizin. D611W displayed the largest quenching of 80%, followed by R601W (67%). It also exhibited the maximum red shift in Trp fluorescence (~14 nm), indicating an exposure of this region to a more hydrophilic environment. Titration experiments performed for each mutant showed a similar affinity for all mutants, except for D611W, which exhibited a significantly lower affinity ($K_d$ ~ 54 μM). Also the maximum change in the collisional quenching constant by acrylamide was noted for D611W ($K_{sv}$ = 11 M$^{-1}$ in the absence of phlorizin and 55 M$^{-1}$ in its presence). Similar results were obtained with phloretin. CD measurements and computer modeling revealed that D611W is positioned in a random coil situated between two $\alpha$-helical segments. By combining gel electrophoresis, enzymatic fragmentation, and matrix-assisted laser desorption ionization mass spectrometry, we also analyzed truncated loop 13 photolabeled with 3-azidophloretin. The attachment site of the ortho-position of aromatic ring B of phloretin was localized to Arg-602. Taken together, these data indicate that phlorizin binding elicits changes in conformation leading to a less ordered state of loop 13. Modeling suggests an interaction of the 4- and 6-OH groups of aromatic ring A of phlorizin with the region between amino acids 606 and 611 and an interaction of ring B at or around amino acid 602. Phloretin seems to interact with the same region of the protein.

In mammals, transepithelial transport of D-glucose is mediated by SGLT1 (sodium/glucose cotransporter-1), which can be found in the brush-border membranes of the small intestine and kidney. The transporter facilitates the effective uptake of glucose into cells driven by the electrochemical potential difference of sodium. Coupling and translocation are supposed to be accompanied by conformational changes in the protein. Such changes could be induced, for example, by Na$^+$, which increases the affinity of the cotransporter for sugar (1, 2). Extensive mutagenesis studies revealed that the N-terminal half of the protein contains the Na$^+$-binding sites, whereas glucose binds and permeates through the C-terminal half of the cotransporter (3, 4). Amino acids 162–173 apparently constitute part of an external Na$^+$ pore in the SGLT1 protein, whereas sugar binding is controlled by Gln-457 and Thr-460 (5, 6).

The cotransport system is inhibited by glucosides with either aromatic or aliphatic aglucon residues. Phlorizin, a β-glucoside of the aromatic compound phloretin, is the most potent competitive inhibitor, with an apparent $K_i$ of 1 μM (7). It is proposed that the phlorizin binding to SGLT1 is a two-step process: rapid formation of an initial collision complex, followed by a slow isomerization process that occludes phlorizin within its receptor site (8). Phlorizin is thereby supposed to bind to both the sugar-binding site and the aglucon-binding site, the latter with a hydrophobic/aromatic surface (9, 10).

Site-directed mutagenesis studies suggest that a hydrophobic region located in the C-terminal loop 13 (amino acids 604–610) is critically involved in the binding of phlorizin (11). The binding of phlorizin to loop 13 could be confirmed in solution by monitoring phlorizin-dependent fluorescence quenching of the endogenous Trp-561. It has been demonstrated further that the phloretin (but not the glucose) moiety of phlorizin interacts with loop 13 (12).

The strong sensitivity of Trp fluorescence intensity to protein microenvironment is routinely exploited to follow a variety of protein conformational changes, e.g. ligand/substrate binding, folding/unfolding, etc. (13). We used this signal to characterize the structure-function relationship of loop 13, with the refinement to place Trp residues at different positions of the molecule as reporter groups. Wild-type truncated loop 13 and six mutants were expressed in Escherichia coli and purified, and the activity and signal produced by each single Trp peptide were monitored. Changes in Trp fluorescence and the different accessibilities of each Trp mutant to acrylamide in the presence and absence of phlorizin were thereby used to probe the sequence-specific interactions. The results show that phlorizin binding affects the various mutants differently. The largest changes were found for D611W. A qualitatively similar effect on D611W was observed with phloretin.

To identify unequivocally the interaction sites of phlorizin during its binding to SGLT1, we also performed photoaffinity labeling of truncated loop 13 with a photoreactive 3-azido analog of phlorizin. Taken together, the data suggest that an insertion of phlorizin between two α-helical segments of loop 13 occurs together with strong interactions between the OH groups of aromatic ring A of phlorizin and the area between amino acids 601 and 611.

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† To whom correspondence should be addressed. Tel.: 49-231-133-2200; Fax: 49-231-133-2299; E-mail: rolf.kinne@mpi-dortmund.mpg.de.

‡ To whom correspondence should be addressed. Tel.: 49-231-133-2000; Fax: 49-231-133-2299; E-mail: rolf.kinne@mpi-dortmund.mpg.de.

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Fig. 1. Effect of phlorizin on fluorescence emission spectra of single Trp mutant proteins. The dotted lines show the corrected spectra, and the solid lines represent the effect of 100 μM phlorizin on the intrinsic fluorescence of each Trp mutant. All spectra were corrected as described under “Experimental Procedures.” The protein samples include Q581W (A), E591W (B), R601W (C), D611W (D), E621W (E), and L630W (F). The percentages of fluorescence quenching for each Trp mutant from three independent experiments are presented under “Results.”
Molecular Biology—Mutagenesis experiments were performed using a Chameleon double-stranded, site-directed mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. The following oligonucleotides with mutated nucleotides (underlined) were used for mutagenesis: Q581W, 5′-GGA GAG GAA GAC ATT TCG GAT CCT AAG AAG AAG-3′; R601W, 5′-AAG AAA GGA TTC TCC TTC GGG CCG GCC TAC CCT CTG-3′; D611W, 5′-CTG TTT TGT CCG ATC AAG ACC AAC TGG GAG GCT GCC ATG-3′; and L630W, 5′-GCC AGC ATG AAC CAC TGG GAG GCT GCC ATG-3′. The Apal site (underlined) was selected in a selection primer as an aid in screening with the oligonucleotide primer 5′-GAA TGG ACA GAC ACC TCC GAG-3′. The PCR product was purified using the normal primers flanking the mutation site to produce an insert with BamHI and EcoRI restriction sites. The resulting 222-bp fragment was ligated into the pCR2.1-TOPO cloning vector and sequenced prior to mutagenesis. The confirmed cDNA was ligated into the pGEX-4T-1 expression vector. The mutant cDNAs were sequenced to verify the desired mutations at various points. The recombinant plasmids were then transformed into BL21(DE3) cells for expression of the mutant truncated C terminus of SGLT1.

The abbreviations used are: PBS, phosphate-buffered saline; Bis-Tris, 2-[bis(2-hydroxyethyl)aminol]-2-(hydroxymethyl)propane-1,3-diol; 3-AP, 3-azidophlorizin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

**Table I**

Apparent affinity of phlorizin for the single Trp mutants of truncated loop 13

| Protein | $K_d$ (µM) |
|---------|------------|
| Q581W   | 18 ± 2     |
| E591W   | 25 ± 11    |
| R601W   | 15 ± 7     |
| D611W   | 54 ± 12    |
| E621W   | 26 ± 8     |
| L630W   | 19 ± 5     |

*All experiments were conducted as described under “Experimental Procedures.” Samples include Q581W (black), E591W (red), R601W (green), D611W (dark blue), E621W (sky blue), and L630W (purple). The percentage of quenching at saturating phlorizin concentrations was calculated from the data, and the values were fitted to a single-binding equation using Prism to calculate the equilibrium dissociation constant ($K_d$) values for all mutants are summarized in Table I.

7.3) for the wild-type and mutant proteins (5 µM) in the absence and presence of 50 µM phlorizin or phloretin. Acrylamide was added from aliquots of an 8 µ stock solution to each protein solution (25 °C) at up to 500 mM. The proteins were incubated with the substrate at room temperature for 2 min prior to collecting the fluorescence spectra.
Fig. 3. **Stern-Volmer plots of Trp fluorescence quenching by acrylamide.** Quenching experiments were conducted as described under "Experimental Procedures." The samples were investigated with (■) or without (●) 50 µM phlorizin. The protein samples include Q581W (A), E591W (B), R601W (C), D611W (D), E621W (E), and L630W (F). The slopes of the best fit lines for each data set (\(K_{SV}\) values) are shown in Table II. The means ± S.D. of two or three independent experiments are given. No difference in quenching was observed for L-Trp (5 µM).
Stern-Volmer quenching of all single Trp mutants of truncated loop 13

Fluorescence parameters were determined from the data shown in Fig. 3.

| Protein   | Phlorizin* | $K_{sv}$  
|-----------|------------|------------|
| Q581W     | –          | 11 ± 3     |
| E591W     | +          | 9 ± 2      |
| R601W     | –          | 25 ± 4     |
| D611W     | –          | 38 ± 5     |
| E621W     | +          | 20 ± 5     |
| L630W     | +          | 39 ± 4     |
| Q581W     | –          | 11 ± 2     |
| E591W     | +          | 55 ± 8     |
| R601W     | –          | 17 ± 3     |
| D611W     | +          | 29 ± 6     |
| E621W     | +          | 10 ± 3     |
| L630W     | +          | 11 ± 4     |

* Quenching experiments were conducted in the presence (++) or absence (—) of 50 μM phlorizin. 

The Stern-Volmer quenching constants were determined from the slopes of the lines of $F_0/F = 1 + K_{sv}[Q]$. Values are the means ± S.D. of two or three independent experiments.

Photoaffinity Labeling of Truncated Loop 13 with 3-Azidoephlorizin—Photoaffinity labeling was performed with the photolabile phlorizin analog 3-azidophlorizin (3-AP) synthesized as reported previously (20, 21). The photolabeling experiments were carried out in 200 μl of PBS (pH 7.3) containing 200 μg of truncated loop 13 protein and 1 mm 3-AP. The mixture was preincubated at room temperature in the dark for 5 min. After incubation, photolysis was carried out in a Rayonet RPR-100 photochemical reactor (Southern New England Ultraviolet Co., Branford, CT) fitted with 16, 2800 Å lamps at 22 °C for 10 min. As a control, the photoprobe was first exposed to UV light and then added to the protein. No labeling was observed in the absence of UV light. After photoaffinity labeling, the protein was precipitated with chloroform/methanol (2:1, v/v). A small part of the protein pellet was solubilized with 0.1% trifluoroacetic acid in 50% acetonitrile for MALDI-TOF mass spectrometry analysis, and the rest of the protein pellet was dissolved in sample buffer for SDS-PAGE. Proteins were separated by SDS-PAGE using NuPAGE 4–12% BisTris gels (Invitrogen). Gels were stained with Coomassie Brilliant Blue G-250 (22). Bands of interest were excised from the gel with a clean razorblade, sliced into 1-mm³ cubes, incubated overnight at room temperature with 200 μl of acetonitrile. All extracts were combined in a fresh tube, flash-frozen, and dried in a centrifugal evaporator. Dried extracts were stored at −20 °C until analyzed. Mass spectra were acquired in the positive ion linear mode on a Voyager DE-PRO MALDI apparatus (PE- Biosystems, Shelton, CA). After mixing the solubilized protein with 2,5-dihydroxybenzoic acid matrix (saturated 2,5-dihydroxybenzoic acid solution in 0.1% trifluoroacetic acid in 50% acetonitrile) for intact truncated loop 13 protein and for acquisition of a mass spectrometric peptide map of the trypsin-digested protein, 0.5-μl aliquots of the generated cleavage products 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 in 50% acetonitrile). Samples were deposited on a MALDI plate and dried at room temperature prior to collecting the spectra.

Circular Dichroism Analysis —The 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 1-mm optical path length quartz cells and 10 μM wild-type loop 13. 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 protein spectra, and the data were corrected for buffer contributions. Measurement was performed under nitrogen flow. The secondary structure percentages were calculated using the K2d computer modeling program. The results are expressed as mean residue ellipticity in units of degrees/cm²/dmol. The spectrum was recorded in 10 mM phosphate buffer at pH 6.8.

RESULTS

Protein Expression and Purification —Proteins were expressed in E. coli and purified to homogeneity with a yield of −2.5 mg/liter culture. Plasmid pGEX-4T-1 containing wild-type truncated loop 13 generates a Trp-free protein; thus, the introduced Trp is the only reporter group for each of the mutant proteins.

Mutants Q581W and E591W: Fluorescence and Effect of Phlorizin —The corrected spectra of Trp fluorescence (λex = 295 nm) of Q581W and E591W are shown in Fig. 1 (A and B, respectively). The emission maxima of both Trp mutants are in the range of 347–350 nm, which is typical for a hydrophilic environment. 100 μM phlorizin was used to monitor the percentage of quenching regarding the various positions of each Trp mutant. Quenching of fluorescence reaching ~54% with a
slight red shift (−3 nm) was observed in the spectrum of Q581W, whereas the fluorescence of E591W was quenched by 58%, accompanied by a more noticeable red shift (−5–6 nm).

**Mutants R601W, D611W, E621W, and L630W:** Fluorescence and Effect of Phlorizin—The corrected Trp fluorescence spectra of R601W, D611W, E621W, and L630W are shown in Fig. 1 (C–F, respectively). The fluorescence maxima for these proteins are in the range of 340–343 nm, indicating a slightly less polar environment compared with Q581W and E591W. The fluorescence of R601W was quenched upon addition of phlorizin by 67% with a 8-nm red-shifted spectrum, D611W by 80% with a maximum red shift of 14 nm, and E621W by 65% with a red shift of 10–11 nm. Fluorescence quenching of L630W was only 18%; however, the same red shift as in E621W was observed. The differences between the fluorescence quenching spectra of the various mutants suggest that different segments of loop 13 react differently to the binding of phlorizin to the loop. Such differences could, however, also be due to differences in the phlorizin affinity of the mutants. We therefore determined the apparent binding affinities.

**Effect of the Mutations on Phlorizin Binding**—Titration of all single Trp mutants with different concentrations of phlorizin is shown in Fig. 2. About 90% of the fluorescence of each mutant was quenched at the highest concentration of phlorizin. The estimated equilibrium dissociation constants for all mutants are presented in Table I. For calculation of binding constants, the peak points with shifting toward longer wavelength were collected upon each addition of phlorizin. The mutant proteins (e.g. Q581W, E591W, R601W, E621W, and L630W) showed values very similar to those of wild-type loop 13 (12), indicating that the replacement of amino acids at these positions with Trp residues does change the ability of the protein to bind phlorizin. However, the D611W mutant has a lower affinity, which has to be taken into account when conformational changes are considered (see below).

**Effect of Phlorizin on Trp Accessibility Monitored by Collisional Quenching**—The collisional quencher acrylamide was used to detect changes in the "availability" of Trp to the surrounding solvent. The Stern-Volmer quenching plots of each mutant in which \( F/F_0 \) is plotted against the acrylamide concentration in the presence and absence of phlorizin were linear and are shown in Fig. 3. The Stern-Volmer constants for all mutants are compiled in Table II. Upon phlorizin binding, the Stern-Volmer constant for Q581W did not change significantly. At 50 μM phlorizin, a non-significant protection effect was noted for mutant Q581W. For the E591W and R601W mutant proteins, the quenching constants started to increase, and an

**TABLE III**

| Fragment | Amino acids | Sequence | Theoretical mass \((M + H)^+\) monoisotopic | Measured mass \((M + H)^+\) | Additional peaks |
|----------|-------------|----------|---------------------------------|-----------------|----------------|
| 1        | 1-3         | PER      | 401.21                          | ND*              |                |
| 2        | 4-6         | NSK      | 348.18                          | ND               |                |
| 3        | 7-9         | EER      | 433.20                          | ND               |                |
| 4        | 10-33       | IDLDAGEEDIQEAP EEATDTEVPK | 2614.18 | 2614.37 |                |
| 5        | 34          | K        | 147.11                          | 147.27           |                |
| 6        | 35          | K        | 147.11                          | 147.27           |                |
| 7        | 36          | K        | 147.11                          | 147.27           |                |
| 8        | 37-40       | GFFR     | 526.27                          | 526.41           |                |
| 9        | 41          | R (602)  | 175.11                          | 175.35           | 624.52b        |
| 10       | 42-53       | AYDLFICGLDQDK | 1387.61 | 1387.63 |                |
| 11       | 54-56       | GPK      | 301.18                          | ND               |                |
| 12       | 57-59       | MTK      | 379.20                          | 380.85           |                |
| 13       | 60-66       | EEEAMK   | 807.35                          | 807.26           |                |
| 14       | 67-68       | LK       | 260.19                          | ND               |                |
| 15       | 69-77       | LTDTSEHPL| 1012.49                         | ND               |                |

* Not determined.

b Denotes a peptide plus label.
from 11 to 31 M increase in the Stern-Volmer quenching constant for D611W in the presence and absence of phloretin is shown in Fig. 4. The linear Stern-Volmer quenching plot of D611W in upon addition of phloretin by 69% with a 9

Fig. 7. Secondary structure of wild-type truncated loop 13 by CD spectroscopy. The CD spectrum was recorded as described under “Experimental Procedures.” The measurement was carried out at 10 μM protein in 10 mM phosphate buffer at 20 °C.

Detection of Truncated Loop 13 Photocross-linked to 3-AP by MALDI Mass Spectrometry—The reaction product of photolabeling of truncated loop 13 with 3-AP was analyzed by MALDI mass spectrometry. Fig. 5 (upper panel) shows the expected mass peak for loop 13 at m/z 8825.60, and Fig. 5 (lower panel) demonstrates photoaffinity labeling of the loop with 3-AP. Peak I corresponds to truncated loop 13 (m/z 8825.60), and peak II corresponds to the photolabeled truncated loop 13 protein (m/z 9275.10). The mass difference between peaks I and peak II (Fig. 5, lower panel) corresponds to the mass of photolyzed 3-azido-phlorizin (m/z 449.5).

MALDI Mass Spectrometry of Loop 13 Photolabeled with 3-AP—As a next step in the identification of the ligand contact points, photolabeled truncated loop 13 was digested in gel with trypsin. After extraction of the peptides from the gel, the resulting mixture was analyzed in MALDI-TOF mode with unlabeled truncated loop 13 as a reference (Table III). The only additional peak observed in the labeled probe was at m/z 624.52 (Fig. 6, lower panel). This can be explained as an adduct peak to m/z 175.11 (corresponding to Arg-602), with a difference of 449.41. The latter is equal to the mass of photolyzed azido-phlorizin. All other peaks appeared also in the control as shown in Fig. 6 (upper panel).

Structural Determination—The CD spectrum of wild-type truncated loop 13 at pH 6.8 is shown in Fig. 7. The CD spectrum shows characteristic minima at 222 and 208 nm, from which the secondary structure was estimated to consist of 37% α-helical residues and 63% random coil. The secondary structure agrees well with calculated values based on the amino acid sequence (14).

DISCUSSION

Elucidation of the structure-function relation of membrane proteins has been hindered by difficulties in expressing the complete protein. We therefore chose to express a fragment of the SGLT1 protein in 10 m M phosphate buffer at 20 °C.

Phloretin-induced Changes in Trp Fluorescence and Accessibility to Acrylamide—To check the effect of phloretin, the aglucon of phlorizin, on Trp quenching and accessibility, the same experiments were also performed for D611W. E621W showed less quenching, and almost no quenching was observed for L630W. These data suggest that the major conformational changes are initiated by phlorizin at and/or in the vicinity of amino acid 611.

Fig. 6. MALDI mass spectrum of in-gel trypsin-cleaved truncated loop 13. 200 μg of truncated loop 13 was photolabeled with 3-AP; proteins were separated by SDS-PAGE, and protein band of interest was excised, destained, and digested in gel with trypsin. The peptides were extracted from the gel pieces with acetonitrile. Subsequently, 0.5-μl aliquots of the generated cleavage products were dispensed onto the sample support, followed by 0.5 μl of α-cyano-4-hydroxycinnamic acid matrix solution; and the MALDI mass spectra were recorded. Upper panel, truncated loop 13 before photoaffinity labeling; lower panel, truncated loop 13 after photoaffinity labeling with 3-AP. The peak indicated by the asterisk clearly indicates the mass shift due to modification of Arg-602 with 3-AP. Intens., intensity.

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Truncation of the molecule did not, however, interfere with the ability of the protein to interact with phlorizin. The phlorizin dissociation constants in our studies are very similar to those determined for the formation of the initial collision complex by Oulianova and Berteloot (9) in intact membrane vesicles and also agree with the observed binding constant for complete loop 13 (12).

To facilitate discussion of the secondary structure of truncated loop 13 and its conformational changes upon phlorizin binding, the predicted conformation based on the amino acid sequence is shown in Fig. 8A. In agreement with this prediction, the maxima of Trp fluorescence of the various mutants differ. For example, mutants 581 and 591 show a spectrum that has a maximum close to 350 nm, suggesting a random coil orientation of the neighboring segments. For the other mutants e.g. 601, 611, 621, and 630, a more hydrophobic environment of...
the Trp can be assumed (blue shift in the maxima), suggesting a location in a more ordered, probably α-helical structure. The presence of these α-helices in solution is confirmed by the CD spectrum, which quite nicely reflects the relative abundance of the various conformations.

Several major spectral changes are induced by phlorizin binding to truncated loop 13. First, a red shift in the fluorescence maximum indicates a transition between an ordered state (hydrophobic) to a less ordered state (more hydrophilic) of the loop. The most evident shift was observed for mutant D611W. Thus, phlorizin binding appears to induce the opening of the region around amino acid 611 of loop 13, which before seemed to be buried between the two α-helices (see model in Fig. 8A).

The shift in the maximum of Trp fluorescence toward a longer wavelength could, however, also be caused by a strong interaction of phlorizin-associated water with the loop. Such a complex between phlorizin and water molecules can be formed by H-bonding between OH groups of phlorizin (e.g. mainly by the 4- and 6-OH groups of ring A and 4-OH of ring B). This suggests the position of Asp-611 in the binding pocket. A critical role of this region of loop 13 in phlorizin binding has also been proposed from studies on the intact carrier using site-directed mutagenesis (11) and from the biophysical studies using isolated loop 13 in solution (12).

The similar red shift in the fluorescence maximum of L630W is difficult to explain by the above assumption. Perhaps in this part of loop 13, bound water molecules situated around the protein contribute mostly to the signal. In other studies, ligand-induced conformational changes were also deduced from the fluorescence spectra. In this instance, both water around the ligand and water around the protein contributed to the shift; the contribution of each is in most cases difficult to estimate (18).

Furthermore, fluorescence quenching was observed. We would like to correlate the quenching with the proximity of the indole ring of Trp to the phlorizin molecule. The main mechanism responsible for fluorescence quenching is charge/energy transfer between phlorizin and Trp residues. Similar effects were observed for collagen fluorescence in the presence of varying concentrations of hypericin (16, 17). The maximum fluorescence quenching at position 611 could then indicate that phlorizin ring A is very close to the free indole ring of tryptophan. For the amino acids located in region 606–609, also an interaction, although with slightly less affinity, would ensue. The binding site is thereby defined. The minimum quenching observed for mutant L630W suggests a positional arrangement of this part of loop far from the binding region. In the case of mutants Q581W and E591W, significant quenching signals in the presence of phlorizin can be attributed to a conformational change that brings this part of loop 13 somehow close to the binding site.

Mutation D611W resulted in a decrease in phlorizin affinity. This can be explained by the fact that, because of the replacement of aspartate and the removal of the negative side chain, the network of neighboring amino acids is disturbed, which plays an important role in establishing the preformed pocket for phlorizin binding during the initial complex formation.

A surprising result of this study is that the region between residues 591 and 621 is more accessible to acrylamide in the presence of phlorizin than in its absence. In our previous studies on phlorizin binding to loop 13, a protection effect of phlorizin was observed, leading to a decreased accessibility of Trp at position 561 and its resonance partner Tyr at position 604 (12). The difference might be due to the fact that, in the former studies, the protein contained a disulfide bond, which can alter the conformational changes significantly. The significant change observed for mutant L630W is in agreement with its position in the α-helical region of the protein and also suggests its location to be far from aromatic ring A of phlorizin compared with the location of Q581W (Fig. 8A). The high accessibility of D611W to acrylamide after phlorizin binding suggests that the interaction with phlorizin occurs in an aqueous environment; hence, no protection effect is observed.

It is interesting to note that phloretin, the aglucon of phlorizin, induced qualitatively similar changes in D611W as phlorizin, although the effects on fluorescence and acrylamide accessibility were smaller. These results suggest that phloretin might bind to the same site as phlorizin. The larger disturbance of the conformation of loop 13 by phlorizin might be explained by the bulky nature of the glucose residue attached to two aromatic rings. If phloretin indeed binds to the same site as phlorizin, the noncompetitive nature of the inhibition of D-glucose transport by phloretin might be due to a close coupling between loop 13 and the sugar-translocating positions of SGLT1. Such coupling can occur by the formation of disulfide bonds between the extramembranous loops; direct evidence for the presence of such bonds has been obtained recently.2 Recently, we also found ligand-induced conformational changes in SGLT1 in intact rabbit renal medullary brush-border membrane vesicles by monitoring the accessibility of extramembranous cysteine residues with polyethylene oxide-maleimide-activated biotin, followed by specific immunodetection of the biotin-coupled protein and by determining binding probability and unbinding force using antibodies against loop 13 attached to an atomic force microscopic cantilever (15).

The conformation of phlorizin in aqueous solutions was previously studied by two-dimensional NMR and pharmacophore analysis by Wieler-Badt et al. (19). Their model indicates that the interactions via hydrogen bonds from the 2-, 3-, 4-, and 6-hydroxyl groups of the pyranoside and the 4- and 6-OH groups of aromatic ring A are essential for phlorizin binding. This information can now be combined with the Trp fluorescence data to estimate the dimensions of the phlorizin-binding and recognition region. We can assume that phlorizin binds to the region very close to position 611 by H-bonding probably with the 4-OH group by acceptor/donor atom O-4 of aromatic ring A. Thus, position 611 is supposed to be very important in phlorizin recognition, as also suggested by the lower affinity of the D611W mutant. The H-bonding of any one hydrophobic amino acid located in region 606–609 with the 6-OH group by acceptor/donor atom O-6 of aromatic ring A of phlorizin also causes a strong interaction.

The combination of photoaffinity labeling, SDS-PAGE, enzymatic fragmentation, and MALDI mass spectrometry analysis with the resulting allocation of the attachment site of the ortho-position of ring B of phlorizin to Arg-602 defines directly the phlorizin-binding site in loop 13. Thus, an interaction of this region with ring B of phlorizin can be assumed. The binding pocket could thereby accommodate the phlorizin molecule in the predicted conformation.

On the basis of the photoaffinity labeling pattern of truncated loop 13 with 3-AP, we could provide a generalized mechanism by which phlorizin inhibits sugar transport by SGLT1. According to our assumption, a phlorizin-binding region in SGLT1 is located between amino acids 601 and 611; in this region, aromatic rings A and B of phlorizin interact with hydrophobic amino acids, and the glucoside moiety of phlorizin is free to interact with sugar interaction sites of SGLT1 (5, 6). Previous mutagenesis studies provided evidence that also Asp-
176 might be involved in determining the overall affinity of phlorizin for the transporter (23). Thus, there seems to be close vicinity between loop 13 and Asp-176, again probably caused by disulfide bonds between various parts of the transporter.

According to our modeling assumptions based on secondary structure prediction and CD analysis, the regions (especially those between residues 599 and 607 and residues 620 and 631) around the binding pocket are in α-helical conformation. In the absence of phlorizin, the region around amino acid 611 appears to interact with part of the α-helical region, in particular with the hydrophobic amino acids between positions 606 and 609. Upon phlorizin binding, this region becomes exposed to an aqueous environment as shown by the direction of the large arrow in Fig. 8A. In addition, the 4-OH group of aromatic ring A comes close to position 611, strongly altering the Trp emission (Fig. 8A). At the same time, aromatic ring B comes conformationally close to position 600. We also propose that phlorizin binding results in reduction of van der Waals forces in the vicinity between loop 13 and Asp-176, again probably caused by disulfide bonds between various parts of the transporter.

The careful secretarial work of Natascha Kist is gratefully acknowledged. We also thank Jonathan W. Mueller for experimental help and suggestions on constructing the schematic model.
Phlorizin Recognition in a C-terminal Fragment of SGLT1 Studied by Tryptophan Scanning and Affinity Labeling
M. Mobeen Raja, Navneet K. Tyagi and Rolf K. H. Kinne

J. Biol. Chem. 2003, 278:49154-49163.
doi: 10.1074/jbc.M306881200 originally published online September 3, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M306881200

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