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Ligand-mediated conformational changes and positioning of tryptophans in reconstituted human sodium/D-glucose cotransporter1 (hSGLT1) probed by tryptophan fluorescence

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Abstract

Recombinant purified human sodium/D-glucose cotransporter1 (hSGLT1) was reconstituted in a functional form into phospholipid vesicles and its conformational states in the absence and presence of ligands and inhibitors were probed by intrinsic tryptophan fluorescence. In the presence of sodium, sugars increase intrinsic fluorescence (maximum 17%) in a saturable manner in the following order α-MDG > D-Glu ≈ D-Gal ≫ D-Man > D-All, with no effect of L-Glu. Apparent affinities ranging from 0.65 to 10.4 mM were observed. In addition, D-Glu increased the accessibility of the Trps to hydrophilic collisional quenchers. On the contrary, the transport inhibitor phlorizin decreased Trps fluorescence in a sodium-dependent manner by 50% with a red shift of 4–6 nm and decreased quencher accessibility, these effects were saturable with a high affinity of 5 μM. Furthermore, the positioning of the tryptophans in the reconstituted transporter was investigated. hSGLT1 Trps fluorescence was reduced by *N*-bromosuccinimide treatment maximally 25% in membranes and 65% in solution. The fluorescence was also significantly but differently quenched by the lipid-soluble spin labeled probes 5-Doxyl-phosphatidylcholine (40%) and 12-Doxyl-phosphatidylcholine (26%). Depth-calculation using the parallax method suggested a location of Trps at an average depth of 10 Å from the center of the bilayer. These studies demonstrate the existence of different conformational states of the membrane-embedded transporter in its glucose-free form, as sodium–glucose-carrier complex and as sodium–phlorizin-carrier complex. They further indicate that most of the Trp residues in hSGLT1 are located in hydrophobic regions of the protein or in contact with the lipid bilayer of the membrane. There, they are located close to the membrane–water interface contributing to the vectorial nature of the transporter.

Keywords: Reconstitution; Human sodium/D-glucose cotransporter 1; Tryptophan fluorescence; Phlorizin; Quencher; Parallax method

1. Introduction

Transporters are the gatekeepers for all cells and organelles, facilitating influx and efflux of crucial compounds such as sugars, amino acids, nucleotides, inorganic ions and drugs. One important example of such transporter is the human sodium/D-glucose cotransporter 1 protein (hSGLT1) a protein central for the homeostasis of glucose, salt and water [1]. hSGLT1 uses the electrochemical potential difference of sodium across the brush border membrane of the intestine and renal proximal tubule to drive the intracellular accumulation of glucose [2–5].

In the absence of 3-dimensional crystal structures of hSGLT1 or its homologues, other biophysical methods like tryptophan fluorescence studies of hSGLT1 provide valuable information on its molecular architecture and substrate and inhibitor binding.

Abbreviations: hSGLT1, Human sodium/D-glucose cotransporter1; D-Glu, D-glucose; Phlz, Phlorizin; α-MDG, α-methyl-D-glucopyranoside; Trp, Tryptophan; KI, Potassium iodide; TCE, Trichloroethanol; 5-Doxyl PC, 1-Palmitoyl-2-stearoyl (5-Doxyl)-*sn*-glycero-3-phosphocholine; 12-Doxyl PC, 1-Palmitoyl-2-stearoyl (12-doxyl)-*sn*-glycero-3-phosphocholine; NBS, *N*-Bromosuccinimide.

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All fluorescence studies on hSGLT1 reported so far were either carried out in *Xenopus* oocytes [6] or in solution [7]. However in solution some transporter proteins can bind ligands from both sides as reported for UhpT transporter of *E. coli* [8] so we decided to perform fluorescence studies of hSGLT1 in a reconstituted form which is more relevant for membrane proteins. On the one hand, aqueous soluble quenchers like iodide, acrylamide, and TCE have been widely employed to provide information of the gross location of tryptophan residues in the complex three-dimensional structure of membrane proteins [9,10]. On the other hand, lipid-soluble membrane-associated quenchers such as bromine atoms [11,12] or nitroxide [13–15] groups covalently linked to fatty acids or phospholipids derived from these fatty acids, have been efficiently used to evaluate the involvement of tryptophan-containing regions in membrane protein interaction. In order to better characterize conformational states of hSGLT1 and to probe the positioning of Trp residues we performed fluorescence studies of hSGLT1 reconstituted into proteoliposomes in its functional state. Fluorescence studies showed that the hSGLT1 intrinsic fluorescence is selectively modified upon interaction of the ion, sugar and/or inhibitor and that most of the Trps reside in hydrophobic environments either within the protein or the phospholipids.

2. Materials and methods

2.1. Materials

All sugars, phloretin, phlorizin, *N*-bromosuccinimide, asolectin soy lecithin, cholesterol, and 2, 2, 2 trichloroethanol were from Sigma (Munich, Germany). Synthetic 1-Palmitoyl-2-stearoyl (*n*-doxyl)-*sn*-glycero-3-phosphocholine (DoxylPC), with the spin labels at the 5- and 12-positions of the *sn*-2-acyl chain, were purchased from Avanti Polar Lipids Inc (Alabama, USA). All other chemicals were of analytical grade and obtained from commercial sources.

2.2. hSGLT1 expression, purification and reconstitution

hSGLT1 was expressed in *Pichia pastoris* and purified to homogeneity by nickel-affinity chromatography, and reconstituted into liposomes according to the method describe in Ref [7].

2.3. Steady-state fluorescence studies

Steady-state fluorescence measurements were done with a Perkin-Elmer LS 50B fluorescence spectrometer (Perkin-Elmer), fitted with a 450W xenon arc lamp at room temperature. A 0.3-cm excitation and emission path length quartz cell was used for all fluorescence measurements. The excitation wavelength was set at 295 nm for selective excitation of Trp. A 290 nm cutoff filter was used to minimize the contribution of scattering signals. Emission spectra were collected from 300–400 nm, averaging six scans. The bandwidths for both excitation and emission monochromators were 5 nm. The emission spectra were corrected for the background and dilution effects, for vesicle blank (scatter), which at the maximal lipid concentration used contributed at most 10% to the total signal, and for the inner-filter effect. The inner-filter effect of substrates used in this study, which is due to their absorbance at the excitation and emission wavelengths range of Trp was subtracted using the following equation:

$$F_{corr} = F_{obs}\, antilog[(A_{ex} + A_{em})/2] \quad (1)$$

Where A_{ex} and A_{em} were the absorbance of the substrates at 295 and emission at 340 nm, respectively, F_{corr} and F_{obs} were the corrected and observed fluorescence intensity. To check whether Eq. (1) works well in our case, titration of L-Trp (1 μM) with the substrates and phlorizin (excitation at 295 nm) was also performed to obtain the correction factor ($C_{ex}=F_{corr}/F_{obs}$). In the range of substrates concentration used, the difference between the C_{ex} obtained through the equation and Trp titration is very small; therefore, we used Eq. (1) to correct the inner-filter effects of substrates.

2.4. Fluorescence quenching analysis by external quencher

Steady-state fluorescence quenching was carried out by measuring the fluorescence intensities at the emission maxima as a function of the quencher concentration. Increasing concentrations of the quencher were added from a concentrated stock solution of the quencher in the buffer. All quencher solutions were freshly prepared and 0.1 mM $Na_2S_2O_3$ was added to the KI stock solution to prevent I_3^- formation. The accessibility of Trp was monitored by analysing the quenching data using a Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}[Q]. \quad (2)$$

Where F_0 and F are the fluorescence intensities in the absence and presence of quencher, respectively, $[Q]$ is the concentration of quenching agent, and K_{SV} is the Stern-Volmer quenching constant. In the case of a purely collisional quenching mechanism, a Stern-Volmer plot of F_0/F vs $[Q]$ gives a linear plot with a slope value equal to K_{SV}.

2.5. Ligand binding assay

The ligand-induced fluorescence quenching as a function of α-methyl-D-glucopyranoside (α-MDG), D-glucose (D-Glu), D-galactose (D-Gal), D-mannose (D-Man), D-allose (D-All), L-glucose (L-Glc), or phlorizin (Phlz) concentration was monitored as described [7]. Increasing amounts of sugars (0.1–20 mM) or phlorizin (1–50 μM) were added to 1 μM reconstituted hSGLT1 in 100 mM sodium phosphate buffer pH 7.4. Apparent binding constants were calculated according to a previously described method [7].

2.6. N-bromosuccinimide modification of hSGLT1 in solution and in proteoliposomes

hSGLT1 (1 μM) reconstituted in liposomes or in solution was subjected to NBS modification using 5-, 10-, 20-, 30-, 40-,

50-, 75-, and 100-fold molar excess of NBS over the protein, using 2 mM stock solution of NBS in the same buffer. The fluorescence intensity at 338 nm was monitored as a function of ratio of NBS/hSGLT1 after excitation at 295 nm. In each case the fluorescence intensity of the same amount of hSGLT1 without NBS modification was used as control.

2.7. Depth measurements using the parallax method

The idea of using localized quenchers in membranes has been extended to allow calculation of the depth of the fluorophore from the center of bilayer [17–20]. In this type of experiment, two sets of samples are prepared. One set contains a fluorophore, a lipid labeled with quencher at one depth, and an unlabeled lipid. The other set contains the same fluorophore, a quencher lipid labeled at different depth, and the unlabeled lipid (Fig. 5). The ratio of fluorescence intensity in a sample from one set to that in a sample from another set is then given by Eq. (3) if equal concentrations of quenchers are present in the two samples.

$$F_1/F_2 = \{(F_1/F_0)/(F_2/F_0)\} = e^{-\pi Rc^2C+\pi Z_{1F}^2C+\pi X^2C}/e^{-\pi Rc^2C+\pi Z_{2F}^2C+\pi X^2C}. \quad (3)$$

Note that all terms denoted L refer to the depths of the quenchers which can be accurately estimated, and thus represent known values. Canceling out, substituting $L_{21}+Z_{1F}$ for Z_{2F}, and rearranging yield Eq. (4),

$$Z_{cF} = L_{cl} + \{[(1-/\pi C)\ln(F_1/F_2) - L_{21}^2]/2L_{21}\}. \quad (4)$$

Average depth of Trps in hSGLT1 reconstituted into POPC membrane was calculated by the parallax method [17,20] using Eq. (4). Once Z_{1F} is known, the fluorophore distance from the center of the bilayer can be calculated from

$$Z_{cF} = Z_{1F} + L_{c1}. \quad (5)$$

Where Z_{cF}=the depth of the fluorophore from the center of bilayer, L_{c1}=the distance of the center of the bilayer from the shallow quencher (5-Doxyl PC in our case), L_{21}=the difference in depth between the two quenchers, and C=the 2-dimensional quencher concentration in the plane of the membrane (molecule/Å^2). Here F_1/F_2 is the ratio of the F_1/F_0 and F_2/F_0 in which F_1 and F_2 are fluorescence intensities in the presence of the shallow (5-Doxyl PC) and deep quencher (12-Doxyl PC) respectively, both at the same quencher concentration C; F_0 is the fluorescence intensity in the absence of any quencher.

An important value needed for the calculation of depth is the distance of the spin labels from the bilayer center (i.e., L_{c1} and L_{c2}). As described in literature [20], considerable data justify placement of spin labels at the same depth as that which would be occupied by the fatty acyl carbon atom to which the spin label is attached if it were in an unlabeled chain. In turn, the position of the carbon atoms in an unlabeled chain has been determined by a series of X-ray, NMR, and neutron diffraction studies, which have revealed the conformation of the polar head group, including an asymmetric disposition of the start of the fatty acyl chain, and the approximate spacing in depth between adjacent carbon atoms, derived directly and through the increase in bilayer thickness with an increase in chain length. Previous studies [18,20] indicate that for the lipids used in this report the hydrocarbon thickness, if defined to be the distance between the 2-position chain carbonyl carbons in opposite leaflets, is about 30 Å and there is a spacing of 0.9 Å between adjacent fatty acyl carbon atoms. Using these values, we can immediately calculate the distances of the spin label groups from the bilayer center (Fig. 5). All the bilayer parameters used were the same as described previously [20].

3. Results

3.1. Trp fluorescence of hSGLT1 reconstituted in proteoliposomes

The polarity of the microenvironment of Trp residues in a protein can be assessed from their fluorescence emission maxima [21]. The corrected emission spectrum of hSGLT1 reconstituted into proteoliposomes is shown in Fig. 1. The fluorescence exhibited an emission maximum at 338–340 nm which indicates that Trp residues responsible for fluorescence in reconstituted hSGLT1 are located in a relatively hydrophobic environment. As compiled in Table 1 in the presence of sodium, sugars that have been identified earlier as substrates for the transporter increased intrinsic fluorescence in a saturable manner, α-methyl-D-glucopyranoside increased fluorescence by 17±2% followed by D-glucose (15±1.5%), D-galactose (14±2.3%), D-mannose (3±0.5%), and D-allose (1±0.2%) with no shift in emission maxima. L-glucose which is not transported by hSGLT1 did not exert any effect on fluorescence. When saturation kinetics were applied the following apparent binding affinities were calculated: α-MDG (0.65±0.1 mM), D-Glu (0.74±0.12 mM), D-Gal (0.76±0.11),

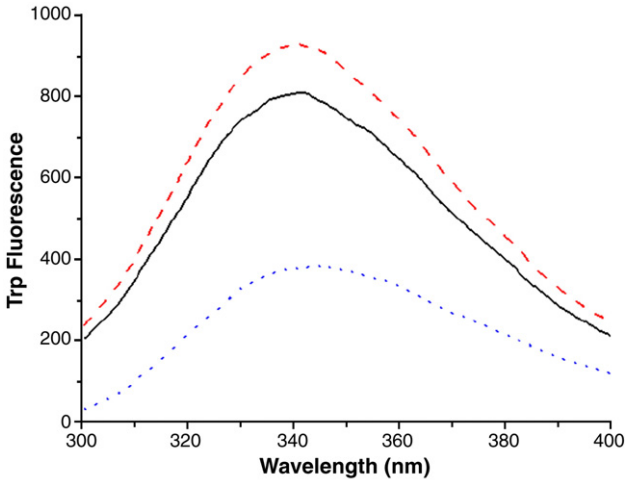

Fig. 1. Intrinsic Trp fluorescence of hSGLT1 in proteoliposomes and effect of D-glucose or phlorizin on fluorescence. For each experiment, 1 μM hSGLT1 reconstituted into proteoliposomes in the ratio of 200:1 was incubated in the absence (solid line), presence of 10 mM D-glucose (dash line), or presence of 100 μM phlorizin (dotted line). The excitation wavelength was 295 nm. The results shown are typical of ≈20 independent experiments.

Table 1
The effect of ligands on the Trp fluorescence of hSGLT1

Ligands	Maximum change in fluorescence (%)	Shifts in maxima	K_d [a] (mM)
α-MDG	+17±2	No	0.65±0.10
D-Glucose	+15±1.5	No	0.74±0.12
D-Galactose	+14±2.3	No	0.76±0.11
D-Mannose	+3±0.5	No	4.3±0.70
D-Allose	+1±0.2	No	10.4±1.0
L-Glucose	No change	No	ND
Phlorizin	−50±5	5±1 (Red)	0.005±0.0008

+ Sign indicates increase in the fluorescence intensity after addition of ligand.
− Sign indicates fluorescence quenching after addition of ligand.
[a] The apparent equilibrium dissociation constant (K_d) was determined as previously described [7].

D-Man (4.3±0.7), and D-All (10.4±1). Phlorizin a well known high affinity inhibitor for hSGLT1 decreased intrinsic fluorescence by 50±5% with a red shift of 4–6 nm in emission maximum (Table1).

3.2. Quenching of Trp fluorescence of hSGLT1 reconstituted in proteoliposomes by KI, acrylamide, or TCE

Trp fluorescence quenching of proteins by KI, acrylamide, or TCE has been widely used for the determination of exposure of Trp residues in membrane proteins to a hydrophilic environment. The effects of all three quenchers in the absence of ligands and in the presence of glucose or phlorizin on the hSGLT1 Trp fluorescence are shown in Fig. 2, the corresponding Stern-Volmer constants are complied in Table 2. In the absence of ligands the small K_{sv} values obtained for all three quenchers reflect a low accessibility of Trp residues to the hydrophilic environment in reconstituted hSGLT1 (Table 2). Interestingly, the K_{sv} values are higher than the values reported for hSGLT1 in detergent solution [7], suggesting a slightly different conformation in the phospholipid bilayer.

In the presence of saturating D-glucose concentrations the K_{sv} values for all three quenchers increased significantly suggesting conformational changes in hSGLT1 after forming the sodium–glucose-carrier complex. These results demonstrate that most of the Trps responsible for hSGLT1 fluorescence reconstituted in proteoliposomes are located within a hydrophobic environment probably buried within the protein. Sugars apparently change the conformation of hSGLT1 in such a way that some Trps move to positions where they become more accessible to external hydrophilic collisional quenchers. Since all the effects could only be observed in the presence of sodium, they appear to be linked to the sodium–sugar cotransport function of the protein. This assumption is supported by the fact that L-glucose, a nontransported sugar did not exert any effect on Trp accessibility (data not shown).

In the titration experiments with the collisional quenchers in the presence of phlorizin a decreased accessibility of Trps was observed (lower K_{sv} values: KI 14%, acrylamide 27%, and TCE 13%). Thus phlorizin binding induces a conformation of hSGLT1 that is quite different from that assumed by the transporter when it interacts with sugar.

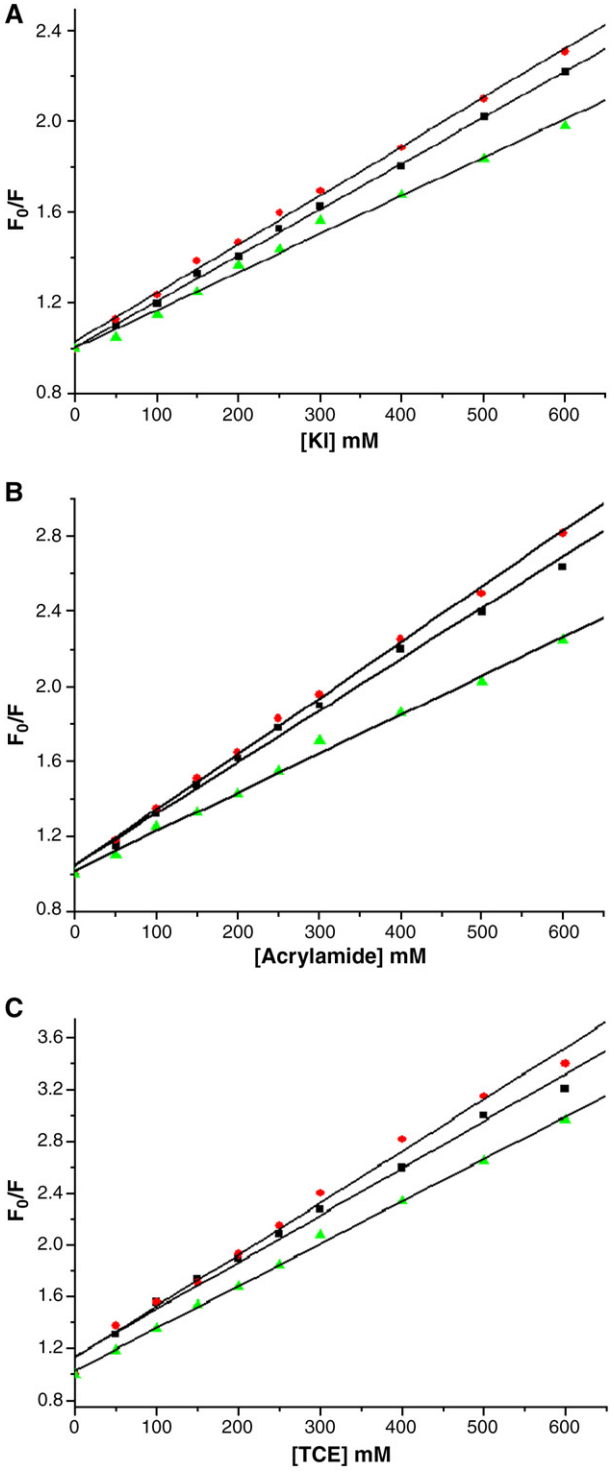

Fig. 2. Quenching of hSGLT1 fluorescence in proteoliposomes using quenchers of varying accessibility. Representative data for Stern-Volmer plots of (A) KI (■), 10 mM D-glucose (●), 100 μM phlorizin (▲). (B) Acrylamide (■), 10 mM D-glucose (●), 100 μM phlorizin (▲). (C) TCE (■), 10 mM D-glucose (●), 100 μM phlorizin (▲). Quenching experiments were performed as described in Materials and methods. The slopes of the best-fit linear regression lines for each data set (K_{SV} values) are shown in Table 2. The mean±S.D. of three independent experiments are given. No difference in quenching was observed for L-Trp.

Table 2
Stern-Volmer quenching constant (K_{SV}) of hSGLT1 reconstituted in proteoliposomes in the presence or absence of D-glucose or phlorizin in the presence of Na^+

Quenchers	K^d_{SV} (M^{-1}) in the absence of ligand[a]	K^d_{SV} (M^{-1}) in the presence of 10 mM D-glucose[b]	K^d_{SV} (M^{-1}) in the presence of 100 μM phlorizin[c]
KI	1.98±0.06	2.10±0.07	1.72±0.04
Acrylamide	2.75±0.04	2.97±0.04	2.01±0.03
TCE	3.69±0.15	3.84±0.15	3.23±0.11

[a]Quenching experiments were conducted in the absence of ligand.
[b]Quenching experiments were conducted in the presence of 10 mM D-glucose.
[c]Quenching experiments were conducted in the presence of 100 μM phlorizin.
[d]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 three independent experiments.

3.3. Quenching of Trp fluorescence of hSGLT1 in solution or reconstituted into proteoliposomes by N-bromosuccinimide

The fluorescence quenching studies of hSGLT1 reported above suggested that after reconstitution the Trps of the cotransporter are deeply buried either within the protein or the liposome phospholipids. We hence decided to further probe the accessibility of the Trp by investigating their chemical modification using *N*-bromosuccinimide. The cotransporter was modified using various molar ratios of NBS to protein, and the reduction in fluorescence emission at 338 nm was monitored after excitation at 295 nm. At an NBS/hSGLT1 ratio of 5 in about 2.5 min 13% of the fluorescence was abolished. At higher ratios and a longer incubation time more Trps were modified. Fig. 3 shows the plot of the residual hSGLT1 fluorescence versus the NBS/hSGLT1 ratio obtained after 5 min when the reaction was complete. Even at 50 times molar excess of NBS, ~75% of fluorescence was still observable, indicating that most of the Trp residues are inaccessible to chemical modification from the extramembranous space. It was also observed that the residual fluorescence had an emission maximum at 334 nm and was 4–6 nm blue shifted, indicating that the residual fluorescence is probably due to Trps, which were in a very hydrophobic environment. In order to differentiate between burial of Trps within the hydrophobic patches of protein itself or within liposomes, we performed NBS oxidation of hSGLT1 in solution. As also shown in Fig. 3 at 5 molar excess of NBS, 30% of Trp fluorescence decreases, as we increase NBS concentration decrease in hSGLT1 Trp fluorescence reaches maximum at 40 molar excess of NBS over hSGLT1. Even hSGLT1 treatment with 100 molar excess of hSGLT1 did not completely diminish its Trp fluorescence. Residual fluorescence of hSGLT1 in solution after NBS treatment shows the same emission maximum as its shows in reconstituted form, which suggest that a significant fraction of Trp residues is buried deep inside hydrophobic parts of the transporter.

3.4. Membrane penetration depths of hSGLT1 Trps in reconstituted liposomes

Membrane penetration depth is an important parameter in the study of membrane protein structure and organization. Knowledge of the precise depth of a membrane-embedded group or molecule often helps define the conformation and topology of membrane probes and protein. In addition, properties such as polarity, fluidity, segmental motion, ability to form hydrogen bonds, and the extent of solvent penetration are known to vary in a depth-dependent manner. The quenching of Trp fluorescence of membrane proteins by lipid incorporated quenchers

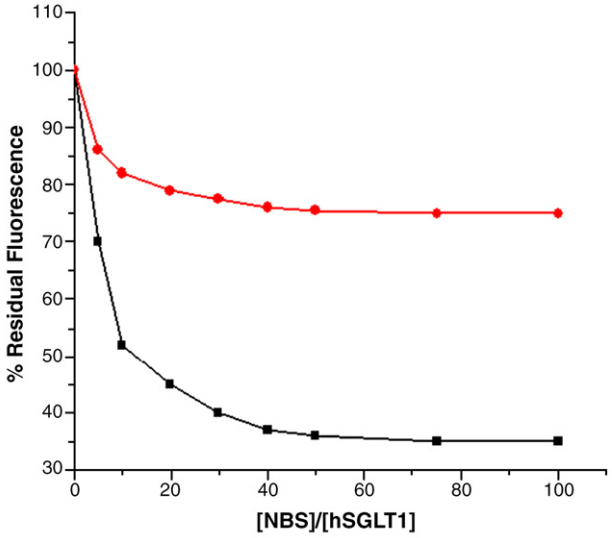

Fig. 3. NBS modification of hSGLT1 Trp residues in solution (■), and in proteoliposomes (●). 1 μM hSGLT1 in solution or in reconstituted in proteoliposomes in the lipid: protein ratio of 200:1 was subjected to NBS modification using 5, 10, 20, 30, 40, 50, 75, and 100-fold molar excess of NBS over the transporter using a 2 mM stock solution of NBS in buffer. The decrease in emission intensity at 338 nm was monitored after 5 min of NBS addition, excitation wavelength used was 295 nm. A plot of the percent residual fluorescence at equilibrium (5 min) as a function of molar ratio of NBS/hSGLT1 is shown.

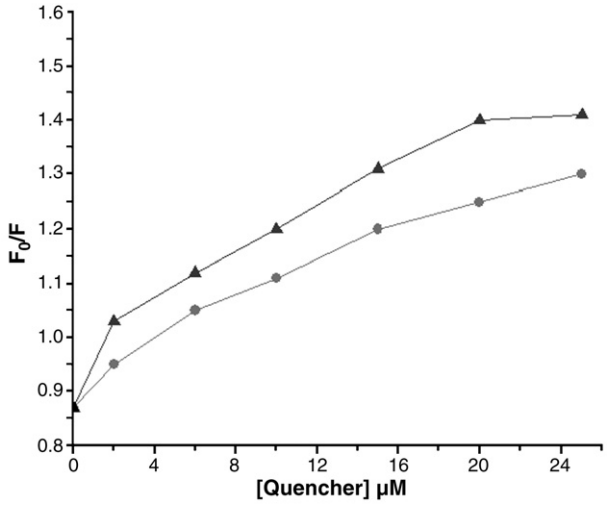

Fig. 4. Fluorescence quenching of hSGLT1 reconstituted into POPC membrane containing different concentration of 5-Doxyl PC (▲), or 12-Doxyl PC (●). The intensity of fluorescence at 338 nm was measured after excitation on 295 nm. The excitation and emission bandwidth was 5 nm. F_0 is the fluorescence intensity in the absence of the quencher, whereas F is the intensity in the presence of quencher. The results shown are typical of ≈5 independent experiments.

has been widely used for evaluating the exposure of Trps to the surrounding phospholipids. Therefore we studied quenching of hSGLT1 fluorescence in liposomes containing 5-Doxyl PC, or 12-Doxyl PC. As shown in Fig. 4 about 40% of the fluorescence was quenched by 5-Doxyl PC, and 26% of the fluorescence by 12-Doxyl PC. Using the parallax method from these results an average depth of Trp residues in the membrane of about 10 Å from the center of the bilayer could be calculated. This value is consistent with the interfacial localization of Trps in hSGLT1 in its reconstituted form.

4. Discussion

Tryptophan residues have been successfully used as fluorescent probes to investigate conformational changes in membrane proteins [6,7,12,14,22–30]. Our recent success in functional hSGLT1 expression and purification from *P. pastoris* and identification of different conformational states of hSGLT1 in solution [7] raised the question whether these conformational changes can also be observed in proteoliposomes in a reconstituted form, an experimental condition closer to the *in vivo* situation of membrane proteins.

In the reconstituted form hSGLT1 is fully functional and shows kinetic parameters which are indistinguishable from hSGLT1 reported in different systems [31–34]. Trp fluorescence of hSGLT1 reconstituted in proteoliposomes exhibited emission maxima at 338–340 nm indicating that most of Trps in hSGLT1 responsible for fluorescence are located in a relatively hydrophobic environment. This conclusion is further supported by the low accessibility of Trp residues for hydrophilic collisional quenchers. In the presence of sodium, sugars that have been identified previously as substrate for the transporter increase intrinsic fluorescence in a saturable manner by a maximum of 17% by α-MDG followed by 15% D-Glu, 14% D-Gal, 3% D-Man and 1% D-All. L-Glu was without effect. Application of saturation kinetics yields binding constants for different sugars in the following order α-MDG > D-Glu ≈ D-Gal > D-Man > D-All. These binding constant mirror the apparent K_m values reported for hSGLT1 in different systems by transport assays [7,35–37]. However, detergent mediated reconstitution of membrane protein into proteoliposomes sometimes leads to scrambled or inside out orientation of the transporter. In our experimental setup the orientation of the transporter is most probably right-side out since sodium-dependent phlorizin-inhibitable D-glucose transport can be observed, a hallmark for the outside face of SGLT1. The transporters in proteoliposomes are reconstituted in right-side orientation was further supported by results of our proteomic studies (Kumar et al., unpublished data), in which Trypsin beads digestion of hSGLT1 in proteolisomes generated peptides from the N-terminal domain, loop 7, loop 9 and loop 13; previous studies reported an extracellular orientation of these parts of the transporter [38,39].

Results from present quenching studies using KI, acrylamide, and TCE suggest that the bulk of Trp residues remains inaccessible to hydrophilic quenchers added externally to the system, and accordingly establish that these Trps are normally located either in the hydrophobic region of the membrane or in a hydrophobic environment within the protein. However in the presence of D-glucose (Fig. 2, and Table 2) a Trp residue (or conceivably, fractional populations of several Trps) does become located into a region which is more accessible to water upon D-glucose binding due to conformational changes — a phenomenon also observed in solution [7]. Whether these Trps are indeed directly involved in sugar binding and/or translocation remains to be established. The changes are nevertheless indicative for a distinct conformation of the sodium–sugar-carrier complex in the membrane. All conformational changes are strictly sodium-dependent as in the presence of other monovalent cations (Li^+ or K^+) we did not observe these changes in the fluorescence properties of the transporter (data not shown).

In the presence of phlorizin, the well established inhibitor of hSGLT1, a drastic decrease of 50% in Trp fluorescence with 4–6 nm red shifts in emission maxima was observed. Presence of a red shift in the fluorescence emission maximum of hSGLT1 after interaction with phlorizin indicates transition of one or more Trp into a hydrophilic environment due to conformational changes in transporter. 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 Trp containing parts of hSGLT1. Such a complex between phlorizin and water molecules can be formed by hydrogen 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). When saturating concentrations of phlorizin were present we observed with all three quenchers a slight protection against quenching with lower quenching constants. The reduction of the fluorescence could indicate that phlorizin was in close contact with Trps. Previous *in vitro* studies on isolated functional domains in solution revealed indeed strong conformational changes in loop 13 related to phlorizin binding that induced reduction in Trp fluorescence [40,41]. Decrease in the Trp fluorescence and quenching could also be the result of a reorientation of transmembrane helices, caused by distinct changes in gross topology of the transporter previously reported by AFM studies [42]. While selective quenching of accessible Trp residues is expected to produce nonlinear Stern-Volmer plots for multi-Trp containing proteins [43], the apparently linear plots exhibited by hSGLT1 may result from the combined effect of selective (downward curvature) and static (upward curvature) quenching. The observed D-glucose-induced increase and phlorizin-induced decrease in the slope of Stern-Volmer plots (Fig. 2) support the notion that transported substrate and inhibitor induced different conformations of the transporter.

The accessibility of the hSGLT1 Trps after reconstitution into liposomes was further investigated by chemical modification using NBS as an oxidant. NBS oxidation of the indole ring of Trp to the nonfluorescent oxindole can be used to assess the relative accessibility of Trp residues in proteins and peptides [44]. Even at 50 times molar excess of NBS, ~75% of fluorescence was still observable, indicating that most of the Trp residues are inaccessible to chemical modification from the outside of the membrane. This result is in agreement with the low degree of quenching exerted by the collisional quenchers. Thus, most of hSGLT1 Trp residues are deeply buried in the

hydrophobic core of the protein interior or of the membrane. A look at the topology model proposed for hSGLT1 (Fig. 5) [38,45] also reveals that only 2 out of the 14 Trps are present in extramembranous loops and thus can be assumed to be in a hydrophilic environment. Quenching of hSGLT1 Trp fluorescence by NBS in solution shows a different pattern as compared to the reconstituted form. In solution approximately 35% of Trp residues in the transporter are deeply buried within the hydrophobic core of the protein itself, since 65% Trp are accessible for NBS oxidation compared to only 25% in the reconstituted form. These results indicate that in a reconstituted form about 40% of Trp residues are inaccessible for NBS modification due to their close contact to in the phospholipids and 35% Trp residues are buried deep inside the hydrophobic core of protein.

The spin label fluorescence quenching approach has several advantages as a method to measure depth, including the following: (1) it is experimentally and analytically straight forward to use; (2) it yields a numerical value for depth (in Å) rather than just a relative location; (3) it requires only small amounts of both fluorescent molecules and lipids; and (4) it is widely applicable to reconstituted systems because, unlike other types of quenchers, spin labels can quench virtually all types of fluorophores, including tryptophans [46]. Furthermore, since quenching by spin labels in membranes is static unless unusually long-lived fluorophores are used [46], the method is very insensitive to fluorescence properties such as lifetime and quantum yield, and therefore, it is unnecessary to measure these parameters. Nevertheless, there are certain limits and precautions that must be considered. One consideration is that an average depth is obtained. It can be presumed that the depths obtained represent at least an average over a couple of angstroms, arising from a distribution of depths of fluorophores and spin labels. Another important concern is the exact depth of the spin label groups. Clearly, the accuracy of these values will limit the accuracy of the values of fluorophores depth determined. Fortunately, several lines of evidence justify placement of the spin labels close to the corresponding position expected in unlabeled PC (Fig. 5).

A schematic diagram illustrating the hypothetical 2-dimensional topology model of hSGLT1 and the parallax method as applied to hSGLT1 is shown in Fig. 5. Using parallax method we calculated the average depth of the membrane-quenchable Trps to be located of about 10 Å from the center of the bilayer. Since for the lipids used in these experiments the thickness of hydrocarbon region is 30 Å [20], a depth of 10 Å from the center of the bilayer is indeed shallow, and actually corresponds to the level of the seventh carbon atom of the fatty acyl chain. This depth thus confirms the quenching pattern by 5-Doxyl PC, and 12-Doxyl PC.

The hSGLT1 thus appears to be another example of a protein in which aromatic residues seems to be sequestered at the

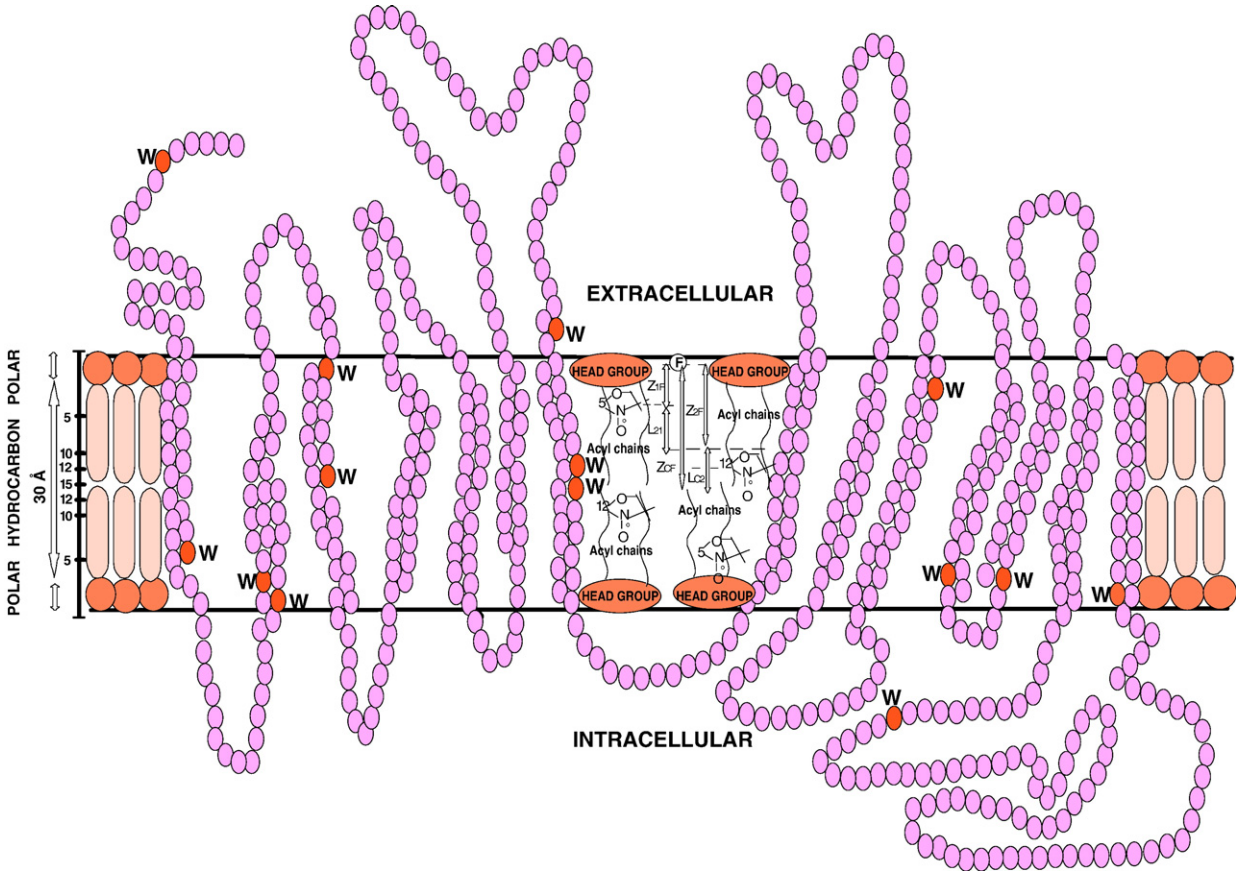

Fig. 5. Schematic diagram illustrating the hypothetical 2-dimensional topology model of hSGLT1 and the parallax method as applied to hSGLT1. hSGLT1 consists of 14 α-helical transmembrane domains with the N and C termini located on the extracellular phase of membrane. Positions of different tryptophan residues are marked in red color.

membrane boundary. In this arrangement Trps in the membrane are oriented in such a fashion that they are present near phospholipids heads groups but buried in the membrane lipid and not accessible to external hydrophilic collisional quenchers. Examples for a similar situation are lactose permease of *Escherichia coli* [47], K^+ channels [48–52], bacterial porins [53], the bacterial photosynthetic reaction center [54], cytochrome *c* oxidase [55], and bacteriorhodopsin [56].

Trp is a unique amino acid in that it has the largest nonpolar surface area and is a polar amino acid due to the presence of indole N–H, which gives it the ability to form an H-bond near the interfacial region. Experiments from different studies suggest that Trp residues have a preference for the lipid head group side of the membrane interface. The preferential location of Trp residues at membrane interface is thought to be due to the aromaticity of the indole moiety and the overall amphipathic nature of Trp. The Trp rich aromatic belt at the membrane interface in transmembrane helices is thought to stabilize the helix with respect to the membrane environment. The amphipathic nature of Trp residue has also been implicated in acting as anchors or floats for membrane proteins inserted into the membrane. This gives stability to the vectorial nature of membrane proteins. Assuming some tilting of the helices [57,58] the topology predictions in the different helices of hSGLT1 seem to be confirmed by these data.

In summary, these studies demonstrate that also reconstituted in a phospholipid bilayer hSGLT1 can assume three different conformational states, one in the absence of glucose, another as sodium–glucose-carrier complex and when the carrier interacts with sodium and phlorizin. Furthermore they confirm topology predictions where the majority of the Trps seems to be buried inside hydrophobic environments either in the protein or in the lipid bilayer. Some of them change their position when the sodium–sugar-carrier complex is formed. Those carrier segments containing Trps in contact with the lipid bilayer can transmit changes in the lipid to the transporter, as previously shown in reconstitution studies [59]. Only a minor portion of the Trps is located extramembraneously and might be involved in substrate and/or inhibitor binding.

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