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Examination of Sodium/Glucose Cotransport by Using a Visible Glucose Analogue†

Brian E. Peerce
Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550
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ABSTRACT: The glucose derivative, 2,2,6,6-tetramethylpiperidine-1-oxylglucose (TEMPO-glucose) was synthesized and examined for its ability to substitute for glucose as a substrate for the intestinal brush border membrane Na^+/glucose cotransporter. TEMPO-glucose inhibited Na^+-dependent phlorizin binding with an apparent K_I of 18 μM and Na^+-dependent glucose uptake with an apparent K_I of 70 μM. The transport competence of TEMPO-glucose was examined by using two measures of transport. The first involved comparing the reversial of trans Na^+ inhibition by D-glucose and TEMPO-glucose. The second directly examined Na^+-dependent TEMPO-glucose uptake by using TEMPO-glucose quenching of intervesicular fluorescein sulfonate fluorescence. Tryptophan fluorescence was sensitive to TEMPO-glucose in a Na^+-dependent, glucose-inhibitable manner. The bulk of these tryptophans appeared to be located in hydrophobic environments based on Cs^+-insensitivity. With the reconstituted cotransporter, TEMPO-glucose, and tryptophan quench reagents, the cotransporter was compared in three transport modes: zero trans uptake, zero trans uptake in the presence of a shunt of membrane potential, and substrate exchange. The results suggest that the cotransporter conformation varies depending on its mode of operation and that TEMPO-glucose may be a useful probe for localizing amino acid residues involved in glucose transport.

A number of amino acids have been identified as important for substrate transport by Na^+/glucose cotransporters on the basis of inhibition of Na^+-dependent glucose uptake by amino acid specific reagents. The amino acids identified include tyrosines (Peerce & Wright, 1985; Lin et al., 1982; Wright & Peerce, 1985), lysines (Weber & Semenza, 1983; Peerce & Wright, 1984; Fernandez et al., 1989), sulfydryls (Klip et

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al., 1979; Biber et al., 1983), and carboxylic acids (Turner, 1986; Weber et al., 1987; Friedrich et al., 1987). Defining the molecular basis of amino acid specific reagent induced inhibition of substrate uptake has been based on the presence or absence of substrate protection. On this basis tyrosines and lysines have been suggested to be located at or near the cotransporter Na^+ site (Peerce & Wright, 1985; Lin et al., 1982) and glucose site (Weber & Semenza, 1983; Peerce & Wright, 1984), respectively. The effects of sulfydryl reagents and carboxylic acid reagents are less clear. Sulfydryl residues that appear to be essential for transport appear to be far removed from the apparent substrate-binding sites (Klip et al., 1979). Neither substrate binding nor substrate-induced conformational changes alter cotransporter sensitivity to sulfydryl specific reagents. Two different classes of carboxylic acid residues have been reported. Carboxylic acid residues have been reported at or near the cotransporter glucose site in renal brush border membranes (Turner, 1986). A second class of carboxylic acids that was not substrate protectable has been reported for the intestinal brush border membrane cotransporter (Weber et al., 1987).

There are two levels of specificity involved in the use of group-specific reagents for examination of the molecular mechanism of Na^+-dependent glucose cotransport. The first involves the reagents. Reagent-induced inhibition of ion-gradient-driven organic substrate uptake may result from increased membrane permeability due to the reaction conditions or side reactions of the reagent. This limitation may be at least partially controlled by using gradient-independent measures of cotransporter activity (Hopfer, 1977; Semenza et al., 1984).

The second level of specificity is more difficult to define. Reagent-sensitive amino acids are broadly defined as at or near the substrate sites on the basis of substrate competition. The inability to specifically define the site of reagent reaction is based on numerous observations of substrate-induced conformational changes (Peerce & Wright, 1984; Kaunitz & Wright, 1984; Hopfer & Groseclose, 1980; Peerce, 1990) and the possibility that substrate-induced conformational changes result in the observed substrate protection. Defining cotransporter activity in terms of substrate uptake is also a limitation to the use of these reagents. Unlike the ion-transporting ATPases, partial reaction assays for Na^+-dependent cotransporters have not been defined. While amino acid specific reagents have provided insights into the molecular mechanism of ion and organic solute transport across membranes, these reagents at best are regional static probes.

In an attempt to better define the sequence of events following substrate binding, a new probe of the cotransporter, 2,2,6,6-tetramethylpiperidine-1-oxylglucose (TEMPO-glucose), was developed (Peerce et al., 1990). This reagent is a fluorescence-quench reagent and can be used as a monitor of glucose transport. This communication compares TEMPO-glucose to phlorizin and glucose as substrates of the cotransporter. Examination of three modes of cotransporter-mediated Na^+/glucose cotransport is also described.

Materials and Methods

TEMPOL was purchased from Molecular Probes, Inc., Eugene, OR. Glucose pentaacetate, titanium tetrachloride, Ag_2Cl_2, Dowex 50 H^+, phlorizin, and all organic solvents were purchased from Aldrich Chemical, Milwaukee, WI. [^{3}H]-Glucose and [^{3}H]phlorizin were purchased from NEN/Du Pont, Wilmington, DE. Valinomycin and protease inhibitors were purchased from Sigma Chemical, St. Louis, MO. Polybuffer 74 was purchased from Pharmacia Chemical, Piscataway, NJ. All other chemicals were purchased from Fisher Chemical, Plano, TX, and were reagent grade or better.

Brush Border Membrane Vesicle Preparation. Ca^{2+}-precipitated brush border membrane vesicles were prepared from rabbit intestinal mucosa was previously described (Peerce & Wright, 1984; Stevens et al., 1982). SDS–BBM vesicles were prepared as previously described (Peerce & Clarke, 1990). Following isolation, the vesicles were resuspended in 300 mM mannitol and 10 mM Hepes/Tris pH 7.5 and stored at liquid N_2 temperatures until needed.

Purification and Reconstitution of the Na^+/Glucose Cotransporter. The intestinal Na^+/glucose cotransporter was purified by chromatofocusing chromatography and Con A Sepharose chromatography as previously described (Peerce & Clarke, 1990) with the addition of the following protease inhibitors during CHAPS solubilization and during chromatofocusing chromatography: 1 μg/mL pepstatin, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 100 μg/mL PMSF. Purification was assayed by Na^+-dependent phlorizin binding of the CHAPS-solubilized cotransporter as previously described (Peerce & Clarke, 1990). The protein used in these studies possessed Na^+-dependent phlorizin binding of 12.6 ± 0.4 nmol/mg of protein.

The Na^+/glucose cotransporter was reconstituted into phosphatidylcholine/cholesterol proteoliposomes as previously described (Peerce & Clarke, 1990). The protein was resuspended in 0.5% CHAPS, 300 mM mannitol, and 10 mM Hepes/Tris, pH 7.5, prior to reconstitution. Proteoliposomes were collected by centrifugation and the pellets resuspended in 50 mM potassium gluconate, 100 mM TMA gluconate, and 25 mM Hepes/Tris, pH 7.5.

Synthesis of Tempo-Glucose. TEMPO-glucose was synthesized from TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) and tetraacetylglucose in chloroform according to the method of Struve and McConnell (1972). Tetraacetylglucose was synthesized from the pentaacetate derivative by using titanium tetrachloride as described by Lemieux (1963). TEMPO-glucose was purified on a 32 cm × 3.5 cm silica gel column eluted with dichloromethane/ethyl ether (8:2). The first fraction was recovered and examined by thin-layer chromatography on silica gel plates with dichloromethane/ethyl ether (8:2) as the mobile phase. A single orange spot was seen with an R_f of 0.44. No reaction was seen with the aniline–diphenylamine–phosphoric acid reagent, which gives positive colored reactions with reducing sugars (Lewis & Smith, 1969), suggesting that the nitroxide spin radical is bound to carbon one of glucose.

Na^+-Dependent Glucose Uptake. Na^+-dependent glucose uptake was performed by using a rapid-mixing/rapid-sampling system as previously described (Stevens et al., 1982). Experiments used 15 μg of reconstituted cotransporter, 25 μM [^{3}H]glucose, and 10-s uptakes at 23 °C. The uptake media consisted of 100 mM NaCl or 100 mM TMACl, 50 mM KCl, 25 mM Hepes/Tris pH 7.5, and 2 μg of valinomycin. The reaction was stopped by a 10-fold dilution with ice-cold stop solution consisting of 300 mM mannitol and 25 mM Hepes/Tris pH 7.5, the mixture was filtered on 0.22 μM Millipore filters, and the filters were washed with an additional 8 mL of stop solution. Filters were counted by liquid scintillation counting. Na^+-dependent uptake was defined as uptake in the presence of Na^+ minus uptake in the presence of TMA^+.

Fluorescence Experiments. Fluorescence experiments were performed on an SLM SPF 500c spectrofluorometer in the ratio mode at 23 °C. Tryptophan fluorescence was excited at 290 nm and the emission monitored continously as a

function of wavelength or measured at 350 nm as a function of time. Slit widths were set at 4 nm.

Experiments examining the effect of I^- and Cs^+ on tryptophan fluorescence of reconstituted cotransporter were performed in 100 mM sodium gluconate or TMA gluconate, 50 mM potassium gluconate, and 25 mM Hepes/Tris, pH 7.5. Some experiments included 2 μg of valinomycin. Proteoliposomes were preincubated in 50 mM potassium gluconate, 100 mM TMA gluconate, and 25 mM Hepes/Tris, pH 7.5, by overnight incubation at 4 °C. I^- and Cs^+ were added from 2 M stocks to minimize dilution effects and varied from 5 to 100 mM to minimize ionic-strength effects. $Na_2S_2O_3$ (1 mM) was added to the I^- stocks to minimize I_3^- formation. Proteoliposome scatter was corrected by comparison to liposomes without protein. Inner filter effects were minimized by maintaining protein absorbance readings of less than 0.02 absorbance units. All results are reported as corrected emission spectra.

Fluorescence quenching experiments were analyzed by the method of Lehrer (1971) with the modified Stern–Volmer equation as previously described (Peerce & Wright, 1987).

$$F_0/\Delta F = 1/f_a K_s[Q] + 1/f_a$$

where F_0 is the fluorescence in the absence of quencher, ΔF is the change in fluorescence on addition of quencher (Q) to a concentration [Q], f_a is the effective maximal fraction of tryptophans accessible to the quencher at an infinite quencher concentration, and K_s is the modified Stern–Volmer quench constant. Plots of $F_0/\Delta F$ vs $1/[Q]$ were linear from 5 to 100 mM Q with a y intercept of f_a^{-1} and a slope of $(f_a K_s)^{-1}$. The presence of 13 tryptophan residues on the intestinal Na^+/glucose cotransporter and the absence of information regarding the number of tryptophans involved in the fluorescence-quench experiments prevent the use of the Stern–Volmer quench constant, K (Eftink & Ghiron, 1981). The tables describing these experiments make use of K_s as has been previously described (Peerce & Wright, 1987).

Experiments examining the time course of TEMPO-glucose quenching of tryptophan fluorescence were performed as described above except that TEMPO-glucose was added from 0.1 M ethanolic stocks. In some experiments examining the different modes of cotransporter transport, overnight preincubation included 100 mM sodium gluconate, 50 mM potassium gluconate, 25 mM Hepes/Tris, pH 7.5, and variable glucose concentrations. Experiments examining the effect of Cs^+ on the time course of tryptophan fluorescence quenching by TEMPO-glucose were performed as described above by using 0.15 M TMA to replace Cs^+ and a final osmolality of 450 mosm. Experiments designed to minimize interliposomal tryptophan fluorescence using Cs^+-equilibrated proteoliposomes were performed following overnight incubation with 0.15 M Cs^+ as described above. The proteoliposomes were then diluted into 0.15 M Cs^+ or 0.15 M TMA^+, 0.1 M Na^+, 50 mM K^+, and 25 mM Hepes/Tris, pH 7.5. All fluorescence-quenching experiments were performed in quadruplicate, and the results shown are typical of four separate experiments.

TEMPO-Glucose Transport. Uptake of TEMPO-glucose was examined by two methods. The first method involved the relief of trans Na^+ inhibition of Na^+-dependent glucose uptake. Reconstituted proteoliposomes were preincubated at 4 °C overnight in 100 mM sodium gluconate, 50 mM potassium gluconate, and 25 mM Hepes/Tris, pH 7.5, in the presence and absence of 1 mM glucose, or 1 mM TEMPO-glucose. Na^+-dependent glucose uptake was determined as described above.

The second method utilized the ability of TEMPO-glucose as a fluorescence-quench reagent. Proteoliposomes were preincubated with 1 mM fluorescein sulfonate, 50 mM KCl, 100 mM TMACl, and 25 mM Hepes/Tris, pH 7.5, for 12 h at 4 °C. Free dye was removed by centrifugation and the pellets resuspended in the incubation medium without dye. The wash step was repeated twice. TEMPO-glucose uptake was determined as Na^+-dependent fluorescein sulfonate fluorescence quenching by comparing fluorescein sulfonate fluorescence in 100 mM NaCl to that in 100 mM TMACl upon addition of TEMPO-glucose. To ensure that TEMPO-glucose was being transported by the cotransporter, the effect of glucose was also examined. All experiments were performed on an SLM SPF 500c spectrofluorometer at 23 °C set in the ratio mode. Fluorescein sulfonate was excited at 490 nm and its emission recorded at 520 nm. Slit widths were set at 2 nm. Proteoliposome scatter was corrected as described above by using proteoliposomes without fluorescein sulfonate.

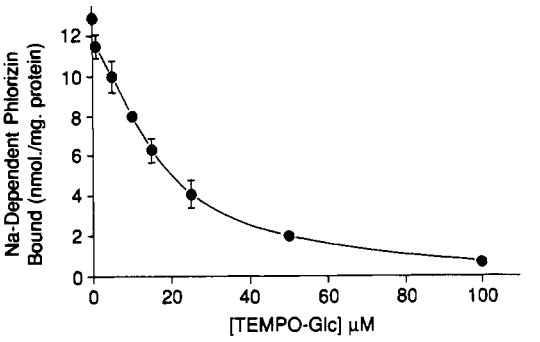

FIGURE 1: Effect of TEMPO-glucose concentration on Na^+-dependent phlorizin binding. A total of 10 μg of Na^+/glucose cotransporter in 0.5% CHAPS and 50 mM Hepes/Tris, pH 7.5, was examined for Na^+-dependent phlorizin binding in 100 mM NaCl, minus 100 mM KCl, 50 mM Hepes/Tris, pH 7.5, and 15 μM [^{3}H]phlorizin as described under Materials and Methods. Results are means ± SE of duplicate determinations and are representative of five experiments.

RESULTS

Transport and Binding of TEMPO-Glucose. Inhibition of Na^+-dependent glucose uptake and Na^+-dependent phlorizin binding were used as measures of TEMPO-glucose's ability to interact with the cotransporter. The results of these studies are shown in Figures 1 and 2.

Figure 1 shows the effect of increasing TEMPO-glucose concentration on Na^+-dependent phlorizin binding by the CHAPS-solubilized cotransporter. In the absence of TEMPO-glucose, 12.9 ± 0.3 nmol of phlorizin/mg of protein bound to the cotransporter. There was a progressive decrease in Na^+-dependent phlorizin bound with increasing TEMPO-glucose concentration. The TEMPO-glucose concentration resulting in half-maximum inhibition was 17 ± 3 μM (n = 3). This value is in good agreement with the apparent K_I for glucose inhibition of Na^+-dependent phlorizin binding of 20–33 μM (Tannenbaum et al., 1977) in the brush border membrane.

The effect of TEMPO-glucose concentration on Na^+-dependent glucose uptake is shown in Figure 2. Figure 2 is a Dixon plot of Na^+-dependent glucose uptake as a function of increasing TEMPO-glucose concentration at 25 μM (open circles, solid line) and 100 μM glucose (solid circles, dashed line). The apparent K_I was 70 ± 10 μM (n = 4).

The experiments described in Figures 1 and 2 indicate that TEMPO-glucose can substitute for glucose on the Na^+/glucose cotransporter. These experiments do not distinguish between competition with respect to binding and transport and competition with respect to binding only. In order to determine if TEMPO-glucose was transported by the Na^+/glucose cotransporter a second series of experiments was performed.

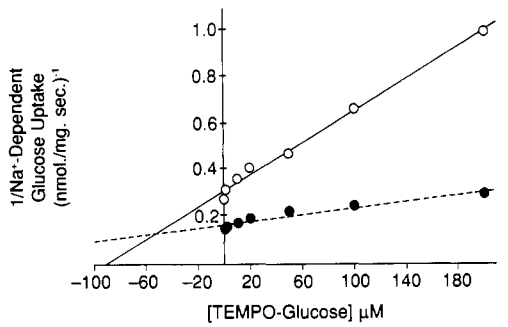

FIGURE 2: Effect of TEMPO-glucose concentration on Na^+-dependent glucose uptake. Proteoliposome reconstituted cotransporter preincubated with 100 mM TMA gluconate, 50 mM potassium gluconate, 25 mM Hepes/Tris, pH 7.5, was examined for Na^+-dependent glucose uptake by using 15 μg of reconstituted protein, 100 mM sodium gluconate or 100 mM TMA gluconate, 50 mM potassium gluconate, 25 mM Hepes/Tris, pH 7.5, 2 μg of valinomycin, and 25 μM [^{3}H]glucose (solid line) or 100 μM (broken line) [^{3}H]glucose. Results are means ± SE of triplicate determinations of glucose uptake in the presence of Na^+ minus uptake in the presence of TMA^+ and are representative of four experiments.

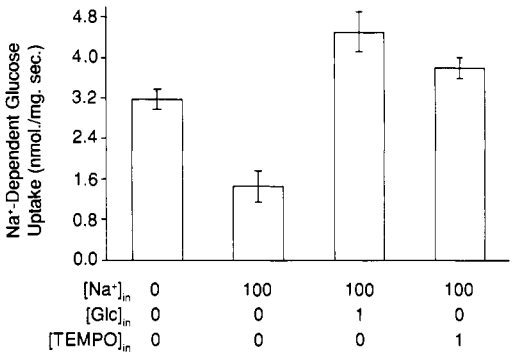

FIGURE 3: Effect of substrates are trans Na^+ inhibition of Na^+-dependent glucose uptake. Cotransporter-reconstituted proteoliposomes were incubated overnight at 4 °C with the indicated substrates, 25 mM Hepes/Tris, pH 7.5, and 50 mM potassium gluconate. Na^+-dependent glucose uptake was determined as described under Materials and Methods. Results are means ± SE of triplicate determinations and are representative of three experiments.

These experiments are shown in Figures 3 and 4.

Cotransporter-Mediated Uptake of TEMPO-Glucose. Figure 3 shows the effect of interliposomal Na^+ on Na^+-dependent glucose uptake. Internal Na^+ inhibited glucose uptake 54% ± 4% (n = 3). Addition of 1 mM glucose to the preincubation medium resulted in a 45% ± 4% stimulation of Na^+-dependent glucose uptake over that seen in the absence of internal Na^+ and glucose (zero trans uptake experiment minus control). Addition of 1 mM TEMPO-glucose to the preincubation medium resulted in a 25% ± 3% (n = 3) increase in Na^+-dependent glucose uptake compared to the control uptake. Only transport-competent substrates relieve trans Na^+ inhibition of Na^+-dependent glucose uptake (Dorando & Crane, 1984), suggesting that TEMPO-glucose was transported by the cotransporter approximately half as well as glucose itself.

The second series of experiments involved the use of intervesicular fluorescein sulfonate fluorescence as an indicator of TEMPO-glucose transport. Fluorescein sulfonate is a poorly permeant nonreactive fluorescent derivative of fluorescein that has been used as a reporter of vesicle swelling (Chen et al., 1988). In these experiments TEMPO-glucose quenching of fluorescein sulfonate fluorescence in the presence and absence of Na^+ and glucose was used to determine cotransporter-mediated TEMPO-glucose transport. The quenching of fluorescein sulfonate fluorescence by TEMPO-glucose in the presence and absence of glucose is shown in Figures 4 and 5.

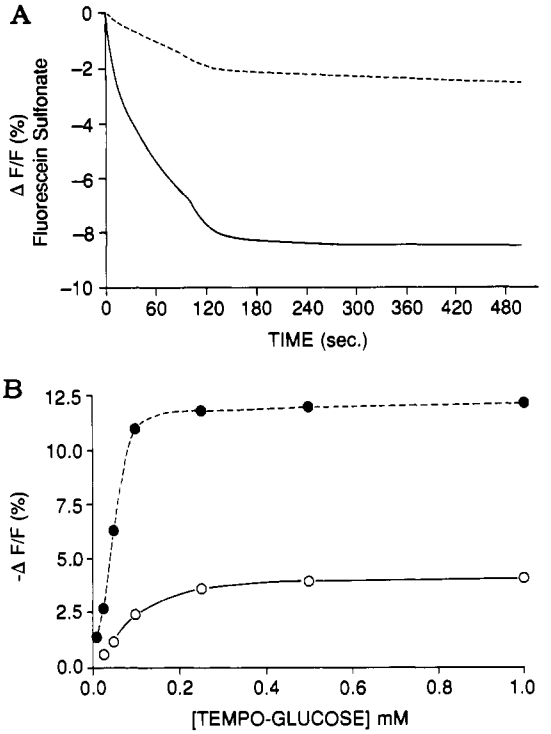

FIGURE 4: Effect of TEMPO-glucose on interliposomal fluorescein sulfonate fluorescence. (A) Cotransporter-reconstituted proteoliposomes were preincubated in 300 mM mannitol, 25 mM Hepes/Tris, pH 7.5, and 1 mM fluorescein sulfonate for 12 h at 4 °C. Following incubation the proteoliposomes were diluted 100-fold with 300 mM mannitol and 25 mM Hepes/Tris, pH 7.5, and centrifuged at 100000g for 60 min. The pellets were resuspended in 300 mM mannitol and 25 mM Hepes/Tris pH 7.5. The centrifugation and wash were repeated twice. A total of 20 μg of protein was added to 100 mM NaCl, 50 mM KCl, 25 mM Hepes/Tris, pH 7.5, and 200 μM TEMPO-glucose in the presence (Broken line) or absence (solid line) 500 μM glucose. Fluorescein sulfonate fluorescence was recorded as described under Materials and Methods. Results are from a single experiment and are representative of triplicate determinations and five separate experiments. (B) The effect of TEMPO-glucose concentration on fluorescein sulfonate fluorescence was determined as described above in the presence (solid line) and absence (broke line) of 500 μM glucose. The fluorescence quenching at 150 s is shown. Results are means ± SE of duplicate determinations and are representative of four experiments.

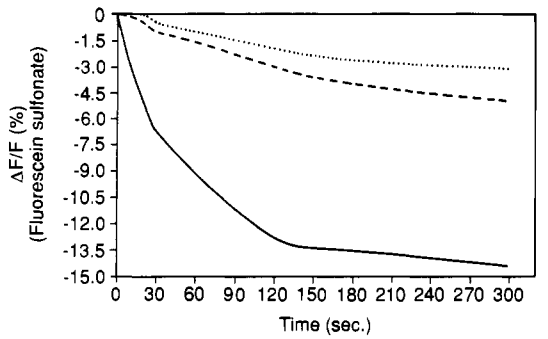

FIGURE 5: Effect of substrates on TEMPO-glucose quenching of intervesicular fluorescein sulfonate fluorescence. A total of 20 μg of the reconstituted cotransporter was added to 100 mM NaCl (solid line) or 100 mM KCl (dotted line) as described in the legend to Figure 4. The dashed line shows the effect of TEMPO-glucose on intervesicular fluorescein sulfonate fluorescence in the presence of 100 mM NaCl and 0.5 mM D-glucose. Results are from a single experiment performed in triplicate and are representative of three separate experiments.

The effect of TEMPO-glucose in 100 mM NaCl in the presence (dashed line) and absence (solid line) of 0.5 mM glucose is shown in Figure 4a. TEMPO-glucose quenched

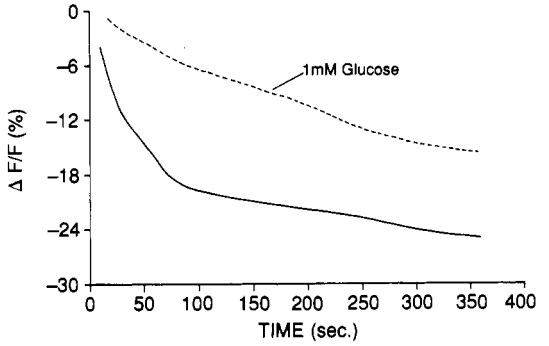

FIGURE 6: Effect of TEMPO-glucose on tryptophan fluorescence. A total of 15–20 μg of reconstituted proteoliposomes was diluted into 100 mM sodium gluconate, 50 mM potassium gluconate, and 25 mM Hepes/Tris, pH 7.5, in the presence (broken line) and absence (solid line) of 1 mM glucose. At time 0, 1 mM TEMPO-glucose was added and the tryptophan emission at 350 nm recorded. Results are from a single experiment and are representative of four experiments performed in triplicate.

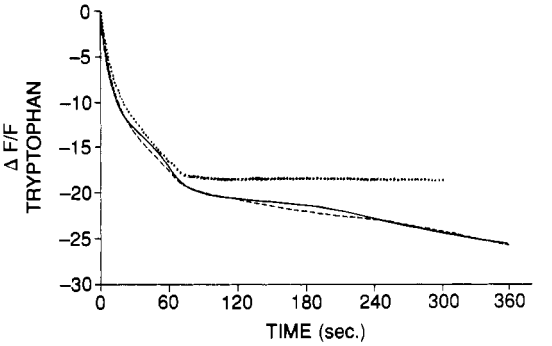

FIGURE 7: Effect of Cs^+ on TEMPO-glucose quenching of tryptophan fluorescence. TEMPO-glucose quenching of tryptophan fluorescence was determined as described in the legend to Figure 6. The solid line is tryptophan fluorescence + 0.15 M TMACl, the broken line is tryptophan fluorescence + 0.15 M Cs^+, and the dotted line is tryptophan fluorescence of proteoliposomes equilibrated with 0.15 M Cs^+ for 12 h at 4 °C + 0.15 M Cs^+. At time 0, 200 μM TEMPO-glucose was added. Results are from a single experiment and are representative of four experiments performed in triplicate.

intervesicular fluorescein sulfonate fluorescence, and this quenching was inhibited by glucose. The apparent $K_{0.5}$ for TEMPO-glucose is 68 ± 8 μM (n = 4) (Figures 4b).

The substrate specificity of TEMPO-glucose quenching of intervesicular fluorscein sulfonate fluorescence was similar to the substrate specificity of Na^+-dependent glucose uptake. Figure 5 compares TEMPO-glucose quenching of intervesicular fluorescein sulfonate in the presence of K^+ (dotted line), and Na^+ (solid line). The figure demonstrates that K^+ may not substitute for Na^+. The broken line shows fluorescein sulfonate fluorescence in the presence of Na^+ and 2 mM glucose. In addition L-glucose had no effect on Na^+ and TEMPO-glucose quenching of intervesicular fluorescein sulfonate fluorescence (data not shown).

These results suggest that TEMPO-glucose is a substrate for the intestinal Na^+/glucose cotransporter, and although it is not transported with the same efficiency as glucose, it may be used as a probe of glucose transport by the reconstituted cotransporter.

Effect of TEMPO-glucose on Cotransport Tryptophan Residues. TEMPO-glucose as a probe of cotransporter-mediated glucose uptake is demonstrated in Figures 6 and 7. Figure 6 shows TEMPO-glucose quenching of tryptophan fluorescence in 100 mM NaCl in the presence (dashed line) and absence (solid line) of 2 mM glucose. Tryptophan fluorescence was quenched by the glucose analogue specifically, where specificity was defined as substrate-sensitive quenching. That TEMPO-glucose quenched tryptophan fluorescence is an indication that there are tryptophan residues close to that portion of the cotransporter involved in glucose binding and transport. The apparent $K_{0.5}$ for TEMPO-glucose quenching of tryptophan fluorescence was 18 ± 3 μM (n = 5).

Tryptophan residues in the hydrophobic portion of the cotransporter should not be sensitive to quenching by the hydrophilic impermeant quench reagent Cs^+. The effect of 0.15 M Cs^+ on subsequent Na^+-dependent glucose-sensitive TEMPO-glucose quenching of tryptophan fluorescence is shown in Figure 7. The addition of 0.15 M Cs^+ (broken line) to the uptake medium had no effect on the rate or magnitude of TEMPO-glucose quenching of tryptophan fluorescence compared to 0.15 M TMA^+ addition. In the absence of valinomycin, the rate constant for TEMPO-glucose quenching of tryptophan fluorescence was 0.027 ± 0.002 s^{-1} (n = 6) and 0.042 ± 0.003 s^{-1} (n = 6) in the presence of valinomycin. In the presence of 0.15 M Cs^+, the rate constants were 0.027 ± 0.003 s^{-1} (n = 5) and 0.044 ± 0.004 s^{-1} (n = 5) in the absence and presence of valinomycin, respectively. Preequilibration of proteoliposomes with Cs^+ appeared to eliminate the tryptophan quench seen at late time points (dotted line).

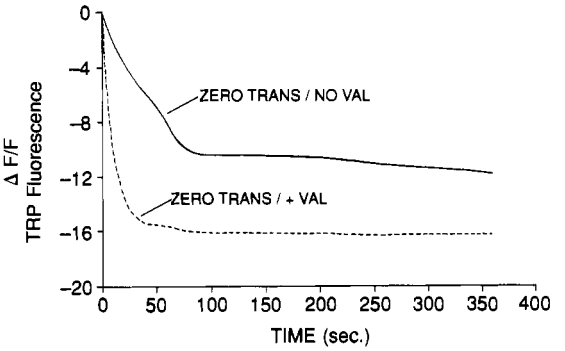

FIGURE 8: Effect of valinomycin on TEMPO-glucose quenching of tryptophan fluorescence. TEMPO-glucose quenching of tryptophan fluorescence was determined as described in the legend to Figure 6. The effect of 1 mM glucose has been subtracted from the results shown. Prior to the addition of TEMPO-glucose, 2 μg of valinomycin was added (broken line). Results are from a single experiment and are representative of four experiments in duplicate.

Examination of Cotransporter Modes by Using TEMPO-Glucose. Three modes of cotransporter activity, zero trans uptake, zero trans uptake plus valinomycin, and activity under substrate exchange conditions, were examined by using TEMPO-glucose. Under conditions of substrate exchange, the internal glucose concentration was 0.1 mM. The results are shown in Figures 8 and 9.

Figure 8 examines the effect of membrane potential on Na^+/glucose cotransport as monitored by tryptophan fluorescence and TEMPO-glucose. TEMPO-glucose transport as monitored by its quenching of tryptophan fluorescence is shown in the presence (dashed line) and absence (solid line) of valinomycin. As expected, the presence of a shunt of membrane potential resulted in a stimulation of transport. Both the magnitude of the quenching as well as the time course were enhanced. Addition of 100 μM glucose and 100 mM Na^+ to the vesicle interior (substrate exchange) also stimulated TEMPO-glucose quenching of tryptophan fluorescence, resulting in a time course similar to that seen with valinomycin (Figure 9). Increasing the glucose concentration to 2 mM inside the vesicle reduced TEMPO-glucose quenching of tryptophan fluorescence to less than 25% of that seen at 0.1 mM glucose, consistent with TEMPO-glucose being a less "efficient" substrate compared to glucose.

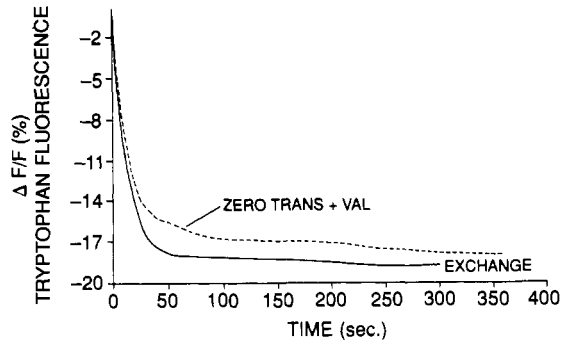

FIGURE 9: Time course of TEMPO-glucose transport under zero trans and substrate-exchange conditions. TEMPO-glucose quench of tryptophan fluorescence was determined as described in the legend to Figure 6 and under Materials and Methods. Results shown have been corrected for the effect of glucose as described in the legend to Figure 6. Solid line, substrate exchange conditions; broken line, zero trans uptake in the presence of 2 μg of valinomycin. Results are from a single experiment and are representative of duplicate determinations and five separate experiments.

Table I: Effect of Cs^+ and I^- on Tryptophan Fluorescence of the Reconstituted Na^+/Glucose Cotransporter[a]

conditions	quench reagent			
	I^-		Cs^+	
	f_a (%)	K_S (M^{-1})	f_a (%)	K_S (M^{-1})
Na^+	34 ± 4	17 ± 3	45 ± 2	18 ± 3
Na^+ + glucose	47 ± 4	18 ± 3	30 ± 3	35 ± 3
Na^+ + TEMPO-glucose	73 ± 7	6 ± 1	38 ± 2	17 ± 2

[a] The results are means ±SE of duplicate determinations and six separate experiments.

Previous results have indicated three distinct cotransporter conformations based on tryptophan fluorescence (Peerce, 1990). Table I examines the proteoliposome-reconstituted cotransporter conformations with respect to I^- and Cs^+ quenching of tryptophan fluorescence. The addition of glucose to the Na^+-bound cotransporter resulted in a 48% increase in I^- quenching and a 32% decrease in Cs^+ quenching of cotransporter tryptophan fluorescence. These results are similar to previous studies examining substrate-induced cotransporter conformational changes by using detergent-solubilized cotransporter (Peerce, 1990).

Substitution of TEMPO-glucose for glucose resulted in a doubling of the I^--accessible tryptophans and an 11% decrease in the Cs^+-accessible tryptophans as compared to the Na^+ conformation. When fully loaded cotransporter conformations were compared and it was assumed that TEMPO-glucose induced a glucose-like conformational change, the fraction of I^--accessible tryptophans increased 50% (from 49–73%) and the fraction of Cs^+-accessible tryptophans increased 30% (from 30–39%) when TEMPO-glucose was substituted for glucose.

Table II compares three modes of cotransporter activity with TEMPO-glucose as a substrate and I^- and Cs^+ as reporters of cotransporter conformation. In the absence of a membrane potential, I^- quenching decreased to 56% in the Na^+ plus TEMPO-glucose-loaded cotransporter while Cs^+ quenching increased to 50%. Under conditions where the cotransporter was always in the fully loaded state (substrate-exchange conditions), I^- quenching was similar to that seen in the Na^+ plus glucose conformation while Cs^+ quenching was similar to that seen in the Na^+ plus TEMPO-glucose conformation.

Discussion

A new probe of the Na^+/glucose cotransporter has been synthesized and its association with the Na^+/glucose cotransporter described. TEMPO-glucose inhibits substrate binding (Figures 1 and 2) and transport (Figures 2–6). This inhibition appears to be competitive with respect to glucose (Figures 2 and 6). These results are consistent with TEMPO-glucose being a transport-competent glucose analogue.

Table II: Effect of Cotransporter Transport Mode on Cs^+ and I^- Quenching of Tryptophan Fluorescence[a]

conditions	quench reagent			
	I^-		Cs^+	
	f_a (%)	K_S (M^{-1})	f_a (%)	K_S (M^{-1})
Na^+ + TEMPO-glucose	73 ± 7	6 ± 1	38 ± 2	17 ± 2
Na^+ + TEMPO-glucose + VAL	56 ± 2	8 ± 2	49 ± 4	6 ± 2
substrate exchange[b]	43.4 ± 4	12 ± 2	38 ± 1	18 ± 2

[a] All results are means ±SE of duplicate determinations and five separate experiments. [b] The following conditions were used in the substrate exchange experiments: $[Na^+]_{in} = [Na^+]_{out}$; $[glucose]_{in} = 0.2$ mM; and $[TEMPO\text{-}glucose]_{out} = 1$ mM.

In addition to being a transport competent glucose analogue, TEMPO-glucose is a fluorescence-quenching reagent (London, 1982). What is known of nitroxide spin labels as fluorescence-quenching reagents is that fluorescence quenching appears to result from the long-lived exicted state of the nitroxide spin radical (100 ns) as compared to that of the common fluorophores (1–10 ns). The physical nature of the quenching process is not well understood; however, at high spin radical concentrations, an upward curvature of the Stern–Volmer plots may suggest a static contribution.

In solution, the effective quenching range for the fluorophore and spin radical has been reported as 5–7 Å. When covalently attached to the membrane lipid, the effective quenching distance has been reported to be approximately 10 Å (London, 1980). These effective quenching distance approximations suggest that this probe of the Na^+/glucose cotransporter is a reporter at the amino acid level.

The TEMPO-glucose-sensitive tryptophans appear to have at least two general distributions. The absence of a measurable effect of Cs^+ on the time course and magnitude of TEMPO-glucose quenching of tryptophan fluorescence (Figure 7) suggests that there was little contribution of extraliposomal hydrophilic tryptophans to the TEMPO-glucose fluorescence quenching. Figure 7 also suggests that there was an interliposomal hydrophilic contribution to the tryptophan fluorescence quenching by TEMPO-glucose at the later time points. This fluorescence quenching was partially glucose sensitive, suggesting a cotransporter contribution unrelated to substrate transport. The fluorescence quenching seen at the early time points (<90 s) appeared to represent primarily hydrophobic tryptophan residues and accounted for approximately 95% of the observed fluorescence quenching at early time points. At later time points, interliposomal hydrophilic tryptophans may have contributed to the observed fluorescence quenching accounting for approximately 25% at 6 min.

Tables I and II examining the cotransporter conformations by using tryptophan fluorescence provide a unique view of Na^+-dependent cotransport. Within the limitations discussed above (TEMPO-glucose transport competence was approximately 50% that of glucose and TEMPO-glucose fluorescence quenching was not limited to hydrophobic tryptophans), some preliminary conclusions concerning cotransporter-mediated glucose transport can be made.

Multiple tryptophan classes were involved in TEMPO-glucose transport regardless of the cotransporter mode. The effective quenching distance of TEMPO-glucose and the effect of TEMPO-glucose on cotransporter fluorescence indicate that

there are at least two classes of hydrophobic tryptophans [membrane potential sensitive and membrane potential insensitive; compare zero trans uptake in the presence and absence of valinomycin (Figure 8)]. Within these classes two subgroups (Cs^+ sensitive or I^- sensitive and both Cs^+ and I^+ sensitive) may be defined on the basis of the limited (12%) overlap of Cs^+ and I^- fluorescence quenching and the effect of valinomycin on the observed K_S for Cs^+. Further definition of the tryptophan classes would require fluorescence lifetimes and a second method of tryptophan selection.

The cotransporter transport mode and the transport of charge affected cotransporter conformation. The cotransporter conformation in the zero trans transport mode was 50% more I^- sensitive than in the substrate-exchange mode. The addition of a shunt of membrane potential decreased I^- fluorescence quenching but increased Cs^+ fluorescence quenching, suggesting different tryptophan environments under zero trans conditions.

The amino acid sequence of the intestinal Na^+/glucose cotransporter has been reported (Hediger et al., 1987). Hydropathy profiles suggest 11 membrane-spanning regions. A single membrane-spanning segment contains multiple tryptophans, segment number 6, within amino acid residues 272–292. Consistent with the assignment of this segment as involved in substrate transport is the observation that Na^+ binding to the cotransporter appeared to be at or near a tyrosine (Peerce & Wright, 1985) and that Na^+ binding to the cotransporter resulted in increased lysine exposure to the solvent (Peerce & Wright, 1987). The amino acid sequence 229–270, which the hydropathy plots suggest are hydrophilic and possibly glycosylated, contains four tyrosines, and amino acid residues 335–424, which are also likely to be hydrophilic and glycosylated, contain multiple lysines. These two hydrophilic segments border membrane-spanning segment 6.

A unique probe of ion-gradient-driven transport has been described. This probe differs from previous protein probes in that it is not static. The probe mimics the transported species, glucose; however, unlike glucose it is visible during transport. As a fluorescence-quenching reagent TEMPO-glucose may be used in conjunction with both extrinsic and intrinsic fluorophores to perform multiple tasks. Substrate transport under near physiological conditions can be more specifically examined. Extrinsic fluorophores can be added and their effect on transport as well as their location on the cotransporter examined. In cases where multiple fluorophores bind, TEMPO-glucose may provide a tool for determining which of these fluorophores is important in substrate transport.

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Registry No. L-Trp, 73-22-3; D-glucose, 50-99-7; TEMPO-glucose, 132802-11-0; fluorescein sulfonate, 105042-60-2.

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