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Examination of the Na^+-Induced Conformational Change of the Intestinal Brush Border Sodium/Glucose Symporter Using Fluorescent Probes†

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ABSTRACT: The Na^+-induced change in conformation of the intestinal brush border glucose carrier has been examined by three procedures. In the first, we have measured the effect of Na^+ on the binding of fluorescein isothiocyanate (FITC) to the glucose site; 100 mM Na increased the specific [blocked by D-glucose, *p*-(chloromercuri)benzenesulfonic acid, and *N*-acetylimidazole] FITC binding to a 75-kilodalton polypeptide 3-fold. In the second series, we have examined the effect of Na^+ on the susceptibility of the fluorescently labeled glucose site [pyrene isothiocyanate (PYTC) labeled] to a hydrophilic quencher (Tl^+); 100 mM NaCl increased the fraction of PYTC sites available to Tl^+ from 32% to 92% and decreased the apparent quenching constant from 94 to 44 M^{-1}. Finally, in the third series, we probed the distribution of tryptophan residues 15-30 Å from the glucose site using a "distant reporter group method", where tryptophan was used an an energy donor to anthracene isothiocyanate bound to the glucose site. Tryptophan quench reagents (I^-, Cs^+, and acrylamide) were then employed to probe the accessibility of the glucose site tryptophans in the presence and absence of sodium. In the absence of Na^+, there were two major classes of glucose tryptophans—exterior surface residues and residues buried in the hydrophobic protein matrix. Na^+ caused a redistribution of the donor tryptophans such that a higher percentage were accessible to I^- (51% vs. 25%) and fewer were accessible to Cs^+ (13% vs. 25%) and acrylamide (27% vs. 57%). These results indicate that during the Na^+-induced conformational change, there is a redistribution of tryptophans at the surface of the protein. These short- and long-range conformational changes induced by Na^+ are consistent with the Na^+-induced increase in accessibility of the glucose site to D-glucose, phlorizin, and FITC.

The Na^+ and glucose binding sites of the intestinal brush border Na^+/glucose cotransporter are located on a 75000-dalton polypeptide (Peerce & Wright, 1984a,b, 1985). Furthermore, Na^+ binding to the carrier produces a conformational change at the glucose site which results in an increase in the affinity for glucose binding (Kaunitz & Wright, 1984; Peerce & Wright, 1984a,b). The conformational change is also observed as a Na^+-dependent quench of fluorescein isothiocyanate (FITC)[1] bound to the glucose site on the carrier.

In the present study, we have used two new isothiocyanate derivatives, 1-pyrene isothiocyanate (PYTC) and 2-anthracene isothiocyanate (AITC), to probe the structure of the glucose site in its two conformations. With PYTC, Tl^+ quenching was used to determine the fraction of pyrenes that are exposed to the external aqueous environment in the presence and absence of Na^+. AITC, on the other hand, was used to monitor the solvent exposure of tryptophan residues close to the glucose site. AITC is a good energy acceptor up to 30 Å away from donor tryptophans. Tryptophan quench reagents (Eftink & Ghiron, 1981) were then employed to examine the solvent exposure and charge surrounding these neighborhood residues in the two conformations of the glucose site.

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[1] Abbreviations: PITC, phenyl isothiocyanate; FITC, fluorescein isothiocyanate; PYTC, pyrene isothiocyanate; AITC, anthracene isothiocyanate; Trp_G, tryptophan residues energy-donating to the glucose site; TFE, 2,2,2-trifluoroethanol; kDa, kilodalton(s); pCMBS, *p*-(chloromercuri)benzenesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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These studies suggest that in response to Na^+, the glucose binding site becomes more solvent exposed and that there is a redistribution and change of access of the tryptophan residues 15–30 Å from the glucose site.

Materials and Methods

Isolation of Brush Border Membranes. Rabbit small intestinal brush borders were prepared by a Ca^{2+} precipitation procedure (Stevens et al., 1982) and were then treated with KSCN to remove core material (Peerce & Wright, 1984a,b). The membranes were 70-fold enriched in alkaline phosphatase and 125-fold enriched in γ-glutamyl transpeptidase and sucrase activities over that seen in the initial homogenate. Following isolation, the vesicles were stored in 300 mM mannitol and 10 mM HEPES/Tris, pH 7.5, at liquid nitrogen temperature until needed. Storage for up to 3 weeks was found not to affect the brush border enzyme activities or Na^+-dependent glucose uptake.

Glucose Transport Assay. Initial rates of Na^+-dependent glucose uptake were assayed by a rapid-mixing/rapid-filtering procedure (Stevens et al., 1982). Na^+-dependent uptake is defined as uptake in the presence of 100 mM *cis* NaCl minus uptake in the presence of 100 mM *cis* KCl. All uptakes were measured at 22 °C following a 3-s exposure to 50 μM D-[^{3}H]glucose and 100 μg of membrane protein.

Fluorescent Labeling of the Glucose Carrier Vesicles. Vesicles to be labeled specifically at the glucose site were pretreated with 2 mM phenylisothiocyanate (PITC) in the presence of 100 mM NaCl, 10 mM glucose, and 50 mM Tris-HCl, pH 9.2, + 2 mM EDTA for 30 min at room temperature in order to derivatize nonspecific sites (Peerce & Wright, 1984ab). Final protein concentration was maintained at 500 μg/mL. The reaction was stopped by the addition of ice-cold 50 mM Tris-HCl, pH 9.2, and 2 mM EDTA and centrifuged for 30 min at 38000*g*. Following PITC pretreatment, vesicles were exposed to 50 μM FITC, AITC, or PYTC in 50 mM Tris-HCl, pH 9.2, + 2 mM EDTA for 15 min at 22 °C in the dark in order to label the glucose site (Peerce & Wright, 1984a,b). The reaction was stopped by the addition of ice-cold 50 mM Tris-HCl, pH 9.2, + 2 mM EDTA, and the mixture was centrifuged for 30 min at 38000*g*. The pellets were resuspended in 300 mM mannitol and 10 mM HEPES/Tris, pH 7.5.

In some experiments, following labeling and resuspension of FITC-labeled vesicles, the labeled vesicles were exposed to FITC for a second 15-min incubation in the presence of varying concentrations of Na^+, K^+, or Li^+, with and without 10 mM glucose as described above. The effect of *N*-acetylimidazole or pCMBS on the Na^+ increment of FITC binding was examined by adding varying amounts of pCMBS or *N*-acetylimidazole for 30 and 60 min, respectively, to 50 mM potassium phosphate buffer, pH 7.5, prior to the second exposure to FITC. The reactions were stopped by the addition of a 20-fold excess of ice-cold buffer, and the vesicles were centrifuged and resuspended as described above. The amount of FITC bound was determined by using the extinction coefficient for FITC of 75×10^3 cm^{-1} M^{-1} and an Aminco-SLM DW2-C dual-beam spectrophotometer, and, where appropriate, the amount of FITC bound to the carrier was determined by subtraction of the total FITC bound from FITC binding in the presence of 100 mM Na and 10 mM glucose.

Fluorescence. Fluorescence experiments were performed on an Aminco-SLM SPF-500 spectrofluorometer at 22 °C set in the ratio mode. The excitation and emission wavelengths were set as follows: for FITC, 492 and 522 nm; for AITC, 337 and 401 nm; and for PYTC, 330 and 383 nm, respectively.

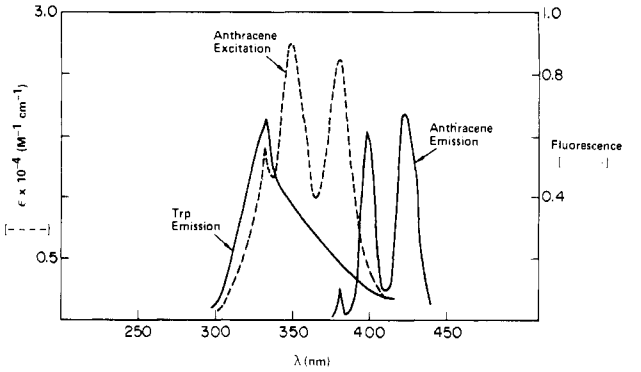

FIGURE 1: Spectral relationship between brush border membrane tryptophans and AITC-labeled membranes. One hundred micrograms of brush border membrane protein was added to 2.5 mL of 50 mM Tris-HCl, pH 7.4, and 2 mM EDTA. Tryptophan fluorescence emission was determined with an excitation of 290 nm. Anthracene isothiocyanate labeled vesicles (100 mg) were added to 2.5 mL of buffer, and emission and excitation spectra were determined. When excitation was scanned, the emission wavelength was set at 401 nm. When emission was scanned, the excitation wavelength was set at 337 nm. Excitation and emission spectra are uncorrected. Solid lines are emission spectra, and the broken line is the excitation spectrum of anthracine. All slits were 2 nm.

For tryptophan fluorescence, the excitation wavelength was set at 290 nm, and the emission was monitored at 338 nm. For typtophan to anthracene energy transfer, the excitation was set at 290 nm, and the emission was monitored at 401 nm. Slit widths for both excitation and emission were set at 2 nm. Fluorescence experiments were performed with 100 μg of membrane protein in 3 mL of 50 mM Tris-HCl, pH 7.5, and 2 mM EDTA. Inner filter effects were at a minimum since the optical density was less than 0.02 ODU. Tl^+ quenching of pyrene fluorescence was performed in 50 mM Tris–nitrate, pH 7.5, + 2 mM EDTA and, where appropriate, in $NaNO_3$ to avoid problems associated with Tl^+ solubility in Cl^--containing solutions.

Tryptophan quench studies were performed in 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 1 M KCl. High salt was added to avoid problems in the determination of quench constants in varying ionic strength media and to minimize the amount of protein required per quench curve. $Na_2S_2O_3$ (1 mM) was added to the KI solutions to avoid formation of I_3^-.

Quenching of fluorescence was analyzed in terms of the modified Stern–Volmer equation (Lehrer, 1971; Lehrer & Leavis, 1978; Eftink & Ghiron, 1981):

$$F_0/\Delta F = 1/f_a K[Q] + 1/f_a \quad (1)$$

where F_0 is the fluorescence in the absence of quencher, ΔF is the change in fluorescence on addition of a quencher (Q) to a concentration [Q], f_a is the effective maximal fraction of the fluorescence accessible to the quencher at infinite quencher concentration, and K is a Stern–Volmer quench constant. Plots of $F_0/\Delta F$ vs. $1/[Q]$ were linear with a y intercept of f_a^{-1} and a slope of $(f_a K)^{-1}$.

To examine tryptophan residues near the glucose site on the transport protein, we have combined tryptophan quench experiments with fluorescence energy transfer. This is a variant of the "distant reporter group method" used by Zukin et al. (1977) to study the salmonella galactose binding protein. A suitable tryptophan energy acceptor is covalently bound to the glucose site, anthracene isothiocyanate, and the sensitized acceptor fluorescence is monitored in the presence and absence of tryptophan quench reagents; i.e., the anthracene is used as a reporter group for the quenching of tryptophans within 15–35 Å of the glucose site.

Figure 1 shows the spectral overlap between brush border membrane tryptophan emission and anthracene excitation. Tryptophan was excited at 290 nm, and tryptophan emission was observed with a peak at 334 nm. The anthracene excitation spectra exhibited a good overlap with tryptophan emission, while the anthracene emission spectra did not. Hence, observing anthracene emission at 401 nm with excitation at 290 nm provides a measure of energy transfer from tryptophan to anthracene.

We have calculated the Forster critical distance, R_0, for tryptophan to anthracene [see Lakowicz (1983)] using the formula

$$R_0 = (9.79 \times 10^3)(J\phi_D\eta^{-4}k^2)^{1/6}\ \text{Å} \quad (2)$$

where J is the spectral overlap integral and ϕ_D the tryptophan quantum yield in the absence of anthracene [0.14 according to the method of Parker and Rees (1968) with quinine sulfate in H_2SO_4 as a quantum counter (Weber & Teale, 1957)]. The orientation factor (k^2) was assumed to be random ($^2/_3$), and the refractive index (η) was assumed to be 1.4. R_0 is 21 Å, which indicates that anthracene is an efficient reporter of tryptophans within 15–30 Å of the glucose site. However, since the number of tryptophan donors is unknown, it is not possible to be more precise about the actual distance between the donors and acceptors.

The experimental approach was then to examine sensitized anthracene emission as a function of tryptophan quenchers in the presence and absence of 100 mM NaCl. As for quenching of bulk membrane tryptophans, the results are analyzed in terms of the modified Stern–Volmer equation (see above). In all cases, plots of $F_0/\Delta F$ vs. $1/[Q]$ were linear over the concentration range examined (0.01–0.1 M).

One can describe the rate of energy transfer (k_t) between tryptophan and anthracene as

$$k_t = \frac{1}{\tau_d}\left(\frac{R_0}{r}\right)^6 \quad (3)$$

and the efficiency of energy transfer, E, as

$$E = \frac{k_t}{1/\tau_d + k_t} \quad (4)$$

and

$$E = 1 - \tau_{da}/\tau_d \quad (5)$$

where τ_d and τ_{da} are the tryptophan lifetimes in the absence and presence of anthracene, respectively, and r is the distance between tryptophan and anthracene.

It can be shown that

$$\tau_{da} = \tau_d\left[1 - \frac{(R_0/r)^6}{1 + (R_0/r)^6}\right] \quad (6)$$

In the absence of quencher in these transfer experiments, this τ_{da} may be substituted for τ_0 in the Stern–Volmer equation, assuming $K_a = k_q'\tau_{da}$, i.e.

$$\frac{F_0}{\Delta F} = \frac{1}{f_a k_q'\tau_{da}[Q]} + \frac{1}{f_a} \quad (7)$$

A plot of $F_0/\Delta F$ vs. $1/[Q]$ still results in a y intercept of $1/f_a$, but the slope is

$$(f_a k_q'\tau_{da})^{-1} = f_a k_q'\tau_d\left(1 - \frac{(R_0/r)^6}{1 + (R_0/r)^6}\right)^{-1} = (f_a X_a)^{-1} \quad (8)$$

Since the apparent quench constant is dependent upon the distance of the donor to the reporter group, as well as the tryptophan quench constants, we have designated it X_a (eq 8) to distinguish it from the usual Stern–Volmer quench constants (K_{SV}, K).

Equation 8 predicts that this distant reporter group method selects for those tryptophan donors between 15 and 30 Å distant from the fluorescence energy acceptor. At these distances, there is sufficient energy transfer for the donated energy to contribute to the sensitized acceptor emission, while at the same time quench reagents can still affect the donation. Donors further away than 30 Å will contribute less than 10% of their energy to the acceptor, and donors closer than 15 Å will contribute almost 90% of their energy to the acceptor, even in the presence of quencher. Therefore, donors >30 Å and <15 Å should contribute little to the observed quenching. The fact that acrylamide can quench almost 100% of the sensitized emission (Table IH) suggests that there is no tryptophan within 15 Å of the acceptor. Since it appears that all energy donors can be quenched by quench reagents, most or all should be 15–30 Å distant from the glucose site.

In this study, we used acrylamide as an efficient neutral collisional quencher ($\gamma = 1$), iodide as an efficient negatively charged collisional quencher ($\gamma = 1$), and cesium as a positively charged collisional quencher ($\gamma = 0.2$) (Eftink & Ghiron, 1981; Lehrer & Leavis, 1978).

In fluorescence quenching experiments, the absorbance of NO_3^- at 338 nm and acrylamide at 290 and 338 nm was corrected by the method of Parker (1968). Inner filter effects were minimized by maintaining absorbances of less than 0.02 ODU for all experiments using tryptophan fluorescence or extrinsic fluorescent probes. Background fluorescence was corrected for by the method of McClure and Edelman (1967). Light scatter was corrected by blanking against deproteinated brush border membrane vesicles. All fluorescent results are reported as uncorrected fluorescence emission spectra.

Membrane disintegration was carried out with TFE (2,2,2-trifluoroethanol, Aldrich, Goldlabel) to examine the effect of hydrophilic quench reagents on groups at the internal surface of the vesicle and at the protein–membrane lipid interface (Shinitzky & Rivnoy, 1977). Vesicles were added to a mixture consisting of 2 volumes of TFE and 1 volume of buffering 150 mM Tris-HCl, pH 7.5, and 6 mM EDTA for 20 min at 22 °C. TFE, like trichloroethanol, is believed to preserve the tertiary structure of proteins in solution, but TFE is more stable in aqueous solutions.

SDS–Polyacrylamide Gel Electrophoresis. SDS–PAGE was performed on 10–15% linear gradient slab gels according to the method of Laemmli as described previously (Peerce & Wright, 1984a,b, 1985).

Enzyme Assays. Brush border marker enzymes (Peerce & Wright, 1984a,b, 1985) were routinely used to monitor vesicle purification.

Results

Fluorescent Probes at the Glucose Site. Figure 2 shows the effect of Na^+ on FITC binding to the glucose site on the glucose carrier. In these experiments, the amount of specific FITC binding [i.e., the difference between FITC binding in the presence and absence of substrates (100 mM NaCl and 10 mM D-glucose)] was first determined following PITC treatment as described under Materials and Methods. The specific FITC binding (0.7 nmol/mg) is similar to that reported previously (Peerce & Wright, 1984b). Membranes were then reexposed to FITC in the presence or absence of 0–100 mM NaCl, KCl, or LiCl. With KCl or LiCl, there was no significant increase in FITC binding, but with NaCl, FITC

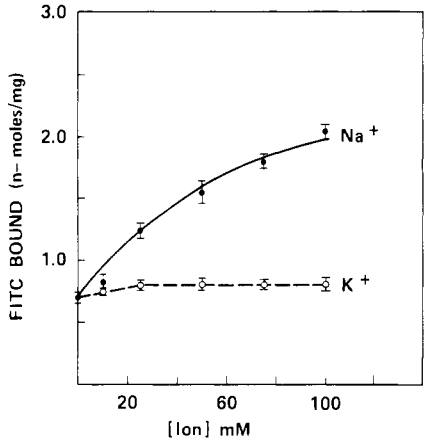

FIGURE 2: Ion dependence of FITC binding. Five hundred micrograms of membrane protein was pretreated with PITC and substrates, followed by reaction with FITC as described under Materials and Methods. Samples were then exposed to FITC a second time in the presence of K^+ (open circles, broken line) or Na^+ (closed circles, solid line) or Li^+ (not shown) in 50 mM Tris-HCl, pH 9.2, and 2 mM EDTA. FITC was determined by the absorbance at 490 nm. Where appropriate, 10 mM glucose, *N*-acetylimidazole, or pCMBS was added during the second incubation with FITC. The results are averages ± standard errors of duplicate determinations. The results are representative of three separate determinations.

binding increased in a saturable manner toward a maximum of 2 nmol/mg; 10 mM D-glucose blocked this Na-dependent increase in FITC binding. The 3-fold increase in specific FITC binding was associated with a small increase in the inhibition of Na^+-dependent glucose transport (from 70 ± 5% to 85 ± 3% in three experiments). SDS–PAGE revealed that the Na^+-induced increase in specific FITC binding was to the same 75-kDa polypeptide previously identified as the glucose carrier (Peerce & Wright, 1984a,b).

N-Acetylimidazole, a tyrosine group specific reagent that reacts with the glucose carrier sodium site (Peerce & Wright, 1984b, 1985), blocks the Na^+-dependent increment in FITC binding with an apparent inhibitor constant of 45 ± 5 μM (n = 3), which is similar to that for inhibition of glucose transport and the Na^+ quench of FITC fluorescence (Peerce & Wright, 1985). pCMBS inhibits the increment in FITC binding 60% with an apparent inhibitor constant of 50 ± 7 μM (n = 4), which again is similar to that for inhibition of transport and quenching (Peerce & Wright, 1985).

These data suggest that the Na^+-induced conformational change of this transport protein triples the number of lysine residues at the glucose sites that are accessible for reaction with FITC. Preliminary energy-transfer studies between the two populations of binding sites using FITC and eosin isothiocyanate indicate that they are at the same sites, i.e., within 10 Å of each other.

Experiments with pyrene and anthracene isothiocyanates demonstrate that these analogues of phenyl and fluorescein isothiocyanates also inhibit Na^+-dependent glucose transport. As shown in Table I, Na^+ produces a specific quenching of PYTC and AITC fluorescence comparable in magnitude to FITC (Peerce & Wright, 1984a,b). The PYTC and AITC quenches are consistently a little smaller than that with FITC, which may be related to differences in their fluorescence lifetimes.

Tl^+ Quenching and Pyrene Fluorescence. We have used thallium quenching of pyrene (Copper & Thomas, 1977) as a probe of glucose site exposure to solvent. Figure 3 shows Tl^+ quench of PYTC bound specifically to the glucose site in Na^+-free buffers. Tl^+ produces a saturable quench of PYTC fluorescence, with a maximum quenching of 20%. In the

Table I: Na^+ vs. K^+ Quench of Fluorescent Probes[a]

fluorophore	$\Delta F/F$ (%) Na^+	$\Delta F/F$ (%) K^+
fluorescein	24 ± 2 (n = 4)	0.8 ± 0.4 (n = 4)
pyrene	18 ± 1 (n = 4)	0.5 ± 0.4 (n = 4)
anthracene	17 ± 1 (n = 3)	0.5 ± 0.3 (n = 3)

[a] 500 μg–1 mg of brush border membrane protein was pretreated with PITC in the presence of substrates and subsequently reacted with the indicated fluorescent isothiocyanate, and Na^+-induced fluorescence quenching was performed as described under Materials and Methods. All quenching was corrected for non-carrier-related quenching and dilution effects by determining quenching of fluorescent isothiocyanate bound in the presence of substrates and K^+-induced quenching, respectively. The results are the means ± standard errors of three determinations, and the number of experiments is shown in parentheses.

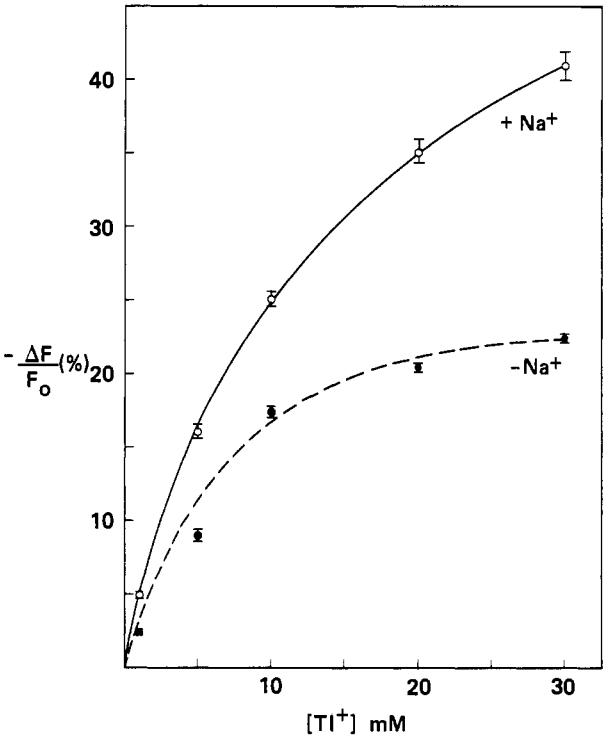

FIGURE 3: Tl^+ quench of PYTC. Protein was pretreated with PITC and substrates, followed by reaction with PYTC as described under Materials and Methods. The PYTC-labeled membranes (100 μg) were added to 50 mM Tris–nitrate, pH 7.4, and 2 nM EDTA. The fluorescence was determined, Tl^+ added, and emission again recorded. Where indicated, 100 mM $NaNO_3$ was added prior to the addition of Tl^+. PYTC was excited at 338 nm, and the emission at 383 nm was recorded. Open circles are Tl^+ quenches after addition of Na^+; closed circles, without Na^+. Results are the averages ± standard errors of duplicate determinations and representative of three separate experiments.

presence of 100 mM $NaNO_3$, the Tl^+ quench increased significantly at all Tl^+ concentrations. At 30 mM Tl^+, close to the limits of $TlNO_3$ solubility, the quenching increased from 20% to 45%. These Tl^+ quenching curves can be fitted by a modified form of the Stern–Volmer equation (Lehrer, 1971), i.e., eq 1.

The modified Stern–Volmer plots ($F_0/\Delta F$ vs. $1/[Q]$) are linear over the concentration range tested, both in the absence and in the presence of Na^+. In four separate experiments, f_a was 0.32 ± 0.03 and K_{SV} was 94 ± 8 M^{-1} in the absence of Na^+, but on the addition of 100 mM $NaNO_+$, f_a increased to 0.92 ± 0.05 and K_{SV} decreased to 44 ± 5 M^{-1}. Na^+, therefore, triples the fraction of PYTC molecules accessible to Tl^+ quenching, and this indicates that Na^+ binding produces a conformational change in the glucose carrier that moves the

Table II: Effect of Tryptophan Quench Reagents on Bulk Tryptophans[a]

quencher	control		TFE	
	K_a (M^{-1})	f_a (%)	K_a (M^{-1})	f_a (%)
I^-	23 ± 2.5 (n = 6)	20 ± 2.5 (n = 6)	15 ± 0.5 (n = 3)	60 ± 2 (n = 3)
Cs^+	60 ± 6 (n = 6)	18 ± 2 (n = 6)	24 ± 2.5 (n = 4)	39 ± 4 (n = 4)
acrylamide	15 ± 2 (n = 4)	79 ± 5 (n = 4)		

[a] The effect of tryptophan quench reagents on bulk tryptophan emission in the absence and presence of TFE was determined as described in Figures 4 and 5. Fractional tryptophan accessibility (f_a) and the apparent quench constant (K_a) were determined by using the modified Stern–Volmer plot. All results are the means ± standard errors of six determinations. The number of experiments for each quench reagent is shown in parentheses.

fluorescent probe from the hydrophobic core to the exterior surface of the plasma membrane. The origin of the decrease in the K_{SV} for Tl^+ on addition of sodium is more ambiguous. A decrease in K_{SV} could be due to a decrease in the lifetime of the pyrene isothiocyanate (τ_0) or a decrease in the bimolecular quench constant (k_q).

Quenching of Membrane Tryptophans. In this series of experiments, the effect of quench reagents on the bulk tryptophans in brush border membranes was examined. As shown in Figure 4, I^- quenches the bulk tryptophans. The quenching curve fits the modified Stern–Volmer equation (eq 1), and the f_a's and K_a's obtained are summarized in Table II. The fractions of the membrane tryptophans accessible to the quenchers (f_a) were I^-, 20%; Cs^+, 18%; and acrylamide, 75%. It should be noted that the hydrophilic quenchers I^- and Cs^+ quenched approximately one-fifth of the tryptophans in native membranes, but upon disintegration of the membrane with TFE, the fractions of tryptophans accessible to I^- tripled, and Cs^+ doubled. Since the brush border membrane permeability to Cs^+ is low (Gunther et al., 1983), the increased Cs^+ quenching suggests that 20% of the total tryptophans are at the outside surface, about 20% are at the intravesicular surface of the membrane, and the remainder are in the hydrophobic core of the membrane. The effect of TFE on I^- may suggest an effect of TFE in addition to membrane disruption. Acrylamide has good access to those hydrophobic tryptophans in the absence of TFE ($f_a \sim 75\%$). The I^- and acrylamide quenching constants and the Cs^+ quenching constant obtained in the presence of TFE (15–24 M^{-1}) yield bimolecular rate constants expected [$(0.2–3) \times 10^{10}$ M^{-1} s^{-1}] for diffusional quenching of tryptophans with lifetimes of 0.5–8 ns. The higher than expected bimolecular rate constants for Cs^+ in intact membranes (2–3-fold greater than in the TFE membranes) could be due to preferential localization of Cs^+ around negatively charged groups close to the exterior tryptophans, but additional measurements are required to confirm this interpretation.

Quenching Tryptophans Close to the Glucose Site. A disadvantage of the tryptophan quench experiments with brush border membranes described above is that they are nonspecific; i.e., it is impossible to assign the results to the tryptophans on a particular membrane protein. Our approach to overcoming this problem is the "distant reporter group method" (see Materials and Methods).

We examined the effect of tryptophan quenchers on the tryptophan residues close to the glucose site (Trp_G) by exciting at 290 nm and recording the anthracene emission at 401 nm. Direct effects of the tryptophan quenching reagents on anthracene fluorescence were checked by examining the effect of KI, CsCl, and acrylamide on anthracene emission when anthracene was excited at 337 nm. I^- at concentrations as high as 400 mM quenched less than 4% of the anthracene fluorescence directly. Cs^+ did not quench anthracene either in the presence or in the absence of Na^+. Acrylamide quenched less than 5% of the anthracene fluorescence in the presence or absence of Na^+.

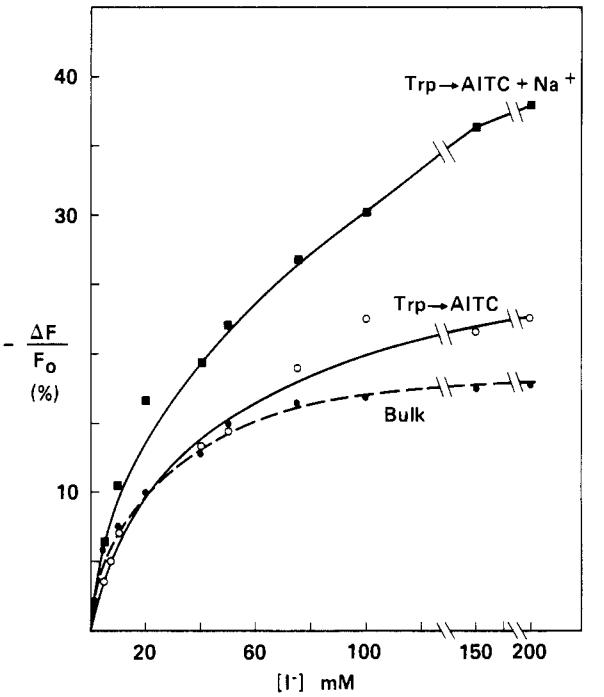

FIGURE 4: Iodide quenching of tryptophan emission. Varying concentrations of I^- were added to 100–150 μg of brush border membranes labeled with AITC in 50 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 500 mM KCl, or 400 mM KCl and 100 mM NaCl. In all experiments, 1 mM $Na_2S_2O_3$ was added to prevent I_3^- formation. I^- senstivity of average tryptophans was determined by measuring the emission at 338 nm with excitation at 290 nm (closed circles, broken line). The senstivity of tryptophans energy donating to AITC was determined by exciting at 290 nm and maintaining the emission of AITC at 401 nm in the absence (open circles) or in the presence (closed squares) of 100 mM Na. The results are representative of six separate determinations.

In these experiments, the strategy was to examine the effect of quenching reagents in the presence and absence of Na^+ on Trp_G in native membranes and in TFE-solubilized membranes. The modified Stern–Volmer plots were linear over the concentration range examined (0.01–0.1 M), indicating that each reagent was apparently quenching just one class of Trp_G sites [however, see Eftink and Ghiron (1981) for qualification of this conclusion].

The results are summarized in Figure 4 and Table III. I^- quenches a fraction of the total Trp_G sites in the absence of Na^+, and that addition of Na^+ doubles the fraction accessible (from 25% to 50%). In TFE-treated membranes, the Na^+ effect on the accessibility of Trp_G was maintained, but the apparent quenching constants decreased.

In the case of Cs^+, the fraction of Trp_G sites accessible to quenching is similar to that for I^- in the absence of sodium (Table III). However, Na^+ actually reduced the fraction of Trp_G sites accessible to Cs^+ in both intact (from 25% to 13%) and solubilized (from 14% to 9%) membranes. These results suggest that the Trp_G sites at the external surface of the protein are equally accessible to Cs^+ and I^- but on changing the

Table III: Effect of Tryptophan Quench Reagents on Tryptophans near the Glucose Site[a]

	control		TFE	
quencher	X_a (M^{-1})	f_a (%)	K_a (M^{-1})	f_a (%)
I^-	56 ± 6 (n = 5)	25 ± 3 (n = 5)	19 ± 2 (n = 3)	28 ± 3 (n = 3)
$+Na^+$	45 ± 6 (n = 4)	51 ± 4 (n = 4)	24 ± 2 (n = 3)	42 ± 4 (n = 3)
Cs^+	64 ± 6 (n = 3)	25 ± 5 (n = 3)	59 ± 3 (n = 3)	14 ± 1 (n = 3)
$+Na^+$	66 ± 10 (n = 3)	13 ± 3 (n = 3)	53 ± 6 (n = 3)	9 ± 0.5 (n = 3)
acrylamide	30 ± 5 (n = 4)	57 ± 2 (n = 4)	16 ± 1 (n = 3)	84 ± 4 (n = 3)
$+Na^+$	60 ± 5 (n = 4)	27 ± 3 (n = 4)	14 ± 1 (n = 3)	100 ± 7 (n = 3)

[a] The effect of tryptophan quench reagents on tryptophan energy donating to AITC was determined as described under Materials and Methods. Fractional accessibility (f_a) and the apparent effective quench constant (X_a) were determined from modified Stern–Volmer plots. All results are means ± standard errors, and the number of experiments for each reagent is shown in parentheses.

conformation of the transport protein by Na^+, the glucose site tryptophans become more accessible to I^- and less accessible to Cs^+.

The Trp_G sites are virtually all accessible to acrylamide in TFE. These observations are consistent with the notion that acrylamide is able to diffuse into the interior of proteins (Eftink & Ghiron, 1981). Surprisingly, in the intact membranes, acrylamide did not reach all the Trp_G sites ($f_a \sim 57\%$), and Na^+ reduced the number of sites accessible to acrylamide (from 57% to 28%). This Na^+ effect was lost upon treating the membranes with TFE (Table III). One interpretation of these observations is that Na^+ changes the conformation of the glucose carrier in the intact membrane such that acrylamide is no longer able to diffuse through the membrane matrix to reach the hydrophobic Trp_G sites. This is supported by the acrylamide quenching constant data: in intact membranes, the acrylamide X_a was 2-fold higher in the presence of Na^+ than in the absence of Na^+.

Cs^+ quenched 25% of the Trp_G with a quenching constant X_a of 60 M^{-1}. In the presence of Na^+, the Cs^+ quenching constant X_a remained unaffected, but the tryptophans accessible were halved. Making the membranes permeable by the addition of TFE had no effect on X_a but did appear to convert the Na-free cotransporter to the Na-bound form. This may relate to an additional effect of TFE to promote α-helix formation (Long et al., 1977).

The I^- susceptibility of Trp_G in the absence of Na^+ was similar to bulk tryptophans. Addition of Na^+ had little effect on X_a but doubled the Trp_G accessible to quencher. The effect of Na^+ was reduced, but not eliminated, in TFE, while X_a decreased by half. The effect of Na^+ on the iodide-susceptible Trp_G suggests two Trp_G conformations which differ primarily in accessibility to quencher. Exposure to TFE results in a third conformation similar to bulk in quenching constant, but with increased quencher accessibility. Since the Na^+ quench is retained in TFE-treated membranes, the tryptophans that we have labeled Trp_G must have a passive role in the Na^+-induced conformational change.

Discussion

Kinetic experiments on the intestinal Na/glucose cotransporter have suggested that binding of Na^+ results in an increase in the carrier affinity for the second substrate, glucose (Kaunitz & Wright, 1984). We have described a Na^+-selective quench of FITC fluorescence when FITC is bound to the glucose site which is consistent with such a Na^+-induced conformational change (Peerce & Wright, 1984a,b). Here we extend our initial observations to the nature of the conformational change at the fluorescent probe binding site and the region neighboring that site.

The nature of the Na^+-induced conformational change may be divided into two classes: those changes occurring at or near the glucose site and those changes occurring at a region of the cotransporter removed from the site. Evidence suggesting that more than the glucose site is involved in the conformational change is indicated by the existence of an SH residue removed from the active sites that is essential for Na-dependent glucose uptake, phlorizin binding, and Na-induced quenching of FITC fluorescence (Semenza et al., 1984; Peerce & Wright, 1984a,b). This view is strengthened by fluorescence energy-transfer experiments (Peerce & Wright, 1986) which indicate that the Na^+ and glucose binding sites are separated by some 35 Å.

At the glucose site, we have observed two major responses to Na^+ binding. There is an apparent increase in the amount of FITC which selectively binds at or near the glucose site. The increase in FITC binding is localized to the 75-kDa polypeptide previously demonstrated to contain the FITC binding polypeptide, and this binding is glucose and *N*-acetylimidazole sensitive (Na^+ site blocker). The increase in FITC binding is also specific for Na^+ as K^+ and Li^+ have no effect, consistent with activation of glucose uptake and phlorizin binding.

The second response is increased solvent exposure of the site. The PYTC fluorescence quench by the heavy atom quencher Tl^+ more than doubles following the addition of Na^+. Modified Stern–Volmer plots of the fluorescence quench indicate that the fraction of accessible pyrenes triples, while the apparent quenching constant is reduced from 94 to 44 M^{-1}. This suggests an increase in the solvent exposure of the site rather than a localized change in the pyrene or the nature of the protein residues near pyrene which may be contributing to the quenching. These results also indicate that pyrene is not freely accessible to the solvent in the absence of Na^+ but rather is embedded in a region of the protein which is inaccessible to solvent.

A method to examine regions of the cotransporter removed from the substrate binding sites was developed to extend our analysis of the protein conformational change beyond the glucose site. This fluorescence energy-transfer method involves excitation of tryptophan residues nonselectively at 290 nm. Then, using the limited nature of tryptophan fluorescence emission, observe only those tryptophans near the glucose site by monitoring the sensitized emission of anthracene bound to the glucose site. The nature of those tryptophans is then examined by using classical tryptophan quenching reagents [see Eftink and Ghiron (1981)] in the presence or absence of Na^+.

Comparison of eq 1 and eq 7 illustrates the difference between classical tryptophan quenching and tryptophan quenching by the "distant reporter group method". Equation 7 contains two contributions to the observed fluorescence decrease: the quench due to added donor quench reagent and a contribution due to acceptor excitation. The effectiveness of the acceptor-induced tryptophan fluorescence quench is dependent on the distance separating the donor/acceptor pair. Measurements of an apparent effective quenching constant

(X_a) do not separate the contribution by each component, and therefore, X_a is not interchangeable with K_{SV} (the Stern–Volmer quenching constant) or K [the effective quenching constant, a Stern–Volmer quenching constant in a heterogeneously emitting system (Eftink & Gihron, 1981)]. The value of X_a is related to K_{SV} and K by eq 8.

This relationship predicts that X_a approaches K_{SV} and K as the distance between fluorophores increases. When the Trp donor and AITC are at R_0, X_a is twice K_{SV}, and at $2R_0$, X_a is K_{SV} (±1.5%). At the level of resolution inherent in the examination of Trp_G, there are two possible sources of change in the apparent effective quenching constant (X_a). The first is due to inherent changes in Trp itself, such as a change in its fluorescence lifetime or a change in its accessibility to quencher. The second source is movement of Trp away from or toward the reporting group or movement of the reporting group away from or toward the donor. Movement toward the glucose site would result in decreased X_a, since a greater percentage of the tryptophan's fluorescence energy would be transferred even in the presence of quencher. Movement away from the reporting group would result in an increase in X_a and a greater effect of quencher with X_a approaching K_{SV}. The source of alterations in tryptophan fluorescence is potentially interesting since it would further define the Na-induced conformational change. At the same time, the inability to ascribe changes in X_a to movement of the reporting or donating groups, or to inherent changes in the donor, does not alter the fundamental conclusions. The effects of I^- and acrylamide in the presence of Na^+ are divergent. If the sole response to Na was movement of the donor or acceptor, then both reagents would show increases in X_a. We are also aware of the complications arising in the interpretation of steady-state tryptophan quench experiments in globular proteins containing an unknown number of tryptophan residues [see, for example, the review by Eftink and Ghiron (1981)]. The bulk brush border tryptophan quenching data are presented to illustrate that Trp_G is a subclass of the bulk tryptophans. We feel that within these constraints, we can draw a number of tentative conclusions about the distribution of tryptophans on the glucose carrier and the Na^+-induced conformational changes relating to the tryptophans close to the glucose binding sites.

First, there are two major distinct populations of tryptophans near the glucose site (Trp_G): (a) those buried in the hydrophobic core of the protein [these are quenched by acrylamide in TFE-treated membranes (Table III, f_a = 100%)] and (b) those on the exterior surface of the membrane that are quenched by the hydrophilic quenchers Cs^+ and I^- (Table III, f_a = 10–50%). All these tryptophans are on the exterior surface as judged by the fact that, unlike bulk tryptophans (Table II), TFE did not increase the fraction accessible to Cs^+.

Acrylamide is a nonpolar collisional quencher that has varied access to tryptophan residues in the hydrophobic core of globular proteins (Eftink & Ghiron, 1981; Eftink & Hagamon, 1985). As with oxygen (Lakowicz & Weber, 1973), acrylamide penetration of proteins is permitted by rapid (nanosecond) fluctuations in the conformation that open and close the protein matrix. The larger acrylamide requires larger fluctuations to gain access, and so there is variation from protein to protein in the ability of acrylamide to quench hydrophobic tryptophans. TFE perturbs the structure of the glucose carrier to expose most of the tryptophans to acrylamide (~84%, Table III).

Second, Na^+ produces marked changes in the accessibility of the Trp_G residues to quenchers. Namely, Na^+ decreases the fraction exposed to Cs^+ and acrylamide and increases the fraction exposed to I^- (Table III). If we assume that the tryptophan energy donors are identical in the two conformational states of the glucose carrier, these changes in tryptophans accessible to I^-, Cs^+, and acrylamide suggest a major reorientation of the protein; e.g., more I^--susceptible tryptophans appear at the exterior surface of the protein, and there is condensation of the protein to restrict the access of acrylamide to hydrophobic tryptophans. Somewhat surprising, however, is the observation that in the presence of Na^+ the fraction accessible to I^- is twice as high as that accessible to acrylamide; i.e., acrylamide is unable to quench all the external surface-exposed tryptophan residues in membranes. Note that this effect is eliminated upon addition of TFE. These differential effects of Na^+ on the I^-, Cs^+, and acrylamide X_a's and f_a's indicate a marked heterogeneity among the tryptophan residues donating to anthracene at the glucose site. The number and location of the tryptophan residues near the glucose site in its two conformations await further structural analysis of this membrane transport protein.

These considerations lead to the conclusion that Na^+ produces rather global conformational changes on the intestinal brush border Na^+/glucose carrier. Binding of Na^+ to the 75-kDa transport protein at a site 30–40 Å away from the glucose binding site (Peerce & Wright, 1986) produces an increase in solvent exposure of the glucose binding site (increase quenching of PYTC by Tl^+, Figure 2) and a redistribution of the tryptophan residues 15–30 Å from the glucose binding site (Figures 4 and 5 and Table III). Similar long-range conformational changes in other transport proteins have been reported. For example, the Na/K- and Ca-ATPases undergo ligand-induced conformational changes that can be detected by changes in tryptic digestion patterns and/or fluorescent signals (Jorgensen, 1985; Karlish, 1981). In bacteria, the distant-reporter group method has also been quite powerful in documenting that sugar binding to receptors produces conformational changes that are propagated over 30–80 Å (Zukin et al., 1979a,b; Zukin, 1979). In the case of the Na/glucose carrier, these conformational changes underlie the Na-dependent increase in affinity for glucose binding, but their role in the translocation process has yet to be resolved. Lifetime measurements of the intrinsic fluorescence and of extrinsic fluorescence probes may provide additional unique information into the conformational changes associated with the binding and transport of Na^+ and glucose across the brush border membrane.

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Novel Fluorescent Phospholipids for Assays of Lipid Mixing between Membranes†

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ABSTRACT: A series of fluorescent phospholipids has been synthesized, by a general and versatile procedure, with various fluorescent groups attached to the methyl-terminal half of one acyl chain in an otherwise normal phospholipid structure. Phospholipids labeled with (dialkylamino)coumarin moieties, and to a slightly lesser extent those labeled with a bimane group, exhibit a strong and stable blue fluorescence in phospholipid dispersions that is relatively insensitive to the physical state of the lipid phase. The fluorescence of these labeled phospholipids is efficiently quenched by resonance energy transfer to lipids labeled with a [[(dimethylamino)phenyl]azo]phenyl or a methyl(nitrobenzoxadiazolyl)amino group when these acceptors are incorporated into the same bilayer as the donor species. Acyl chain labeled phospholipid probes, both of whose chains are at least sixteen carbons in length, exchange extremely slowly between lipid vesicles (<1% exchange/h). These properties allow various donor–acceptor combinations of probes to be employed in sensitive and reliable assays of lipid mixing accompanying membrane fusion. We demonstrate that, in two particularly demanding applications (assays of the calcium-mediated coalescence of phosphatidylserine vesicles and of the proton-triggered coalescence of phosphatidylethanolamine vesicles), some combinations of acyl chain labeled probes offer substantial advantages over the commonly used *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine/*N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine pair to monitor accurately the progress of lipid mixing between vesicles.

The fusion of a variety of artificial and biological membranes has become an object of intensive study in recent years. Research in this area has been advanced considerably by the development of sensitive fluorometric assays that can monitor either intermixing of the aqueous compartments bounded by two initially distinct membranes or the intermixing of lipids that occurs when two membranes coalesce [for a review, see Düzgünes and Bentz (1986)].

In principle, assays of the mixing of fluorescent lipid probes between membranes can provide a convenient and sensitive method to monitor interactions between two membranes during the process of fusion. For some systems, this approach is more practical than measurements of aqueous contents mixing for monitoring certain aspects of membrane fusion, as lipid-mixing assays are not complicated by the effects of membrane leakiness, and they do not require prior manipulation of the internal aqueous compartments bounded by both membranes (Bental et al., 1984; Morris & Bradley, 1984; Harmsen et al., 1985). Moreover, assays of lipid mixing can be profitably employed to provide information complementary to that provided by contents-mixing assays in the study of systems for which both types of assays are feasible (Ellens et al., 1985; Düzgünes et al., 1985; Leventis et al., 1986).

While a number of assays have been devised to monitor membrane coalescence through lipid mixing, often with considerable success (Gibson & Loew, 1979a; Vanderwerf & Ullman, 1980; Owen, 1980; Struck et al., 1981; Uster & Deamer, 1981; Morgan et al., 1983; Gad & Eytan, 1983; Hoekstra et al., 1985; Pryor et al., 1985; Parente & Lentz,

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