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Changes in the Intrinsic Fluorescence of the Human Erythrocyte Monosaccharide Transporter upon Ligand Binding†

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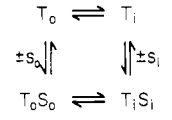
ABSTRACT: The effect of ligands on the tryptophan fluorescence of the purified monosaccharide transporter from human erythrocytes has been investigated. Cytochalasin B, D-glucose, and ethylideneglucose quench the fluorescence of the protein at longer wavelengths by 17%, 13%, and 8%, respectively. Propyl glucoside, another ligand, has no effect on the protein fluorescence. Values of the dissociation constants for cytochalasin B, D-glucose, and ethylideneglucose were determined from the concentration dependence of fluorescence change; these agree with the values obtained from the effects of these compounds upon the binding of [^{3}H]cytochalasin B measured by equilibrium dialysis. There is no correlation between the effect of each ligand on the fluorescence of the transporter and the conformational state expected for its complex on the basis of other evidence. The fact that the quenching is greatest at longer wavelengths suggests that an exposed tryptophan residue(s), possibly located at the ligand binding sites, is the perturbed one.

Most of the information concerning the steady-state kinetics (Geck, 1971; Regen & Tarpley, 1977; Foster & Jacquez, 1976), inhibitor specificity (Barnett et al., 1975), and substrate binding properties (Gorga & Lienhard, 1981) of the monosaccharide transporter of the human erythrocyte is consistent with an alternating conformation model for transport (Vidaver, 1966; Baldwin & Lienhard, 1981). According to this model, the transporter alternates between a conformation in which there is a substrate binding site at the external surface (T_o) and a conformation in which there is a site at the cytoplasmic surface (T_i). Translocation of D-glucose and other substrates occurs when the occupied transporter (T_oS_o, T_iS_i forms) undergoes the conformational change. The postulated species and reactions are summarized in the following scheme:



The monosaccharide transporter has been shown to be a transmembrane protein that does not undergo complete rotation across the bilayer (Baldwin et al., 1980); consequently, the transition between the two conformers is envisioned as a limited structural rearrangement occurring within a protein that has a largely fixed orientation in the bilayer.

Now that the monosaccharide transporter is available in purified form (Sogin & Hinkle, 1980; Baldwin et al., 1980; Baldwin & Baldwin, 1981), it should be possible, if the alternating conformation model is correct, to obtain direct evidence for the occurrence of the two conformations and to determine the values of the rate and equilibrium constants for their interconversion, in both the unoccupied and occupied states. As an initial step in this project, we describe herein changes in the intrinsic fluorescence of the transporter that occur upon ligand binding.

Experimental Procedures

Materials. 4,6-*O*-Ethylidene-α-D-glucose (ethylideneglucose) was purchased from Aldrich; *n*-propyl β-D-glucopyranoside (propyl glucoside) was synthesized after the method of Barnett et al. (1975), as described previously (Gorga & Lienhard, 1981). Both of these sugars were purified by chromatography on cellulose and by treatment with charcoal (Gorga & Lienhard, 1981). D-Glucose (ACS grade from Fisher), L-glucose (Pfanstiehl), cytochalasin B (Aldrich), [^{3}H]cytochalasin B (New England Nuclear), and 21,22-dihydrocytochalasin B (a generous gift from Dr. D. C. Aldridge of ICI Ltd.) were each used as obtained.

Transporter. The glucose transporter was purified from human erythrocyte membranes by a modification (Baldwin & Baldwin, 1981; S. A. Baldwin, J. M. Baldwin, and G. E. Lienhard, unpublished results) of the method described by Baldwin et al. (1980). In this procedure, erythrocyte membranes that have been depleted of peripheral proteins are extracted with 1.35% octyl glucoside, and the transporter is isolated by chromatography of the extract on DEAE-cellulose. The transporter, which, together with a portion of the erythrocyte lipids, is not adsorbed by the DEAE-cellulose, is reconstituted into bilayers through the removal of the detergent by dialysis, without the addition of exogenous phospholipid. This method results in a preparation containing about 130 μg of protein and 400 μg of erythrocyte phospholipid per mL in 100 mM NaCl/50 mM Tris-HCl/1 mM EDTA, pH 7.4, which is frozen in liquid nitrogen and stored at −70 °C.

We have examined the form of the purified, reconstituted transporter by gel filtration on a calibrated column of Bio-Gel

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A150m (Baldwin et al., 1980). Sixty-five percent of the preparation elutes at the void volume and so is in membranous species of greater than 2200-Å diameter; the other 35% exists in species of about 1000-Å diameter. The simultaneous uptake of 100 mM D- and L-glucose by the preparation at 25 °C has been measured (Baldwin et al., 1981). The half-times for equilibration were about 8 and 20 min, respectively. The amount of uptake of each sugar at equilibrium was only about 15% of that expected for a mixture of vesicles of 2200- and 1000-Å diameter. This finding suggests that the membranous species are largely leaky vesicles and unsealed sheets; such structures would be expected at the high ratio of protein to lipid in the preparation.

The transporter preparations used in this study bound 13 nmol of cytochalasin B/mg of protein, as determined by amino acid analysis. Elsewhere (Baldwin & Baldwin, 1981; S. A. Baldwin, J. M. Baldwin, and G. E. Lienhard, unpublished results) we report that partial denaturation of the transporter unavoidably occurs while it is solubilized in detergent during purification and that fully active transporter would be expected to bind about 21 nmol of cytochalasin B/mg. Thus, the preparations used were about 60% active, and it is worth noting that if it were possible to obtain fully active transporter, the changes in fluorescence observed would probably be correspondingly larger.

Fluorescence Measurements. Fluorescence measurements were made with a Perkin-Elmer–Hitachi MPF-2A fluorescence spectrophotometer. The wavelength for excitation was set at 295 nm in order to minimize the contribution of tyrosine residues to the fluorescence (Weber & Teale, 1965); the bandwidth of the excitation beam was 5 nm. The bandwidth of the emission beam was either 6 or 12 nm, depending upon the protein concentration. A filter cutting off at 290 (for complete spectra) or 310 nm (for titrations at 380 nm) was placed in the emission beam in order to reduce the intensity of the peak due to scattering of the excitation beam. The sample compartment of the instrument was maintained at 25 °C with a circulating water bath.

Titration of the transporter with each ligand was accomplished by making sequential additions from a stock solution of the ligand in buffer to the transporter, such that the total increase in volume was less than 15%. After each addition, the contents of the cuvette were mixed by means of a small magnetic stir bar, and the fluorescence emission at 380 nm was monitored for 2 or 3 min. The reading was constant over this period. This wavelength was chosen because, in preliminary experiments, the largest percentage change in fluorescence for each ligand that caused a change was in the region of 380 nm. In each experiment, a control in which aliquots of buffer, instead of ligand solution, were added to the cuvette was performed, in order to determine the fluorescence changes that occurred as the result of dilution of the protein, as well as any drift of the instrument over the period of measurement. It was found that after the fluorescence values of these control mixtures had been corrected for dilution, the values were between 98% and 100% of the initial value. For each titration, the fluorescence values in the presence of ligand have been corrected for dilution of the protein, and the differences between the corrected values and the initial value in the absence of ligand have been expressed as percentages of the initial value.

The fluorescence intensity at 380 nm and the absorbance at 295 nm of each ligand alone were measured at the highest concentration used in the titration. The values were such that the small contribution of the ligand to the overall fluorescence intensity at 380 nm (between 0% and 3% of the total value) was more or less compensated for by the small decrease in fluorescence (between 0% and 3% of the total value) expected for the internal filter effect from the absorbance at 295 nm. For example, the fluorescence intensity of 500 mM D-glucose was 2.0% of that of the transporter under the conditions of the titration (see the legend to Figure 3). On the other hand, the absorbance of 500 mM D-glucose at 295 nm was 0.028, and this absorbance should have led to a 3.2% decrease in fluorescence due to the internal filter effect (Parker, 1968). Because in each case these small changes compensated for one another so that the expected overall change in fluorescence intensity was a decrease of less than 2% at the highest ligand concentration, no corrections have been made for these effects.

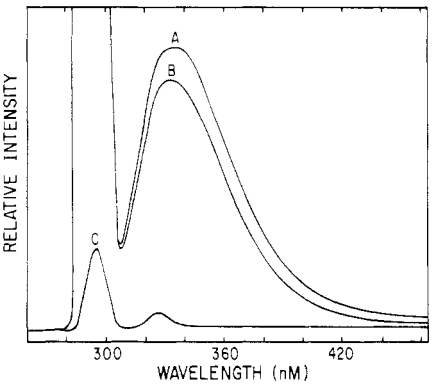

FIGURE 1: Fluorescence emission spectra of the transporter in the absence (A) and presence (B) of cytochalasin B (2.4 μM). The concentration of protein was 13 μg/mL ($[T]_{total}$ = 0.16 μM), and the emission bandwidth was 12 nm. The spectrum of transporter in the presence of 2.4 μM dihydrocytochalasin B was identical with that in the absence of cytochalasin B (A). Curve C is the spectrum of buffer alone.

In one control experiment, complete spectra of the transporter and of the transporter with a high concentration of each compound tested as a ligand were taken within 5 min of the addition of the compound and 1.5 h thereafter. The percentage change in the fluorescence intensity found with each compound was the same after 1.5 h as within 5 min. Thus, in agreement with our expectation from the characterization of the transporter preparation, the binding sites are sufficiently accessible so that equilibrium is approached within 5 min.

Other Methods. The dissociation constants for cytochalasin B and the various sugars at 22–24 °C were also determined by equilibrium dialysis with [^{3}H]cytochalasin B according to methods that we have previously described (Zoccoli et al., 1978; Gorga & Lienhard, 1981). In the case of each sugar, the inhibitory effect of five different concentrations on the binding of [^{3}H]cytochalasin B at a single low concentration (5×10^{-8} M) was determined; the dissociation constant of the sugar was determined from a plot of the ratio of the free concentration of cytochalasin B to the bound concentration against the concentration of sugar (Gorga & Lienhard, 1981).

The buffer used throughout this study was 100 mM NaCl/1 mM EDTA/50 mM Tris-HCl, pH 6.8, at 25 °C. Protein was determined by the Petersen (1977) modification of the Lowry procedure.

Results

Cytochalasin B Binding. Figure 1 (curve A) shows the fluorescence emission spectrum of the reconstituted glucose transporter. The spectrum is characterized by two major peaks, one at 295 nm that is due to scattering of the excitation beam and one, centered at 336 nm, that is due to the tryptophan fluorescence of the protein. Curve B in Figure 1 shows

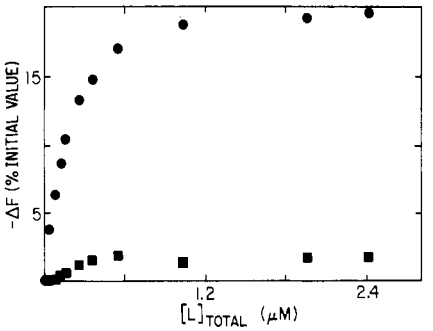

FIGURE 2: Concentration dependence of the fluorescence changes observed with cytochalasin B (●) and dihydrocytochalasin B (■). The initial concentration of protein was 14 μg/mL. Sequential additions of a 40 μM stock solution of each compound were made as described under Experimental Procedures, and the fluorescence was monitored at an emission bandwidth of 12 nm. The small changes observed with dihydrocytochalasin B were similar to those observed when buffer alone was added.

the spectrum of the transporter in the presence of a saturating concentration of cytochalasin B. The peak of this spectrum is shifted about 3 nm toward shorter wavelengths, and the peak height is diminished by approximately 10%, with respect to the spectrum of the unliganded transporter. This cytochalasin B induced fluorescence change is saturable and specific (Figure 2). Dihydrocytochalasin B, which differs from cytochalasin B only by the reduction of a single double bond and which does not bind to the transporter (dissociation constant greater than 20 μM) (Lin et al., 1978; Lin & Lin, 1978), does not alter the fluorescence of the transporter (Figure 2).

The dissociation constant (K_L) for cytochalasin B binding was determined by plotting the data in Figure 2 according to eq 1 (plot not shown). This equation describes a linear plot

$$\frac{F_{TL} - F}{F - F_T}[L]_{total} = \frac{F_{TL} - F}{F_{TL} - F_T}[T]_{total} + K_L \qquad (1)$$

for the case of a simple bimolecular association equilibrium in which a significant fraction of the ligand is bound (Secemski et al., 1972). F_{TL}, F_T, and F are the fluorescence intensities of the ligand–transporter complex, the free transporter, and the mixture, respectively; $[L]_{total}$ and $[T]_{total}$ are the total concentrations of ligand and transporter, respectively. For the latter value, we have used the concentration of cytochalasin B binding sites determined by equilibrium dialysis. The value of K_L obtained from the plot was 0.9×10^{-7} M; it agrees reasonably well with the value of 1.3×10^{-7} M that was obtained by Scatchard plot analysis of [^{3}H]cytochalasin B binding to the same preparation of transporter (data not shown) and with other values in the literature (Sogin & Hinkle, 1978; Baldwin et al., 1980).

Sugar Binding. The binding of D-glucose to the transporter causes a change in fluorescence that is similar to but smaller than that seen with cytochalasin B (spectra not shown). In this case there is a 7% decrease in peak height and about a 2-nm shift in the wavelength of maximum emission. This change is also saturable and specific (Figure 3). L-Glucose, which is a very slowly transported substrate (Lin et al., 1978; Lacko et al., 1972) and has a minimal inhibitory effect upon cytochalasin B binding (Zoccoli et al., 1978), has no effect on the fluorescence of the transporter (Figure 3).

Ethylideneglucose and propyl glucoside, sugar derivatives that each bind to the transporter (Gorga & Lienhard, 1981; Sogin & Hinkle, 1980), gave results that differ from one another (Figure 4). Ethylideneglucose causes a decrease in the fluorescence intensity of the transporter and a shift in the wavelength of maximum emission that are qualitatively similar

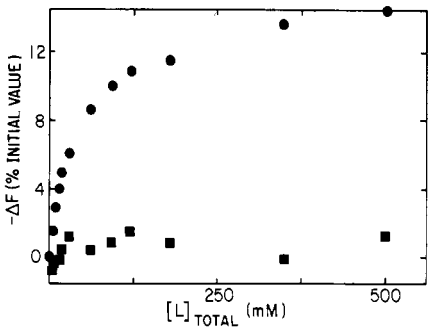

FIGURE 3: Concentration dependence of the fluorescence changes observed with D-glucose (●) and L-glucose (■). The initial concentration of protein was 155 μg/mL. Sequential additions of a 4 M stock solution of each sugar were made, and the fluorescence was monitored as described under Experimental Procedures. The change observed with L-glucose is similar to that seen with buffer alone.

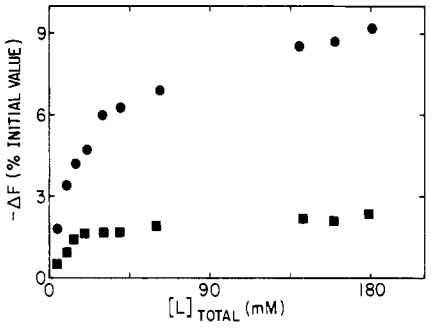

FIGURE 4: Concentration dependence of the fluorescence changes observed with ethylideneglucose (●) and propyl glucoside (■). The initial concentration of protein was 155 μg/mL in the experiment with ethylideneglucose and 128 μg/mL in the case of propyl glucoside. The stock solutions for the titrations were 1.74 M ethylideneglucose and 2.0 M propyl glucoside. The changes observed with propyl glucoside are not significantly different from the changes seen with additions of buffer alone.

Table I: Fluorescence Changes and Dissociation Constants for Ligand Binding to the Glucose Transporter[a]

compd	$-\Delta F_{max}$[b] (%)	K_L[c] (mM) fluorescence	K_L[c] (mM) inhibition
cytochalasin B	17.3	0.9×10^{-4}	1.3×10^{-4}
D-glucose	13.4	48	45
ethylideneglucose	8.3	16	23
propyl glucoside	0.9[d]		34

[a] The values for ΔF_{max} and K_L from fluorescence change are average ones from duplicate titrations done at different times. The individual values were within 10% of the average ones in the case of ΔF_{max} and 25% in the case of K_L. [b] The maximum decrease in fluorescence intensity at 380 nm. [c] The dissociation constant determined from the concentration dependence of the fluorescence change or from the inhibition of [^{3}H]cytochalasin B binding (see the text). [d] At 180 mM propyl glucoside; not significantly different from the changes observed with additions of buffer alone.

to, but smaller than, those seen with cytochalasin B and D-glucose. Propyl glucoside, on the other hand, does not change the fluorescence of the transporter.

The values of the dissociation constants for D-glucose and ethylideneglucose have been obtained from the data by plotting it (plots not shown) according to eq 2, which describes the case

$$\frac{F_{TL} - F}{F_{TL} - F_T}[L]_{total} = K_L \frac{F - F_T}{F_{TL} - F_T} \qquad (2)$$

in which only a small fraction of the ligand is bound (Secemski et al., 1972). The values are given in Table I. They are in

reasonable agreement with the values of the dissociation constants determined by the inhibition of [^{3}H]cytochalasin B binding under the same conditions (Table I). The data for the inhibition of [^{3}H]cytochalasin B binding are not presented here, since the results were very similar to those for sugar binding in a different buffer and at a different temperature reported previously (Gorga & Lienhard, 1981).

Discussion

The transporter is a protein of approximately 50000 molecular weight (Gorga et al., 1979) that contains about seven tryptophan residues (S. A. Baldwin and G. E. Lienhard, unpublished results). The fact that the maximum of the fluorescence emission spectrum is 336 nm indicates that the spectrum is a composite one of tryptophan residues in nonpolar environments, which are known to emit maximally at about 330 nm, and of exposed residues in polar environments, which emit maximally at 340–350 nm (Burstein et al., 1973). The ligands cytochalasin B, D-glucose, and ethylideneglucose each shift the wavelength of maximum emission of the transporter slightly to the blue and quench the fluorescence most strongly at longer wavelengths. The difference spectra (not presented) show a maximum in the region of 350 nm. These results thus suggest that the binding of the ligands selectively reduces the quantum yield from one or more of the exposed tryptophan residues. The simplest structural interpretation is that the binding sites for these ligands, which would be expected to be exposed to the aqueous phase, contain a tryptophan residue(s). The quenching of the tryptophan upon ligand binding would then be due either to a conformational change in which a quenching group(s) of the protein is (are) more favorably oriented or possibly to a direct interaction with the ligand (Lehrer, 1976). Of course, more complicated interpretations cannot be excluded.

Our rationale for examining the effects of ethylideneglucose and propyl glucoside on the fluorescence of the transporter was that these compounds fix the transporter in conformations with outward and cytoplasmic-facing substrate sites, respectively (Barnett et al., 1975; Baker et al., 1978; Devés & Krupka, 1978). We were interested in determining whether there is a correlation between the effects of various compounds on the fluorescence and the conformational states that are expected for the transporter–ligand complexes on the basis of other evidence (either a conformation with an outward-facing sugar site or one with a cytoplasmic-facing site).

In fact there does not appear to be a correlation. The available evidence suggests that cytochalasin B and D-glucose, the effects of which are similar to that of ethylideneglucose rather than propyl glucoside, form complexes in which the ligand site faces the cytoplasm. In the case of cytochalasin B, steady-state kinetic data (Jung & Rampal, 1977; Basketter & Widdas, 1978; Devés & Krupka, 1978) indicate that the binding site probably overlaps with that of the cytoplasmic-facing D-glucose site, although it is possible that cytochalasin B binds to an allosteric site (Krupka & Devés, 1980). In the case of D-glucose, the fact that both propyl glucoside and D-glucose accelerate the rate of inactivation of transport by 1-fluoro-2,4-dinitrobenzene, whereas ethylideneglucose slows the rate (Barnett et al., 1975), has suggested that the predominant complex with D-glucose is the T_iS_i form (see the introduction). Since the intrinsic fluorescence will be determined by the detailed interactions in each complex, the lack of a correlation is not surprising.

The changes in the fluorescence reported here should prove useful for investigating the kinetics of association of the various ligands with the purified transporter. This investigation may, in turn, provide information concerning the postulated kinetic mechanism for transport (see the introduction).

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