Monitoring Conformational Change in the Human Erythrocyte Glucose Carrier: Use of a Fluorescent Probe Attached to an Exofacial Carrier Sulfhydryl†

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ABSTRACT: Several fluorescent sulfhydryl reagents were tested as probes for assessing substrate-induced conformational change of the human erythrocyte glucose carrier. Of these, 2-(4′-maleimidylanilino)-naphthalene-6-sulfonic acid (Mal-ANS) inhibited 3-*O*-methylglucose transport most strongly and specifically labeled a previously characterized exofacial sulfhydryl on the glucose carrier. Analysis of equilibrium cytochalasin B binding in cells treated with Mal-ANS suggested that the inhibition of transport was due to a partial channel-blocking effect, and not to competition for the substrate binding site or to hindrance of carrier conformational change. In purified glucose carrier prepared from cells labeled on the exofacial sulfhydryl with Mal-ANS, a blue shift in the peak of fluorescence indicated that the fluorophore was in a relatively hydrophobic environment. Mal-ANS fluorescence in such preparations was quenched by ligands with affinity for the outward-facing carrier (ethylidene glucose, D-glucose, and maltose), but not by inhibitors considered to bind to the inward-facing carrier conformation (cytochalasin B or phenyl β-D-glucoside). The effect of ethylidene glucose appeared to be related to an interaction with the glucose carrier, since the concentration dependence of ethylidene glucose-induced quench correlated well with the ability of the sugar analog to inhibit cytochalasin B binding to intact cells. The hydrophilic quenchers iodide and acrylamide decreased carrier-bound Mal-ANS fluorescence, resulting in downward-curving Stern–Volmer plots. Whereas ethylidene glucose enhanced iodide-induced quench, it had no effect on that of acrylamide. These results suggest differential accessibility of charged and uncharged quenchers to the fluorophore, and that the change to an outward-facing conformation induced by ethylidene glucose may affect the charge distribution in the neighborhood of the exofacial carrier sulfhydryl.

The sequences of several proteins mediating facilitated diffusion of glucose have been elucidated by molecular cloning techniques (Mueckler et al., 1985; James et al., 1989; Thorens et al., 1988; Kayano et al., 1988). A prominent structural feature of members of this transporter family is the presence of multiple membrane-spanning α-helical domains (Mueckler et al., 1985), which probably interact to form the hydrophilic channel for passage of sugar molecules (Gould & Bell, 1990). The mechanism of sugar gating has not been determined, but several types of biophysical data derived from analysis of the human erythrocyte carrier (GLUT1) suggest that it is accompanied by physical conformational changes in the protein. Thus, sugar analogs and specific transport inhibitors have been shown to quench intrinsic tryptophan fluorescence of the carrier (Gorga & Lienhard, 1982; Appleman & Lienhard, 1985; Carruthers, 1986a,b; Appleman & Lienhard, 1989; Pawagi & Deber, 1990), to allosterically inhibit D-glucose binding to the transport protein assessed with ^{1}H-NMR spectroscopy (Wang et al., 1986), to increase the α-helical content of the carrier protein (Pawagi & Deber, 1987, 1990), and to modify the rate of tryptic digestion of the carrier protein (Gibbs et al., 1988; Karim et al., 1987; King et al., 1991). Taken together, these data strongly suggest that the sugar gating mechanism involves distinct and functionally relevant conformational states.

Additionally, the exposure of an exofacial sulfhydryl on the erythrocyte carrier protein (Batt et al., 1976; Abbott & Schachter, 1976; Roberts et al., 1982) also appears to be modulated by ligand or inhibitor binding (Krupka, 1985; May, 1985; Krupka & Devés, 1986; May, 1989b–d). In the presence of cytochalasin B, which binds to the inward-facing carrier form (Devés & Krupka, 1978), this sulfhydryl is less reactive with impermeant sulfhydryl reagents (Batt et al., 1976; Roberts et al., 1982; May, 1989b,c). On the other hand, the disaccharide maltose, which does not enter erythrocytes (May, 1987) and thus binds only to the outward-facing carrier in intact cells, enhances exofacial sulfhydryl reactivity with such agents (Krupka, 1985; May, 1988a–c). The maltose results also suggest that the sulfhydryl is not located in the substrate binding site. Indeed, it may not be required for transport, since its labeling by *N*-[^{3}H]ethylmaleimide is half-maximal at concentrations of the reagent severalfold less than those required for inhibition of transport (May, 1989a).

If the exofacial sulfhydryl is sensitive to changes in carrier conformation, and if when alkylated it does not necessarily inhibit transport-associated conformational changes, it may serve as a useful reporter site for such changes. Therefore, in the present work we have evaluated the reactivity of the exofacial carrier sulfhydryl with fluorescent sulfhydryl reagents and have found that 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid (Mal-ANS)[1] reacts with the exofacial sulfhydryl and that once it reacts, it undergoes fluorescence

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[1] Abbreviations: Mal-ANS, 2-(4′-maleimidylanilino)naphthalene-6-sulfonic acid; 1,5-I-AEDANS, 5-[[[(iodoacetyl)amino]ethyl]amino]-naphthalene-1-sulfonic acid; Cys-Mal, bis(maleimidomethyl) ether–L-cysteine; PBS, phosphate-buffered saline; EGlc, 4,6-*O*-ethylideneglucose; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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quenching in a manner sensitive to changes in carrier conformation.

EXPERIMENTAL PROCEDURES

Materials. The fluorescent probes Mal-ANS, 5-[[[(iodoacetyl)amino]ethyl]amino]naphthalene-1-sulfonic acid (1,5-I-AEDANS),[1] and didansyl-L-cystine were obtained from Molecular Probes, Inc. Unlabeled and ^{35}S-labeled bis-(maleimidomethyl) ether–L-cysteine (Cys-Mal)[1] were prepared as previously described (May, 1989b), the latter using L-[^{35}S]cysteine (1.1 Ci/mmol) purchased from ICN.

Cell and Membrane Preparation. Freshly prepared glucose-depleted human erythrocytes (May, 1988b) were suspended in 12.5 mM sodium phosphate buffer containing 150 mM NaCl (PBS).[1] Cell lysis and preparation of white ghosts followed the method of Fairbanks et al. (1971), with subsequent protein depletion as described by Gorga and Lienhard (1981). Purified glucose carrier, or band 4.5 in the nomenclature of Fairbanks et al. (1971), was prepared from protein-depleted ghosts by the method of Baldwin et al. (1982). This transporter preparation has been shown to consist largely of leaky vesicles and unsealed sheets, with both cytoplasmic and exofacial sides exposed to the aqueous solution (Appleman & Lienhard, 1985).

Reaction of Mal-ANS with Intact Erythrocytes. Stock solutions of Mal-ANS were prepared just before each experiment by dissolving the reagent in dimethyl sulfoxide and diluting with PBS so that the concentration of dimethyl sulfoxide never exceeded 0.5% in the presence of cells. Intact erythrocytes were incubated in the dark for 30–45 min at 37 °C with the indicated Mal–ANS concentration (expressed per volume of extracellular space). The cell suspension was washed free of unreacted Mal-ANS by three centrifugation washes in 5 volumes of PBS and adjusted to the appropriate hematocrit prior to use.

Fluorescence Measurements. Fluorescence measurements were made with an ISS Greg 200 multifrequency phase fluorometer in the single photon counting mode using ISSPC-Spectra Software, Version 2 (Urbana, IL). The bandwidth was 5 nm. Spectra were recorded using 5-s integrations at 1-nm intervals with smoothing. Spectra were not corrected for variation in detector response with change in wavelength.

Fluorescence studies were performed in a 1-mL volume at room temperature using either protein-depleted ghosts or purified band 4.5 suspended in buffer containing 100 mM NaCl/50 mM Tris-HCl/1 mM EDTA (Tris/NaCl/EDTA buffer), pH 7.4. Titration quenching experiments involved sequential additions of reagent with a micropipet, thorough mixing with the contents of the cuvette, and fluorescence measurements in duplicate or triplicate over 5-s data acquisition periods. The cuvette was not removed from the sample holder during the additions, and the overall dilution did not exceed 10% of the initial sample volume. Fluorescence intensities with each addition were expressed relative to the initial fluorescence of the sample and corrected for dilution and for a small amount of photobleaching observed with reacted Mal-ANS. This correction was accomplished by comparison to an identical sample titrated with a nonquenching control solution.

Sugars used in quenching experiments were diluted from 1–2 M stock solutions in Tris/EDTA/NaCl buffer, with the exception of phenyl β-D-glucopyranoside, which was diluted from 0.2 M stock. Several lots of 4,6-*O*-ethylidene-α-D-glucose (EGlc)[1] contained a small amount of fluorescent contamination (excitation 328 nm, emission peak at 450 nm). When present, this was corrected for as follows. A solution of Mal–ANS which had been reacted with *N*-acetyl-L-cysteine (in a 3-fold molar excess of Mal-ANS) was prepared to a fluorescence intensity similar to that of the experimental sample and titrated in an identical manner with EGlc, and the fluorescence attributable to that contaminating the sugar was subtracted from the corresponding value of the experimental titration. In lots of the sugar containing a substantial amount of fluorescent contamination, the latter was partially removed by chromatography as described by Gorga and Lienhard (1981).

Measurement of iodide-induced fluorescence quenching was carried out as follows. Five molar solutions of KI and NaCl were prepared just before each experiment, each with the addition of $Na_2S_2O_3$ to 10^{-4} M (Lehrer, 1971). Sequential additions of the quencher solution were made to the sample, and a minimum of two 5-s fluorescence acquisitions were recorded. Correction for dilution, ionic strength, and photobleaching was performed by titrating a paired sample in an identical manner with the NaCl solution. For each concentration of KI, the fractional quench was calculated by dividing the quenched reading (F) by the initial sample reading (F_0), with correction of F for quench observed in the paired NaCl-treated sample. The fluorescence intensity of Mal-ANS-labeled glucose carrier was not appreciably affected by ionic strength. The value F_0/F was derived by taking the inverse of the fractional quench (Lehrer, 1971). Acrylamide was diluted from a freshly prepared 8.3 M stock solution. Acrylamide titrations were corrected for dilution only. Since acrylamide does not absorb significantly at 328 nm, no correction was required for an inner filter effect.

Fluorescence quench data involving either KI or acrylamide were expressed according to the Stern–Volmer relationship modified to incorporate ground-state heterogeneity in fluorophore exposure, but not a static quenching component (Eftink & Ghiron, 1976, 1981; Stryjewski & Wasylewski, 1986):

$$\frac{F_0}{F} = \frac{1 + K_{SV}(Q)}{f_0 + f_0 K_{SV}(Q) + f_i} \qquad (1)$$

where f_0 is an unquenchable component of the fluorescence, f_i is the fraction of total fluorescence which can be quenched, and K_{SV} is the collisional quench constant. Two assumptions were made, based on observations in the current system: lack of a major component of static quench, and the presence of two interconvertible states of a single fluorophore attached to the glucose carrier: one accessible and the other inaccessible to the quenching agent. The data were fit using a nonlinear curve-fitting program available in the scientific graphics package Sigmaplot 4.1 (Jandel Scientific, Inc.).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE).[1] Linear SDS–PAGE was carried out by the method of Laemmli (1970) as previously detailed (May, 1986). Exponential gradient gels were prepared as described by Hennessy and Scarborough (1989) with minor modifications (May et al., 1990).

Other Assays. Zero-trans entry of 3-*O*-methylglucose was measured using a previously described transport assay in intact erythrocytes (May, 1988b), except that 3-*O*-[^3H]methyl-α-D-glucose was used instead of ^{14}C-labeled sugar. Equilibrium binding of [^3H]cytochalasin B to intact cells was measured at room temperature as previously detailed (May, 1988a, 1989a). Binding data were treated by the method of Scatchard as modified by Rosenthal (1967). The GSH content of intact erythrocytes was measured with the method of Hissin and

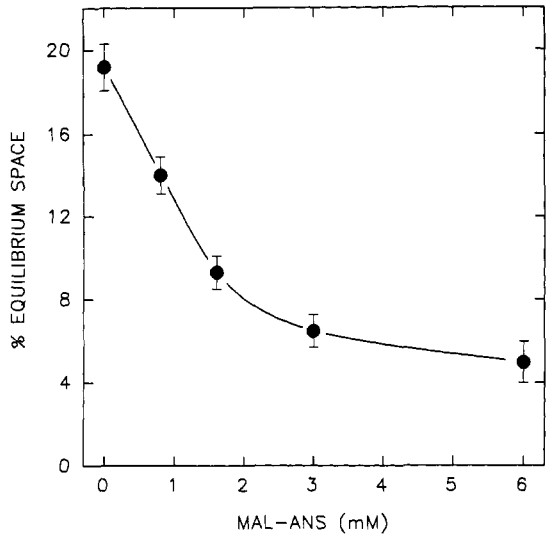

FIGURE 1: Inhibition of 3-*O*-methylglucose uptake by Mal-ANS. Erythrocytes (0.8 mL) at a 20% hematocrit were incubated for 30 min with the indicated extracellular concentration of Mal-ANS, washed three times by centrifugation in 6 volumes of PBS, and resuspended to a 20% hematocrit for the transport assay. Data from three experiments are expressed as a percent of equilibrium 3-*O*-methylglucose space (30 min at 37 °C) attained after 30 s of transport at 3–4 °C.

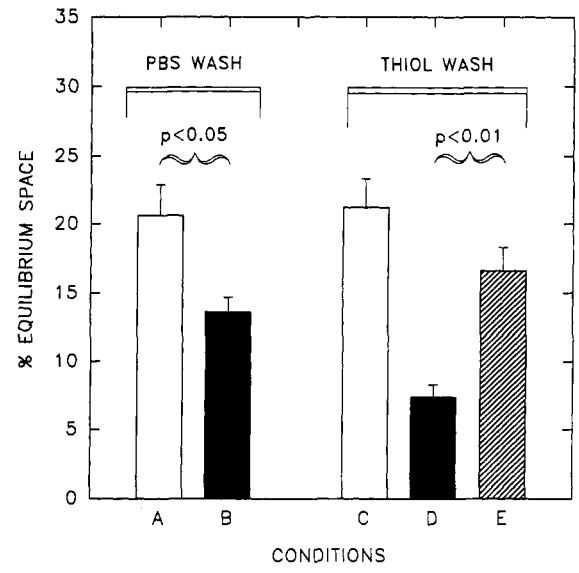

FIGURE 2: Protection from Mal-ANS-induced transport inhibition by blockade of the exofacial carrier sulfhydryl with DTNB. Bars A and B: Erythrocytes (0.8 mL) at a 20% hematocrit were incubated for 30 min at 37 °C without (bar A) for with (bar B) 4 mM DTNB. Samples were washed three times by centrifugation in 5 volumes of PBS (PBS WASH) and resuspended to the initial hematocrit for the transport assay. Bars C–E: Cells prepared as above were treated for 30 min at 37 °C with 4 mM DTNB (bar C), 1.3 mM Mal-ANS (bar D), or first with 4 mM DTNB for 30 min followed by 1.3 mM Mal-ANS for an additional 30 min, all at 37 °C. Cells were then washed three times in 5 volumes of PBS containing 10 mM cysteine (THIOL WASH) and resuspended to the original hematocrit for the hexose transport assay. The results shown are from 3 experiments.

Hilf (1976). Protein was determined by the BCA method (Pierce Chemical Co.). Data are expressed as mean ± standard error for the indicated number of experiments, except where indicated. Statistical comparisons were made using the paired Student's *t*-test.

RESULTS

Transport Inhibition by Fluorescent Sulfhydryl Reagents. Several sulfhydryl-reactive fluorescent probes were tested for their ability to inhibit 3-*O*-methylglucose uptake into erythrocytes. Following incubation under the conditions outlined in the legend to Figure 1, the iodoacetyl derivative 1,5-I-AEDANS had no effect on transport at concentrations below 5 mM (not shown); above 5 mM there was progressive cell lysis. The disulfide didansyl-L-cystine irreversibly inhibited transport with a half-maximal effect at about 3 mM (not shown); however, it also caused cell lysis at concentrations of 5 mM and greater. The maleimide derivative Mal-ANS was a more potent transport inhibitor, irreversibly inhibiting transport half-maximally between 1 and 2 mM (Figure 1), without evidence of cell lysis at the concentrations used. On the basis of these results, Mal-ANS was used in all subsequent studies.

Cell Penetration by Mal-ANS. The ability of Mal-ANS to penetrate intact erythrocytes was assessed by measuring the content of GSH in cells treated with the agent. Incubation with Mal-ANS concentrations of up to 6 mM for 30 min at 37 °C, followed by removal of unreacted reagent with three 5-volume centrifugation washes, had no effect on the intracellular GSH content of erythrocytes (data not shown). In other experiments not shown, electrophoretic gels of leaky ghosts (Fairbanks et al., 1971), which had been prepared from cells treated with Mal-ANS in a similar fashion, showed no evidence of fluorescent labeling of cytosolically exposed membrane or cytoskeletal proteins (e.g., spectrin, actin, and glyceraldehyde-3-phosphate dehydrogenase). These results suggest that Mal-ANS appears to penetrate erythrocytes very little under the conditions used.

Specificity of Mal-ANS for the Exofacial Carrier Sulfhydryl. The possibility that Mal-ANS inhibits hexose transport by attaching to a group other than a sulfhydryl was explored with the use of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB).[1] This reagent reacts by disulfide interchange with absolute specificity for sulfhydryls (Jocelyn, 1987). It has been shown not to penetrate human erythrocytes, but to inhibit hexose transport, very likely by reacting with the exofacial sulfhydryl on the glucose carrier (May, 1989d). As previously observed (May, 1989d), DTNB irreversibly inhibited erythrocyte hexose transport (bars A and B of Figure 2). When cells were washed with buffer containing 10 mM cysteine instead of PBS (bars C–E of Figure 2), the transport inhibition by DTNB was reversed, as expected for a disulfide interchange mechanism (bar C). Transport inhibition by Mal-ANS, on the other hand, was not reversed by thiol washes (bar D). When cells were treated first with DTNB and then with Mal-ANS, followed by thiol washes, the inhibitory effect of Mal-ANS was significantly reduced (bar E). Our interpretation of this result is that DTNB protected the exofacial carrier sulfhydryl from reaction with Mal-ANS, indicated by a return toward basal transport rates following DTNB removal with the thiol washes. Although an allosteric effect of DTNB is possible, this does not seem likely since DTNB was previously shown not to affect carrier conformation when measured by cytochalasin B binding to erythrocytes (May, 1989d).

Further evidence that Mal-ANS had reacted with superficial sulfhydryls on erythrocytes and with the exofacial carrier sulfhydryl in particular is provided in Figure 3. In this experiment, [^{35}S]Cys-Mal was used to label superficial sulfhydryls on intact cells (May, 1989b). Compared to untreated control cells (lane A), pretreatment of cells with 1.3 mM Mal-ANS caused a substantial decrease in labeling of all proteins, including the glucose carrier in the band 4.5

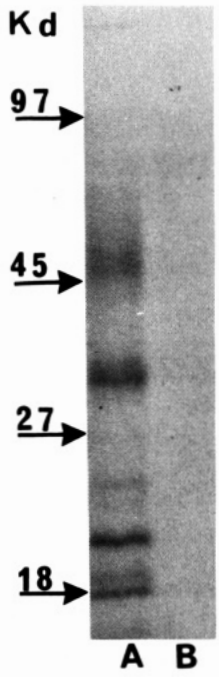

FIGURE 3: Inhibition of [^{35}S]Cys-Mal labeling of exofacial erythrocyte sulfhydryls by Mal-ANS. Washed erythrocytes (2 mL) at a 50% hematocrit were incubated without (lane A) or with 1.3 mM Mal-ANS (lane B) for 30 min at 37 °C, followed by labeling with [^{35}S]Cys-Mal (0.6 mM, 550 Ci/mol) under the same conditions for an additional 30 min. The cells were washed three times by centrifugation in 5 volumes of PBS, hypotonically lysed, and depleted of extrinsic membrane proteins. Fifty micrograms of ghost protein from each treatment was subjected to SDS–PAGE on a 10–15% gradient gel. The gel was stained with Coomassie brilliant blue R-250, dried, and exposed to Kodak XAR film for 5 days. The locations of molecular weight markers are indicated.

region, in the autoradiogram of the electrophoretic gel of protein-depleted ghosts (lane B). Previous studies (May, 1989b) have shown that the labeling of the glucose carrier under the same conditions occurs almost completely on the exofacial sulfhydryl of the glucose carrier. Prevention of [^{35}S]-Cys-Mal labeling of the carrier by Mal-ANS indicates that it likely also reacted with the exofacial carrier sulfhydryl.

Specificity of Mal-ANS for the exofacial carrier sulfhydryl was also suggested by the results of differential labeling studies. It has previously been shown that treatment of erythrocytes with cytochalasin B (as opposed to the inactive cytochalasin E) substantially protects the exofacial carrier sulfhydryl from subsequent reaction with impermeant sulfhydryl reagents (Abbott & Schachter, 1976; May, 1989a–c). Erythrocytes were treated with a differential labeling protocol similar to that used previously (May, 1989c), with the results shown in Figure 4. Paired aliquots of cells were incubated with either 25 μM cytochalasin B or E (the former to protect the exofacial carrier sulfhydryl), followed by blockade of unprotected exofacial sulfhydryls by reaction with 5 mM DTNB, washes in PBS to remove the cytochalasins and unreacted DTNB, addition of 100 μM phloretin to enhance exposure of the exofacial sulfhydryl (May, 1989b,c), and finally labeling with 2.5 mM Mal-ANS. Purified glucose carrier prepared from these cells showed severalfold greater Mal-ANS fluorescence when the exofacial carrier sulfhydryl was initially protected by cytochalasin B as opposed to cytochalasin E (Figure 4).

Finally, the following studies were performed in order to determine whether it was the exofacial and not some other sulfhydryl on the hexose carrier which had reacted with Mal-ANS. Glucose carrier was prepared from cells which had been labeled with 1.2 mM Mal-ANS. Electrophoresis of Mal-

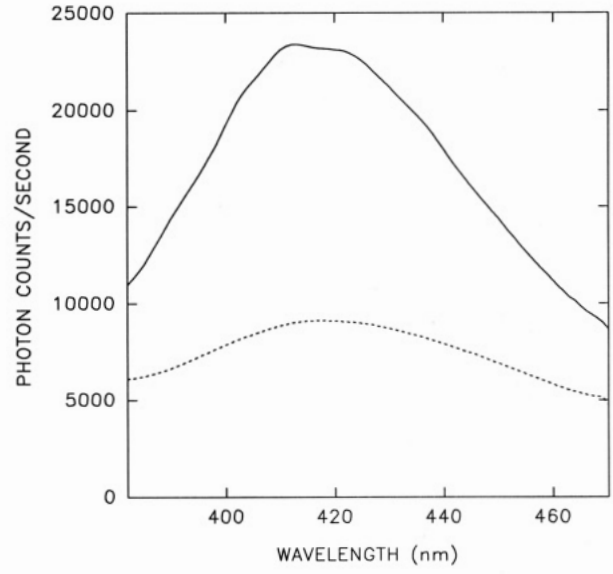

FIGURE 4: Cytochalasin B protection of the exofacial carrier sulfhydryl from labeling by Mal-ANS. Intact erythrocytes (1 mL) at a 50% hematocrit were incubated for 5 min at 37 °C with either cytochalasin B (solid line) or cytochalasin E (dashed line) at concentrations of 25 μM. DTNB was added to both samples to a final extracellular concentration of 5.2 mM, and the incubation was continued for an additional 30 min at the same temperature. The cells were then washed four times in 4 volumes of PBS, resuspended to a 50% hematocrit, treated with 100 μM phloretin, and labeled with 2.5 mM Mal-ANS for 30 min at 37 °C. Purified glucose carrier was prepared from these cells and resuspended to a concentration of 50 μg/mL, and spectra were measured as described under Experimental Procedures.

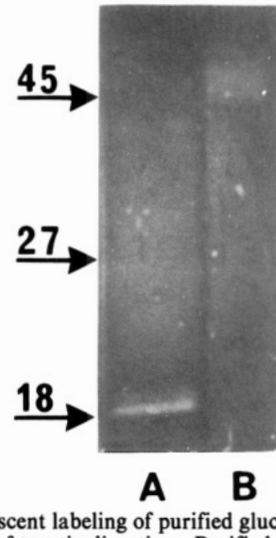

FIGURE 5: Fluorescent labeling of purified glucose carrier by Mal-ANS and effects of tryptic digestion. Purified glucose carrier was prepared from erythrocytes labeled with 1.2 mM Mal-ANS, suspended to a protein concentration of 260 μg/mL in 1 mM EDTA and 50 mM Tris-HCl buffer, and treated with trypsin at 200 μg/mL for 30 min at 37 °C (lane A) or with the same volume of buffer (lane B). Digestion was terminated with the addition of soybean trypsin inhibitor to a concentration 5-fold that required to inhibit trypsin action, the sample was dissolved in electrophoresis sample buffer, and 100 μg was subjected to SDS–PAGE on a 10% acrylamide gel. The gel was illuminated with long-wave UV light (USV-21, Ultra-Violet Products, Inc., San Gabriel, CA) and photographed. The locations of prestained molecular weight markers are indicated.

ANS-labeled carrier showed fluorescent labeling restricted to the broad M_r = 45 000–65 000 glucose carrier region (Figure 5,lane B). However, when the labeled carrier preparation was subjected to exhaustive tryptic digestion before electrophoresis, a single membrane-retained fragment was observed,

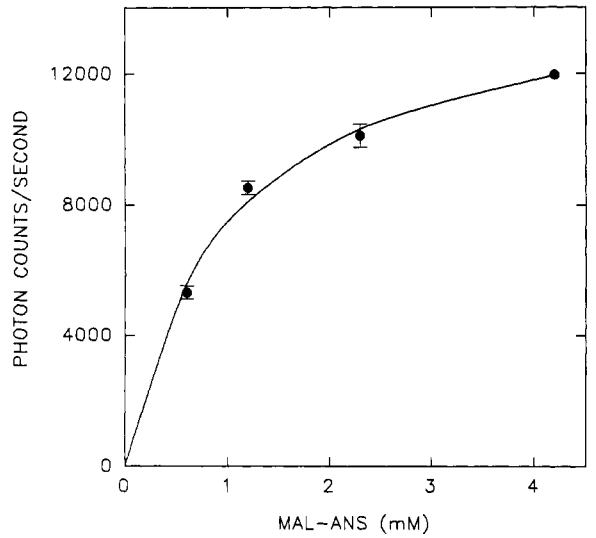

FIGURE 6: Dose–response curve of Mal-ANS fluorescent labeling of the glucose carrier. Erythrocytes were labeled with the indicated concentration of Mal-ANS as described under Experimental Procedures, followed by preparation of the glucose carrier. Fluorescence measurements were taken at an excitation wavelength of 328 nm and an emission wavelength of 416 nm on solutions of 100 μg/mL of the carrier preparation. Data from two determinations on separate membrane aliquots from one experiment are shown as mean ± one standard deviation.

which migrated as a sharp band at M_r = 18 000–19 000 (Figure 5, lane A). The faint band at M_r = 23 000–24 000 in this gel was not a consistent finding in others and was felt to represent a partially cleaved fragment (Cairns et al., 1984; Tai & Carter-Su, 1988). Since previous studies (Deziel et al., 1985; May et al., 1990) indicated that the exofacial sulfhydryl is the *only* carrier sulfhydryl labeled in the M_r = 18 000–19 000 tryptic fragment, these results confirm the specificity of Mal-ANS for the exofacial carrier sulfhydryl in the purified carrier preparation.

The selective fluorescent labeling of the band 4.5 carrier region demonstrated in Figure 5 was saturable, as shown in Figure 6. The concentration of half-maximal reactivity was between 1 and 2 mM, in reasonable agreement with the Mal-ANS concentration producing half-maximal transport inhibition (Figure 1). Unfortunately, UV spectroscopy was not sensitive enough to allow calculation of the stoichiometry of Mal-ANS labeling of the glucose carrier preparation under such conditions.

Effects of Mal-ANS on Cytochalasin B and Sugar Binding to the Hexose Carrier of Intact Erythrocytes. Cytochalasin B binding has been frequently used to assess the relative affinities of sugar substrates for the carrier (Gorga & Lienhard, 1981; Abbott et al., 1986; Helgerson & Carruthers, 1987). For sugars with greater affinity for one face of the carrier or the other, inhibition of cytochalasin B binding will also reflect changes in carrier conformation (Gorga & Lienhard, 1981; Helgerson & Carruthers, 1987; Carruthers & Helgerson, 1991). We therefore used cytochalasin B binding as a measure of changes in carrier structure or conformation in intact erythrocytes which had been treated with Mal-ANS.

Our previous results suggested that the exofacial sulfhydryl was most reactive with sulfhydryl reagents when the carrier is in an outward-facing conformation (May, 1989b–d). Since cytochalasin B binds to an inward-facing conformation (Devés & Krupka, 1978; Krupka & Devés, 1986) and indeed protects the exofacial sulfhydryl from reaction with impermeant maleimides (Batt et al., 1976; Roberts et al., 1982; May, 1989b,c), a substantial conformational change resulting from alkylation of the exofacial sulfhydryl with Mal-ANS would be expected to affect cytochalasin B binding. However, as shown in Table I, 1.3 mM Mal-ANS had no effect on either the apparent affinity (K_D) or total binding (B_0) of cytochalasin B. This concentration of Mal-ANS decreased rates of 3-*O*-methylglucose transport by about 50% (Figure 1) under similar conditions. Thus, the carriers coupled to Mal-ANS were able to reorient (presumably inwardly) and bind cytochalasin B normally, indicating that Mal-ANS attached to the exofacial carrier sulfhydryl may in fact be a good reporter of carrier conformational change.

Table I: Effect of Mal-ANS Labeling of Intact Erythrocytes on Equilibrium Cytochalasin B Binding and Its Inhibition by Competing Sugars[a]

(A) Scatchard Analysis				
measured parameters	control	*p*	Mal-ANS	*N*
K_D (nM)	142 ± 10	NS	123 ± 7	3
B_0 (pmol/10^9 cells)	76 ± 7	NS	73 ± 4	3
(B) Apparent Inhibitor Constants				
ligand	control	*p*	Mal-ANS	*N*
D-glucose	11 ± 0.6	<0.01	20 ± 0.7	4
maltose	35 ± 5.7	<0.02	85 ± 8.3	6
4,6-*O*-ethylideneglucose	10 ± 0.7	<0.01	28 ± 3.2	6
phenyl β-D-glucoside	1.6 ± 0.6	NS	1.3 ± 0.4	4

[a] Erythrocytes at a 20% hematocrit were incubated 30 min at 37 °C with or without 1.3 mM Mal-ANS as indicated, washed three times in 5 volumes of PBS, and resuspended to a 10% hematocrit for assay of equilibrium cytochalasin B binding. In panel A the binding data were treated by Scatchard analysis. In panel B cells were incubated with tracer [^{3}H]cytochalasin B (final concentration = 10 nM), along with increasing concentrations of the indicated sugars inclusive of the observed K_i value. The apparent K_i values were calculated from linear least-squares analysis of Dixon plots. "*N*" indicates the number of experiments performed.

On the other hand, ligands known to bind preferentially to the outward-facing form of the carrier [maltose and EGlc (Barnett et al., 1975)] or to both faces (D-glucose) showed decreased ability to displace cytochalasin B in erythrocytes treated with 1.3 mM Mal-ANS compared to controls (Table I). The apparent affinity of phenyl β-D-glucoside, which binds selectively to the inward-facing carrier conformation (Barnett et al., 1975), was not affected by pretreatment of cells with Mal-ANS (Table I). Although Mal-ANS does not appear to affect the ability of the carrier to change conformation and bind cytochalasin B, its presence on the exofacial carrier sulfhydryl selectively hinders the binding of or conformational changes induced by outward-binding analogues, as opposed to inward-binding analogues.

Fluorescence Studies. The reaction of Mal-ANS with *N*-acetyl-L-cysteine produced a derivative with wavelength of maximal emission 445–450 nm in aqueous solution (Figure 7). The wavelength of maximal emission for purified glucose carrier reacted with Mal-ANS was significantly blue-shifted, to 412–418 nm in most studies (Figure 7). When the carrier preparation was solubilized with 0.1% sodium dodecyl sulfate, the wavelength of maximal emission was red-shifted to 430–435 nm and the fluorescence intensity was decreased by 11% (Figure 7). These results suggest a relatively hydrophobic environment for Mal-ANS bound to the exofacial carrier sulfhydryl, with increased solvent exposure and quenching upon detergent-mediated solubilization and denaturation.

The effects of several transport inhibitors on the fluorescence intensity of Mal-ANS bound to the purified glucose carrier were assessed. Maltose and D-glucose at 100 mM caused a

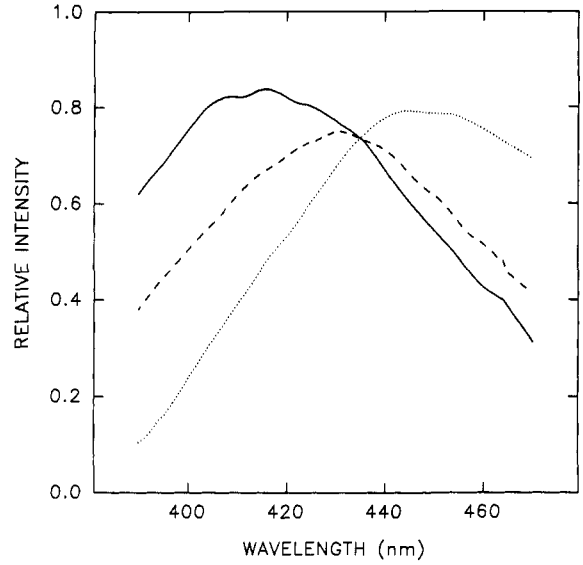

FIGURE 7: Emission spectra of Mal-ANS derivatives. The dotted line shows the emission spectrum of 10 μM Mal-ANS which had been reacted with a 3-fold molar excess of *N*-acetyl-L-cysteine in Tris/NaCl/EDTA buffer. Glucose carrier prepared from cells labeled with 1.5 mM Mal-ANS as described under Experimental Procedures was suspended to a concentration of 15 μg/mL. An emission spectrum was obtained (solid line), 20% sodium dodecyl sulfate was added to a final concentration of 0.1%, and a repeat scan was obtained (dashed line).

5–7% decrease in Mal-ANS fluorescence compared to controls of L-glucose and sucrose, respectively (not shown). No significant differences in quench were noted for cytochalasin B (2 μM) or phenyl β-D-glucoside (20 mM). On the other hand, the fluorescence quench induced by EGlc was substantial and is shown in Figure 8. In panel A, quenching by 40 mM EGlc of Mal-ANS-reacted carrier is compared to that of L-glucose. A quench of about 13% was observed in this experiment, which was not accompanied by any detectable shift in the wavelength of maximal emission. Titration with increasing amounts of EGlc quenched Mal-ANS fluorescence in a saturable manner (Figure 8B), with a total decrease in fluorescence intensity of 12–20%, in different preparations. The normalized data from three experiments were fit to a one-site binding equation, yielding an apparent K_D of 31 ± 5.6 mM, which agrees well with that determined in the cytochalasin B binding studies of Table I.

Fluorescence Quenching by Iodide and Acrylamide. The hydrophilic quenchers iodide and acrylamide were used to probe further the extent of aqueous exposure of Mal-ANS attached to the exofacial carrier sulfhydryl, and whether this was affected by EGlc. Titration of the soluble conjugate between Mal-ANS and *N*-acetyl-L-cysteine with increasing amounts of iodide resulted in linear Stern–Volmer plots, with apparent K_{SV} values of 0.3–0.6 M^{-1} (not shown). In contrast, titration of Mal-ANS coupled to the glucose carrier consistently produced downward-curving plots, as shown for L-glucose in Figure 9. Stern–Volmer plots from a representative titration experiment for L-glucose (60 mM) and EGlc (30 and 60 mM) are illustrated in Figure 9, with quenching parameters shown in Table II. The fitted lines of Figure 9 were derived from nonlinear curve fits to the model described under Experimental Procedures. EGlc clearly potentiated the iodide-induced quench, as reflected in the presumed model by increases in both the K_{SV} and to a lesser extent the fractional exposure of the fluorophore (f_a). Results of other similar experiments confirmed these observations, although the relative shift in calculation of K_{SV} and f_a due to EGlc was

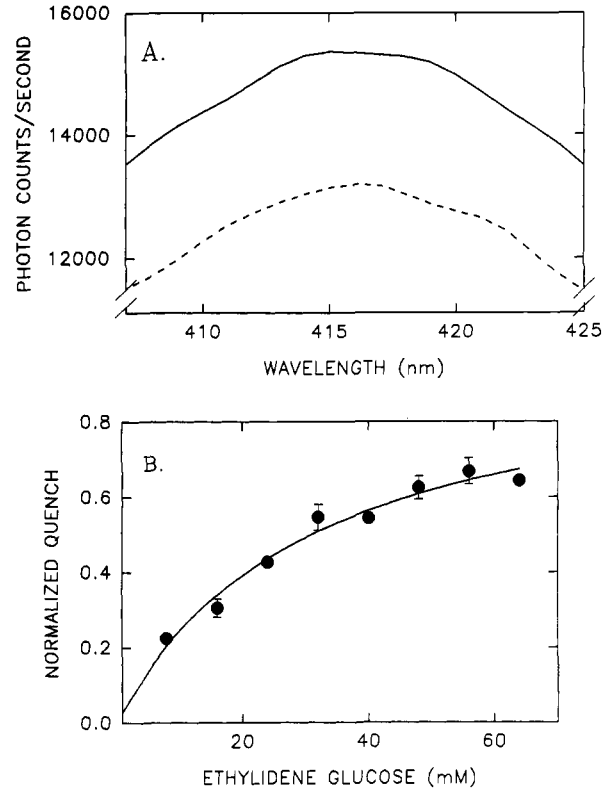

FIGURE 8: Quenching of Mal-ANS fluorescence by EGlc. Glucose carrier was prepared from cells labeled by 4 mM Mal-ANS by the method described under Experimental Procedures and resuspended to a concentration of 20 μg/mL. Panel A shows emission scans of membranes treated with 40 mM L-glucose (solid line) or with 40 mM EGlc (dashed line). In panel B, titration with the indicated concentration of EGlc was performed as described under Experimental Procedures, and fluorescence at a fixed emission wavelength of 416 nm was measured after each addition. The data from three experiments (±one standard deviation) using different membrane preparations are expressed as percent quench normalized to the initial fluorescence intensity. The combined data were fit to a simple one-site binding isotherm indicated by the solid line.

variable. Nonetheless, the K_{SV} for samples not treated with sugar consistently ranged from 3 to 10 M^{-1}, considerably greater than that for *N*-acetyl-L-cysteine-coupled Mal-ANS in aqueous solution.

Quenching of Mal-ANS fluorescence by acrylamide also resulted in a slightly downward-curving Stern–Volmer plot, as shown in Figure 10. In this experiment, typical of two others performed, the calculated parameters were $f_0 = 0.56 \pm 0.06$; $f_a = 0.44 \pm 0.05$; and $K_{SV} = 2.8 \pm 1.0$ (mean ± SE). The quench parameters suggested a greater fractional exposure of the fluorophore to acrylamide than to iodide. Additionally, the acrylamide-induced quench was unaffected by addition of EGlc (Figure 10). Titration of *N*-acetylcysteine with acrylamide over the same concentration range resulted in a linear Stern–Volmer plot, with a K_{SV} of 3.2.

DISCUSSION

An exofacial or outwardly exposed sulfhydryl group on the human erythrocyte glucose carrier protein is reactive with a variety of sulfhydryl reagents, most of which inhibit transport (Abbott & Schachter, 1976; Roberts et al., 1982; Krupka, 1985; May, 1988b,d). Proteolytic studies have indicated that this sulfhydryl corresponds to one of the three cysteines in the C-terminal half of the carrier sequence (Deziel et al., 1985; May, 1989b,c; May et al., 1990), possibly Cys-429 (May et al., 1990). As discussed in the introduction, the reactivity of

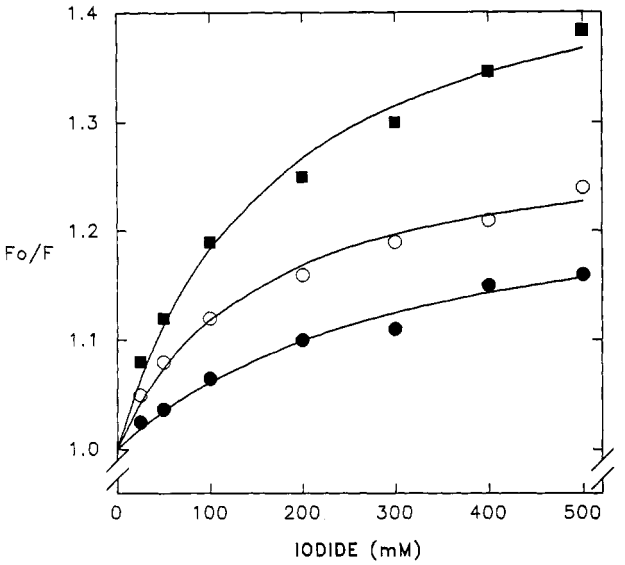

FIGURE 9: Iodide quenching of Mal-ANS coupled to the exofacial carrier sulfhydryl. Purified glucose carrier was prepared from erythrocytes labeled with Mal-ANS as described under Experimental Procedures and resuspended to a protein concentration of 15 μg/mL. Samples were treated with 60 mM L-glucose (closed circles, ●), 30 mM EGlc (open circles, ○), or 60 mM EGlc (closed squares, ■). Sequential titrations to the concentrations of iodide indicated were performed and the fluorescence intensity measured at 416 nm. An identical sample was titrated in a similar manner with NaCl, and the resulting change in fluorescence intensity was used to correct the iodide-titrated sample before calculation of F_0/F. The symbols represent the actual data, whereas the lines are derived from nonlinear curve-fitting of eq 1 as described under Experimental Procedures. The data shown are from a representative experiment.

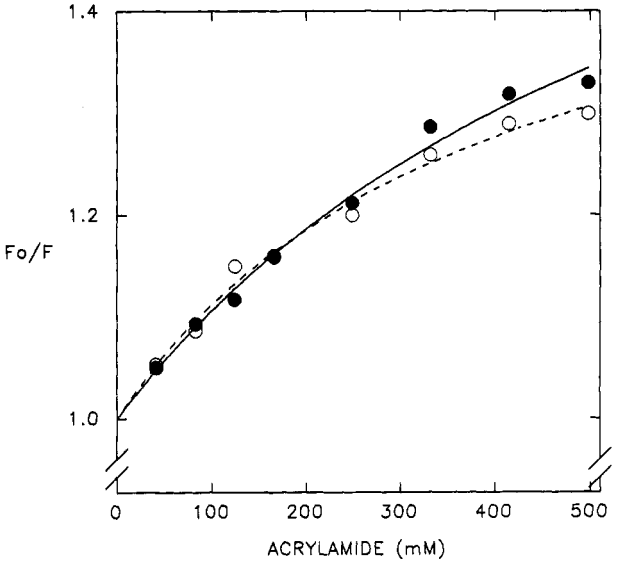

FIGURE 10: Quenching of Mal-ANS fluorescence by acrylamide. Conditions were identical to those noted in the legend to Figure 9, except that the control sample was treated with 20 μL of Tris/NaCl/EDTA buffer (closed circles, ●), and the EGlc sample was treated with EGlc to a final concentration of 60 mM (open circles, ○). Following initial measurement of fluorescence, samples were titrated with increasing amounts of acrylamide to the concentrations indicated. Correction for dilution and a small amount of photobleaching was accomplished by titrating a paired sample with NaCl. The fitted lines were derived from nonlinear regression to eq 1 as described under Experimental Procedures.

Table II: Calculated Quench Parameters for Data of Figure 9[a]

sugar	concn (mM)	K_{SV} (M^{-1})	f_0[b]	f_a[c]
L-glucose	60	4.0 ± 1.9	0.8 ± 0.03	0.2 ± 0.03
EGlc	30	8.8 ± 3.2	0.77 ± 0.02	0.23 ± 0.01
EGlc	60	9.0 ± 3.4	0.67 ± 0.02	0.33 ± 0.02

[a] Parameters are shown ± SE derived from the curve-fitting routine. [b] Fraction of fluorescence not quenched by iodide. [c] Fraction of fluorescence quenched by iodide.

this sulfhydryl is increased by ligands which selectively bind to the form of the carrier with a substrate binding site facing outward, suggesting that its exposure results from a conformational change in the protein. In the present work we sought to react this sulfhydryl with a fluorescent probe in order to assess its aqueous exposure and to use it as a reporter site for measuring changes in carrier conformation.

Of several potential fluorescent sulfhydryl reagents tested, Mal-ANS was the most potent glucose transport inhibitor, as effect which was not reversed by washes or associated with significant cell penetration by the reagent. Under the conditions utilized, Mal-ANS reacted with the previously studied exofacial sulfhydryl, such that, in purified glucose carrier prepared from cells treated with Mal-ANS, the exofacial appeared to be the only sulfhydryl in the protein so modified.

Transport inhibition by Mal-ANS could not be attributed to inhibition of a transport-associated conformational change, but was best explained by a channel-blocking effect impairing access of substrate to the sugar binding site (Falke & Chan, 1986). This conclusion is derived from cytochalasin B binding experiments presented in Table I. Cytochalasin B is considered to bind selectively to the carrier in a form with substrate binding site facing inward, since it inhibits glucose efflux competitively, but glucose influx noncompetitively (Devés & Krupka, 1978). More important for the present discussion, the cytochalasin B binding site appears to be responsive to changes in carrier conformation, since the binding of maltose to the external substrate binding site in intact cells decreases binding of cytochalasin B to the carrier (May, 1988a; Carruthers & Helgerson, 1991). Since the parameters of equilibrium cytochalasin B binding were unaffected by the presence of Mal-ANS, and since affinity of the substrate analog phenyl β-D-glucopyranoside for the inward-facing carrier conformation (Barnett et al., 1975) were not affected by Mal-ANS treatment, the alkylation does not appear to inhibit transport by causing a major change in conformational stability. Although the ability of the carrier to shift to an inward-binding conformation is not affected, a more subtle conformational change impairing substrate translocation cannot be ruled out. The Mal-ANS-induced fall in apparent affinity for the transported D-glucose (Table II) is in accord with this interpretation.

On the other hand, we also found that both maltose and EGlc, nontransported analogs with high selectivity for the outward-facing form of the carrier, were 2- to 3-fold less effective in inhibiting cytochalasin B binding when the exofacial sulfhydryl was alkylated by Mal-ANS. Based on these results, another explanation for the observed inhibition of 3-*O*-methyglucose transport is a partial blockade of access of sugar to the outward-facing substrate binding site, possibly due to steric hindrance (Falke & Chan, 1986). Reaction of Mal-ANS at the substrate binding site seems unlikely, since as noted previously, maltose binding actually accelerates reaction of the exofacial sulfhydryl with certain sulfhydryl reagents (Krupka, 1985; May, 1988b, 1989c). Thus, despite inhibition of transport, the exofacial sulfhydryl reacted with Mal-ANS may be useful as a reporter group of conformational change of the carrier protein.

When Mal-ANS was bound to the exofacial sulfhydryl of the purified glucose carrier, its wavelength of maximal fluorescence was substantially blue-shifted compared either to that of Mal-ANS reacted with *N*-acetyl-L-cysteine in aqueous solution or to that of Mal-ANS-reacted carrier which had been solubilized by SDS (Figure 7). By analogy with previous studies in which Mal-ANS was allowed to react with the (Na,K)-ATPase (Gupte & Lane, 1979), this suggests a relatively hydrophobic environment for carrier-bound Mal-ANS, possibly with the naphthalene ring buried in a hydrophobic cleft between adjacent transmembrane α-helices.

EGlc induced a pronounced quench in fluorescence intensity of Mal-ANS bound to the exofacial carrier sulfhydryl (Figure 8). The extent of quench correlated well with the ability of the sugar to inhibit cytochalasin B binding in cells previously reacted with Mal-ANS (Table I). From this we infer that the sugar-induced fluorescence quench was related to a change in the conformation of the carrier. Both D-glucose and maltose also quenched the fluorescence of Mal-ANS when coupled to the glucose carrier, although considerably less strongly than did EGlc. The small quenching effect of D-glucose could be due to the fact that binding of transported sugar to both sides of the carrier induces a relatively small change in carrier conformation, since the carrier exists mostly in an inward-facing conformation under these conditions (Lowe & Walmsley, 1986). The maltose-induced fluorescence quench may have been lessened because it has access to both carrier faces in unsealed membranes, although it clearly has different effects on endogenous tryptophan fluorescence than does D-glucose (Pawagi & Deber, 1990). Also, the decrease in sugar affinity caused by the reaction of Mal-ANS with the exofacial sulfhydryl is expected to have a relatively greater impact for maltose at 100 mM than for higher affinity sugars at the same concentration. The finding that neither the sugar analog phenyl β-D-glucoside nor cytochalasin B (both bind to the inward-facing carrier conformation) affected Mal-ANS fluorescence may relate at least in part to the fact that at 20–23 °C about 60–80% of carriers have an inward-facing conformation already (Lowe & Walmsley, 1986). Failure of cytochalasin B or phenyl β-D-glucoside to quench Mal-ANS fluorescence is consistent with the lack of an effect of Mal-ANS on the apparent affinity of the carrier for these agents measured in the cytochalasin B binding experiments of Table I.

The EGlc-induced quench of Mal-ANS fluorescence mirrors that found in previous studies, in which the glucose analog also quenched endogenous tryptophan fluorescence in similar preparations of glucose carrier (Gorga & Lienhard, 1982; Appleman & Lienhard, 1985, 1989). In those studies, the concentration-dependent quench of tryptophan fluorescence was attributed to a shift of the carrier to an outward-facing conformation having greater aqueous exposure of otherwise buried tryptophan(s) (Gorga & Lienhard, 1982; Appleman & Lienhard, 1985, 1989). Indeed, Lienhard and colleagues have used rapid stopped-flow kinetic measurements to follow this quenching phenomenon (Gorga & Lienhard, 1982; Appleman & Lienhard, 1985, 1989) and have shown that it correlates very well with a single half-turnover time for the carrier derived from transport kinetic studies (Lowe & Walmsley, 1986, 1987). In contrast to the results with Mal-ANS, in previous studies cytochalasin B caused a substantial quench of intrinsic tryptophan fluorescence (Gorga & Lienhard, 1982; Appleman & Lienhard, 1985, 1989; Carruthers, 1986a,b). This may relate to a greater sensitivity of the fluorescence of the involved tryptophan(s) than of Mal-ANS fluorescence to a change in carrier conformation, or to the possibility that the binding of cytochalasin B induces a selective quench of one or more nearby tryptophans. Furthermore, there is evidence that cytochalasin B does not simply bind to the inward-facing substrate binding site, but itself may induce an allosteric inhibitory effect on transport (Devés & Krupka, 1978).

Titration of Mal-ANS-reacted glucose carrier with iodide caused a quench in fluorescence, resulting in a downward-curving Stern–Volmer plot (Figure 9). Such curves have been considered to indicate ground-state heterogeneity of fluorophore exposure in proteins (Eftink & Ghiron, 1976, 1981). Since we feel that Mal-ANS is substantially bound only to the exofacial sulfhydryl of the glucose carrier, the simplest model for analysis of the experiment is one in which bound Mal-ANS is accessible to iodide when the carrier is in an outward-facing conformation, but inaccessible when the latter is in an inward-facing conformation, presumably buried in a hydrophobic domain (Lehrer, 1971; Stryjewski & Wasylewski, 1986). Although this model is appealing in its simplicity and fits the experiments well in some respects, two additional observations suggest that a more complex model will be required.

First, despite uncertainty in its actual value (a coefficient of variation of 30–50%), the estimated K_{SV} of the iodide-quenched species was clearly greater than that of Mal-ANS-conjugated *N*-acetyl-L-cysteine in solution. Similarly, it was found that the effect of EGlc on iodide quench was due to increases in both exposure of the fluorophore (f_a) and in its susceptibility to quench (K_{SV}). Under our hypothesis, the former would be caused by an EGlc-induced shift of the carrier to an outward-facing conformation, which in turn would enhance iodide access to carrier-bound Mal-ANS. The increase in K_{SV} requires additional explanation. At present we can only speculate that the high K_{SV} of a fraction of Mal-ANS molecules might be due to the presence of nearby positive charges on the carrier protein, which could enhance quench either by localizing iodide ions in the vicinity of the fluorophore, or by neutralizing repulsive negative charges on the Mal-ANS molecule. In this regard, Lehrer (1971) did find that iodide was substantially more effective in quenching the fluorescence of tryptophan-containing poly(L-lysine) than of a similar tryptophan-containing peptide composed of poly-(L-glutamate). This possibility will require further study.

Second, the hydrophilic but uncharged acrylamide produced Stern–Volmer plots which were only slightly downward-curving and which were unaffected by EGlc (Figure 10). The presence of static quenching, which is frequently observed for acrylamide, could have attenuated the downward curvature somewhat (Eftink & Ghiron, 1981). The lack of an effect of EGlc on acrylamide-induced quench may indicate increased accessibility of the uncharged acrylamide (compared to iodide) to the presumably "buried" naphthalene ring of Mal-ANS. Nonetheless, more complex models must also be considered to include accessible and inaccessible components in each conformation of the carrier.

It is also relevant to consider the effects of iodide and acrylamide in quenching intrinsic tryptophan fluorescence of the glucose carrier. In the studies of Pawagi and Deber (1990), titration of the glucose carrier preparation with either iodide or acrylamide resulted in linear Stern–Volmer plots. Maltose enhanced iodide-induced quench, and D-glucose decreased it. Although not directly comparable to the present results, they also suggest the existence of ligand-induced conformational change in the carrier. In conclusion, a sulfhydryl known to

be exposed on the outward-facing erythrocyte glucose carrier reacts with the fluorescent sulfhydryl reagent Mal-ANS such that changes in its fluorescence may be useful for monitoring carrier conformation. We are currently undertaking time-resolved fluorescence studies to better define the relationship between changes in carrier-bound Mal-ANS fluorescence and those in carrier conformation upon binding of ligands.

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Registry No. Mal-ANS, 71936-81-7; Cys-Mal, 124505-88-0; 3-*O*-methylglucose, 146-72-5; ethylidene glucose, 13224-99-2; D-glucose, 50-99-7; maltose, 69-79-4; cytochalasin B, 14930-96-2; phenyl β-D-glucoside, 1464-44-4.