Tryptic Digestion of the Human Erythrocyte Glucose Transporter: Effects on Ligand Binding and Tryptophan Fluorescence†

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ABSTRACT: The conformation of the human erythrocyte glucose transport protein has been shown to determine its susceptibility to enzymatic cleavage on a large cytoplasmic loop. We took the converse approach and investigated the effects of tryptic digestion on the conformational structure of this protein. Exhaustive tryptic digestion of protein-depleted erythrocyte ghosts decreased the affinity of the residual transporter for cytochalasin B by 3-fold but did not affect the total number of binding sites. Tryptic digestion also increased the affinity of the residual transporter for D-glucose and inward-binding sugar phenyl β-D-glucopyranoside but decreased that for the outward-binding 4,6-*O*-ethylidene glucose. These results suggest that tryptic cleavage stabilized the remaining transporter in an inward-facing conformation, but one with decreased affinity for cytochalasin B. The steady-state fluorescence emission scan of the purified reconstituted glucose transport protein was unaffected by tryptic digestion. Addition of increasing concentrations of potassium iodide resulted in linear Stern–Volmer plots, which were also unaffected by prior tryptic digestion. The tryptophan oxidant *N*-bromosuccinimide was investigated to provide a more sensitive measure of tryptophan environment. This agent irreversibly inhibited 3-*O*-methylglucose transport in intact erythrocytes and cytochalasin B binding in protein-depleted ghosts, with a half-maximal effect observed for each activity at about 0.3–0.4 mM. Treatment of purified glucose transport protein with *N*-bromosuccinimide resulted in a time-dependent quench of tryptophan fluorescence, which was resolved into two components by nonlinear regression using global analysis. Tryptic digestion retarded the rate of oxidation of the more slowly reacting class of tryptophans. Thus, tryptic digestion caused the residual transporter to assume an inward-facing conformation different from that induced by transporter ligands.

Despite recent advances in our understanding of the primary and higher order structure of the family of proteins that mediate facilitated diffusion of glucose, little is known about the actual transport mechanism. Kinetic studies using the GLUT1 or human erythrocyte transporter indicate that transport is accompanied by a conformational change in which the protein alternates from an outward-facing to an inward-facing form as glucose enters the cell (Barnett et al., 1975; Lowe & Walmsley, 1986, 1987). The kinetic data suggesting a conformational change with substrate binding or transport are also supported by results from biophysical studies using the techniques of fluorescence spectroscopy (Gorga & Lienhard, 1982; Carruthers, 1986; Appleman & Lienhard, 1989), ^{1}H NMR spectroscopy (Wang et al., 1986), hydrogen (Jung et al., 1986) or deuterium exchange (Alvarez et al., 1987), and circular dichroism (Pawagi & Deber, 1987, 1990).

Besides affecting the membrane-spanning hydrophobic regions of the GLUT1 protein, the transport-induced conformational changes also involve the large cytoplasmic loop and cytoplasmic C-terminal tail. Work from several laboratories suggests that transporter conformation modifies the susceptibility of these regions to proteolytic cleavage. Outward-binding glucose analogs such as 4,6-*O*-ethylideneglucose (EGlc),[1] maltose, and bis(mannose) derivatives retard proteolytic cleavage of the cytoplasmic loop of the transporter (Holman & Rees, 1987; Gibbs et al., 1988; King et al., 1991; Asano et al., 1992). Conversely, nontransported inward-binding analogs such as propyl β-D-glucopyranoside or phenyl β-D-glucopyranoside (PGlc) either have no effect (Gibbs et al., 1988) or increase the rates of cleavage (King et al., 1991). The transported sugar D-glucose uniformly accelerates the rate of enzymatic cleavage of GLUT1 (Gibbs et al., 1988; King et al., 1991; Asano et al., 1992).

Once the transporter has been cleaved with trypsin, available evidence suggests that it can no longer transport glucose (Masiak & LeFevre, 1977). However, the trypsin-cleaved transporter, most of which remains embedded in the plasma membrane, retains D-glucose-inhibitable cytochalasin B binding activity (Cairns et al., 1984, 1987; Karim et al., 1987). Whereas the affinity of the cleaved protein for cytochalasin B is decreased, its affinity for D-glucose is increased severalfold (Cairns et al., 1987). These observations suggest that proteolytic cleavage may provide a useful tool for study of structure–function relationships of the transporter.

Endogenous tryptophan fluorescence has been used to follow changes in conformation of the transport protein, as noted above. At room temperature or below, kinetic studies suggest that most of the transporters in human erythrocytes exist in an inward-facing conformation, with or without bound glucose (Lowe & Walmsley, 1986). Under such conditions, addition of a substrate analog with selectivity for the outward-facing carrier form, such as EGlc (Barnett et al., 1975), quenches

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[1] Abbreviations: DNSB, dimethyl(2-hydroxy-5-nitrobenzyl)sulfonium bromide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EGlc, 4,6-*O*-ethylideneglucose; PGlc, phenyl β-D-glucopyranoside; SDS, sodium dodecyl sulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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the fluorescence of a portion of the transporter tryptophans (Gorga & Lienhard, 1982; Carruthers, 1986; Appleman & Lienhard, 1989). The most likely explanation of these data is that EGlc causes a shift from an inward- to an outward-facing conformation and increases the solvent exposure of one or more tryptophans on the protein. Pawagi and Deber (1990) have provided further support for this interpretation using dynamic fluorescence quenching techniques. They found that both of the hydrophilic quenchers iodide and acrylamide decreased tryptophan fluorescence in preparations of purified erythrocyte transporter by as much as one-third. When maltose was present, the quench induced by either agent was increased even further. Since the binding of maltose appears to induce an outward-facing conformation of the transporter (King et al., 1991), these data suggest that one or more of the glucose transporter tryptophans has greater aqueous exposure when the carrier is in an outward-facing conformation.

In the present work we used tryptic digestion to cleave the human erythrocyte glucose transporter and assessed the resulting changes in conformation/structure by monitoring cytochalasin B binding, endogenous tryptophan fluorescence, and the susceptibility of tryptophans to oxidation by *N*-bromosuccinimide (NBS). Our results suggest that tryptic cleavage of the transporter at the C-terminal tail and cytoplasmic loop produces an inward-facing conformation of the residual membrane-bound protein, but one distinct from that induced by inward-binding sugars.

EXPERIMENTAL PROCEDURES

Materials. NBS and dimethyl(2-hydroxy-5-nitrobenzyl)-sulfonium bromide (DNSB) were from Sigma. Radionuclides were obtained as follows: 3-*O*-[^{3}H]methyl-D-glucose from New England Nuclear and [4-^{3}H]cytochalasin B from Amersham.

Cell and Membrane Preparation. Protein-depleted ghosts (Gorga & Lienhard, 1981) and purified glucose transporter (Baldwin et al., 1982) [band 4.5 in the nomenclature of Fairbanks et al. (1971)] were prepared from freshly obtained human erythrocytes according to procedures described previously (May, 1988). Protein was determined by the BCA method (Pierce Chemical Co.).

Transport and Binding Assays. The uptake of tracer amounts of 3-*O*-[^{3}H]methyl-D-glucose into intact erythrocytes was measured as previously detailed (May, 1988). Equilibrium binding of [4-^{3}H]cytochalasin B to protein-depleted ghosts was measured as follows. Ghosts at a protein concentration of 150–200 μg/mL were incubated on ice with mixing for 30 min with concentrations of [^{3}H]cytochalasin B ranging from 10 to 3000 nM in a total volume of 0.4 mL of 50 mM Tris-HCl buffer, pH 7.4. A 100-μL aliquot of the membrane suspension was removed for liquid scintillation counting in 5 mL of Omni-Fluor (Packard Instrument Co.). The remaining membranes were centrifuged in a Fisher 235 microfuge at 12 400 rpm for 15 min at 4 °C. An additional 100-μL aliquot of the supernatant was removed for scintillation counting. The amount of radioactivity bound was taken as the difference between total and unbound cytochalasin B in each sample. Preliminary studies showed that essentially all of the cytochalasin B binding was inhibited by 500 mM D-glucose, so that no correction for nonspecific binding was necessary. The binding data were analyzed by Scatchard analysis according to the method of Rosenthal (1967).

In experiments in which the affinity of a sugar for the transporter or its fragments was measured, protein-depleted ghosts were incubated for 5 min on ice with the sugar, followed by addition of [^{3}H]cytochalasin B to a final concentration of 10 nM (tracer alone), and the binding assay was performed as described above. Sugar concentrations were chosen to bracket the expected K_i value for the sugar. The binding data were analyzed using a plot of free/bound [^{3}H]cytochalasin B *vs* the inhibitor concentration to derive the apparent K_i according to the procedure of Gorga and Lienhard (1981).

Tryptic Digestion of Protein-Depleted Ghosts and Purified Reconstituted Glucose Transporter. Protein-depleted erythrocyte ghosts at a concentration of 1–2 mg/mL protein were incubated in the presence or absence of 20 μg/mL trypsin for 1 h at 37 °C, after which time the digestion was terminated with the addition of a 3-fold excess of soybean trypsin inhibitor. The ghosts were washed twice by centrifugation at 29000*g* at 3 °C in 10 volumes of the Tris-HCl buffer and resuspended to a protein concentration of 150–200 μg/mL for the cytochalasin B binding assay.

Tryptic digestion of purified glucose transporter was performed by incubating the reconstituted transporter at a protein concentration of 200 μg/mL in 50 mM Tris-HCl containing 150 mM NaCl and 1 mM EDTA, pH 7.4, with 20 μg/mL trypsin for 1 h at 37 °C. The digestion was terminated by addition of a 3-fold excess of soybean trypsin inhibitor. The preparation was then diluted 10-fold in the Tris–NaCl–EDTA buffer and centrifuged at 38 000 rpm for 30 min in a Beckman Ti 70.1 rotor. The centrifuge wash was repeated once. The membranes were resuspended to a concentration of 0.5–1 mg/mL and stored at –20 °C until use.

Fluorescence Measurements. Tryptophan fluorescence of the purified glucose transporter preparation was monitored with a SPEX Fluorolog spectrofluorometer and DM3000 software (SPEX, Edison, NJ). Measurements were made in the photon-counting mode with slit widths of 2–5 mm, bandwidths of 5 nm, and an excitation wavelength of 295 nm. In a typical experiment, the sample volume was 1 mL and contained 16–20 μg of purified glucose carrier protein in 50 mM Tris-HCl, 150 mM NaCl, and 1 mM EDTA buffer, pH 7.4. Fluorescence emission scans were performed at 1-s integration times and were not corrected for variation in detector response with wavelength. For time-based studies, the fluorescence of a glucose transporter preparation was measured at an emission wavelength of 330 nm. In iodide quenching studies, the baseline fluorescence of a 1-mL sample was monitored for 10–15 s, followed by titration with 10–20-μL aliquots of 5 M KI (or NaCl) over 5 min to a final concentration of 500 mM. The decline in fluorescence in NaCl-treated samples (due to dilution and a small amount of photobleaching) was used to correct the KI-titrated samples. Both KI and NaCl solutions contained 0.1 mM $Na_2S_2O_3$. The data were plotted according to the Stern–Volmer method (Eftink & Ghiron, 1981).

Titrations with NBS were performed as follows: The fluorescence of the sample in a 1-mL volume was recorded for 10–15 s, followed by addition of 10 μL of a 40 mM solution of NBS with vigorous mixing (final concentration 0.4 mM). The change in fluorescence of the sample was then recorded over a 5–10-min period. NBS caused a 12–15% quench of tryptophan fluorescence at the first time point measured (12 s). This was due largely to an inner filter effect but also to oxidative quenching that had occurred before the earliest time point was taken. For display and curve-fitting, the data were normalized to the fluorescence observed at the first time point taken. The resulting quench curves were fit by nonlinear regression using the method of global analysis (Beechem, 1992). This approach uses the Marquardt–Levenberg algo-

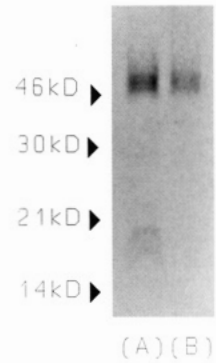

FIGURE 1: Inhibition of [^{3}H]cytochalasin B labeling of the trypsin-cleaved transporter by D-glucose. Trypsin-digested protein-depleted erythrocyte ghosts (0.1 mg/mL) were incubated without (lane A) or with 100 mM D-glucose (lane B) for 5 min at 3 °C, followed by addition of 30 μCi of [^{3}H]cytochalasin B (7.3 μM), incubation for 15 min at 3 °C, and photolysis for 5 min with a short-wave UV lamp (Model UVS-11, Ultra-Violet Products, Inc., San Gabriel, CA). The membranes were diluted in 170 volumes of 100 mM Tris-HCl, pH 7.4, and centrifuged at 29000*g* for 45 min, and the resulting pellet was stored at –20 °C until electrophoresis. Ghost protein (50 μg) from each treatment was subjected to SDS–PAGE and fluorography as described under Experimental Procedures. The locations of prestained molecular mass markers are indicated.

rithm for nonlinear fitting but gains additional resolving power by linking multiple data sets via common fitting parameters. Rigorous error estimates for a selected fitting parameter are obtained by performing multiple nonlinear analyses over a range of parameter values inclusive of the recovered minimum. All other parameters are allowed to vary freely to obtain the minimum possible value for the chi-square statistic. A plot of the minimum chi-square as a function of the fixed parameter value provides a rigorous confidence interval based on the *F* statistic (Box, 1960). A confidence interval of 67% was considered a significant difference.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE). SDS–PAGE was performed according to the method of Laemmli (1970) using the Tris–tricine gel system of Schägger and von Jagow (1987) with 4% stacking and 10% resolving acrylamide minigels. Fluorography of tritium-containing gels was carried out according to the method of Bonner and Laskey (1974). Following impregnation with the fluorophore, the gel was stained with Coomassie Brilliant Blue G-250, dried, and exposed to Kodak XAR film for 2–3 weeks at –70 °C. Immunoblotting of the C-terminal transporter tail was performed as described previously (May et al., 1990) using 0.45-μm poly(vinylidene difluoride) microporous membrane (Immobilon-P, Millipore Corp., Bedford, MA) in the transfer step.

Statistics. Data are expressed as mean ± standard error for the indicated number of experiments, except where indicated. Statistical comparisons were made using the nonpaired Student's *t*-test.

RESULTS

Effects of Tryptic Digestion of Protein-Depleted Ghosts on Cytochalasin B Photolabeling and Binding. We first wished to confirm that the C-terminal half of the transporter remaining in the membrane following tryptic cleavage retains the ability to bind cytochalasin B and D-glucose. Following mild tryptic digestion, [^{3}H]cytochalasin B photolabeled the native band 4.5 transporter as well as M_r = 18 000–20 000 fragments of the C-terminal transporter in protein-depleted ghosts, as shown in the fluorogram of Figure 1. Photolabeling

Table I: Effect of Tryptic Digestion of Protein-Depleted Erythrocyte Ghosts on Equilibrium Cytochalasin B Binding and Its Inhibition by Competing Sugars[a]

A. Scatchard Analysis

measured parameter	control	*N*	*p*	trypsin	*N*
K_D (nM)	144 ± 21	4	<0.05	368 ± 78	5
B_0 (nM)	0.5 ± 0.047	4	NS	0.56 ± 0.1	5

B. Apparent Inhibitory Constants

ligand (mM)	control	*N*	*p*	trypsin	*N*
D-glucose	21 ± 3	3	<0.001	3 ± 0.2	4
4,6-*O*-ethylideneglucose	15 ± 1.7	2	<0.01	84 ± 7.4	3
phenyl β-D-glucopyranoside	1.8 ± 0.06	2	<0.01	0.7 ± 0.13	4

[a] Equilibrium cytochalasin B binding to protein-depleted erythrocyte ghosts was measured as described under Experimental Procedures. In (A) the binding data were treated by Scatchard analysis. In (B) cells were incubated with tracer [^{3}H]cytochalasin B (final concentration 10 nM) and with increasing concentrations of the indicated sugars inclusive of the observed K_i value. An *N* value indicates the number of experiments performed for each treatment.

of both the uncleaved transporter and the fragments was inhibited by D-glucose. A concentration of 100 mM D-glucose inhibited photolabeling of the residual broad band 4.5 transporter by about 80% on the basis of densitometric scanning of the fluorogram of Figure 1 but almost completely prevented photolabeling of the M_r = 18 000–20 000 fragments. This suggests a higher affinity of these fragments for D-glucose than that of the native protein.

To assess directly the extent of tryptic cleavage under the conditions used in the cytochalasin B binding and fluorescence experiments reported below, protein-depleted ghosts and purified glucose transporter were first photolabeled with [^{3}H]cytochalasin B and then digested with trypsin. Since cytochalasin B does not affect the rate of tryptic cleavage of the transport protein at physiologic temperatures (Gibbs et al., 1988), this seemed to be a reasonable approach with which to assess the extent of tryptic cleavage. In results not shown, subsequent electrophoresis and fluorography produced the expected labeled M_r = 18 000–20 000 fragment for both preparations. Densitometric scanning of the fluorogram from such a preparation typically showed about 80% or greater cleavage of the transport protein. Additionally, since the M_r =18 000–20 000 fragment was not detected in immunoblots using an antibody directed against this portion of the molecule (not shown), the observed fragment corresponds to the C-terminal half of the transporter molecule without the cytoplasmic tail.

Equilibrium binding of cytochalasin B to protein-depleted ghosts was then measured to determine whether tryptic digestion modifies carrier conformation. Tryptic digestion of protein-depleted ghosts decreased the K_D of cytochalasin B binding 2–3-fold but was without effect on the total number of binding sites (Table I). The affinity of the trypsin-treated transporter for sugars was assessed by their ability to inhibit binding of a low concentration of [^{3}H]cytochalasin B. Using this method, the apparent affinity for D-glucose was increased about 7-fold by trypsin (Table I), consistent with the results of the cytochalasin B photolabeling studies shown in Figure 1. During transport D-glucose necessarily binds to both conformations of the transporter, so that a change in its apparent affinity cannot distinguish between inward and outward orientation of the transporter. Therefore, side-selective sugar analogs were used to determine whether tryptic digestion might stabilize a single conformation in the residual protein. As shown in Table I, the apparent affinity of EGlc was decreased about 5-fold by tryptic digestion, whereas that

of PGlc was increased 2–3-fold. Since EGlc selectively binds to the outward-facing and PGlc to the inward-facing transporter (Barnett et al., 1975), our results are compatible with the notion that tryptic digestion leaves the residual membrane-bound transporter fragments in an inward-facing form. In fact, as suggested by one of the reviewers of this work, the residual affinity of the trypsin-treated protein for EGlc could be due mostly to its weak binding to the inward-facing sugar binding site.

Effects of Tryptic Digestion on Endogenous Tryptophan Fluorescence. To increase the transporter-specific fluorescence signal, all fluorescence studies were performed using purified transport protein after functional reconstitution into endogenous erythrocyte lipids, rather than protein-depleted ghosts, in which the predominant protein is the band 3 anion transporter (Baldwin et al., 1979). Proteolytic studies have shown that this purified band 4.5 preparation is largely transporter and is about 80% accessible from either face of the lipid membrane (Appleman & Lienhard, 1985).

Although most of the transporter was cleaved under the tryptic digestion conditions used, there were no significant differences in the steady-state fluorescence emission scans of uncleaved and trypsin-cleaved transporter preparations (results not shown). This indicates that no tryptophans were lost as a result of the digestion. It also shows that the conformational change expected as a result of tryptic digestion had no net effect on the environment or aqueous exposure of fluorescing tryptophans in transporter fragments retained in the vesicles. Similarly, tryptic digestion had no effect on iodide-induced fluorescence quenching. Titration of a purified transporter preparation resulted in linear Stern–Volmer plots, but neither the K_{SV} nor the Y-intercept values were different in trypsin-treated preparations (not shown). The K_{SV} for control was 0.54 M^{-1}, and that for trypsin-treated membranes was 0.57 M^{-1}. We therefore took another approach to enable us to determine whether tryptic digestion might affect tryptophan environment.

The oxidant NBS is known to quench tryptophan fluorescence in proteins by oxidizing tryptophan residues (Ramachandran & Witkop, 1967). We reasoned that NBS would have access tryptophans in proportion to their aqueous exposure, so that the rate of NBS-induced tryptophan oxidation might provide a more sensitive measure of conformational change than that available from the steady-state emission spectrum alone. However, it was first important to determine whether NBS interacts with the transporter. This was done by studying its effects on glucose transport and cytochalasin B binding to the transporter.

Inhibition of 3-O-Methylglucose Transport and Cytochalasin B Binding by NBS. Treatment of intact erythrocytes with increasing concentrations of NBS resulted in irreversible inhibition of 3-*O*-methylglucose uptake (Figure 2). Over the narrow concentration range depicted, transport was inhibited in a linear dose-dependent manner, with a half-maximal effect between 0.3 and 0.5 mM NBS. Relatively low NBS concentrations were employed, since above 3 mM hemolysis of intact cells was observed. Because of this, and because the extent of transport inhibition was variable from experiment to experiment, further studies were performed with protein-depleted erythrocyte ghosts.

As shown in Figure 3, equilibrium binding of cytochalasin B to protein-depleted ghosts was irreversibly inhibited by NBS, since the inhibition persisted after removal of the unreacted reagent by centrifugation washes. The inhibition was complete at 0.6 mM NBS and half-maximal at about 0.3 mM. Since

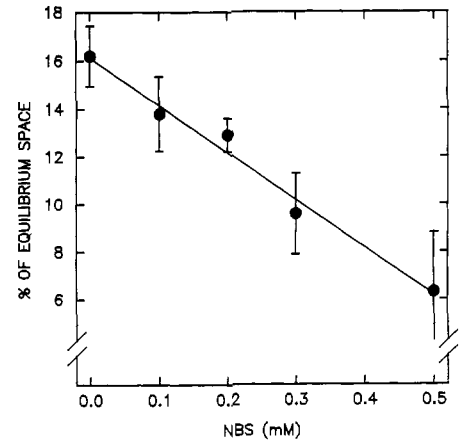

FIGURE 2: Inhibition of 3-*O*-methylglucose uptake by NBS. Erythrocytes (0.8 mL) at a 20% hematocrit were incubated for 30 min with the indicated concentration of NBS, washed three times by centrifugation in 6 volumes of PBS, and resuspended to a 20% hematocrit for the transport assay. The space occupied by 3-*O*-methylglucose following a 30-s transport assay at 3 °C is expressed as a percent of equilibrium 3-*O*-methylglucose space (30 min at 37 °C). The data shown are from one of three experiments performed.

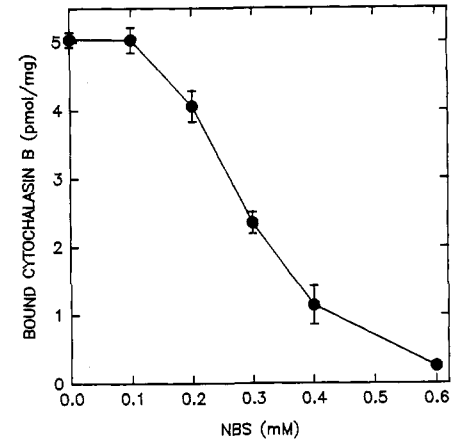

FIGURE 3: Inhibition of cytochalasin B binding to protein-depleted erythrocyte ghosts by reaction with NBS. Ghosts at a concentration of 200 μg/mL were incubated for 30 min at 23 °C with the indicated concentration of NBS, washed twice by centrifugation in 3 volumes of 50 mM Tris-HCl buffer, pH 7.4, and resuspended to 200 μg/mL for the cytochalasin B binding assay as described under Experimental Procedures. The data shown are from four experiments.

NBS may react with several amino acids besides tryptophan, including tyrosine and cysteine (Ramachandran & Witkop, 1967), the possibility that the observed inhibitory effects were due to reaction of one or more cysteines was investigated. This was accomplished by protecting transporter sulfhydryl groups by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) before NBS treatment. DTNB is specific for sulfhydryls (Jocelyn, 1987), and we have previously shown that it can protect against alkylation of an exofacial carrier sulfhydryl by impermeant maleimides in intact cells (May, 1989a,b). As depicted in Figure 4, 4 mM DTNB lacked a significant irreversible effect on cytochalasin B binding by itself but did partially prevent the inhibitory effects of NBS. Therefore, the inhibitory effects of NBS on cytochalasin B binding appear to be due both to oxidation of an exofacially exposed cysteine on the transporter (May, 1989a,b) and to effects on other amino acids, presumably trypophans.

Under certain conditions NBS can cleave the peptide bond on the carboxyl side of tryptophans (Patchornik et al., 1960;

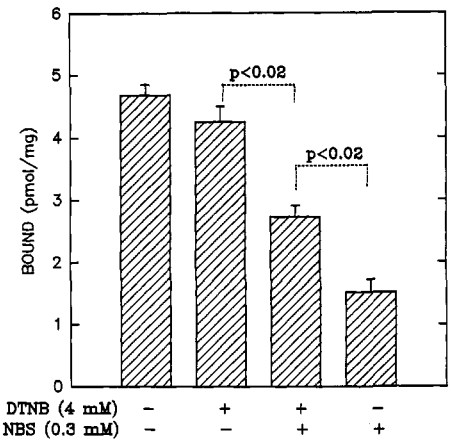

FIGURE 4: Partial protection by DTNB from NBS-induced inhibition of cytochalasin B binding to protein-depleted ghosts. Protein-depleted ghosts (200 μg/mL) were incubated without or with 4 mM DTNB for 30 min at 23 °C, treated as noted with 0.3 mM NBS, washed twice by centrifugation at 29000*g* in 3 volumes of Tris buffer, and resuspended to 200 μg/mL for cytochalasin B binding as described under Experimental Procedures. The data shown are from four experiments.

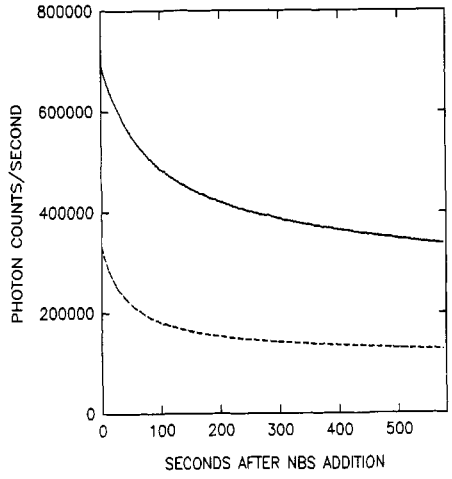

FIGURE 5: NBS quenching of tryptophan fluorescence of purified glucose transport protein and the effects of SDS. In the upper curve, purified glucose transport protein was treated with 0.4 mM NBS and the subsequent decline in fluorescence measured as described under Experimental Procedures. In the lower curve, the same concentration of the transporter preparation was solubilized by the addition of SDS to a final concentration of 0.1% before addition of NBS and measurement of the time-dependent quench in fluorescence.

Ramachandran & Witkop, 1967). Therefore, evidence for damage to the transporter protein was sought by reacting protein-depleted ghosts with NBS under the conditions employed for cytochalasin B binding, followed by SDS–PAGE of the washed membranes, electrotransfer to poly(vinylidene difluoride) membranes, and immunoblotting with a C-terminal-specific antibody. At concentrations below 2 mM no cleavage of the protein could be detected, although there was some fragmentation apparent above 3 mM (results not shown).

Effects of NBS on Glucose Transporter Tryptophan Fluorescence. Treatment of purified reconstituted glucose transporter with 0.4 mM NBS resulted in a time-dependent curvilinear decline in tryptophan fluorescence intensity (upper curve of Figure 5). About 30–40% of the tryptophan fluorescence was not quenched by this concentration of NBS. To determine whether the unquenchable fluorescence was due to tryptophans "buried" in the protein core or due to depletion of the reagent, a transporter preparation was first solubilized with SDS and then treated with NBS. SDS at a concentration

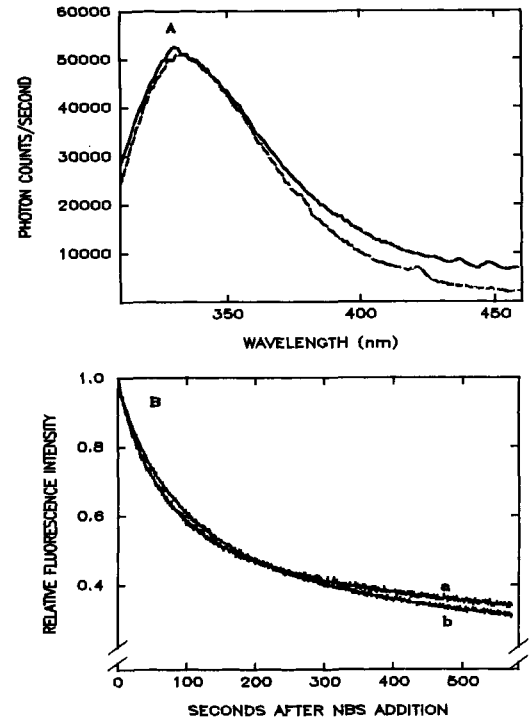

FIGURE 6: Effects of octyl β-D-glucoside on the emission spectrum and NBS-induced quench of purified glucose transporter. (A) An emission scan was taken of an aliquot of purified transporter (20 μg/mL) (solid line). Octyl β-D-glucoside was then added to a final concentration of 40 mM and the scan repeated (dashed line). The scans were corrected for buffer fluorescence, and that of octyl β-D-glucoside was corrected for dilution and for a small amount of fluorescence present in the sugar solution. (B) NBS-induced quench of untreated (curve a) transporter and transporter that had been solubilized in 40 mM octyl β-D-glucoside (curve b). The fluorescence data were linked to those for tryptic digestion of Figure 7 and fit to the model described in the text using global analysis. The line through the data points represents the fitted results.

of 0.1% caused clearing of the slightly opalescent membrane solution and an initial 50% quench in tryptophan fluorescence compared to the sample before solubilization (Figure 5, lower curve). Subsequent addition of NBS to a concentration of 0.4 mM again caused a curvilinear decline in the residual tryptophan fluorescence, which was calculated to result in the eventual loss of about 90% of the original fluorescence signal. Thus, denaturation of the transporter with SDS exposed a substantial portion of the tryptophan fluorescence to quenching by water, and most of that remaining was accessible to oxidation by NBS.

The results with SDS can be contrasted with the effects of solubilizing the transporter preparation with 40 mM octyl β-D-glucoside. Despite the fact that octyl β-D-glucoside also cleared the membrane solution, it had little effect on the tryptophan emission scan of the preparation (Figure 6 A). There was typically a 2–5-nm shift in the peak of maximal emission to a longer wavelength, suggesting that some change in the tryptophan environment had occurred. Subsequent NBS treatment of the octyl β-D-glucoside-solubilized transporter preparation (Figure 6B) caused a decline in tryptophan fluorescence similar to that resulting from NBS treatment alone, accompanied by a small increase in the ultimate quench produced by NBS. These results are compatible with the notion that the transporter was not substantially denatured by octyl β-D-glucoside.

Whereas tryptic digestion had little effect on the tryptophan emission spectrum, it did retard the time-dependent NBS quenching of tryptophan fluorescence (Figure 7). Since the

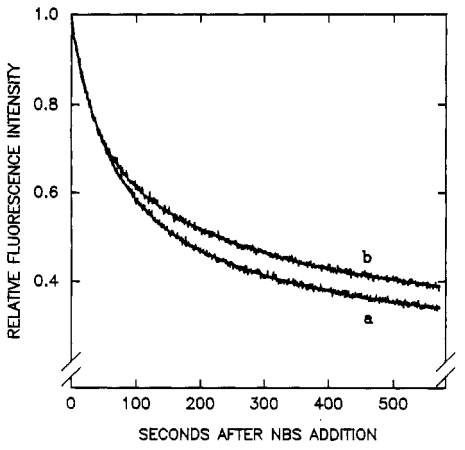

FIGURE 7: Effects of tryptic digestion of NBS-induced quench of glucose transporter tryptophan fluorescence. Curve a represents data from untreated membranes. Curve b shows membranes at the same concentration that were digested with trypsin, as described under Experimental Procedures, and resuspended to a protein concentration of 20 μg/mL. The data from both curves shown in this figure and from the NBS quench curve in the presence of octyl β-D-glucoside from Figure 6 were linked in global analysis and the resulting fits shown as lines through the data.

Table II: Fitted Parameters from Global Analysis of NBS Oxidation Time Course Curves[a]

parameter (from eq 1)	control	trypsin
a	0.34	0.35
k_1	0.0037	0.0036
b	0.34 (0.31–0.38)	0.29 (0.27–0.3)*
k_2	0.020 (0.019–0.022)	0.026 (0.023–0.029)*
c	0.30 (0.27–0.31)	0.34 (0.32–0.36)*

[a] Global analysis of the linked data from Figures 8 and 9. Shown are the results of control and trypsin digested samples only. The data were normalized to the first fluorescence reading after NBS addition, and the values for amplitude are shown relative to unity. An asterisk (*) denotes a significant difference from control NBS oxidation at a 67% confidence interval (the ranges of these intervals are shown in parentheses following the parameter values). The confidence intervals were derived from an F-test of the chi-square distribution in the error analysis routine.

data showing effects of octyl β-D-glucoside (Figure 6) and trypsin (Figure 7) on NBS-induced quench were taken from the same membrane preparation, the data sets were linked in the nonlinear fitting routine using global analysis. The NBS-induced decline in tryptophan fluorescence was best fit by a model with double-exponential decay and an asymptote according to

$$y = ax^{-k_1 t} + bx^{-k_2 t} + c$$

The equation parameters for the normalized results of trypsin treatment shown in Figure 7 are provided in Table II, along with the relevant confidence intervals. The two rate constants (k_1 and k_2) for either control or trypsin treatment were resolved by the fitting procedure at 95% confidence levels, indicating two classes of reactive tryptophans. There was also a component of tryptophan fluorescence that was inaccessible to NBS (parameter c). Each component accounted for about one-third of the initial fluorescence signal in the control preparation. Tryptic digestion caused a decrease in both the amplitude and the rate constant of the slower-reacting component of tryptophan fluorescence, as well as a compensatory increase in the residual fluorescence not quenched by NBS (i.e., the asymptote, parameter c). These differences were significant at the 67% confidence level (Table II). Although there was variability in the NBS kinetic parameters derived from different membrane preparations, within an experiment the effect of trypsin was restricted to the slower-reacting component of tryptophan fluorescence.

In an attempt to determine which tryptophans on the transporter might have increased aqueous exposure, a transporter preparation was labeled with DNSB. Upon reaction with tryptophan this reagent develops increased UV absorption at 415 nm (Barman & Koshland, 1967; Horton & Tucker, 1970). The reagent has also been recently shown to inhibit erythrocyte glucose transport in a sulfhydryl-independent manner (Lowe et al., 1991). The purified transporter was first treated with DTNB (to protect exposed sulfhydryls), followed by a 30-min incubation with 1 mM DNSB, centrifugation washes to remove unreacted reagent, tryptic cleavage, and electrophoresis in 5-mm tube gels. These gels were scanned on a linear transport, and the absorption at 415 nm was compared to the location of molecular weight standards electrophoresed in the same experiment. Following subtraction of background absorption present in an untreated transporter preparation, it was apparent that labeling had occurred in both the N- and C-terminal fragments of the transporter (results not shown).

We also investigated the possibility that NBS quenching of tryptophan fluorescence might be sensitive to sugar-induced conformational changes of the carrier. This was tested by preincubating the purified glucose transporter with carrier-specific ligands, followed by addition of NBS and recording of the subsequent decline in tryptophan fluorescence. However, none of the ligands tested, including D-glucose (50 mM), maltose (100 mM), ethylideneglucose (50 mM), and cytochalasin B (10 μM), had a significant effect on the rate of loss of tryptophan fluorescence (results not shown). This suggests that stabilization of the carrier in either an outward- or inward-facing conformation by substrate or ligand binding does not affect NBS access to or reaction with the involved tryptophan(s).

DISCUSSION

In this work we have used tryptic digestion of the human erythrocyte glucose transport protein to study effects of conformational change induced by an agent other than a sugar. Exhaustive digestion of erythrocyte membranes with any of several enzymes cleaves the transporter on its cytoplasmic or endofacial surface, producing two major fragments on SDS–PAGE: a broadly migrating glycosylated M_r = 30 000 N-terminal band and an M_r = 18 000–20 000 C-terminal band (Cairns et al., 1984; Shanahan & D'Artel-Ellis, 1984). The former has been shown to correspond to amino acid residues 1–212 and the latter to residues 265–456 in the protein (Mueckler et al., 1985; Cairns et al., 1987). Evidence from labeling studies suggests that whether entering or leaving the cell D-glucose binds to the C-terminal half of the transporter molecule (Holman & Rees, 1987; Cairns et al., 1984; Shanahan & D'Artel-Ellis, 1984). Karim et al. (1987) have shown as well that the inhibition by D-glucose of cytochalasin B photolabeling to this C-terminal fragment persists even after tryptic cleavage, which we have confirmed in the present work (Figure 1). The latter results also favor a D-glucose binding site on the C-terminal half of the transporter molecule. A remaining caveat, of course, is that the two major portions of the transporter may still be functionally combined in the lipid bilayer following tryptic digestion, so that the inhibitory effect of D-glucose on cytochalasin B binding may be allosteric rather than direct.

As originally reported by Cairns et al. (1984, 1987) and also confirmed in the present work (Table I), tryptic cleavage

of the glucose transport protein markedly decreases its affinity for cytochalasin B, whereas the apparent affinity for D-glucose increases severalfold. The latter conclusion derives from an indirect measure of D-glucose affinity, derived from its ability to inhibit the binding of cytochalasin B to a transporter with a lower cytochalasin B affinity. Nonetheless, since cytochalasin B binding sites are not lost because of tryptic digestion, the competition between D-glucose and cytochalasin B should still provide a measure of glucose affinity. Our data using side-selective sugar analogs (Table I) show that once cleaved by trypsin, the remaining transporter is stabilized in a conformation with decreased affinity for outward-binding sugars but with increased affinity for the natural ligand D-glucose and for those sugars that bind predominantly to the inward-facing conformation. On the basis of similar reasoning, Oka et al. (1990) have suggested that the rabbit brain glucose transporter lacking the C-terminal tail due to mutagenic truncation is locked in an inward-facing conformation. Trypsin may produce a similar shift to an inward-facing conformation. However, the decrease in affinity of the trypsin-cleaved transporter for cytochalasin B suggests that such cleavage may remove portions of the protein required for ligand binding, may distort the cytochalasin B binding site, or may cause an inward-facing conformation different from that of the intact transporter. The notion that an inward-facing conformation results from tryptic cleavage is compatible with previous data reviewed in the introduction showing that this conformation is at least permissive for cleavage with trypsin and other enzymes. Taken together with our results, these observations indicate that an inward-facing conformation not only facilitates tryptic cleavage but also is the measurable conformation assumed by the residual membrane-bound transporter fragments following such cleavage.

Despite the known sensitivity of transporter tryptophan fluorescence to changes in conformation, the presumed conformational change induced by tryptic cleavage did not substantially affect either the steady-state tryptophan fluorescence emission spectrum or the iodide-induced fluorescence quenching. The failure to detect changes may have several explanations: (1) the unloaded transporter was already mostly facing inward (70–80%) at room temperature (Lowe & Walmsley, 1986); (2) tryptic cleavage was not complete; (3) the cleaved transporter was still functionally associated in the membrane; or (4) there were compensatory changes that canceled any effects. Nonetheless, tryptic digestion did slow the rate of tryptophan oxidation by NBS.

NBS is an oxidant with high specificity for tryptophan (Imoto, 1972), which has been used to map the location of aqueous-exposed tryptophans in several proteins, including lysozyme (Hayashi et al., 1965; Peterman & Laidler, 1979, 1980), chymotrypsin (Peterman & Laidler, 1979), apocytochrome *c* (Peterman & Laidler, 1979), and the L-arabinose-binding protein of *Escherichia coli* (Parsons & Hogg, 1974). We found that low concentrations of NBS reacted with the transporter, reflected both in inhibition of transport in intact erythrocytes (Figure 2) and in decreased cytochalasin B binding to protein-depleted membranes over the same concentration range (Figures 4–6). NBS also oxidized transporter tryptophans, since it quenched endogenous tryptophan fluorescence of a purified transporter preparation (Figures 7–9).

The rate of fluorescence quenching by NBS has been suggested to correlate with the extent of aqueous exposure of the involved tryptophan(s) (Frazier et al., 1973). In our studies, nonlinear regression analysis of quench curves derived from purified glucose transporter reacted with NBS indicated that the data were well fit by a double-exponential decay to an asymptote (Figures 8 and 9). This suggests that there are three major classes of reactive tryptophans in the transporter. One class reacts rapidly and presumably has substantial aqueous exposure, a second reacts more slowly, and a third remains unreactive in the reconstituted preparation. Most of the latter class can be exposed when the transporter is solubilized by 0.1% SDS (Figure 5), although not by the nondenaturing detergent octyl β-D-glucoside (Figure 6). Addition of 0.4 mM NBS to a purified transporter preparation that had been digested with trypsin resulted in a decrease in the rate of oxidation of the slowest-reacting class of tryptophans (Figure 7; Table II). This suggests that the effect of tryptic digestion was to shift this class of tryptophans to an environment with even less exposure to the bathing medium than that before trypsin treatment. Such an effect is consistent with the tendency for trypsin to induce an inward-facing conformation in the residual protein, since transporter tryptophans are generally less exposed to the aqueous medium in this conformation (Gorga & Lienhard, 1982; Pawagi & Deber, 1990). Although the time-dependent quench in tryptophan fluorescence caused by NBS was affected by tryptic digestion, side-specific ligands were without effect on NBS-induced quench. Thus, trypsin may cause an inward-facing conformation in what fragments of the transporter remain in the membrane, but this conformation does appear to differ in certain respects from that related to the transport process.

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