Biochimica et Biophysica Acta, 599 (1980) 699—714

BBA 78775

THE MONOSACCHARIDE TRANSPORT SYSTEM OF THE HUMAN ERYTHROCYTE

ORIENTATION UPON RECONSTITUTION

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(Received October 18th, 1979)

Key words: Cytochalasin B; Hexose transport; Membrane orientation; Phospholipid vesicle; Reconstitution; (Erythrocyte membrane)

Summary

Treatment of intact human erythrocytes with trypsin had no effect upon either the rate of hexose transport or the binding of cytochalasin B to the transport system. In contrast, proteolysis of inside-out vesicles prepared from human erythrocyte membranes inactivated both hexose transport and cytochalasin B binding. When purified hexose transporter, reconstituted into phospholipid vesicles of undetermined size, was treated with trypsin, approx. 50% of the cytochalasin B binding activity was lost. This loss correlated with a decrease in the amount of the transporter polypeptide, as assayed by gel electrophoresis. These results show that the orientation of the transporter can be established through trypsin treatment in conjunction with cytochalasin B binding. Small unilamellar vesicles containing transporter were prepared by sonication of larger species and by a cycle of cholate solubilization and removal of the detergent. In the former case, the transporter orients almost randomly, whereas in the latter approx. 75% of the transporters have the cytoplasmic domain external.

Introduction

The human erythrocyte contains a transport system for D-glucose and other monosaccharides that catalyzes the equilibration of these substrates across its membrane [1]. Recently a protein that appears to be this transporter has been isolated by others [2,3] and ourselves [4]. Upon its incorporation into phospholipid vesicles this protein catalyzes the transport of D-, but not L-glucose ([2,3]; Baldwin, J.M., Lienhard, G.E. and Baldwin, S.A., unpublished results).

In addition, the reconstituted transporter exhibits another property of the transport system, which is a high affinity for the compound cytochalasin B, a potent inhibitor of transport [4,5].

It is becoming a generalization that an integral membrane protein, such as a transport system, possesses a stable orientation in the direction perpendicular to the bilayer, and that in biological membranes this orientation, relative to the cytoplasmic surface, is the same for all copies of the protein [6,7]. A question that thus arises concerning the glucose transporter reconstituted into vesicles is what fraction of the transporters are oriented with the extracellular domain exposed to the external medium and what fraction with the cytoplasmic domain exposed. Lin and Spudich [8] reported briefly that trypsin treatment of erythrocyte ghosts inactivated the binding of cytochalasin B to the transporter, whereas treatment of intact erythrocytes had no effect on binding. In this paper, we describe the elaboration of this finding and its use to determine the orientation of the purified, reconstituted transporter *.

Materials and Methods

Materials

[^{3}H]Cytochalasin B, from New England Nuclear, was purified by thin-layer chromatography [9]. L-[1-^{3}H]Glucose was a product of New England Nuclear; L-[U-^{14}C]sorbose and [^{14}C]EDTA were from Amersham. Unlabeled cytochalasins B and E were obtained from Aldrich Chemical. Soybean phospholipids, from Associated Concentrates, were acetone-washed without the use of antioxidants [10]. 1,2-Dioleoyl- and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (dioleoyl and dimyristoyl phosphatidylcholine) were obtained from Dr. G. Smith, Department of Biochemistry, University of Cambridge and Sigma, respectively. A stock solution of sodium cholate, prepared from purified cholic acid [11], was a gift of Dr. B. Trumpower. Bovine pancreatic trypsin and lung aprotinin were gifts of Boehringer-Mannheim; α-chymotrypsin was a product of Worthington. Stock solutions of the proteases in 1 mM HCl were prepared just prior to use. Uniform latex particles (794, 85 ± 5.5 and 38 ± 7.5 nm) were purchased from Dow Diagnostics. Nikkol (octaethyleneglycol *n*-dodecyl ether, $C_{12}H_{25}$-$(OCH_2CH_2)_8OH$) was a product of Nikko Chemicals Ltd., Tokyo. Outdated units of human blood and packed cells in citrate/phosphate/dextrose were kindly provided by the blood bank of the Mary Hitchcock Memorial Hospital, Hanover, NH.

Preparations

Erythrocyte membranes (ghosts) were prepared from washed cells according to the method of Steck and Kant [12]. Membranes that lacked polypeptide bands 1, 2, 4.1, 4.2, 5 and 6 [13] (referred to as protein-depleted membranes) were obtained by releasing these peripheral proteins with alkali [14]. In our procedure ghosts, at 4 mg/ml in 5 mM sodium phosphate, pH 8, are mixed with 5.3 vols. of 2 mM Na_2EDTA/0.2 mM dithiothreitol/15.4 mM NaOH, at 1°C.

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* A preliminary report of these findings was presented in abstract form at the XI International Congress of Biochemistry, 1979, Toronto.

After 10 min, the membranes are separated by centrifugation at 19 000 rev./min for 15 min in a Sorvall SS-34 rotor and washed twice with 50 mM Tris-HCl (pH 7.4 at 3°C). A scan of the SDS-polyacrylamide gel after electrophoresis of the protein-depleted membranes has been published [4].

Inside-out vesicles were prepared from erythrocyte ghosts by the method of Steck and Kant [12]. The sidedness of these preparations was determined by assaying the activity of acetylcholinesterase, an externally oriented enzyme in cells, in the absence and presence of Triton X-100 [12]. Trypsin treatment of the inside-out vesicle preparations destroyed the acetylcholinesterase activity that was found in the absence of Triton, but it had no effect on the additional activity that was found upon Triton-induced lysis of the vesicles. Thus, the inside-out vesicles in the preparations must remain impermeable to both trypsin and acetylthiocholine (the substrate used in the assay) during trypsin digestion.

Purified, reconstituted transporter

The purification and reconstitution of the hexose transporter were carried out as previously described [4], except that the soybean phospholipids used in the reconstitution procedure were added from a stock solution of 20 mg/ml in 5% Triton X-100, to give a final concentration of 2 mg/ml before removal of the detergent with Bio-Beads SM2. A typical preparation contained approx. 35 μg/ml of transporter protein and 1.4 mg/ml phospholipid after the reconstitution. In preparations designated Triton-DOPC, dioleoyl phosphatidylcholine (DOPC) was substituted for soybean phospholipids in the reconstitution.

More recently, we have substituted the non-ionic detergent, Nikkol, for Triton throughout the purification procedure, and have as well reconstituted with dioleoyl phosphatidylcholine. These preparations are designated Nikkol-DOPC. Nikkol, which was used at the same concentration as Triton, solubilizes approx. 1.6 times as much transporter from the protein-depleted membranes and thus results in a corresponding increase in yield. SDS-polyacrylamide gel electrophoresis of the Nikkol-DOPC preparation shows a pattern (Fig. 5) very similar to that of the Triton-soybean phospholipid one [2,4]. Scatchard analysis of the binding of [^{3}H]cytochalasin B to the Nikkol-DOPC material gave values of $1.1 \cdot 10^{-7}$ M for the dissociation constant of cytochalasin B at 22°C and pH 6, and 10.2 nmol of sites per mg protein. The value of the dissociation constant is similar to that reported previously for our preparation with Triton and soybean lipids, whereas the specific activity is approx. 50% higher [4].

Generally the preparations of purified, reconstituted transporter were stored frozen at −70°C. Occasionally, the transporter was used as soon as it was isolated, without freezing. These have been referred to as frozen-thawed and unfrozen, respectively. Over 90% of the cytochalasin B binding activity in the frozen-thawed preparations could be pelleted by centrifugation at 150 000 × g for 1 h, whereas a smaller portion of the unfrozen preparations sedimented. Centrifugation provided a convenient method for concentrating the transporter and changing the medium. All the experiments described in Table I were performed with centrifuged transporter, resuspended by hand homogenization.

For some experiments the transporter was further dispersed by sonication. A bath sonicator (Ultrasonic Instruments, power supply G-80-80-1, tank T-80-80-

IRS), equipped with a constant temperature coil, was used; sonication was carried out at 25°C, unless noted otherwise, in a capped 13 × 100 mm test-tube under N_2. The cytochalasin B binding activity was partially inactivated by sonication; for example, under the conditions described in the legend of Fig. 6, 60% of the activity was lost.

Cytochalasin B binding

Intact cells. The binding of cytochalasin B to intact cells was assayed by incubating cells (50 μl of a 25% suspension) with [^{3}H]cytochalasin B and cytochalasin E (50 μl of $6 \cdot 10^{-8}$ M [^{3}H]cytochalasin B/$4 \cdot 10^{-5}$ M cytochalasin E) in 153 mM NaCl/5 mM sodium phosphate (pH 7.0) at 22°C for 3 min and then separating the cells by brief centrifugation [8]. Aliquots of the supernatant were counted to determine the concentration of free cytochalasin B; the concentration of bound cytochalasin B was calculated by subtraction of the free cytochalasin B from the total concentration, which was ascertained by counting an aliquot of the stock solution. Cytochalasin E was included to block the binding of cytochalasin B to the small fraction of high-affinity sites in the erythrocyte that are not located on the transporter [15]. Cytochalasin E does not bind to the transporter [15,16].

Ghosts and inside-out vesicles. Binding to these preparations was measured by incubating the suspension (100 μl) with [^{3}H]cytochalasin B and cytochalasin E (1 μl of $3 \cdot 10^{-6}$ M [^{3}H]cytochalasin B/2.5 mM cytochalasin E in ethanol) at 22°C for 1 min and then separating the membranes by centrifugation at 15 000 rev./min for 5 min in the Sorvall SS-34 rotor [8]. The total and free concentrations of cytochalasin B were measured by counting aliquots taken before and after centrifugation, respectively. The binding of cytochalasin B to protein-depleted membranes was assayed in a similar way, except that cytochalasin E was omitted, since these membranes have lost the high-affinity sites unrelated to transport [4].

Purified, reconstituted transporter. The association of cytochalasin B with the purified, reconstituted transporter was assayed by equilibrium dialysis, with $3 \cdot 10^{-8}$ M [^{3}H]cytochalasin B, according to the method described previously [9]. Before dialysis, the samples were adjusted to pH 6.0 with 0.5 M NaH_2PO_4. Unless it is noted otherwise, in the experiments with trypsin the trypsin inhibitor, aprotinin [17], was added at a concentration in μg/ml twice that of trypsin, to both the mixture containing trypsin and the control before the pH adjustment. At the concentrations used, aprotinin had no effect on the cytochalasin B binding activity.

For all the measurements of cytochalasin B binding made in the several ways described above, parallel measurements of binding in the presence of 400 mM D-glucose were also made. The value of the ratio of bound cytochalasin B to free cytochalasin B in the presence of D-glucose was then subtracted from the value of this ratio in the absence of D-glucose. The binding in the presence of D-glucose is due to low-affinity sites unrelated to transport, and this subtraction corrects for their contribution to the overall value of the bound-to-free ratio [9]. The corrected ratio is equal to the ratio of the concentration of cytochalasin B binding sites on transporters to the dissociation constant for cytochalasin B, and is thus proportional to the concentration of sites [9]. We

refer to the corrected ratio as specific [Bound CB]/[Free CB] in the figures and as cytochalasin B binding activity in the text. With the exception of the instances in which tryptic inactivation was nearly complete, the percentage of the uncorrected ratio due to nonspecific binding was less than 25%. The determinations of cytochalasin B binding activity were always carried out in duplicate. These normally agreed to within ±5% of the average value, which is given in Results.

For Scatchard plot analysis of cytochalasin B binding to the purified, reconstituted transporter, binding was measured by equilibrium dialysis over a range of cytochalasin B concentrations. Correction for nonspecific binding was made in a different way which is outlined in Results.

Other procedures

The initial rates of uptake of L-[^{14}C]sorbose and L-[^{3}H]glucose into cells and inside-out vesicles were determined through procedures that involve separation from the medium by centrifugation for the cells and by filtration for the vesicles. These are given in detail elsewhere [18,19].

The Bio-Gel A-150 m columns were calibrated with uniform latex particles eluted in buffer containing 0.1% Triton [20] and with small unilamellar vesicles of dimyristoyl phosphatidylcholine (25 nm [21]), eluted without detergent. The void and total volumes were taken as the positions of elution of large latex beads (794 nm) and of β-mercaptoethanol. The beads were detected by absorbance at 300 nm, the vesicles by analysis for phosphorus, and the thiol by a colorimetric test [22].

SDS-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli [23]. Samples for electrophoresis (100-μl) received 10 μl of 500 mM Tris-HCl/10 mM EDTA/10% SDS (pH 6.8), 5 μl of 100 mM dithiothreitol and 10 μl of 20% SDS, and then were immediately placed at 100°C for 5 min. After cooling, 10 μl of 150 mM *N*-ethylmaleimide, 10 μl of 84% sucrose, and 1 μl of 12 mg/ml pyronin Y were added. Gels were stained with Coomassie brilliant blue R-250 by the method of Steck and Yu [14]. The relative amounts of the individual polypeptides were estimated from spectrophotometric scans of the gels according to the method given in Ref. 18.

Protein, phospholipid and radioactivity were measured by the procedures given previously [4,9]. When inorganic phosphate was present, the lipid was first extracted with chloroform/methanol (2 : 1, v/v).

Results

Cytochalasin B binding to intact erythrocytes and inside-out vesicles

Treatment of intact red cells with trypsin caused no significant decrease in the D-glucose-inhibitable cytochalasin B binding activity (Fig. 1). On the other hand, digestion of inside-out vesicles prepared from erythrocyte membranes leads to a rapid loss of activity (Fig. 1). The residual activity of the inside-out vesicle preparation (25% of the initial value) is accounted for by contamination with right-side-out vesicles, which amounted to 30% of the total as determined by assay for the accessibility of acetylcholinesterase.

We have also treated erythrocyte membranes (ghosts) and protein-depleted

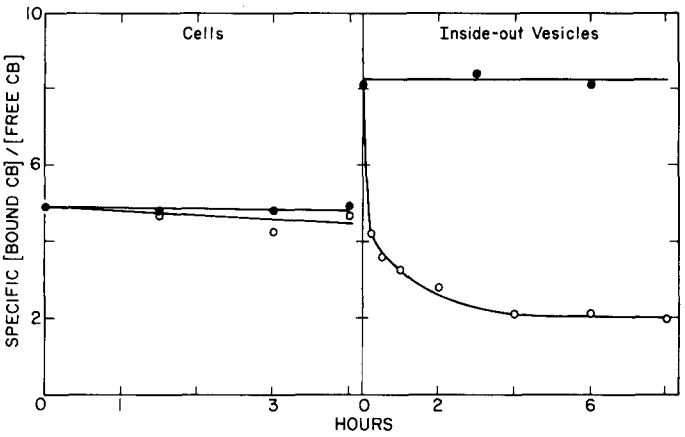

Fig. 1. The effect of trypsin treatment upon cytochalasin B binding by intact cells and inside-out vesicles. Cells at 25% hematocrit, which is approx. 2 mg/ml in membrane protein [18], were incubated in 153 mM NaCl/5 mM sodium phosphate (pH 7.0) at 25°C without (•) and with (○) 200 μg/ml trypsin. Inside-out vesicles, at 1.4 mg protein/ml, were treated in 0.5 mM sodium phosphate (pH 7.2) at 25°C without (•) and with (○) 140 μg/ml trypsin.

membranes with trypsin under conditions similar to those given in the legend of Fig. 1. Upon limit digestion, 87 and 77% of the cytochalasin B binding activity were lost, respectively. The small remaining fraction of activity may be due to sealed right-side-out vesicles in the preparation.

These results thus confirm the conclusion (Lin and Spudich [8]) that the cytochalasin B binding site associated with the glucose transporter is susceptible to proteolytic inactivation only at the cytoplasmic surface of the membrane. Moreover, since cytochalasin B is a relatively nonpolar molecule that readily penetrates the erythrocyte membrane [24], the binding site may be located on the cytoplasmic surface of the transporter. The kinetics of inhibition of transport by cytochalasin B are consistent with this location [25].

Hexose transport by cells and inside-out vesicles

L-Sorbose enters erythrocytes and inside-out vesicles largely by the monosaccharide transport system [18,19], whereas L-glucose is a much poorer substrate for the system [26] that may enter largely by other pathways. The difference between the initial rates of uptake of L-sorbose and L-glucose thus provides a convenient assay for the transport activity of the system [18,19]. We have employed this assay to examine the effect of trypsin treatment on the transport activity.

Cells which have been subjected to the protease show no change in the uptake of either sugar (Fig. 2). In contrast, proteolysis of the inside-out vesicles has several effects. Firstly, the uptake at zero time, which is probably due to a combination of binding to the filter, binding to the outer surface of the vesicles, and rapid uptake into a small fraction of very leaky vesicles, is increased. Secondly, the rate of entry of L-glucose is increased, probably as the result of enhanced 'nonspecific' permeability of the membrane. Thirdly, the rate of entry of L-sorbose is decreased. The net result is that the transport activity of the system, as defined above, is only approx. 5% of that in the absence

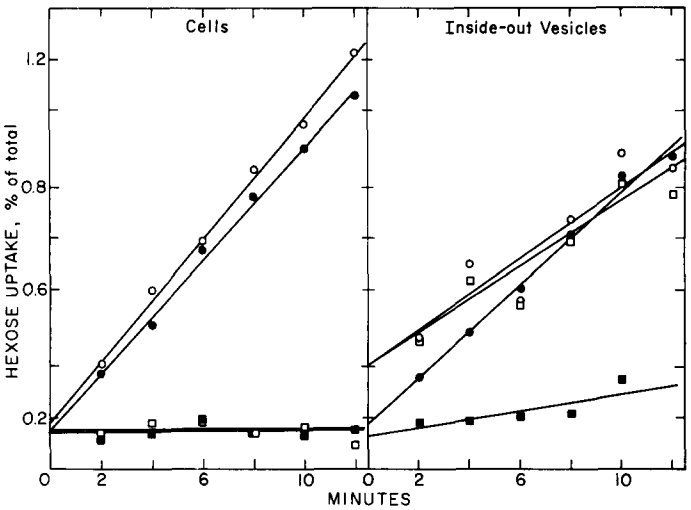

Fig. 2. The effect of trypsin treatment on hexose transport by cells and inside-out vesicles. The cells and inside-out vesicles described in Fig. 1 were used for transport measurements after 4 h of incubation at 25°C. Uptake was initiated by adding to the mixtures a small aliquot of L-[^{3}H]glucose and L-[^{14}C]-sorbose such that the final concentration of each was 0.5 mM for the cells and 0.1 mM for the inside-out vesicles, and was carried out with the mixtures at 12.5°C. The points are averages from duplicate experiments; these agreed to within ±5% for the cells and generally to within ±15% for the inside-out vesicles, and the lines are the best fits according to least-squares linear regression. Unproteolyzed (●, ■); proteolyzed (○, □); L-sorbose (○, ●); L-glucose (□, ■).

of proteolysis.

A somewhat larger remaining activity was expected, since this inside-out vesicle preparation contained approx. 30% right-side-out vesicles. The discrepancy is probably the result of error in the value of the transport activity for the proteolyzed vesicles which showed more scatter in the uptake plots. In a repetition of the experiments described here, with a preparation of inside-out vesicles which contained 22% right-side-out vesicles according to acetylcholinesterase accessibility, we found 26% of the cytochalasin B binding activity and 18% of the transport activity remained after limit trypsin digestion. Thus, proteolysis at the cytoplasmic surface destroys the transport function as well as the cytochalasin B binding function. These results confirm the findings of Masiak and LeFevre [39].

Cytochalasin B binding by the purified, reconstituted hexose transporter

Proteolysis of the purified, reconstituted transporter resulted in a rapid loss of 40—60% of the cytochalasin B binding activity (Fig. 3 and Table I). The remaining activity was resistant to the prolonged action of a high concentration of trypsin (Table I, 5).

Our method for reconstitution of the transporter after purification is to remove the detergent with polystyrene beads in the presence of added phospholipid [4]. We have not examined the nature of the lipid structures obtained by this procedure. However, others have reported that it yields, largely [27] or in part [28], unilamellar vesicles of diameter 200 nm and less. Subsequent freezing has been found to convert these to much larger vesicles [2,27] and brief sonication of the larger vesicles disperses them as unilamellar

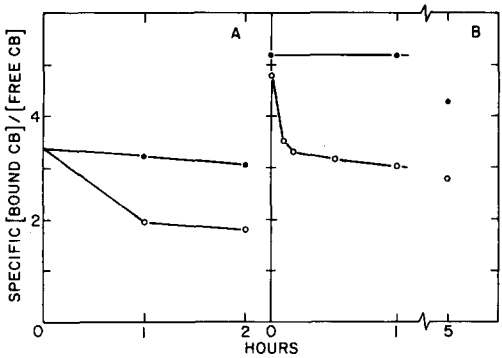

Fig. 3. The effect of trypsin treatment on the binding of cytochalasin B to the purified, reconstituted transporter. (A) Transporter at 70 μg/ml in 5 mM sodium phosphate/160 mM NaCl (pH 7.0) was treated for 1 h with 2 μg/ml trypsin (○, 1 h) and subsequently for 1 h with 4 μg/ml trypsin (○, 2 h), at 22°C. (B) Transporter at 140 μg/ml in 5 mM sodium phosphate (pH 7.0) was digested with 1.5 μg/ml trypsin for various periods (○). The corresponding control mixtures (closed symbols) lacked trypsin. The digestions were stopped by the addition of aprotinin.

vesicles of diameter 200 nm and less [2]. The susceptibility of the transporter to trypsin inactivation in each of these states (unfrozen, frozen-thawed, frozen-thawed-sonicated) is roughly the same and amounts to 40—60% of the activity (Table I, 1—3). This finding is most readily explained by the assumption that the preparations are largely sealed unilamellar vesicles containing almost randomly oriented transporters, but it is also consistent with multilamellar vesicles with the inside-out orientation predominantly for transporters in the outer shell.

TABLE I

EFFECT OF TRYPSIN ON CYTOCHALASIN B BINDING BY THE PURIFIED, RECONSTITUTED HEXOSE TRANSPORTER

Purified, reconstituted transporter, which had been subjected to the treatments described, was then treated with trypsin at 22—25°C in 5 mM sodium phophate (pH 7) (5P7) or in 5P7/160 mM NaCl for periods of 1—6 h, and assayed for cytochalasin B binding. The remaining binding activity is expressed as a percentage of the value for the control mixture that lacked trypsin.

Prior treatment	Transporter (μg/ml)	Trypsin (μg/ml)	Buffer	% binding activity
1. Unfrozen	105	1.5	5P7/NaCl	55
	53	10		62
2. Frozen-thawed	140	1.5	5P7/NaCl	63
	45	10		50
3. Frozen-thawed-5 min sonication	45	10	5P7/NaCl	40
4. Unfrozen-2 min sonication	105	1.5	5P7/NaCl	50
5. Frozen-thawed	140	1.5	5P7	64
	175	1.9		62
	140	30 *		50
6. Unfrozen (Triton-DOPC)	38	1.5	5P7/NaCl	47
7. Frozen-thawed (Nikkol-DOPC)	216	4	5P7	53

* Cytochalasin B binding was measured by equilibrium dialysis without addition of aprotinin.

Another aspects of our reconstitution procedure is that it is carried out in 100 mM NaCl/50 mM Tris-HCl (pH 7.4) [4]. Vesicles with this internal medium would be expected to undergo osmotic lysis in the 5 mM sodium phosphate which was used for some digestions. However, resealing must occur rapidly, since reconstituted transporter in 5 mM sodium phosphate was no more sensitive to proteolysis than that in 5 mM sodium phosphate/160 mM NaCl (Table I, 2 and 5).

It was possible to obtain more extensive inactivation of the cytochalasin B binding activity by freezing, thawing, and sonicating in the presence of trypsin. Transporter (40 μg/ml in 5 mM sodium phosphate/160 mM NaCl (pH 7.0)) was sonicated for 2 min, frozen in solid CO_2/ethanol, and allowed to thaw at room temperature. This operation was repeated and followed by a final 1 min of sonication. Trypsin (10 μg/ml) was either included or added afterwards, and digestion was carried out for 1 h at 22°C. The binding activity of the mixture containing trypsin during the initial manipulations was 14% of that of the corresponding control mixture that lacked trypsin, whereas 54% of the activity was retained in the mixture that received protease after the pretreatments. This finding indicates that the partial inactivation found under the conditions described in Table I is not due to the presence of a form of the transporter that is intrinsically trypsin-resistant. Presumably, trypsin is introduced into vesicles during freezing, thawing, and sonication.

Most of our experiments have been performed with transporter purified in Triton X-100 and incorporated into soybean phospholipids, which are largely a mixture of phosphatidylcholine (40%) and phosphatidylethanolamine (33%) [29]. Transporter which had been purified in Triton X-100 and reconstituted in dioleoyl phosphatidylcholine (Table I, 6), as well as transporter which had been purified in Nikkol and reconstituted in this lipid (Table I, 7), showed the same degree of inactivation.

Scatchard plot analysis of cytochalasin B binding

The assay of cytochalasin B binding activity provides values for the ratio of the total concentration of high-affinity, transporter-related sites ($[R_t]$) to the dissociation constant for these sites (K_d) (see Materials and Methods and Ref. 9). In order to determine whether the effect of trypsin was on the concentration of sites or on the dissociation constant, the dependence of binding on cytochalasin B concentration was determined. The Scatchard plots of the uncorrected data (Fig. 4) are slightly curved, since nonspecific absorption of cytochalasin B, largely to the phospholipid, as well as specific binding to the transporter occurs [4]. The raw data were corrected for nonspecific binding according to a procedure which we have described in detail previously [9]. This procedure optimizes the fit of the corrected data, by least-squares linear regression, to the simple Scatchard equation for a single set of high-affinity sites. It does so through choice of the best value for the constant, small contribution of nonspecific binding to the observed ratios of bound to free cytochalasin B.

The linear plots of the corrected data are shown in Fig. 4. These yield values of $1.02 \cdot 10^{-7}$ M for K_d and $3.92 \cdot 10^{-7}$ M for $[R_t]$ in the case of the unproteolyzed transporter, and $1.22 \cdot 10^{-7}$ M for K_d and $2.90 \cdot 10^{-7}$ M for $[R_t]$ in the case of the partially inactivated system. Thus, for this preparation, which

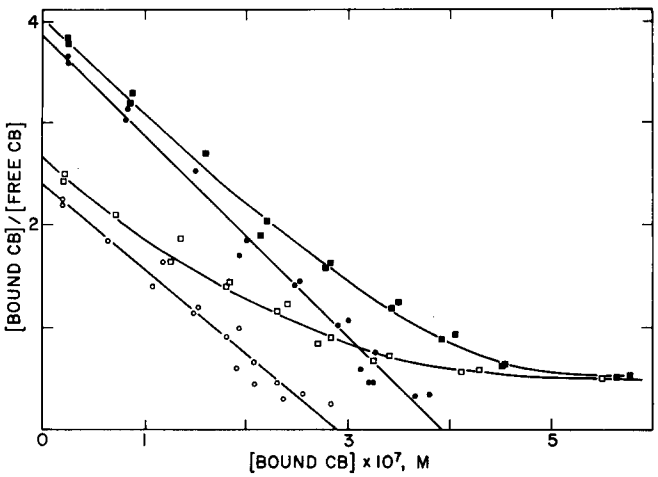

Fig. 4. Scatchard plots for the binding of cytochalasin B to the purified, reconstituted transporter, with and without proteolysis. Transporter, at 175 μg/ml in 5 mM sodium phosphate (pH 7.0), was treated for 3 h at 22°C with 1.9 μg/ml trypsin. Digestion was terminated with 3.8 μg/ml of aprotinin. The sample was diluted with an equal volume of buffer and used for the measurements of cytochalasin B binding. A control mixture that lacked trypsin and aprotinin was handled in the same way. Both the uncorrected values (■, □) and the values corrected for nonspecific binding (●, ○) are shown. Intact (■, ●); proteolyzed (□, ○).

exhibited 62% of the binding activity after trypsin treatment in the standard assay, the results indicate that 74% of the cytochalasin B binding sites remain, with an association constant which is 84% of the initial value. It is worth noting that changes of ±7% in the values for K_d and $[R_t]$ are possible without substantially decreasing the values of correlation coefficients for the plots of the corrected data, through the choice of somewhat different values for the contribution of nonspecific binding. Because of this uncertainty, because of the results with intact cells and inside-out vesicles, and because of the outcome of SDS gel electrophoresis described below, we believe that most, if not all, of the loss in binding activity is due to a decrease in the number of sites.

SDS-polyacrylamide gel electrophoresis of purified, reconstituted transporter

SDS-polyacrylamide gel electrophoresis of purified transporter shows a broad band with a mobility corresponding to an average apparent molecular weight of 55 000 [2,4] (Fig. 5). The broadness of this peak is largely due to heterogeneity in the carbohydrate linked to the transporter polypeptide (Ref. 5, Gorga, F.R. and Lienhard, G.E., unpublished results). In addition, there are present some high molecular weight species, which arise from aggregation of the transporter polypeptide (Ref. 5, Gorga, F.R. and Lienhard, G.E., unpublished results) and a small peak of erythrocyte membrane band 7 polypeptide (nomenclature according to Ref. 13), which amounts to approx. 2% of the total protein on the basis of Coomassie blue staining.

After proteolysis there is a marked reduction in the amount of the transporter polypeptide and its aggregates that corresponds closely to the loss of cytochalasin B binding activity. In the case of the example presented in Fig. 5, which was obtained with a Nikkol-DOPC preparation, proteolysis decreased the

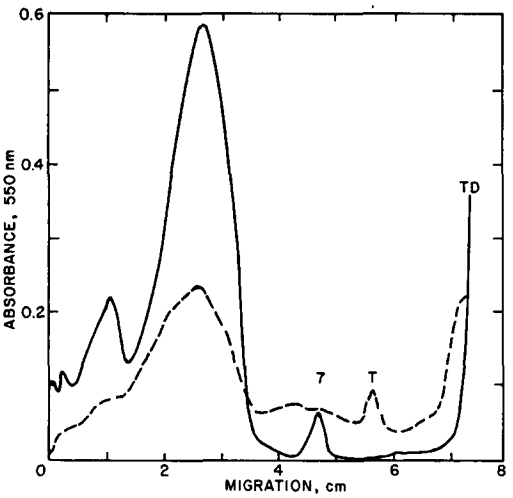

Fig. 5. SDS-polyacrylamide gel electrophoresis of untreated (———) and trypsin-treated (- - - - -) transporter. Purified, reconstituted transporter (Nikkol-DOPC), at 220 μg/ml, was incubated for 6 h at 22°C, with either 4 μg/ml trypsin or no addition. Aprotinin (8 μg/ml) was added to the mixtures, and samples were taken for electrophoresis and cytochalasin B binding. A sample containing 11 μg protein was applied to each gel. 7, T and TD designate band 7, trypsin and tracking dye, respectively.

cytochalasin B binding activity to 53% of the control value and decreased the amount of transporter polypeptide, as measured by the intensity of the Coomassie blue staining, to 46% of the control value. Similar results were obtained with transporter which was purified in Triton and incorporated into soybean phospholipids. These findings thus indicate that substantial fragmentation of the transporter accompanies the inactivation of cytochalasin B binding, and that the remaining activity is associated with an apparently intact polypeptide.

Some of the fragments of the proteolyzed transporter appear as a broad stain in the region for polypeptides of molecular weight 40 000 and less. Electrophoresis of the supernatant and pellet obtained by centrifugation of the digest showed that most of this material remains associated with the membrane. Since the carbohydrate is linked to the extracellular portion of the polypeptide (Gorga, F.R. and Lienhard, G.E., unpublished results) and since tryptic cleavage occurs at the cytoplasmic portion, the larger fragments from proteolysis are expected to be heterogeneously glycosylated and so run as an even broader band than the parent polypeptide.

Proteolysis with chymotrypsin

We have also examined the effect of chymotrypsin on both the cytochalasin B binding activity and the electrophoretic pattern of the purified, reconstituted transporter, under conditions similar to those given for trypsin. The results were virtually the same as those obtained with trypsin.

Orientation of the purified transporter reconstituted in small unilamellar vesicles

Each molecule of purified transporter incorporated into unilamellar vesicles

would be expected to have one of two orientations relative to the plane of the bilayer: either the same as that in the intact erythrocyte, with the extracellular domain exposed, or the same as that in inside-out vesicles, with the cytoplasmic domain exposed. The findings described above indicate that the fraction of transporters oriented with the cytoplasmic domain exposed is equal to the portion of the cytochalasin B binding activity that is inactivated by trypsin. We have used this method to determine the orientation of the transporter in small unilamellar vesicles that have been prepared in two ways.

One way was sonication of the purified, reconstituted transporter [30], followed by gel filtration. Approximately half the phospholipid was converted to vesicles of less than 85 nm diameter (Fig. 6). The distribution of both the protein and the cytochalasin B binding activity paralleled that of the phospholipid. Thus, there is no selective association of transporter with lipid structures of a certain size. The peak fractions of small vesicles (44—52 ml) were combined, and portions were treated with 1 and 2 μg/ml of trypsin. The cytochalasin B binding activity in the mixture with 1 μg/ml dropped to 65 and 57% of its initial value after 1 and 3 h of digestion, respectively; that in the mixture with 2 μg/ml fell to 64 and 62% of the initial value after 3 and 5 h of treatment, respectively. Thus, approx. 40% of the transporter in these vesicles is oriented with its cytoplasmic surface external.

The second method of preparing small unilamellar vesicles was to solubilize the purified, reconstituted transporter with cholate, to sediment insoluble material, and then to remove the detergent from the supernatant with Sephadex G-50 (see the legend of Fig. 7) [31]. The vesicles obtained by this

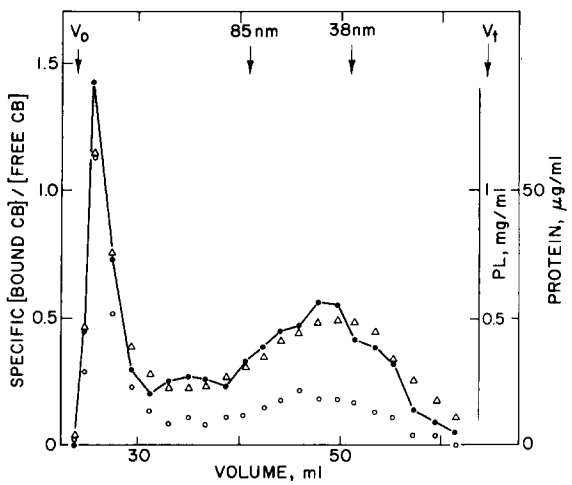

Fig. 6. Gel filtration of vesicles obtained upon sonication. Purified, reconstituted transporter, which had been frozen and thawed, at 260 μg/ml in soybean phospholipids at 9.0 mg/ml, was sonicated in 160 mM NaCl/1 mM EDTA/5 mM dithiothreitol/5 mM sodium phosphate (pH 7.0) for 8 min. A portion (1.8 ml) was applied to a column (1.6 × 34 cm) of Bio-Gel A-150 m and eluted with the same buffer at a flow rate of 4 ml/h, at 4°C. Fractions were analyzed for cytochalasin B binding activity (●), phospholipid (△) and protein (○). Approx. 75% of the initial amount of each was recovered. V_o, V_t, 85 nm and 38 nm designate the void volume, the total volume, and the peak positions for latex beads of these diameters, respectively.

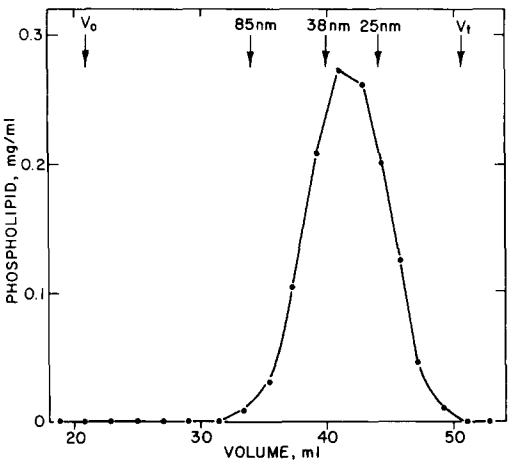

Fig. 7. Gel filtration of vesicles prepared by cholate solubilization and gel filtration. Purified, reconstituted transporter (Nikkol-DOPC, frozen-thawed), at 270 μg/ml with 8 mg/ml phospholipid, in 115 mM NaCl/1.1 mM EDTA/2.2 mM dithiothreitol/50 mM Tris-HCl (pH 7.0) was sonicated for 10 s at 5°C. The mixture was made 2.1% in sodium cholate by addition of an aliquot from a 21% (w/v) stock solution, shaken for 30 min at 4°C, and then centrifuged at 180 000 × *g* for 1 h. Supernatant (0.75 ml) was applied to a Sephadex G-50 (fine) column (1 × 30 cm), equilibrated with 105 mM NaCl/1.0 mM EDTA/2.0 mM dithiothreitol/45 mM Tris-HCl (pH 7.4) and eluted with the same buffer (7 ml/h) at 4°C. The peak fractions of vesicles were located by their absorbance at 425 nm. These were combined and 2.0 ml were applied to a column of Bio-Gel A-150 m (1 × 59 cm) and eluted at 1 ml/h with the buffer given above. The profile of phospholipid is shown. Designations as in Fig. 6.

procedure were examined by gel filtration on Bio-Gel A-150 m (Fig. 7). They are all small, with an average diameter of 32.5 nm. Approx. 50% of the protein and phospholipid treated with cholate was recovered in the vesicles from the Sephadex chromatography; approx. 35% was not solubilized and an additional 15% was lost on the column. The cytochalasin B binding activity of the vesicles per mg of protein was approx. 50% that of the original material. This loss of activity is probably due to partial inactivation while in the solubilized state, a process that we have established in the case of Triton X-100 and Nikkol (Baldwin, J.M., Lienhard, G.E. and Baldwin, S.A., unpublished results).

Treatment of the small vesicles from the cholate solubilization with trypsin led to the loss of the considerably more than half of the cytochalasin B binding activity (Fig. 8). In the case of the example shown in Fig. 8, the residual activity amounted to 16% of the control value. The percentages found in similar experiments with three other preparations of vesicles were 31, 29 and 20%. SDS-polyacrylamide gel electrophoresis showed that this greater loss of activity correlated with the disappearance of a larger fraction of the transporter polypeptide.

Thus, these results suggest that in the small vesicles obtained after cholate solubilization approx. 75% of the transporter polypeptides are oriented with the cytoplasmic portion external. In order to test the alternative, trivial explanations that the vesicles are largely unsealed or break open during the trypsin treatment, we have examined the fate of an impermeant internal marker. Vesicles were prepared with [^{14}C]EDTA in the medium, and the amount of EDTA within the vesicles was measured (Fig. 8). The amount was

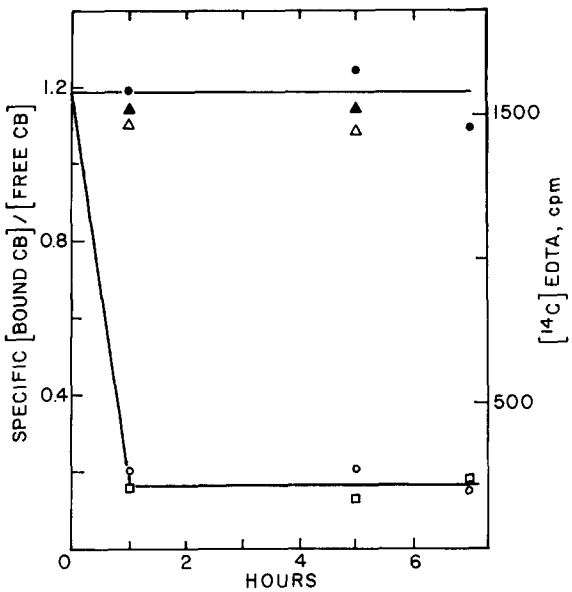

Fig. 8. Effect of trypsin on cytochalasin B binding by the hexose transporter reconstituted into small unilamellar vesicles. Vesicles were prepared by the passage of cholate-solubilized transporter (Nikkol-DOPC) through Sephadex G-50 according to the procedure described in the legend of Fig. 7. These were dialyzed at 4°C against 105 mM NaCl/1.0 mM EDTA/50 mM Tris-HCl (pH 7.4), and then were treated at 22°C for various periods without (●), with 3 μg/ml (○) and 6 μg/ml (□) trypsin. Another preparation of vesicles was made in the same way with [^{14}C]EDTA ($2.7 \cdot 10^7$ cpm/ml) in the cholate solubilization mixture and the Sephadex G-50 column. These vesicles at 1.2 mg phospholipid per ml were treated without (▲) or with 6 μg/ml trypsin (△), and the intravesicular EDTA in 100-μl aliquots was determined at various times by separation of the vesicles from the medium with a gel filtration procedure [38] (right-hand ordinate).

equivalent to an internal volume of 0.58 μl per mg dioleoyl phosphatidylcholine. It remained constant over a 5 h period, with or without trypsin present (Fig. 8). The internal volume associated with protein-free phosphatidylcholine vesicles of 32.5 nm diameter can be calculated to be approx. 0.65 μl per mg of phospholipid [32]. This agreement between the observed and calculated values for the internal volume indicates that the vesicles are largely sealed.

It is worth noting that the number of functional transporter molecules per small vesicle can be estimated as follows. The concentration of cytochalasin B binding sites can be calculated from the specific ratio of bound to free cytochalasin B and the dissociation constant for cytochalasin B [9]. The concentration of vesicles can be estimated from the phospholipid concentration (g/l) and the calculated molecular mass of a 32.5 nm vesicle of dioleoyl phosphatidylcholine (5 600 000 daltons [32]). Under our conditions there was approximately one cytochalasin B binding site per two vesicles. The vesicles that contain transporter must be sealed, since preliminary results show that they selectively take up D-glucose to the expected level (Baldwin, J.M., Lienhard, G.E. and Baldwin, S.A., unpublished results).

Discussion

The results presented here show that trypsin proteolysis in conjunction with cytochalasin B binding is a convenient method to determine the exposure of

the cytoplasmic domain of the erythrocyte hexose transporter. When this method was applied to transporter incorporated into small unilamellar vesicles by solubilizing with cholate and then removing the cholate through gel filtration, approx. 75% of the transporter was found to be oriented with the cytoplasmic domain exposed. The occurrence of asymmetry was not unexpected, since other integral membrane proteins reconstituted into small vesicles by similar procedures involving cholate have been found to be asymmetrically oriented [33,34].

Without more information on the pathway of vesicle formation, it is not possible to understand the basis for the asymmetry. There appear to be several possible explanations. As a prelude to the formation of a vesicle, a protein molcule may be located in a planar patch of phospholipid bilayer surrounded at its perimeter with cholate [35]. As further cholate is removed, this planar patch of bilayer curves so that portions of its edge merge, and upon loss of even more cholate, a sealed vesicle forms. In this case, the direction of curvature may simply be determined by the relative steric bulk of the cytoplasmic and extracellular domains of the protein [34]. Alternatively, vesicles of phospholipid alone may form first. Subsequently, a complex of protein, phospholipid and detergent may insert into (fuse with) the vesicles. It is easy to imagine that insertion (fusion) via one region of the protein is favored, and indeed this situation provides the basis of a method for reconstituting integral proteins in one orientation [36,37]. In any event, it is worth noting that the ratio of approx. 3 : 1 for orientation reported here corresponds to a difference of only 650 cal/mol at 25°C between the free energy changes associated with the two orientations in the rate or equilibrium process that determines orientation.

The finding that the transporter has a different distribution of orientations in the small unilamellar vesicles obtained through sonication of larger structures is not inconsistent with the results for the vesicles from cholate solubilization. Firstly, the vesicles obtained by sonication are larger (30—80 nm vs. 32.5 nm). Secondly, since the pathway for vesicle formation is different in the two cases, there is no reason why the orientational distribution must be the same even in vesicles of the same size, unless one hypothesizes that each method gives rise to the equilibrium state.

Preliminary results on the transport function of the small vesicles obtained by cholate solubilization show that these transport D-, but not L-glucose (Baldwin, J.M., Lienhard, G.E. and Baldwin, S.A., unpublished results). However, the specific transport activity of the transporter reconstituted in this way is only a small fraction of the value expected on the basis of its activity in the intact erythrocyte. Kasahara and Hinkle have reported a similar finding for the transport activity of purified, reconstituted transporter [2]. It may be that additional components are required for full transport activity. In this paper we have referred to the purified protein as the transporter largely for the sake of convenience in expression; in the current state of affairs the term, cytochalasin B binding component, would be more accurate, since the stoichiometry of this interaction approaches unity [4,5]. The transport activity of the purified transporter as well as the effect of trypsin upon it are now under investigation and will be the subject of a future report.

Acknowledgements

This research was supported by Grant GM 22996 from the National Institutes of Health and by a Faculty Research Award to G.E.L. from the American Cancer Society.

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