Binding of Cytochalasin B to Human Erythrocyte Glucose Transporter†

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ABSTRACT: Cytochalasin B, a potent inhibitor of D-glucose transport systems, binds to the glucose transporter purified from human erythrocytes as described previously [Kasahara, M., & Hinkle, P. C. (1977) *J. Biol. Chem. 252*, 7384]. The transporter binds 9.2 ± 1.3 nmol of cytochalasin B/mg of protein with a dissociation constant of 0.18 μM. The binding is competitively inhibited by D-glucose (K_i = 43 mM). Phloretin, diethylstilbestrol, maltose, 6-*O*-propyl-D-galactose, propyl β-D-glucopyranoside, and dithiothreitol were also linear competitive inhibitors of cytochalasin B binding. The propyl sugars have been shown to inhibit transport from either the plasma or cytoplasma side of the membrane, respectively. The binding of cytochalasin B to the isolated transporter was inhibited by both propyl sugars.

The D-glucose transporter of the human erythrocyte membrane has been solubilized with Triton X-100 and purified by DEAE[1]-cellulose chromatography using reconstitution of D-glucose permeability in liposomes as an assay for activity (Kasahara & Hinkle, 1976, 1977). The transporter is a glycoprotein with an apparent molecular weight of 55 000 as determined by polyacrylamide gel electrophoresis with sodium dodecyl sulfate.

Cytochalasin B is a potent inhibitor of D-glucose transport in erythrocytes (Taverna & Langdon, 1973; Taylor & Gagneja, 1975; Lin et al., 1974; Jung & Rampal, 1977) and also binds to another protein which is removed from ghosts by washing with EDTA at low ionic strength (Pinkofsky et al., 1978). Possibly as many as three classes of saturable binding sites have been detected in human erythrocytes (Pinkofsky et al., 1978). The binding to the first class (consisting of two-thirds of the sites) is reversible by D-glucose. Binding to the second class is reversible by cytochalasin E. No inhibitor of binding was found to the third class.

As part of our characterization of the D-glucose transporter, we have studied the binding of cytochalasin B to the purified protein and the effects of other substrates and inhibitors related to glucose transport on the binding of cytochalasin B. Preliminary results were published (Sogin & Hinkle, 1978, 1979; Sogin & Telford, 1978).

Experimental Procedures

Materials. Cytochalasin B was purchased from Aldrich Chemical Co., [^{3}H]cytochalasin B was from New England Nuclear, phloretin was from ICN Pharmaceuticals, and diethylstilbestrol and dithiothreitol were from Sigma Chemical Co. Propyl β-D-glucopyranoside and 6-*O*-propyl-D-galactose were synthesized as described by Barnett et al. (1975). The D-glucose transporter was prepared as described earlier (Kasahara & Hinkle, 1977) and as modified (Sogin & Hinkle, 1978).

Protein Determination. The large amount of lipid present in the samples of purified protein interfered with the determination of protein content by the method of Lowry et al. (1951) or Furchter & Crestfield (1965). Therefore the following procedure was used. Samples (0.4 mL) were made 0.2 M with respect to NaCl, and 50 μL of a solution of 2 mg of sodium deoxycholate/mL and 50% Cl_3CCOOH was added to precipitate the protein (Bensadoun & Weinstein, 1976). The lipid was extracted 4 times with 1 mL of ether–choloroform–methanol (6:2:1). After each extraction the sample was centrifuged at 2000 rpm, the organic layer was removed, and 50 μL of 0.2% deoxycholate and 50% Cl_3CCOOH was added. The ether prevented the extraction of protein and decreased the density of the organic phase such that it floated on top of the aqueous phase. The aqueous phase was heated 15 min at 70 °C to remove the dissolved solvents and then centrifuged to form a pellet. The pellet was washed with 5% methanol in ether to remove the deoxycholate and Cl_3CCOOH and then transferred to polypropylene tubes for alkaline hydrolysis followed by determination of total amino acids with ninhydrin (Fruchter & Crestfield 1965; Moore, 1968). Since the precipitate is insoluble, no other method for determination of protein was satisfactory.

Equilibrium Dialysis. Each chamber (up to 72) of the apparatus for equilibrium dialysis (Riverside Scientific, Seattle, WA) was separated by a circular piece of dialysis membrane (12 000 M_r cutoff). A 50-μL sample containing protein in 10 mM sodium 3-(*N*-morpholino)propanesulfonate, pH 7.5, was added to one side, and 50 μL of [^{3}H]cytochalasin B in the same buffer was added to the other side. To bring the volumes of both chambers to 100 μL, we added 50 μL of either buffer or buffer containing one of several inhibitors to both sides. The chambers were allowed to equilibrate at room temperature for 5 h after the last chamber was loaded. At the end of this period, 80-μL samples were removed from each chamber, and radioactivity was determined in a Beckman LS-100 or 7000 scintillation counter with either Amersham ACS or National Diagnostic Liquiscint counting scintillants. The binding activity of the protein was found to be stable during the course of the experiment. The half-time for equilibration of cytochalasin B was 27 min and of glucose was 17 min. Data for each concentration of competing inhibitor were plotted as described by Scatchard (1949) and lines fitted by linear regression.

Reaction of Cytochalasin B with DTT. Cytochalasin B was incubated for 6 h with 10 mM DTT followed by addition of a 10-fold excess of iodoacetate to react with the DTT. The samples were then extracted with ethyl acetate, dried, and analyzed by thin-layer chromatography on silica gel plates (Lin et al., 1974). Unreacted cytochalalsin B was easily extracted and had an R_f of ~0.5, while the reacted material (as de-

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[1] Abbreviations used: DTT, dithiothreitol; Cl_3CCOOH, trichloroacetic acid; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

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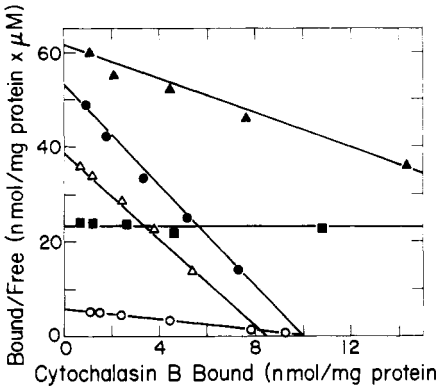

FIGURE 1: Binding of cytochalasin B to glucose transporter. Chambers were filled as described. Purified protein at 30 μg/mL (●); purified protein at 30 μg/mL reconstituted into liposomes (0.2 mg of asolectin/mL) (▲); liposomes without protein (0.2 mg of asolectin/mL) for which a protein concentration was taken to be 30 μg/mL for display purposes (□); reconstituted vesicles corrected for nonspecific binding (△); Triton extract for which the amount bound plotted on the *x* axis has been multiplied by 10 for display purposes (○).

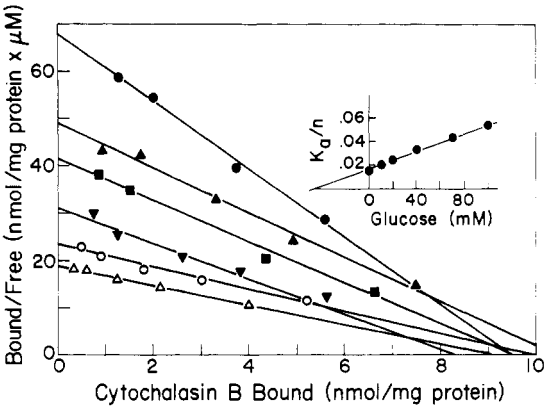

FIGURE 2: Inhibition of binding of cytochalasin B by glucose. Protein concentration was 30 μg/mL for glucose at 0 mM (●), 10 mM (▲), 20 mM (□), and 40 mM (▼), 60 μg/mL for glucose at 70 mM (○), and 120 μg/mL for glucose at 100 mM (△).

termined during the reaction at pH 12) extracted poorly and remained at the origin. Radioactivity for all liquid and chromatographed fractions was determined to calculate the percentage of each product.

Results

As shown by the Scatchard plot in Figure 1, cytochalasin B binds to detergent-free extracts of alkaline-washed ghosts, purified protein, and vesicles reconstituted with purified protein. For all three preparations we observed a single class of saturable binding sites. The binding of cytochalasin B to the purified transporter in vesicles of endogenous lipid showed a dissociation constant of 0.19 μM and an extent of 9.9 nmol/mg of protein. The mean dissociation constant from seven experiments with four different preparations was 0.18 ± 0.03 μM, and the mean extent was 9.2 ± 1.3 (range 8–11) nmol/mg or protein.

The detergent-free Triton extract of the alkaline-treated vesicles showed a dissociation constant of 0.16 μM for binding of cytochalasin B and an extent of 1 nmol/mg of protein (Figure 1). (The binding of cytochalasin B to protein could not be determined in the presence of detergents because the inhibitor bound nonspecificially to detergent micelles.) Thus the DEAE-cellulose chromatography step between the Triton extract and the purified fraction brought about a 10-fold purification. The purified protein also had ~12-fold higher specific D-glucose transport activity in the reconstituted vesicles than the Triton extract, although the transport activity was less than expected based on glucose transport rates in whole cells (Kasahara & Hinkle, 1976). More recent studies have shown that when assayed under optimal conditions the equilibrium exchange transport activity of the reconstituted transporter is 5 times the specific activity of ghosts (Wheeler & Hinkle, 1980).

Liposomes without protein prepared by the freeze–thaw method of reconstitution also bind cytochalasin B (horizontal line, Figure 1), but this binding was not saturable in the concentration range studied. Reconstituted vesicles with the glucose transporter show saturable binding to the protein and nonsaturable binding to the lipid. When the nonspecific binding at each concentration of free cytochalasin B was substracted from the total, the corrected line gave an extent of 8.5 nmol/mg of protein and a dissociation constant of 0.22 μM. If no correction was made, the values were 35 nmol/mg and 0.58 μM, respectively. In control experiments with protein heated at 70 °C for 30 min a small amount of nonspecific binding was observed which would decrease both the dissociation constant and extent for specific binding by 5%. This small amount of binding is expected since the purified protein contains lipid (10:1 lipid to protein). In additional experiments we found that treatment of the purified protein with trypsin eliminated the specific binding of cytochalasin to the protein, giving results similar to that observed for heat-denatured protein.

The correction for nonspecific binding of cytochalasin B to lipids is also necessary for measurements of specific binding sites of plasma membranes. Binding of cytochalasin B to plasma membranes isolated from HeLa cells showed a linear uncorrected Schatchard plot at concentrations below 0.6 μM with a K_D of 0.66 μM and an extent of 0.18 nmol/mg of protein. After correction for nonspecific binding by subtracting the binding to heat-denatured membranes at the same free cytochalasin B concentration, the K_D was 0.22 μM and the extent 0.04 nmol/mg of protein.

As shown in Figure 2, D-glucose is a linear competitive inhibitor of cytochalasin B binding to the purified transporter. The different extents of binding at different glucose concentrations are not significant. A plot of apparent extent of cytochalasin B binding vs. glucose concentration is linear and gives an inhibition constant for glucose of 43 mM. Previous studies with erythrocyte ghosts showed values of 40–60 mM for glucose reversible sites (Jung & Rampal, 1977).

Many compounds have been examined for their ability to inhibit either binding of cytochalasin B or hexose transport in erythrocytes (Jung & Rampal, 1977; Jung, 1974; Krupka, 1971, 1972; LeFevre & Marshall, 1959; Jung et al., 1971). We have examined the effect of several of these on binding of cytochalasin B to the purified transporter. All the compounds tested were linearly competitive inhibitors of binding where the extents of binding remained constant. The inhibition constants (Table I) were determined from secondary plots (Figure 3) and are consistent with reported values as described below.

The inhibition constant for phloretin was higher than reported by others (Jung & Rampal, 1977; LeFevre, 1959). This discrepancy is probably due to the large amount of lipid present in the sample. Phloretin bound to lipid with a K_D of ~10 μM and an extent of 1 molecule of phloretin bound per 4–10 molecules of lipid (Verkmam & Solomon, 1979; Jennings & Solomon, 1976). The lipid present in the purified transporter samples is sufficient to provide ~200 μM binding sites for

Table I: Survey of Inhibitors of Cytochalasin B Binding[a]

inhibitor	K_i	coeff of fit	K_i for transport	K_i, other methods
glucose	43 mM	0.99		52–60 mM (Lin et al., 1974)
phloretin	24 μM	0.99	2.4 μM (LeFevre & Marshall, 1959)	4.7–6.9 μM (Jung & Rampal, 1977)
diethylstilbestrol	17 μM	0.97	4.6 μM (Jung et al., 1971)	12 μM (Jung & Rampal, 1977)
maltose	120 mM	0.99	14 mM (Krupka, 1972)	75 mM (Krupka, 1972)
6-*O*-propyl-D-galactose	52 mM	0.99	17.2 mM (Barnett et al., 1975)	
propyl β-D-glucopyranoside	21 mM	0.99	9 mM (Barnett et al., 1975)	
DL-dithiothreitol	0.93 mM	0.99		
L-dithiothreitol	0.67 mM	0.94		

[a] The inhibition constants (K_i) obtained from Figure 3 are given together with the coefficient of fit from the least-squares fit to a straight line for each set of data. For comparison, the values (K_i) for inhibition reported by various methods are also given.

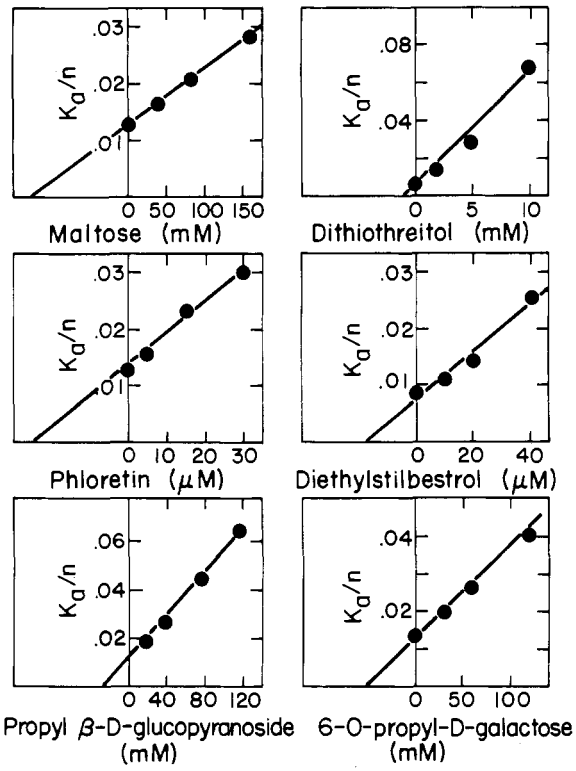

FIGURE 3: Inhibition of cytochalasin B binding. K_{app}/extent vs. inhibitor concentration is shown. The buffer was sodium 3-(*N*-morpholino)propanesulfonate, pH 7.5, for all experiments except for that with dithiothreitol, which was 10 mM sodium 2-(*N*-morpholino)ethanesulfonate, pH 6. All lines were determined by linear regression.

phloretin under the conditions used. Using this information, we calculate that the free phloretin concentration is ~10-fold lower than the concentrations shown in Figure 3, and therefore the true inhibition constant is not significantly different from previously reported values (LeFevre & Marshall, 1959; Jung & Rampal, 1977). A similar correction is probably applicable to the diethylstilbestrol concentrations.

The apparent affinity of maltose for the glucose transporter has been studied by its ability either to inhibit transport or to protect against the inactivation of the carrier by fluorodinitrobenzene (Krupka, 1971, 1972). A fivefold difference was reported for the affinity by these two methods, and the value we obtained is closer to that reported for the inactivation studies.

Dithiothreitol, which has not previously been reported to be an inhibitor of sugar transport or binding of cytochalasin B, was remarkably effective with a K_i of 0.9 mM. Similar results were obtained with the L isomer (K_i = 0.7 mM) and the DL mixture of DTT. The inhibition is not due to the thiol group since 10 mM β-mercaptoethanol did not inhibit binding of cytochalasin B. Cytochalasin B does react with thiols (Lienhard & Wardzala, 1976). However, when a solution of 10 mM dithiothreitol and 0.24 mM cytochalasin B was incubated for 6 h, at 25 °C and at pH 6.0, <5% of the cytochalasin B reacted. The oxidized form of DTT is not an effective inhibitor with a K_i of >20 mM (data not shown).

The sugar derivatives 6-*O*-propyl-D-galactose and propyl β-D-glucopyranoside have been reported to inhibit glucose transport on the outside and inside of the erythrocyte, respectively (Barnett et al., 1975). Both compounds were simple competitive inhibitors of cytochalasin binding to the purified transporter (K_i's of 52 and 21 mM, respectively, Figure 3 and Table I). All three carbohydrates examined had inhibition constants for competition with cytochalasin B that were higher than those reported for their inhibition of transport. The transporter is in vesicles of endogenous lipid and probably has a random orientation in the membrane, but this should not complicate the results with propyl sugar derivatives because they are quite permeant and should equilibrate across the membrane during the equilibrium dialysis.

Discussion

The D-glucose transporter, isolated on the basis of reconstitution of D-glucose permeability in liposomes, binds cytochalasin B with an extent of 7.5–11 nmol/mg of protein and a dissociation constant of 0.18 μM. Baldwin et al. (1979) have reported binding of 6.7 nmol of cytochalasin B/mg of protein to a similar preparation of the transporter. The apparent molecular weight of the protein is 55 000 based on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. We estimated that the molecular weight of the peptide was ~45 000 based on the carbohydrate content (15%) and the amino acid analysis (Sogin & Hinkle, 1978). In addition, we have confirmed recent analyses using endo-β-galactosidase from *Escherichia freundii* to remove most of the carbohydrate from the protein followed by polyacrylamide gel electrophoresis showing a sharper band of the polypeptide at 46 000 molecular weight (Mueller et al., 1979; Gorga et al., 1979). If this estimate of the molecular weight is correct, then the binding of cytochalasin B ranged from 0.34 to 0.5 mol/mol. This result suggests that either the active unit is a dimer or there is some inactive protein in the preparation.

Jung & Rampal (1977) estimate that there are 1.2×10^5–1.5×10^5 cytochalasin B binding sites reversible by glucose per cell, which corresponds to 0.3–0.4 nmol/mg of protein based on 0.66 pg of membrane protein/cell (Juliano, 1973). The alkaline treatment removes about half the membrane protein from ghosts (Steck & Yu, 1973) to give a maximum expected binding of ~0.8 nmol/mg of protein. We found that alkaline-washed membranes bound 1.3 nmol of cytochalasin B/mg of protein (results not shown), and detergent-free Triton extracts of these vesicles bound ~1 nmol of cytochalasin B/mg of protein. The cytochalasin B binding studies thus indicate

a threefold purification of the glucose transporter by the alkaline wash step and a 10-fold purification by the DEAE-cellulose column chromatography step. A similar conclusion was drawn from the original reconstitution studies where the specific activity of the DEAE-cellulose purified protein was 12 times greater than the specific activity of Triton extracts of the washed vesicles (Kasahara & Hinkle, 1977).

The binding of cytochalasin B to the isolated transporter was competitively inhibited by phloretin, diethylstilbestrol, D-glucose, DTT, and other sugars. Studies of D-glucose transport in liposomes reconstituted with the isolated transporter orginally showed no inhibition by phloretin (Kasahara & Hinkle, 1977), but this finding was in error probably because of binding of phloretin to the large amount of lipid present. Recent experiments have demonstrated phloretin to be an inhibitor of the reconstituted system (Wheeler & Hinkle, 1980), as was reported by Kahlenberg & Zala (1977). The results presented suggest that the protein responsible for binding cytochalasin B and the specific transport of glucose are the same, a protein from the region 4.5 [nomenclature as described by Fairbanks et al. (1971)].

The glucose transporter binds propyl β-D-glucopyranoside and 6-*O*-propyl-D-galactose, which have been shown to specifically inhibit transport on either the inner or outer side of the membrane (Barnett et al., 1975), respectively. These results can be explained by either of two models. Barnett et al. (1975) suggested that this difference in specificity for sugar binding from the two sides of the transporter could reflect a conformational change, related to sugar transport, and that there are two asymmetric conformational states of the transporter, but only one binding site. Alternatively there could be multiple binding sites which are mutually exclusive so that binding to one site causes a conformational change that prevents binding at other sites. We found, however, that all sugars which inhibit glucose transport also inhibit binding of cytochalasin B to the purified transporter, and no evidence was obtained for more than one binding site.

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