Journal of Supramolecular Structure 8:447–453 (1978)
Normal and Abnormal Red Cell Membranes 169–175

Characterization of the Glucose Transporter From Human Erythrocytes

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The D-glucose transporter from human erythrocytes has been purified and reconstituted by Kasahara and Hinkle (J Biol Chem 252:7394–7390). Using a similar purification scheme, we have isolated the protein with 65% of the extracted phospholipid at a lipid-protein ratio of 14:1 by weight. The K_D (0.14 μM) and extent (11 nmoles/mg protein) for binding of ^{3}H-cytochalasin B was determined by equilibrium dialysis. Glucose was a linear competitive inhibitor of binding of cytochalasin B, with an inhibition constant of 30 mM. To further characterize the protein, samples were filtered in the presence of sodium dodecyl sulfate (SDS) through Sepharose 6B to remove 95% of the lipid followed by filtration of Sephadex G150 to remove the remaining lipid and a contaminating amount of a minor, lower-molecular-weight protein. This preparation contains only 24% acidic and basic amino acids. The protein also contains 5% neutral sugars (of which 3% is galactose), 7% glucosamine, and 5% sialic acid.

Key words: membrane proteins, transport proteins, glucose transport, reconstitution of glucose transport, purification of glucose transporter, cytochalasin B

Recent studies [1, 2] of isolation and reconstitution of the D-glucose transporter from human erythrocytes have shown that it is a glycoprotein with apparent molecular weight of 55,000. Similar conclusions were reached by others using labeling techniques [3, 4]. Now that the protein has been purified, specific questions can be asked about its structure and mechanism using direct chemical measurements. We have begun to answer some of these questions by determining the amino acid and carbohydrate composition of the protein and its ability to bind inhibitors of glucose transport.

METHODS

Materials

Diethylaminoethanol (DEAE) cellulose was purchased from Whatman, phloretin from ICN Pharmaceuticals, cytochalasin B from Aldrich, ^{3}H-cytochalasin from New

Received for publication April 24, 1978; accepted May 15, 1978.

0091-7419/78/0804-0447$01.70

England Nuclear, and Bio Beads from Bio Rad. Outdated human blood was a gift from the American Red Cross, Syracuse, New York.

Analytical Methods

Protein was hydrolyzed [5] and analyzed for amino acids on a Durrum D-500 analyzer. Content of neutral sugars was determined by the phenol-sulfuric acid method [6], and their relative amounts by gas-liquid chromatography of the trimethylsilylated derivatives [7–8]. Sialic acid content was estimated by the resorcinol procedure [9]. Protein was estimated by the method of Lowry et al [10] with bovine serum albumin as a standard and found to agree well with the amount of protein determined from amino acid analysis. Protein fractions were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis using the low-ionic-strength system of Fairbanks, Steck, and Wallach [11] or by gradient gels using the buffer system of Laemmli [12] which is similar to that described by Mueller and Morrison [13].

RESULTS

Purification of Glucose Transporter

The procedure reported earlier for purifying the protein has been modified slightly to increase the yield and to decrease the time required to produce amounts of protein required for chemical characterization. Erythrocyte ghosts [14] are washed with 0.1 mM Na EDTA (pH 11.2) [15] rather than the low- and high-salt wash used previously [2, 16]. An extract of these alkaline vesicles at 1 mg protein per milliliter is made from 10 mM Tris-HCl (pH 7.5) and 0.5% Triton X-100. The extract is centrifuged at 100,000g for 45 min, and 90% of the supernatant from each tube is retained. The remaining supernatants are combined and centrifuged again as above. The second centrifugation is required to obtain maximum recovery of protein with minimum contamination from the light pellet. The supernatants are combined and the Tris-HCl concentration is increased to 50 mM. The sample is then applied to a column (45% of the volume of the sample) of DEAE cellulose equilibrated with 50 mM Tris-HCl (pH 7.5) 0.5% Triton X-100. The effluent is applied directly to a column of Bio Beads to remove the Triton [17]. The column of DEAE cellulose was eluted with three sample volumes of the equilibration buffer. The combined cloudy fractions are concentrated sixfold by placing the sample in a dialysis bag and burying in sucrose. The sample is then dialyzed against 2 mM Na MOPS* (pH 7.5) to remove the sucrose, and finally the sample is lyophilized. The fraction obtained has a protein/lipid ratio of 1:14 and contains 65% of the extracted phospholipid. Deviations from the procedure outlined above, or starting with untreated ghosts, usually results in impure preparations or no protein at all. The capacity of the column is much greater than would be required to bind the protein applied. Other proteins, such as band 3, are not completely removed by the column if the flow rate is too fast (protein should be in contact with the resin about 45 min) or if insoluble material from the pellet is included.

Removal of Lipid

To permit chemical characterization of the protein, the lipid was removed by gel filtration of 0.5% SDS as shown in Figure 1. The first column, Sepharose 6B, removed about 90% of the lipid. The protein elutes at a position consistent with a molecular weight much higher than 55,000, which is the value estimated by SDS polyacrylamide gel electrophoresis. Filtration on Sephadex G-150 removes the remaining phospholipid and a small

*MOPS–3-(N-morpholino) propanesulfonate.

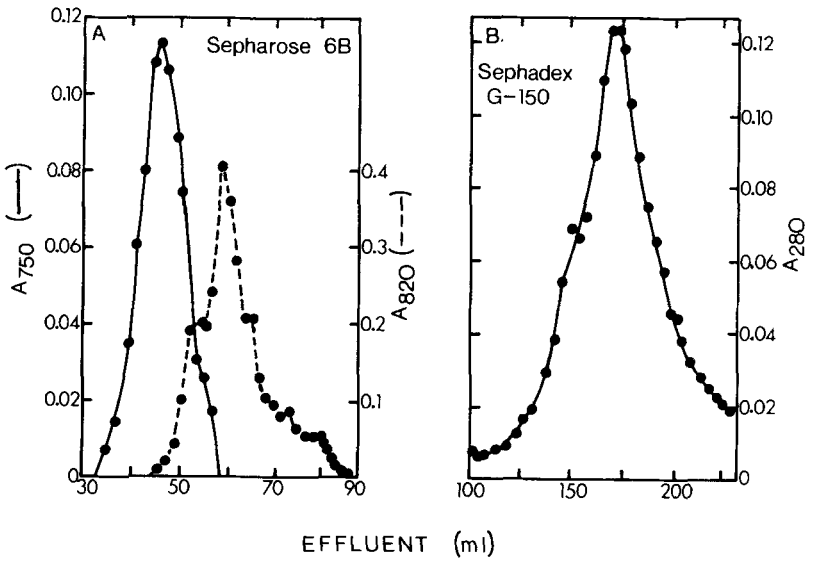

Fig 1. Removal of lipid by gel filtration. A: Purified protein (4 mg with associated lipid) was concentrated to 4 ml and made 5% in SDS and 20 mM in DTT. The sample was centrifuged at 20,000g and applied to a column (1.5 × 42 xm) of Sepharose 6B equilibrated and developed with 0.5% SDS at 37°. The solid is from absorbance at 750 nm after determination of protein of an aliquot (25 μl) in the Lowry procedure [10]. The dashed line is due to absorbance at 820 nm when aliquots (25 μl) were analyzed for phospholipid [28]. The absorbance at 750 nm has been corrected for the contribution of color from the phospholipid. Typically 5–10% of the absorbance at 820 nm is observed at 750 nm during the Lowry procedure even though no protein is shown present. B: The fractions containing protein from A were pooled and concentrated with a Millipore Molecular Separator and then applied to a column (2.5 × 90 cm) of Sephadex G-150 equilibrated and developed with 0.5% SDS at room temperature. The line is for absorbance at 280 nm due to protein. Various regions of the peak were pooled, since the material eluting latest contained protein from band 7.

amount of protein band 7 (Fairbanks' nomenclature [11]) present in the sample. Again the protein behaves as though it were of a molecular weight much greater than 55,000, eluting just after the void volume of the column.

Chemical Composition

The amino acid composition of the purified protein is given in Table I. The protein is relatively hydrophobic, containing 24% charged residues, 39% polar residues, and 37% residues of intermediate polarity. Peaks were detected at the positions of methionine sulfoxide and sulfone. As the peak at the sulfoxide position did not disappear after performic acid oxidation, it is unlikely that this amino acid is responsible for the peak. The peaks may be due to esters formed from the hydrolysis products of the carbohydrate and one or more of the amono acids. The carbohydrate composition for one preparation is also given in Table I.

Gel Electrophoresis

The protein appears as a broad, skewed band on SDS polyacrylamide gel electrophoresis (Fig 2). The broadness of the band might be due to anomolous behavior of the protein on gels or to hetergeneous carbohydrate content. By running the protein in one direction using the discontinuous buffer system of Laemli [12] in SDS on a 7.5% slab gel

TABLE I. Composition of Glucose Transporter*

Amino acid	Mole %	Residues/45,000 MW
Cys	2.0	8.3[a]
Asp	5.44	22.6
Thr	5.4	22.5
Ser	7.56	31.5
Glu	9.67	40.3
Pro	4.78	19.9
Gly	9.35	39.0
Ala	8.0	33.4
Val	8.63	36.0
Met	2.10	8.8
Ile	6.88	28.7
Leu	11.06	46.1
Tyr	2.72	11.0
Phe	7.28	30.3
His	1.18	4.9
Lys	3.57	14.9
Arg	4.10	17.1

Carbohydrate	g/100g Protein
Glucosamine	7
Galactosamine	None detected
Glucose	0.25
Galactose	3.0
Mannose	1.2
Fucose	0.5
Sialic acid	~ 5

*Amino acid composition was determined using a Durrum D-500 after hydrolysis in 6N HCl at 110° for 22, 46, and 72 h. All time points were done in duplicate. The amounts of unstable amino acids were corrected for destruction by extrapolating to time 0. Valine and isoleucine values are those found at the longest hydrolysis period. Total neutral carbohydrate was determined by phenol-sulfuric acid and identity by gas chromatography (after hydrolysis in 1 N H_2SO_4 followed by trimethylsilylation). Amino sugars were determined by amino acid analysis and corrected for destruction. Sialic acid estimated by resorcinol reaction.

[a]Determined as cysteic acid after oxidation with performic acid.

and then rerunning the sample perpendicularly to the original direction of migration on a gradient gel using the same buffer system, we obtained a curved line (not shown), as would be expected if each component in the band remigrates to a position consistent with its apparent molecular weight in the first dimension. With use of the discontinuous buffer system, apparent dimers of the protein were occasionally observed. These dimers were stable as they migrated in the second dimension to a position consistent with their migration in the first dimension. They were usually not seen in the low-ionic-strength system for SDS polyacrylamide gel electrophoresis [11]. The protein would not enter gels of a greater percentage of acrylamide than 7.5% when the discontinuous buffers were used.

Binding of Cytochalasin B

Cytochalasin B is an effective inhibitor of glucose transport in erythrocytes [21–22] and in the reconstituted system [2, 16]. We have measured by equilibrium dialysis the

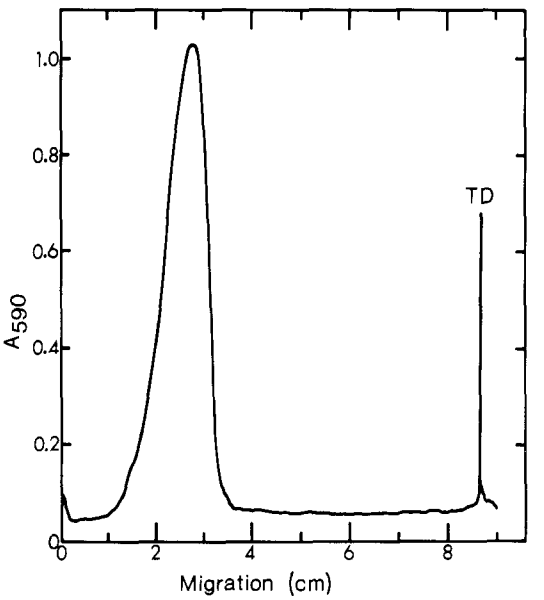

Fig 2. Absorbance scan of SDS polyacrylamide gels. Purifed protein (20 μg) free of lipid and band 7 was applied to a 5.6% polyacrylamide gel [11]. The tracking dye (TD), pyronin Y, was marked with a fine wire. Band 3 migrated about 1.6 cm in a similiar gel.

binding of ^{3}H-cytochalasin B to the purified but not delipidated protein, since removal of lipid requires denaturing conditions. The protein binds 0.5 mole of cytochalasin B per peptide chain (11 nmoles/mg protein) with a dissociation constant of 0.11 μM. About 65% of the total cytochalasin binding sites in the Triton extract are found in the purified protein fraction, which is similar to the recovery of activity for glucose transport measured in the reconstituted system [1, 2].

The binding assay can also be used to determine the inhibition constants for substitutes and inhibitors of glucose transport. By measuring the inhibition of cytochalasin B binding by these compounds, it is possible to determine dissociation constants indirectly which cannot be determined directly due to their low affinities (dissociation constants 2–200 mM [25–27]). As shown in Figure 3, glucose is a linear competitive inhibitor with respect to cytochalasin B and has an inhibition constant of 30 mM. Maltose and phloretin have also been examined and are both linear competitive inhibitors with inhibition constants of 110 mM and 17 μM respectively.

DISCUSSION

Purification

In continuing the work reported earlier [1, 2, 16] we have examined the purification procedure and have improved it while trying to understand why it is successful. Once extracted, the glucose transporter appears to be in a lipid-protein-detergent complex which is depleted of all proteins except the glucose transporter as the Triton extract is passed through the DEAE cellulose column.

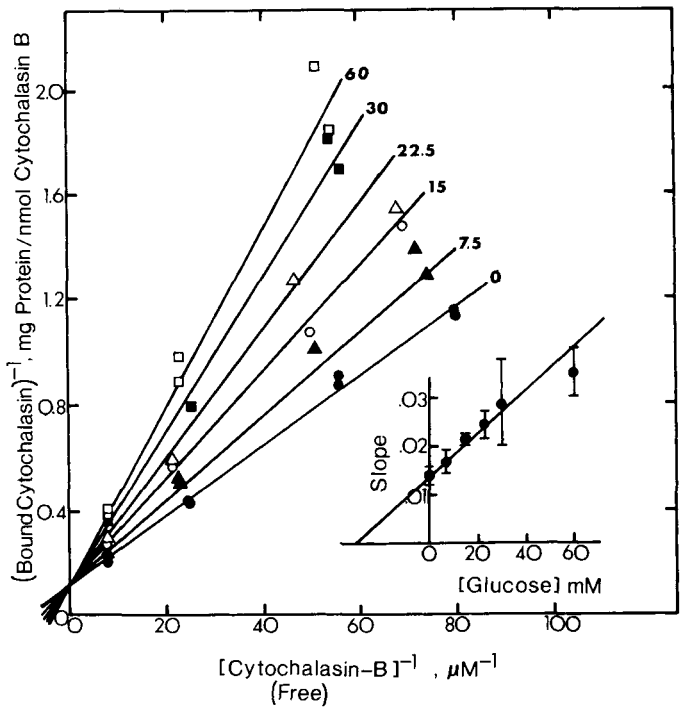

Fig. 3. Inhibition of binding of cytochalasin B by glucose. Protein at 35 μg/ml was placed in one chamber and ^{3}H-cytochalasin B at various concentrations was placed on the other chamber, separated from the first by a dialysis membrane. Glucose was varied from 0 to 60 mM. The chambers were allowed to equilibrate 3 h at 22°. Aliquots were then counted in a liquid scintillation counter to determine the bound and free concentrations of cytochalasin B. The positions of the lines were determined by a least squares fit of the data for each concentration of glucose to equation for a rectangular hyperbola. The standard errors for the slopes for each line are indicated by the error bars in the replot of the slopes.

Physical Characterization

The physical characterization of the protein has been hampered by its insolubility and its tendancy to aggregate even under denaturing conditions. While the amino acid composition is not particularly unusual, it does point toward a relatively hydrophobic molecule. The carbohydrate content may be variable and thus responsible for the broadness of the band on gels. It may also contribute to the anomolous behavior of the protein in gel filtration on Sepharose 6B and Sephadex G-150. The protein also can form dimers on gels, a property which has also been observed with glycophorin [18–20].

Binding of Cytochalasin B

The binding constant for cytochalasin B is similar to those values reported for binding to ghosts [21–23], while the extent per milligram of protein is 15–25 times higher than with ghosts [20, 21–24]. The amount of binding would seem to indicate that either the protein acts as a dimer or our preparation is only 50% pure. An additional indication that the transporter is a dimer was obtained from the freeze-fracture electron microscopy, which showed particles with a diameter of 62 Å, corresponding to a molecular weight of 110,000, twice the estimated molecular weight observed on gels (Telford J, unpublished results). Although it is tempting to conclude that the protein is a dimer in the membrane, additional chemical studies are required to conclusively demonstrate this point.

ACKNOWLEDGMENTS

This work was supported by NIH grant CA-14454 and NS 05667.

We are indebted to Drs William H. Stein and Stanford Moore, in whose laboratory amino acid analyses were performed.

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