Isolation and Reconstitution of the Intestinal Na+/Glucose Cotransporter*

Brian E. Peerce and Rebecca D. Clarke
From the Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550

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The intestinal Na+/glucose cotransporter was isolated from brush border membrane vesicles using a three-step procedure and Na+-dependent phlorizin binding as the measure of brush border membrane enrichment. The initial step was to treat the Ca²⁺-precipitated brush border membrane vesicles with 0.02% sodium dodecyl sulfate (SDS) followed by sucrose gradient centrifugation which resulted in a 5-fold enrichment of the Na⁺/glucose cotransporter. The second step was chromatofocusing chromatography over the pH range from pH 7.4 to pH 4.0. This step resulted in an additional 20-fold purification as compared with the SDS-brush border membrane vesicle protein which served as the starting material. The final step was affinity chromatography on con A-Sepharose which resulted in a 5-fold enrichment of the chromatofocused cotransporter. This fraction consisted of a single 75-kDa polypeptide on SDS-polyacrylamide gel electrophoresis upon staining with silver. On the basis of these criteria it appears that a protocol for the isolation of the Na⁺/glucose cotransporter has been developed.

Transport proteins are normally present as minor components of the total cell protein and only marginal components of the membrane on which they reside. This is certainly true of the class of membrane transport proteins designated Na⁺-dependent cotransporters. In spite of this a number of transport proteins have been isolated and functionally reconstituted from brush border membrane vesicles using a three-step procedure and Na⁺-dependent phlorizin binding as the measure of carrier mediated transport. Partially characterized conformational change upon addition of Na⁺/glucose cotransporter. The second step was chromatofocusing chromatography over the pH range from pH 7.4 to pH 4, and affinity chromatography on concanavalin A. In the accompanying report a second substrate-induced conformational change is reported which appears to be a candidate for the transport committing step (14).

Materials and Methods

All chemicals were purchased from Fisher and were reagent grade or better. Electrophoresis supplies were purchased from Bio-Rad. Phlorizin was purchased from Aldrich. [³H]Glucose and [³H]phlorizin were purchased from Du Pont-New England Nuclear.

Preparation of Brush Border Membrane Vesicles—Brush border membrane vesicles were prepared by Ca²⁺ precipitation of intestinal mucosal scrapings as described previously (5, 15). Following isolation, brush border membrane vesicles were resuspended in 100 mM sodium chloride; BBM, brush border membrane; BBMV, brush border membrane vesicle.

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1 The abbreviations used are: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; ConA, concanavalin A; TMA-Cl, trimethylammonium chloride; BBM, brush border membrane; BBMV, brush border membrane vesicle.
min. The protein was then centrifuged for 60 min at 100,000 $\times g$ and the supernatants collected. The soluble protein was applied to a 40 x 1.5-cm chromatofocusing column equilibrated previously with 20 mM imidazole-HCl, pH 7.4, 100 $\mu$g/ml PMSF, and 1% CHAPS. The column was run as described previously using a flow rate of 20 ml/h (10). Ten-ml fractions were collected and analyzed for $A_{280}$ and pH. Fractiion were pooled, dialyzed against 5 mM Hepes/Tris, pH 7.5, +100 $\mu$g/ml PMSF for 48 h with at least four buffer changes, and lyophilized.

**ConA Chromatography—**Chromatofocusing fractions eluting between pH 5.8 and pH 5.2 were resuspended in 0.5 $M$ NaCl, 0.1 $M$ Tris Cl, pH 7.2, 100 $\mu$g/ml PMSF, and 0.5% CHAPS (ConA column buffer) and passed through a Brown 3000 sonicator at 4 °C. The ConA column equilibrated with ConA buffer. The protein was applied at a flow rate of 4 ml/h. Fractions eluting from the column were collected and examined for protein by $A_{280}$. When the $A_{280}$ stabilized at 0.02 or less (fraction C-1A), 100 $\mu$g glucose was added to the ConA column buffer and fractions collected (fraction C-2A). The fractions eluting before and after the addition of glucose were collected, dialyzed for 48 h against 5 mM Hepes/Tris, pH 7.4, with four buffer changes, and lyophilized.

**Brush Border Marker Enzyme Assays—**Alkaline phosphatase was assayed using p-nitrophenyl phosphate as substrate (5, 17). y-Glutamyl transpeptidase was determined by the method of Glassman and Neville (18). Sucrase was determined by the method of Dahlqvist (19). Glutamyl transpeptidase was determined using the Bio-Rad assay with y-globulin as standard (20) or the SDS-micro-Lowry procedure (21) for assay of chromatofocusing column and ConA column fractions.

**Reconstitution of Na+/glucose Carrier—**ConA fraction 2 (C-2A) was reconstituted into 80% phosphatidylcholine, 20% cholesterol liposomes using the detergent dialysis method of Roach (23) and an Amicon MMC dialysis system. Phosphatidylcholine and cholesterol were solubilized in chloroform and the chloroform blown off with $N_2$. The subsequent film was resuspended in 0.5% CHAPS, 50 mM KC1, 100 mM TMA-Cl, 0.1 mM MgCl$_2$, 0.1 mM CaCl$_2$, and 10 mM Hepes/Tris, pH 7.5 (reconstitution buffer), by brief sonication (4 X 4 s) in a Branson 3000 sonicator at 4 °C. The Na+-dependent glucose carrier was then solubilized and placed in the Amicon MMC with a 10,000-Da cut-off filter at 4 °C. One liter of reconstitution buffer without CHAPS was passed through the sample under pressure. The resultant cloudy suspension was collected, diluted with 200 ml of reconstitution buffer without CHAPS, and centrifuged at 100,000 X g for 40 min. The resultant pellets were resuspended in reconstitution buffer without CHAPS and stored at liquid $N_2$ temperature until needed.

**Na+-dependent Glucose Uptake—**Na+-dependent glucose uptake was examined using a rapid mixing/rapid filtering procedure and buffer without CHAPS and stored at liquid $N_2$ temperature until addition of sucrose from a 50% stock buffered with 25 mM Hepes/Tris, pH 7.5, +100 $\mu$g/ml PMSF for 48 h with at least four buffer changes, and lyophilized.

**SDS-BBMV Vesicles—**Treatment of Ca$^{2+}$-BBMV protein with 0.02% SDS resulted in three fractions. The first fraction consisted of soluble protein comprising the bulk of the protein added to the sucrose gradient. The second fraction did not enter 35% sucrose. This material is referred to as the interface protein and comprised approximately 20% of the Ca$^{2+}$-BBMV protein. The third fraction pelleted through the 35% sucrose and comprised approximately 2% of the applied protein.

The interface protein was 4-fold enriched in the brush border enzyme markers alkaline phosphatase and sucrase. y-Glutamyl transpeptidase activity was 3-fold enriched. The difference in relative enrichments of these marker activities may be traced to higher yields of alkaline phosphatase and sucrase (Table I). Na+-dependent glucose and phlorizin binding activities were also enriched relative to Ca$^{2+}$-BBMV. Na+-dependent glucose cotransporter was 5-fold enriched following treatment with SDS using either measure of cotransporter activity. Approximately 90% of the Na+-dependent phlorizin binding was recovered in the interface protein.

**RESULTS**

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TABLE I
Enrichment of brush border enzyme markers and Na+-dependent glucose cotransporter

<table>
<thead>
<tr>
<th>Marker</th>
<th>Fraction</th>
<th>Ca²⁺-BBMV</th>
<th>SDS-BBMV</th>
</tr>
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<tbody>
<tr>
<td>Protein (mg)</td>
<td>600 ± 28 (n = 14)*</td>
<td>106 ± 18 (n = 14)</td>
<td></td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>4 ± 0.4</td>
<td>21 ± 4</td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase (µmol/mg/h)</td>
<td>28 ± 6 (n = 14)</td>
<td>129 ± 20 (n = 14)</td>
<td></td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>70 ± 5</td>
<td>77 ± 5</td>
<td></td>
</tr>
<tr>
<td>Enrichment</td>
<td>26 ± 6</td>
<td>129 ± 11</td>
<td></td>
</tr>
<tr>
<td>Sucrase (units/mg)</td>
<td>658 ± 80 (n = 5)</td>
<td>2760 ± 100 (n = 5)</td>
<td></td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>80 ± 5</td>
<td>81 ± 6</td>
<td></td>
</tr>
<tr>
<td>Enrichment</td>
<td>24 ± 7</td>
<td>101 ± 5</td>
<td></td>
</tr>
<tr>
<td>Na⁺/Glucose cotransporter</td>
<td>25 ± 6 (n = 14)</td>
<td>335 ± 25 (n = 14)</td>
<td></td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>90 ± 5</td>
<td>92 ± 5</td>
<td></td>
</tr>
<tr>
<td>Enrichment*</td>
<td>5.4 ± 0.4</td>
<td>5.2 ± 0.5</td>
<td></td>
</tr>
</tbody>
</table>

* n is the number of determinations performed in duplicate or triplicate for Na⁺/glucose cotransporter activities.

Enrichments of Na⁺-dependent glucose cotransporter are expressed relative to Ca²⁺-BBMV protein. All other enrichments are expressed relative to the initial homogenate.

Fig. 1. Effect of SDS on Ca²⁺-BBMV protein. Coomassie Blue staining profile of brush border membrane protein run on a 7.5% reducing gel according to the method of Laemmli (20). Lane 1, SDS pellet from sucrose step gradient following treatment with 0.02% SDS as described under "Materials and Methods." Lane 2, SDS interface protein. Lane 3, Ca²⁺-BBMV protein which served as the starting protein. Results are from a single SDS preparation and are representative of eight separate preparations.

Concentrations of SDS on Na⁺-dependent phlorizin binding and Na⁺-dependent glucose uptake shown in Table I.

Chromatofocusing Chromatography on SDS-BBMV Protein—CHAPS-solubilized SDS-BBMV protein was fractionated according to isoelectric point using chromatofocusing chromatography over the pH range from pH 7.4 to pH 4. An elution profile is shown in Fig. 2. Two hundred milligrams of SDS-BBMV protein separated into seven major protein peaks (solid line, closed circles). The peaks eluting between pH 5.8 and pH 5.2 have been shown to contain the intestinal Na⁺/glucose cotransporter under these conditions (16). Examination of this peak by SDS-PAGE indicated that this fraction contained a 75-kDa polypeptide and two other polypeptide bands (data not shown). On the basis of Coomassie Blue staining, the 75-kDa polypeptide represents 25 ± 5% of the protein of this fraction.

Affinity Chromatography on ConA-Sepharose—Fraction 3/4 (eluting between pH 5.8 and pH 5.2) from the chromatofocusing column was subsequently fractionated on a concanavalin A column by standard methods. Fig. 3 is an elution profile from this column. Fraction 3/4 resolved into two fractions: 1) fraction 1 (C-1A), which eluted from the column with the running buffer, and fraction 2 (C-2A), which eluted upon addition of 100 mM glucopyranoside to the running buffer. This second fraction represented 18 ± 4% of fraction 3/4 protein applied to the column. Further washing with 100 mM mannopyranoside followed by 100 mM borate, pH 7.4, failed to release any more protein.

Fig. 4 is an SDS-PAGE gel of two C-2A fractions from two separate ConA column runs. A single polypeptide band of 75 kDa stains with silver (lanes 1 and 2). Lanes 3 and 4 are electrophoresis sample buffer without protein and represent nonspecific staining by silver.

Table II is a summary of the purification of the intestinal Na⁺/glucose cotransporter using soluble Na⁺-dependent phlorizin binding to assay cotransporter enrichment. Following treatment with 0.02% SDS, the cotransporter was approximately 5-fold enriched relative to Ca²⁺-BBMV vesicles. Chromatofocusing chromatography resulted in a 20-fold purification of the cotransporter relative to the SDS-BBMV protein applied to the column. Affinity chromatography on ConA resulted in a further 5-fold purification of chromatofocusing fraction 3/4. The final purification resulting in 13.2 nmol of phlorizin bound per mg of protein is 98% of the theoretical maximum for phlorizin binding, assuming that the cotrans-
Na⁺-dependent Glucose Carrier

1734

0154

5 10 15 20 25

Fraction ::

FIG. 3. Elution profile of C 3/4 protein from ConA column.

Chromatofocusing fraction 3/4 was subjected to affinity chromatography on a ConA column as described under "Materials and Methods." Results shown are from a single experiment and are representative of four individual column runs.

Lane # 1 2 3 4

FIG. 4. SDS-PAGE of ConA fraction C-2A. 25 μg of ConA fraction 2 was run on a 10% polyacrylamide gel as described under "Materials and Methods." Following electrophoresis the gel was stained with silver according to the method of Merrill et al. (22). Lane 1, ConA fraction 2 (12-14-88); Lane 2, ConA fraction 2 (1-6-89); Lane 3, sample buffer no protein; Lane 4, sample buffer no protein.

 porter is a 75-kDa polypeptide and that 1 mol of phlorizin binds to 1 mol of cotransporter (3, 26).

Uptake of Glucose by Na⁺/Glucose Cotransporter Reconstituted into Proteoliposomes—Fig. 5 shows the [³H]glucose uptake by ConA fraction 2 reconstituted into 80% phosphatidylcholine, 20% cholesterol liposomes. The open circles, solid line is uptake in the presence of Na⁺, and the closed circles, dashed line is uptake in the presence of TMA⁺. Uptake in the presence of Na⁺ had a 20-fold overshoot above equilibrium uptake. Equilibrium uptake was 2 nmol/mg protein whether Na⁺ or K⁺ was used in the uptake media. The kinetics of Na⁺-dependent glucose uptake were also examined and the results are shown in Table III. Analysis of the data using Wolfe-Augustinin-Hofstee plots and a nonlinear regression program indicated that the apparent $K_m$ for glucose is 46 ± 5 μM and the $J_{\max}$ is 25 ± 5 (n = 4).

Table II

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein</th>
<th>Na⁺-dependent Phlorizin bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺-BBMV</td>
<td>2400 (n = 10)</td>
<td>0.025 ± 3 (n = 10)</td>
</tr>
<tr>
<td>SDS-BBMV</td>
<td>400 (n = 8)</td>
<td>0.135 ± 20 (n = 8)</td>
</tr>
<tr>
<td>C 3/4</td>
<td>16 ± 3 (n = 8)</td>
<td>2.8 ± 0.3 (n = 8)</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>0.67 ± 0.10</td>
<td>74 ± 6</td>
</tr>
<tr>
<td>Enrichment</td>
<td>112 ± 15</td>
<td></td>
</tr>
<tr>
<td>C-1A</td>
<td>8 ± 2 (n = 6)</td>
<td>1.4 ± 0.2 (n = 6)</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>0.33 ± 0.05</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>Enrichment</td>
<td>56 ± 9</td>
<td></td>
</tr>
<tr>
<td>C-2A</td>
<td>2 ± 0.5 (n = 6)</td>
<td>13.2 ± 0.4 (n = 6)</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>0.08 ± 0.01</td>
<td>44 ± 10</td>
</tr>
<tr>
<td>Enrichment</td>
<td>528 ± 16</td>
<td></td>
</tr>
</tbody>
</table>

*Two chromatofocusing fractions 3/4 are combined per ConA column run, and the numbers shown are from two column runs.

Table III

| Kinetics of fraction C-2A reconstituted into proteoliposomes |
|---|---|---|
| $J_{\max}$ (nmol/mg/s) | 25 ± 5 (n = 4) |
| $K_m$ (μM) | 46 ± 4 (n = 4) |
| % inhibition by Phlorizin* | 98 ± 5 (n = 3) |

* n is the number of experiments performed in triplicate.

indicated that the apparent $K_m$ for glucose is 46 ± 5 μM and the $J_{\max}$ is 25 nmol/mg protein/s ± 3 nmol/mg protein/s.

Osmotic Sensitivity of Na⁺-dependent Glucose Uptake in Proteoliposomes—The Na⁺-dependent glucose uptake by reconstituted C-2A protein was examined for sensitivity to external osmotic strength. These results are shown in Table III. More than 90% (94 ± 3%, n = 3) of the Na⁺-dependent glucose uptake was osmotically sensitive indicating that less than 10% of the observed uptake was the result of glucose binding to the proteoliposomes. These results indicate that the cotransporter is reconstituted and operating in a manner analogous to that seen in the native membrane.
The intestinal Na⁺/glucose cotransporter is a minor component of the enterocyte cell protein and only one of multiple Na⁺-coupled organic solute transporters of the enterocyte brush border membrane. Determination of the molecular mechanism of cotransporter mediated glucose uptake requires isolated transport competent cotransporter. The procedure described here results in a fraction which appears to be transport competent, retains its native brush border membrane characteristics for substrate specificity and inhibitor sensitivity, and is apparently 98% Na⁺/glucose cotransporter.

The isolation protocol involves three post-Ca²⁺ precipitation steps. A pre-chromatographic purification step (negative purification by the detergent, SDS) was found helpful to maximize the amount of cotransporter applied to the chromatography columns. This step resulted in a 5-fold cotransporter enrichment with only a 10–20% loss of cotransporter and in a protein fraction which retained Na⁺-dependent cotransport.

The second post-Ca²⁺ precipitation step was chromatofocusing chromatography. As shown in Fig. 2 CHAPS-solubilized SDS BBM protein was resolved into seven fractions. Fractions 3 and 4 were found to contain Na⁺-dependent phlorizin binding. These fractions were not found to differ significantly on SDS-PAGE, (data not shown) although fraction 3 was found to possess 48% more Na⁺-dependent phlorizin binding. To maximize cotransporter yield these fractions were routinely combined.

Following chromatofocusing, chromatography fraction 3/4 was found to contain two to four contaminants in addition to the 75-kDa polypeptide thought to be the Na⁺/glucose cotransporter (1–4, 6, 27). To eliminate these polypeptides, a ConA chromatographic step was employed to further purify fraction 3/4. Table II and Fig. 4 summarize cotransporter purification. Fraction 2, which eluted from the ConA chromatography column following addition of 100 mM cis-1,2-toluene-3,4-diisocyanate, was seen as a single 75-kDa polypeptide on SDS-PAGE following a 30-s exposure to label under voltage-clamped conditions. Osmotic strength was varied by addition of sucrose. Results shown are means ± S.E. of a single experiment and triplicate determinations. Results are representative of three separate experiments.

**FIG. 6. Osmotic sensitivity of Na⁺-dependent glucose uptake by reconstituted ConA fraction 2.** Na⁺-dependent glucose uptake was determined from the difference in [³H]glucose uptake by 10 µg of reconstituted C-2A protein in the presence of a 100 mM cis-NaCl gradient minus uptake in the presence of a 100 mM cis-TMA-CI gradient following a 30-s exposure to label under voltage-clamped conditions. Osmotic strength was varied by addition of sucrose. Results shown are means ± S.E. of a single experiment and triplicate determinations. Results are representative of three separate experiments.

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The isolation protocol involves three post-Ca²⁺ precipitation steps. A pre-chromatographic purification step (negative purification by the detergent, SDS) was found helpful to maximize the amount of cotransporter applied to the chromatography columns. This step resulted in a 5-fold cotransporter enrichment with only a 10–20% loss of cotransporter and in a protein fraction which retained Na⁺-dependent cotransport.

The second post-Ca²⁺ precipitation step was chromatofocusing chromatography. As shown in Fig. 2 CHAPS-solubilized SDS BBM protein was resolved into seven fractions. Fractions 3 and 4 were found to contain Na⁺-dependent phlorizin binding. These fractions were not found to differ significantly on SDS-PAGE, (data not shown) although fraction 3 was found to possess 48% more Na⁺-dependent phlorizin binding. To maximize cotransporter yield these fractions were routinely combined.

Following chromatofocusing, chromatography fraction 3/4 was found to contain two to four contaminants in addition to the 75-kDa polypeptide thought to be the Na⁺/glucose cotransporter (1–4, 6, 27). To eliminate these polypeptides, a ConA chromatographic step was employed to further purify fraction 3/4. Table II and Fig. 4 summarize cotransporter purification. Fraction 2, which eluted from the ConA chromatography column following addition of 100 mM cis-1,2-toluene-3,4-diisocyanate, was seen as a single 75-kDa polypeptide on SDS-PAGE following a 30-s exposure to label under voltage-clamped conditions. Osmotic strength was varied by addition of sucrose. Results shown are means ± S.E. of a single experiment and triplicate determinations. Results are representative of three separate experiments.

**FIG. 6. Osmotic sensitivity of Na⁺-dependent glucose uptake by reconstituted ConA fraction 2.** Na⁺-dependent glucose uptake was determined from the difference in [³H]glucose uptake by 10 µg of reconstituted C-2A protein in the presence of a 100 mM cis-NaCl gradient minus uptake in the presence of a 100 mM cis-TMA-CI gradient following a 30-s exposure to label under voltage-clamped conditions. Osmotic strength was varied by addition of sucrose. Results shown are means ± S.E. of a single experiment and triplicate determinations. Results are representative of three separate experiments.
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B E Peerce and R D Clarke

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