'Particulate' Hexokinase Activity in Rat Intestine

THE COMPARATIVE CONTRIBUTIONS OF MITOCHONDRIA AND BRUSH-BORDER MEMBRANES

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The activity of hexokinase was examined in brush-border membranes purified from rat intestine. Compared with homogenates, purified membranes exhibited a 20-fold increase in sucrase specific activity and a 15-fold decrease in hexokinase specific activity, which argues against the structural localization of hexokinase within the brush-border membrane.

The last 10 years have witnessed the demise of the phosphorylation/dephosphorylation hypothesis as the basis for active transport of glucose by intestinal cells (Crane, 1968). Although it is established that glucose transport is a step distinct from phosphorylation in those mammalian cells which have been studied, still it is possible that most of the phosphorylation occurs on or near the cytoplasmic face of the plasma membrane and that increased rates of phosphorylation may increase retention and utilization of glucose in the absence of increased transport (Czech, 1976). Characteristic of the lack of understanding of the role of phosphorylation in glucose utilization in the intestine is the continuing uncertainty as to whether or not intestinal hexokinase (EC 2.7.1.1) is a brush-border-membrane enzyme.

van den Berg & Hülsmann (1971), isolating uncharacterized rat brush-border membranes by the sonication method of Harrison & Webster (1964), claimed that 65% of the total cellular hexokinase was in the membranes. Srivastava *et al.* (1968), studying guinea-pig intestinal mucosa, found 10.4% of the cellular hexokinase and 10.9% of the cellular succinate dehydrogenase (EC 1.3.99.1) in a 1500g–10 min pellet. Jones & Mayer (1973), using rat intestinal epithelial cells isolated by sonication in iso-osmotic medium, reported 29.7% of the cellular hexokinase and 40% of the total succinate dehydrogenase in a 500g–10 min pellet. Adhikari & Srivastava (1973), studying rat intestine, found 39% of the cellular hexokinase and 10% of the succinate dehydrogenase in a 1500g–10 min pellet. Although hexokinase appears in the 'brush-border and nuclear fraction' in all of these studies, its presence could have reflected mitochondrial contamination.

The aim of the present study was to measure the activity of hexokinase in a brush-border-membrane

preparation rich in conventional markers, such as sucrase (EC 3.2.1.26), and shown to be free of significant mitochondrial contamination by enzymic and electron-microscopic criteria.

Methods

Adult male Sprague–Dawley rats weighing 200–300g were starved overnight before study and were killed by cervical dislocation. The entire small intestine was quickly dissected free, and the lumen washed in cold 0.9% NaCl. The mucosal scrapings were weighed and homogenized in 75 vol. of cold 5.0mM-EDTA/1.0mM-dithiothreitol, pH 7.4, in a Waring Blendor at Variac setting 90 for 30 s at 4°C.

The homogenates obtained were transferred to silicone-treated plastic centrifuge tubes and all subsequent steps were carried out at 4°C. The homogenate was centrifuged at 500g for 15 min in an International refrigerated PR-2 centrifuge (r_{av} , 16.25 cm), yielding a low-speed pellet. This was washed three times by resuspension in 10 vol. of 5.0mM-EDTA/1.0mM-dithiothreitol, pH 7.4, and centrifugation at 500g for 15 min as above. Supernatants from each step were combined and centrifuged at 23 500g for 30 min in a Sorvall RC2-B centrifuge in a fixed-angle SS34 head (r_{av} , 7.3 cm) to provide a microsomal pellet and a postmicrosomal supernatant. Pellets of the various fractions were suspended in 5.0mM-EDTA/1.0mM-dithiothreitol, pH 7.4, and frozen before assay, except for the materials used in assays of hexokinase activity, which were performed on the day of killing.

In addition to the preparation of conventional subcellular fractions, highly purified brush-border membranes were isolated from the low-speed pellet by the method of Forstner *et al.* (1968).

Preparations of brush-border membranes were checked as a routine at various stages of the purification by phase-contrast microscopy, and final preparations containing more than 0–2 nuclei per high-powered ($\times 400$) field were rejected from consideration.

Sucrase activity was assayed by the method of Dahlqvist (1964). Hexokinase was assayed as described by van den Berg & Hülsmann (1971). Succinate dehydrogenase was determined by the method of Pennington (1961). Glucose 6-phosphatase (EC 3.1.3.9) was assayed by the method of Baginski *et al.* (1974). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V powder pfs A4503; Sigma Chemical Co., St. Louis, MO, U.S.A.) as a standard.

Electron-microscopic studies were performed as described by Dolin *et al.* (1975).

The possible interference of brush-border membranes with the assay system for hexokinase was studied by adding 0.10 ml of membranes (0.15 mg of protein) to a standard hexokinase assay mixture containing the following reagents in a final volume of 1.0 ml: 46 mM-Tris/HCl, pH 7.4, 10 mM-MgCl₂, 5.0 mM-ATP, 0.4 mM-NADP⁺, 3.0 units of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (Sigma Chemical Co.) and 0.03 unit of yeast hexokinase (EC 2.7.1.1) (Sigma Chemical Co.), where 1 unit is defined as a rate of 1 μ mol/min. The reaction was initiated by adding 0.10 ml of glucose in 46 mM-Tris/HCl, pH 7.4 (final glucose concentration was 33 mM), and the reaction rate followed by recording the change in E_{340} in a Gilford recording spectrophotometer at 25°C. Control values were obtained by substituting 0.10 ml of Tris/HCl buffer for the membranes and measuring the activity of the yeast hexokinase alone.

Results

Subcellular distributions of the various marker

enzymes and their recoveries in each fraction are given in Table 1. These results reflect the choice of conditions designed to minimize the mitochondrial contamination of the low-speed pellet, rather than to increase the yield of brush-border membranes. In purified membranes the specific activity of sucrase was 3.28 μ mol/min per mg of protein (Table 2), representing a 20-fold purification with respect to homogenate, and purified membranes were free of mitochondrial contamination as examined by electron microscopy. Judged by the activity of succinate dehydrogenase, the amount of mitochondrial contamination of the final membranes does not exceed 0.3% of the homogenate enzyme.

Similarly, membranes are essentially free of hexokinase activity. Table 2 shows that the hexokinase specific activity decreases approximately 15-fold during the purification procedure. The initial low-speed pellet contains only 0.4–2.1% of the activity found in the homogenate with a fivefold decrease in specific activity; the low-speed supernatant, in contrast, contains 96.7% of the homogenate hexokinase activity. Even when highly purified membranes are prepared (Table 2) from the low-speed pellet with the higher amount of hexokinase activity (2.1%), the final amount of the enzyme in the membranes is negligible. After the low-speed pellet was suspended in 90 mM-NaCl, filtered through glass-wool, and then collected by centrifugation at 500g (as for the low-speed pellet), only 0.03% of the total hexokinase activity remained in the membrane fraction. The specific-activity ratio hexokinase/sucrase fell from 0.280 in the homogenate to 0.001 in the purified membranes, a 280-fold decrease.

Although the brush-border membranes contain only 2.4% of the total glucose 6-phosphatase activity, enzyme specific activity is increased fivefold relative to the homogenate, an amount possibly sufficient to interfere with the assay of hexokinase. On that point, a sample of brush-border membranes equal to that used in the routine assay of hexokinase, when added

Table 1. Subcellular location of hexokinase in intestinal mucosa

The upper value for each entry represents enzyme specific activity expressed as μ mol of substrate metabolized/min per mg of protein; that for protein entry represents total homogenate protein (mg) per experiment. The lower value (in parentheses) represents enzyme or protein recovery as percentage of total homogenate value.

Sample	Protein	Sucrase	Hexokinase	Succinate dehydrogenase	Glucose 6-phosphatase
Homogenate	442 (100.0%)	0.149 (100.0%)	0.0361 (100.0%)	0.0217 (100.0%)	0.0127 (100.0%)
Low-speed pellet	(4.2%)	0.780 (22.3%)	0.0034 (0.4%)	0.0098 (1.9%)	0.0291 (9.8%)
Mitochondria (22500g–30 min)	(41.1%)	0.258 (71.2%)	0.0268 (30.5%)	0.0539 (102.1%)	0.0146 (47.4%)
Microsomal fraction (105200g–2 h)	(13.6%)	0.153 (14.0%)	0.0159 (6.0%)	0.0073 (4.6%)	0.0090 (9.6%)
High-speed supernatant	(60.1%)	0.000 (0.0%)	0.0290 (48.1%)	0.0006 (1.7%)	0.0051 (36.0%)

Table 2. *Distribution of hexokinase activity during the purification of brush-border membranes*

The upper value for each entry represents enzyme specific activity expressed as μmol of substrate metabolized/min per mg of protein; that for protein entry represents total homogenate protein (mg) per experiment. The lower value in parentheses represents enzyme or protein recovery as percentage of total homogenate value. The low-speed pellet is suspended in 3 vol. of 90 mM-NaCl and left for 30 min at 0°C, to allow nuclear aggregation and sedimentation. The upper phase is poured over glass-wool and the pellet filtrate (containing membranes) is centrifuged at 500g for 20 min, yielding brush-border supernatant and membrane fractions.

Sample	Protein	Sucrase	Hexokinase	Succinate dehydrogenase	Glucose 6-phosphatase
Homogenate	840 (100.0%)	0.164 (100.0%)	0.0456 (100.0%)	0.0274 (100.0%)	0.0160 (100.0%)
Low-speed pellet (500g-15 min)	(7.5%)	0.513 (23.5%)	0.0128 (2.1%)	0.0234 (6.4%)	0.0269 (12.6%)
Low-speed supernatant	(82.8%)	0.161 (81.6%)	0.0533 (96.7%)	0.0355 (107.2%)	0.0219 (113.5%)
Pellet filtrate	(1.4%)	1.664 (14.7%)	0.0286 (0.91%)	0.0312 (1.6%)	0.0512 (4.6%)
Brush-border supernatant	(1.1%)	0.616 (4.1%)	0.0264 (0.62%)	0.0536 (2.1%)	0.0140 (1.0%)
Brush-border membranes	(0.5%)	3.281 (9.5%)	0.0029 (0.03%)	0.0204 (0.3%)	0.0794 (2.4%)

to an assay system containing a known amount of yeast hexokinase, decreased the apparent activity by 18% (9.74 to 8.04 nmol/min).

Discussion

The current study indicates that rat intestinal hexokinase exists in both a 'particulate' and a soluble form. Virtually all of the 'particulate' hexokinase activity is associated with mitochondria. Purified brush-border membranes contain only 0.03% of the homogenate hexokinase activity. In addition, the specific-activity ratio hexokinase/sucrase is decreased 280-fold during purification, arguing strongly against any important structural association of hexokinase with the brush border.

Anderson & King (1975), in a study of rat jejunal mucosa, claimed that 10% of the homogenate hexokinase activity was recovered in a washed 800g-15 min pellet; membranes were purified from the pellet by the method of Forstner *et al.* (1968). Although recovery data were not given, hexokinase specific activity was decreased sevenfold in purified membranes compared with the homogenate. Calculations based on the data of Anderson & King (1975) show that the specific-activity ratio hexokinase/sucrase falls from 0.056 in the homogenate to 0.001 in the purified membranes, a 56-fold decrease, compared with a 280-fold decrease in the present study.

Weiser *et al.* (1971) referred to the absence of hexokinase from brush-border membranes, prepared by the method of Forstner *et al.* (1968), but, in their study, recovery data were not given and several factors could have inadvertently led to the

apparent loss of hexokinase activity. Hexokinase might have been displaced from the membrane by NaCl, as has been observed with mitochondrial hexokinase (Rose & Warms, 1967); in the present study 96.7% of the total hexokinase remained in the low-speed supernatant before treatment with NaCl, making solubilization of the enzyme an unlikely explanation for the low hexokinase activity in the membranes. Further, the assay of hexokinase activity might have been hampered by the destruction of the reaction product (glucose 6-phosphate) by phosphatases purified with the brush borders (Forstner *et al.*, 1968). In our experiments a marked excess of glucose 6-phosphate dehydrogenase was used to 'trap' the glucose 6-phosphate; this (3.0 units/ml) was the concentration used by van den Berg & Hülsmann (1971) and ten-fold greater than that used by Weiser *et al.* (1971). The recovery of 36% of the glucose 6-phosphatase activity in the high-speed supernatant is not altogether surprising, since microsomal fractions homogenized and centrifuged in hypo-osmotic EDTA-containing buffers tend to remain fragmented and buoyant. The observed decrease in activity of yeast hexokinase assayed in the presence of brush-border membranes may reflect either membrane phosphatases or direct interference of membranes with the hexokinase reaction; nevertheless, such a small effect (18%) will not change the conclusions of this study. Thus hexokinase is not a structural component of the intestinal brush border.

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