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ANOMERIC PREFERENCE OF GLUCOSE UTILIZATION IN HUMAN ERYTHROCYTES LOADED WITH GLUCOKINASE

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Human erythrocytes were loaded with homogeneous rat liver glucokinase by an encapsulation method based on hypotonic hemolysis and isotonic resealing. As assayed at 10 mM glucose, glucokinase and hexokinase activities in glucokinase-loaded erythrocytes were 218 and 384 nmol/min/gHb, respectively; whereas hexokinase activity in both intact and unloaded red cells, which contain no glucokinase activity, was about 400 nmol/min/gHb. No difference in the rate of lactate production from glucose anomers between intact and unloaded erythrocytes suggested that the encapsulation procedure itself did not affect glucose utilization in red cells. Alpha-anomeric preference in lactate production from glucose was observed in glucokinase-loaded erythrocytes, whereas the β anomer of glucose was more rapidly utilized than the α anomer in intact and unloaded erythrocytes. The results indicate that the step of glucose phosphorylation determines the anomeric preference in glucose utilization by human erythrocytes, since glucokinase and hexokinase are α- and β-preferential, respectively, in glucose phosphorylation. © 1990 Academic Press, Inc.

It has been documented that the utilization of α-D-glucose differs from that of β-D-glucose in rat pancreatic islets (1), rat erythrocytes (2), rat hepatocytes (3), rat adipocytes (4), rat brain cells (5), and human erythrocytes (6, 7). The α-anomeric preference of glucose utilization in rat islets, rat erythrocytes, rat hepatocytes, and rat adipocytes was suggested to be attributable mainly to the α-stereospecificity of glucose-6-phosphate isomerase (EC 5.4.1.9) and phosphoglucomutase (EC 5.3.2.2) (4, 8); and the preference for β-D-glucose in rat brain cells, to the higher velocity for the phosphorylation of the β anomer by hexokinase (EC 2.7.1.1) (5).

Of various kinds of cells, human erythrocytes seem to be the most suitable for an investigation into the mechanism of the anomeric preference in glucose utilization, since glucose metabolism in human red

Abbreviation used: KRP buffer, Krebs-Ringer phosphate buffer.

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cells has been extensively studied and it is easy to collect the cells in large amount. We reported that human erythrocytes produce lactate more rapidly from β- than from α-D-glucose when incubated for 3 min at 37 °C (6). Malaisse-Lagae *et al.* (7) reported that the production of ^3H_2O from β-D-[5-^{3}H]glucose is, as a rule, faster than that from the corresponding α-anomer when measured for 60 min at 9 °C. Earlier we proposed the view that the β-anomeric preference of glucose utilization in human red cells may be caused by the β-preferential phosphorylation of glucose by hexokinase, which is the only glucose-phosphorylating enzyme in human erythrocytes and which is believed to be a rate-limiting enzyme in human red cell glycolysis (9). In an attempt to examine the validity of this view, in the present study we loaded red cells with glucokinase, an isozyme of hexokinase that phosphorylates α-D-glucose more rapidly than β-D-glucose at physiological glucose concentrations (10, 11). We hypothesized that human erythrocytes containing glucokinase would metabolize the α anomer of glucose faster than the β anomer if the step of glucose phosphorylation is a determinant of the anomeric preference in glucose utilization.

MATERIALS AND METHODS

Glucokinase was purified from rat liver extracts by chromatography steps using DEAE-cellulose (Whatman), Phenyl-Sepharose CL-4B (Pharmacia), DEAE Affi-Gel Blue (Bio-Rad), and glucosamine-linked gel (12). The enzyme was judged to be completely homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (5-15 % gradient gel) performed according to the method of Laemmli (13). Purified glucokinase had a specific activity of 290 units/mg protein and was used at a concentration of 54 units/ml in buffer A (pH 7.0) consisting of 20 mM sodium phosphate, 250 mM KCl, 1 mM EDTA dipotassium salt, 1 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM glucose, 5 % (v/v) glycerol, and 0.05 % (w/v) bovine serum albumin.

Phosphorylation of glucose anomers by rat liver glucokinase was determined fluorometrically by the method of Meglasson and Matschinsky (10), except that the pH of the reaction medium was changed from 7.6 to 7.4 and the reaction was conducted for 3 min at 37 °C.

Encapsulation of glucokinase in human erythrocytes was achieved by the hypotonic dialysis-isotonic resealing method (14) essentially according to the procedure of Magnani *et al.* (15). Briefly, plasma and buffy coat were removed after centrifugation of fresh human blood for 5 min at 1,200 x g. The erythrocytes were washed twice with 5 mM sodium phosphate buffer (pH 7.4) containing 154 mM NaCl and 5 mM glucose and then resuspended in the same buffer to give a cell suspension of 70 % hematocrit. An aliquot (0.8 ml) of the cell suspension was placed in dialysis tubing (Viskase, molecular size cut-off, 12-14 kDa). Glucokinase solution (30 μl, 54 units/ml) was added to the suspension, which was subsequently dialyzed with gentle rotation (15 rpm) for 35 min at 4 °C against 50 ml of a hypotonic medium (pH 7.4) consisting of 10 mM sodium phosphate, 10 mM sodium bicarbonate, and 20 mM glucose.

For control (unloaded) experiments, 30 μl of buffer A was used instead of glucokinase solution. The hemolysate was collected and 1 vol. of a resealing solution (pH 7.4) consisting of 5 mM adenine, 100 mM inosine, 100 mM sodium pyruvate, 100 mM sodium phosphate, 100 mM glucose, and 2.05 M NaCl was added to 10 vol. of hemolysate. Resealing of lysed erythrocytes was accomplished by incubation for 20 min at 37 °C. The resealed erythrocytes were washed three times at 37 °C with Krebs-Ringer phosphate buffer (KRP buffer: 15 mM sodium phosphate, 4 mM KCl, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, and 125 mM NaCl [pH 7.4]) supplemented with 0.2 mM glucose, and resuspended in the same buffer to give a hematocrit value of 30-40 %.

Suspensions of intact erythrocytes at a 30-40 % hematocrit were prepared by washing fresh human erythrocytes three times at 37 °C with KRP buffer containing 0.2 mM glucose and by resuspending in the same buffer. To measure lactate production from glucose, aliquots (100 μl) of each of the suspensions of intact, unloaded, and loaded erythrocytes were put into plastic microtubes (full capacity: 2 ml), preincubated for 2 min at 37 °C, and added with 20 μl of either α- or β-D-glucose solution (60 mM in KRP buffer) prepared just before use. After incubation for 3 min at 37 °C, the reaction was stopped by addition of 120 μl of 0.6 N $HClO_4$. In blank experiments, 20 μl of KRP buffer was added instead of glucose anomer solutions, and the reaction was immediately stopped by addition of 0.6 N $HClO_4$ (120 μl). Denatured proteins were sedimented by centrifugation for 5 min at 10,000 x g, and the supernatants were used for the lactate assay.

For measurement of lactate, 120 μl of the supernatant was mixed with 1 ml of 0.5 M glycine/0.4 M hydrazine buffer (pH 9.0) containing 2.4 mM NAD and 7.2 units/ml lactate dehydrogenase (EC 1.1.1.27, from pig heart, Oriental Yeast) and incubated for 30 min at 37 °C (16). Fluorescence of NADH produced was measured and sample fluorescence was corrected by subtraction of the value obtained for the reagent blank.

For measurement of hexokinase and glucokinase activities, intact, unloaded, and loaded erythrocytes were washed three times with 0.9 % NaCl and resuspended in the saline to give a 50 % hematocrit. An aliquot (20 μl) of each suspension was mixed with 180 μl of a stabilizing solution consisting of 0.7 mM 2-mercaptoethanol and 2.7 mM neutralized EDTA, and the mixture was frozen in a dry ice-acetone bath and then thawed in water at room temperature according to the method of Beutler (17). The hemolysate was centrifuged at 10,000 x g at 4 °C for 10 min, and the supernatant obtained was used for the assay of glucose-phosphorylating activities. Assays were performed at 37 °C in 100 mM Tris-HCl buffer (pH 7.4) containing 100 μg/ml bovine serum albumin, 1 unit/ml glucose-6-phosphate dehydrogenase (EC 1.1.1.49, from *Leuconostoc mesenteroides*, Oriental Yeast), 0.5 or 10 mM glucose, and the hemolysate supernatant (50 μl) in a total volume of 1.0 ml. The rate of increase of absorbance at 340 nm was recorded at a full-scale reading of 0.2 OD unit. The hexokinase activity at a glucose concentration of 10 mM was 114 and 115 % of the activity at 0.5 mM glucose in intact and unloaded erythrocytes, respectively, which lack glucokinase activity. These values are similar to that (112 %) estimated from the Michaelis-Menten equation providing that the Km for glucose is 65 mM (18). Slightly higher observed values (mean value, 114.5 %) than the estimated value (112 %) may be due to the partial relief by glucose of glucose 6-phosphate inhibition of hexokinase (19). Based on these considerations, the glucokinase activity was estimated by subtraction of 1.145 times the activity at 0.5 mM glucose from the activity at 10 mM glucose.

Hemoglobin was assayed with a ferricyanide-cyanide reagent kit available commercially.

The statistical analyses were performed by Student's *t*-test.

RESULTS

Glucose phosphorylation by pure rat liver glucokinase occurred with cooperative rate dependence on glucose concentration and with higher velocities for α-D-glucose than for the β anomer at physiological glucose concentrations (Fig. 1). At 10 mM glucose, phosphorylation of the α anomer was about 1.3 times faster than that of the β anomer. The present data on anomeric preference at physiological glucose concentrations agree well with the result reported by Meglasson and Matschinsky (10). Discrepancy in the extent of α-anomeric preference between the present data and our previous data (see Ref. 11) may be due to the difference in the method used.

After the hemolysis/resealing procedure for encapsulation of glucokinase, 4-9 % of the total hemoglobin in the erythrocyte suspension was found in the supernatant. Glucose phosphorylation activities determined at 0.5 mM glucose, however, were not significantly different among intact, unloaded, and loaded erythrocytes when expressed based on hemoglobin content (Table 1), indicating that the hexokinase concentration calculated on this basis was almost the same in these three erythrocyte preparations. The glucokinase and hexokinase activities at 10 mM glucose in loaded erythrocytes were estimated to be 218 and 384 nmol/min/gHb, respectively.

Lactate production from glucose anomers in unloaded erythrocytes was quite similar to that in intact cells, and the rate of lactate production from β-D-glucose was significantly higher than that from the α anomer in

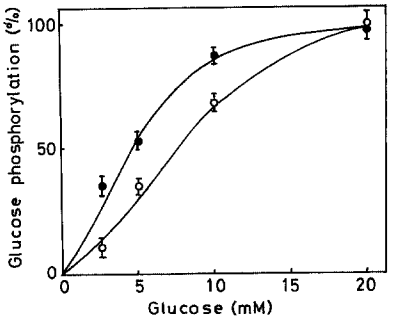

Fig. 1. Phosphorylation of α-D-glucose (●) and β-D-glucose (○) by glucokinase purified from rat liver. Results are expressed as percentages of the glucose phosphorylation rate (0.81 nmol/3 min) observed with 20 mM β-D-glucose.

Table 1. Glucose-phosphorylating activity in intact, unloaded, and glucokinase-loaded erythrocytes

Erythrocytes	Glucose concentration (mM)	Glucose phosphorylation (nmol/min/gHb)
Intact	0.5	348 ± 22
	10	395 ± 8
Unloaded	0.5	350 ± 23
	10	403 ± 2
Loaded	0.5	335 ± 35[§]
	10	602 ± 86*

Glucose-phosphorylating activities of hemolysates obtained from intact, unloaded, and glucokinase-loaded erythrocytes were measured at glucose concentrations of 0.5 and 10 mM. Values are means ± S.D. of 5-6 determinations.

[§]Not significantly different from the activity at 0.5 mM glucose in unloaded erythrocytes.

*$P<0.001$, compared with the activity at 10 mM glucose in unloaded erythrocytes.

both intact and unloaded erythrocytes (Table 2). Erythrocytes loaded with glucokinase produced more lactate than both intact and unloaded cells. In loaded erythrocytes, lactate production from α-D-glucose was significantly higher than that from the β anomer.

Table 2. Lactate production from glucose anomers in intact, unloaded, and glucokinase-loaded erythrocytes

Erythrocytes	Lactate production (nmol/min/gHb)		Ratio (β-Anomer/α-Anomer)
	α-Anomer	β-Anomer	
Intact	182 ± 9	216 ± 12*	1.19
Unloaded	187 ± 14	216 ± 8*	1.16
Loaded	311 ± 7[#]	287 ± 9*, [#]	0.92

Lactate production from 10 mM α- or β-D-glucose for 3 min at 37 °C was measured as described under Materials and Methods. Values for lactate production are means ± S.D. of 5-6 determinations.

*$P<0.001$, compared with α-anomer.

[#]$P<0.001$, compared with value for unloaded cells.

DISCUSSION

Specific activity of hexokinase in unloaded erythrocytes was very similar to that in intact erythrocytes (Table 1), ruling out any selective loss of hexokinase during the encapsulation procedure. Magnani *et al.* (15) found in the experiment for loading hexokinase into human erythrocytes that not only hexokinase but also all other glycolytic enzymes were well retained in unloaded erythrocytes throughout the hemolysis/resealing procedure. These data are in agreement with our findings that lactate production from α- or β-D-glucose in unloaded erythrocytes was indistinguishable from that in intact erythrocytes (Table 2). This suggests that the encapsulation procedure itself does not affect the glycolytic pathway in erythrocytes.

Human erythrocyte hexokinase was reported to phosphorylate β-D-glucose more rapidly than α-D-glucose: v_β/v_α (at 10 mM and at 37 °C)=1.16 by Fujii *et al.* (6); v_β / v_α (at 1 mM and at 10 °C)=1.33-1.40 by Malaisse-Lagae and Malaisse (20). The β/α ratio (1.19) for lactate production from glucose determined in intact erythrocytes at 10 mM glucose and at 37 °C is quite similar to the β/α ratio (1.16) for glucose phosphorylation measured previously by us under the same conditions (6).

Human erythrocyte hexokinase is severely inhibited by intracellular compounds such as glucose 6-phosphate, glucose 1,6-bisphosphate, and ATP (19), whereas rat liver glucokinase is not so sensitive to inhibition as erythrocyte hexokinase (21). Therefore, hexokinase activity, but not glucokinase activity, in hemolysates varies with the concentration of hemolysate used for the enzyme assay, making it difficult to accurately determine the anomeric preference in glucose phosphorylation in red cells containing both hexokinase and glucokinase. This is the reason why we did not determine a β/α ratio for glucose phosphorylation in glucokinase-loaded erythrocytes.

When human erythrocytes were loaded with rat liver glucokinase, both glucose phosphorylation and glucose utilization were significantly increased when assayed at 10 mM glucose. The increase in glucose utilization in glucokinase-loaded erythrocytes compared with that in unloaded erythrocytes is reasonably attributable to the action of the glucokinase encapsulated into erythrocytes. Lactate production from α-D-glucose was more markedly increased than that from β-D-glucose (124 vs. 71 nmol/min/gHb) by encapsulation of glucokinase (Table 2). The α/β ratio (1.7) for lactate production increment was not so different from, though not the same as, that (1.3) for glucose phosphorylation by glucokinase at a glucose concentration of 10 mM (Fig. 1). The most

important thing shown in Table 2 is that α-preferential glucose utilization was observed in erythrocytes loaded with glucokinase, whereas β-D-glucose was more rapidly utilized than the α anomer in intact and unloaded erythrocytes containing hexokinase, but not glucokinase. This result indicates that the step of glucose phosphorylation is a determinant for the anomeric preference in glucose utilization by human erythrocytes.

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