

## Anomeric Specificity of Human Liver and B-cell Glucokinase: Modulation by the Glucokinase Regulatory Protein

Philippe Courtois, Frédéric Bourcet, Abdullah Sener, and Willy J. Malaisse<sup>1</sup>

Laboratory of Experimental Medicine, Brussels Free University, 808 Route de Lennik, B-1070 Brussels, Belgium

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The anomeric specificity of the wild-type recombinant forms of human liver and B-cell glucokinase was investigated using radioactive anomers of D-glucose as tracers. With D-glucose at anomeric equilibrium and at 30°C, the maximal velocity, Hill number, and  $K_m$  amounted, respectively, to 16  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ , 1.8 and 6.9 mM in the case of liver glucokinase, and 7.3  $\mu\text{mol min}^{-1} \text{mg}^{-1}$ , 2.0 and 7.1 mM in the case of B-cell glucokinase. Whether at 20–22 or 30°C, the maximal velocity, Hill number, and  $K_m$  were significantly lower with α-D-glucose than with β-D-glucose in both liver and B-cell glucokinase. As a result of these differences, the reaction velocity was higher with α-D-glucose at low hexose concentrations, while the opposite situation prevailed at high hexose concentrations. In the presence of 0.2 mM D-fructose 6-phosphate, the glucokinase regulatory protein caused a concentration-related inhibition of D-glucose phosphorylation, such an effect fading out at high concentrations of either D-glucose or glucokinase relative to that of its regulatory protein. The phosphorylation of α-D-glucose by liver glucokinase appeared more resistant than that of β-D-glucose to the inhibitory action of D-fructose 6-phosphate, as mediated by the glucokinase regulatory protein. Such a phenomenon failed to achieve statistical significance in the case of the B-cell glucokinase. It is proposed that this information, especially the novel findings concerning the anomeric difference in both Hill number and sensitivity to the glucokinase regulatory protein, should be taken into account when considering the respective contributions of α- and β-D-glucose to the overall phosphorylation of equilibrated D-glucose by glucokinase. © 2000 Academic Press

**Key Words:** human liver and B-cell glucokinase; D-glucose phosphorylation; anomeric specificity.

The anomeric specificity of liver and pancreatic islet glucokinase was examined by several investigators (1–5). Interest in this matter is motivated *inter alia* by the knowledge that, under physiological conditions, α-D-glucose is a more potent insulin secretagog than β-D-glucose (6–10). Because of the key role played by glucokinase in the regulation of D-glucose metabolism in islet insulin-producing cells (11), the question was raised whether the anomeric specificity of B-cell glucokinase may indeed account for the higher insulinotropic capacity of α-D-glucose (4, 5). Moreover, in non-insulin-dependent diabetes mellitus, the preference of the B-cell secretory response for α- relative to β-D-glucose is often decreased and may even be inverted (12–16). Although such a perturbation, which can be reproduced in normal rats through sustained hyperglycemia caused by either the administration of diazoxide (17) or partial pancreatectomy (18), appears usually attributable to glycogen accumulation in the pancreatic islet cells (19), a mutation of the glucokinase gene resulting in an altered anomeric behavior of the enzyme, was also considered as a possible and exceptional cause for the anomeric malaise in insulin secretion (20).

In intact B-cells, however, the phosphorylation of D-glucose is not ruled solely by the cytosolic concentration of the hexose and the intrinsic properties of glucokinase and hexokinase. It can also be affected by a number of other factors, such as the concentration of ATP and D-glucose 6-phosphate (21, 22), the binding of the hexokinase isoenzymes to mitochondrial porin (23–25), the modulation of glucokinase activity by its reg-

<sup>1</sup> To whom correspondence should be addressed. Fax: +32-2-5556239.

ulatory protein (26), and the intracellular translocation of the latter enzyme in glucose-stimulated B-cells (27, 28).

In the present study, therefore, we have reinvestigated the anomeric specificity of glucokinase, with emphasis on its possible modulation by the glucokinase regulatory protein. Our experiments take advantage, first, of the recent availability of the wild-type recombinant forms of both liver and B-cell human glucokinase (29) and, second, from the preparation of the pure anomers of  $^3\text{H}$ - and  $^{14}\text{C}$ -labeled D-glucose (30).

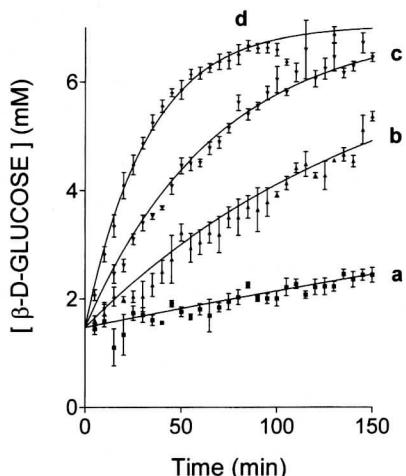
## MATERIALS AND METHODS

**Materials.** The wild-type recombinant forms of human liver glucokinase (purity, 75%) and B-cell glucokinase (purity, 50%), as well as the purified glucokinase regulatory protein (31) from rat liver were kindly provided by Prof. E. Van Schaftingen (Laboratory of Physiological Chemistry, Université Catholique de Louvain, Brussels, Belgium). The unlabeled D-glucose anomers used to measure D-glucose anomeration were purchased from Sigma (St. Louis, MO). Both D-[U- $^{14}\text{C}$ ]glucose and D-[5- $^3\text{H}$ ]glucose were purchased from New England Nuclear (Boston, MA). Unlabeled  $\alpha$ -D-glucose and the labeled D-glucose anomers ( $\alpha$ - and  $\beta$ -D-[U- $^{14}\text{C}$ ]glucose and  $\alpha$ - and  $\beta$ -D-[5- $^3\text{H}$ ]glucose) were prepared and their purity assessed as previously described (30). No anomeric cross-contamination of these preparations, or of commercial unlabeled  $\beta$ -D-glucose, could be detected.

**Methods.** In order to measure the half-life of D-glucose anomeration,  $\alpha$ -D-glucose (Sigma Chemical Co., St. Louis, MO) was dissolved (11.1 mM) in 1.0 ml of either  $\text{H}_2\text{O}$  or a Hepes-NaOH buffer (50 mM, pH 7.1) identical to that otherwise used to measure D-glucose phosphorylation (see below). The total length of incubation was increased from 25 min at 37°C to 100 min at 30°C and 200 min at 20, 10, or 4°C. The concentration of  $\beta$ -D-glucose was measured extemporaneously by the glucose oxidase method (EC 1.1.3.4; Glucose Analyzer 2; Beckman Instruments, Fullerton, CA) in 10- $\mu\text{l}$  samples removed at 2- to 5-min intervals from the incubation medium.

The phosphorylation of D-glucose was conducted in a reaction mixture (0.2 ml) adapted from Veiga-da-Cunha *et al.* (29) and consisting of Hepes-NaOH buffer (50 mM, pH 7.1) containing 4 mM MgCl<sub>2</sub>, 50 mM KCl, 1 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 1 mM ATP, and increasing concentrations of D-glucose (2.5 to 100 mM). The experiments with equilibrated D-glucose were conducted in the presence of a fixed concentration of D-[U- $^{14}\text{C}$ ]glucose (3  $\mu\text{Ci}/\text{ml}$ ). The experiments with the D-glucose anomers were conducted at a fixed specific radioactivity, namely  $146 \pm 7 \mu\text{Ci}/\text{mmol}$  ( $n = 17$ ) for  $\alpha$ - and  $\beta$ -D-[U- $^{14}\text{C}$ ]glucose and  $76.4 \pm 2.3 \mu\text{Ci}/\text{mmol}$  ( $n = 6$ ) for  $\alpha$ - and  $\beta$ -D-[5- $^3\text{H}$ ]glucose. The stock solutions of glucokinase were diluted in the same buffer, except for the absence of ATP and D-glucose. The final concentration of the enzyme, as judged from both the protein concentration of the stock solution and the purity of the enzyme, relative to protein content, ranged from 0.07 to 1.03  $\mu\text{g}/\text{ml}$  in the case of liver glucokinase and 0.06 to 0.98  $\mu\text{g}/\text{ml}$  in the case of B-cell glucokinase. The glucokinase regulatory protein was tested in the presence of 0.2 mM D-fructose 6-phosphate. After 6 to 60 min incubation at room temperature (20–22°C) or 30°C, labeled D-glucose 6-phosphate was separated from its precursor by ion exchange chromatography (32). All measurements were made in triplicate.

At 20–22 and 30°C, respectively, the length of incubation (10 and 6 min) was such that the fraction of each anomer that had been converted to the other anomer at the end of the incubation period represented 13.7–16.8% of the equilibrium value for such a fraction.



**FIG. 1.** Generation of  $\beta$ -D-glucose from  $\alpha$ -D-glucose (11.1 mM; commercial source) over 150 min incubation at increasing temperatures (a, 4°C; b, 10°C; c, 20°C; and d, 30°C). Mean values ( $\pm$ SEM) refer to two to six individual experiments. The curves were calculated from the experimental data according to the equation  $(Y_{\max} - Y) = (Y_{\max} - A) \cdot e^{-kt}$ , in which  $Y_{\max}$  represents the equilibrium value for  $\beta$ -D-glucose concentration (taken as 7.1 mM) and  $A$  the zero time ordinate. The coefficient of correlation between experimental and calculated values amounted to 0.837 (a), 0.955 (b), 0.975 (c), and 0.970 (d).

**Analysis and presentation of results.** The measurements made in each individual experiment were expressed relative to their overall mean value which was used as the reference velocity in further calculation. From these normalized data, the mean values ( $\pm$ SEM) were then calculated for each group of identical experiments. The results were eventually reconverted to absolute values after multiplication by the mean of the reference velocities in each series of experiments (33). A comparable procedure was applied in the experiments dealing with the anomeric specificity of D-glucose phosphorylation, a distinct reference velocity being used for each anomer in each individual experiment.

Mathematical and statistical analysis was made using Prism version 2.0 from GraphPad (San Diego, CA). The determination of the maximal velocity ( $V_{\max}$ ), Hill number ( $n$ ), and the substrate concentration yielding half-maximal velocity ( $K_m$ ) from the reaction velocity ( $v$ ) measured at each hexose concentration was based on the equation  $\log_{10}[v/(V - v)] = n \cdot \log_{10}S - n \cdot \log_{10}K_m$ .

The ratio obtained, for a given variable and in each individual experiment, with  $\alpha$ -D-glucose and  $\beta$ -D-glucose is referred to as the paired  $\alpha/\beta$  ratio.

All results, including those already mentioned, are presented as mean values ( $\pm$ SEM) together with the number of individual observations ( $n$ ). The statistical significance of differences between mean values was assessed by use of Student's *t* test.

## RESULTS

### Anomerization of D-Glucose

As shown in Fig. 1, the half-life for the anomeration of  $\alpha$ -D-glucose (Sigma Chemical Co., St. Louis, MO) increased from about 23 min at 30°C to 47, 107, and 536 min as the temperature was decreased to 20, 10, and 4°C, respectively. Virtually identical results were

obtained when the  $\alpha$ -anomer (11.1 mM) was dissolved in either  $H_2O$  or the Hepes-NaOH buffer (pH 7.1) otherwise used for measuring D-glucose phosphorylation. The results illustrated in Fig. 1 indicate that the commercial preparation of  $\alpha$ -D-glucose was contaminated by about 13%  $\beta$ -D-glucose (1.5 mM). Such a value, which was obtained by extrapolation to time zero of the measurements made at 4°C, was comparable at all temperatures tested (including 37°C, data not shown). It largely exceeded the value (less than 0.1 mM) possibly attributable to the conversion of  $\alpha$ -D-glucose to  $\beta$ -D-glucose during the time ( $12 \pm 1$  s;  $n = 6$ ) required for the assay of  $\beta$ -D-glucose, which was conducted at 37°C by the glucose oxidase method. Because of such a contamination, all further experiments were conducted with the mixtures of unlabeled and radioactive pure  $\alpha$ - or  $\beta$ -D-glucose prepared in our laboratory.

#### Phosphorylation of Equilibrated D-Glucose

In all experiments, either D-[U- $^{14}C$ ]glucose or D-[5- $^3H$ ]glucose was used as the tracer molecule. When compared within the same experiments, the reaction velocity measured with D-[5- $^3H$ ]glucose averaged  $102.0 \pm 6.9\%$  ( $n = 8$ ) of the paired value found with D-[U- $^{14}C$ ]glucose.

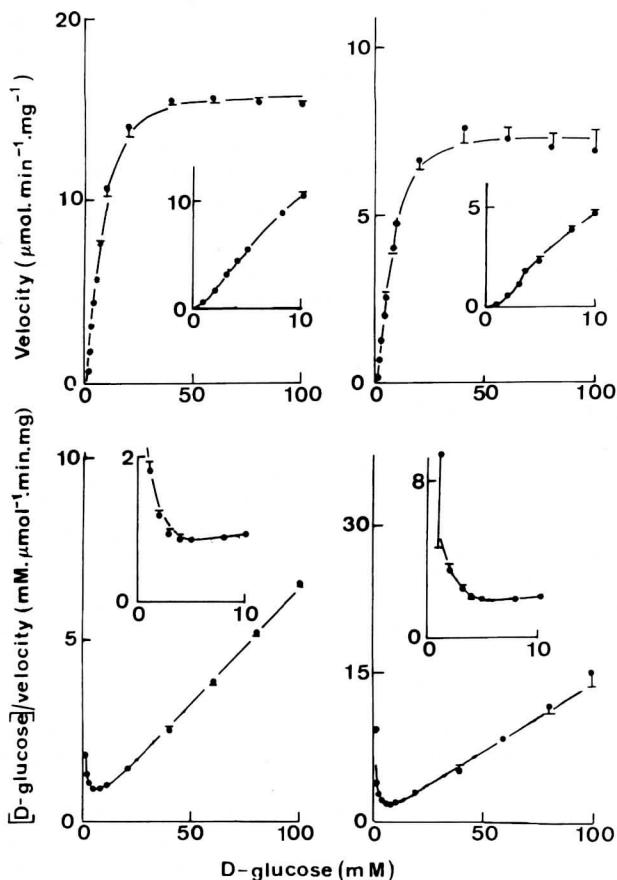
Whether in the case of the liver or B-cell enzyme, the phosphorylation of equilibrated D-glucose (5–10 mM) at 30°C was proportional to both the length of incubation (15 to 60 min) and concentration of glucokinase (0.06 to 0.44  $\mu\text{g}/\text{ml}$ ), with variation coefficients of 11.9% ( $n = 18$ ) and 6.9% ( $n = 12$ ), respectively.

When the phosphorylation rate of equilibrated D-glucose, as catalyzed by liver glucokinase, was measured over 30 min incubation at 30°C, the reaction velocity at increasing concentrations of the hexose (1.0 to 100.0 mM) yielded a typical sigmoidal pattern in Cartesian coordinates and curvilinear pattern in a Hanes-Woolf plot (Fig. 2, left). The maximal velocity amounted to  $15.8 \mu\text{mol min}^{-1} \text{mg}^{-1}$ , the Hill number to 1.79, and the  $K_m$  to 6.90 mM.

A comparable situation prevailed in the case of B-cell glucokinase (Fig. 2, right). The  $V_{\max}$ , Hill number, and  $K_m$  were close to  $7.3 \mu\text{mol min}^{-1} \text{mg}^{-1}$ , 1.96, and 7.09 mM, respectively. Except for the maximal velocity, these values were not significantly different from those found with the liver enzyme. In both cases, the Hill number was significantly higher than unity ( $P < 0.05$  or less).

#### Phosphorylation of D-Glucose Anomers

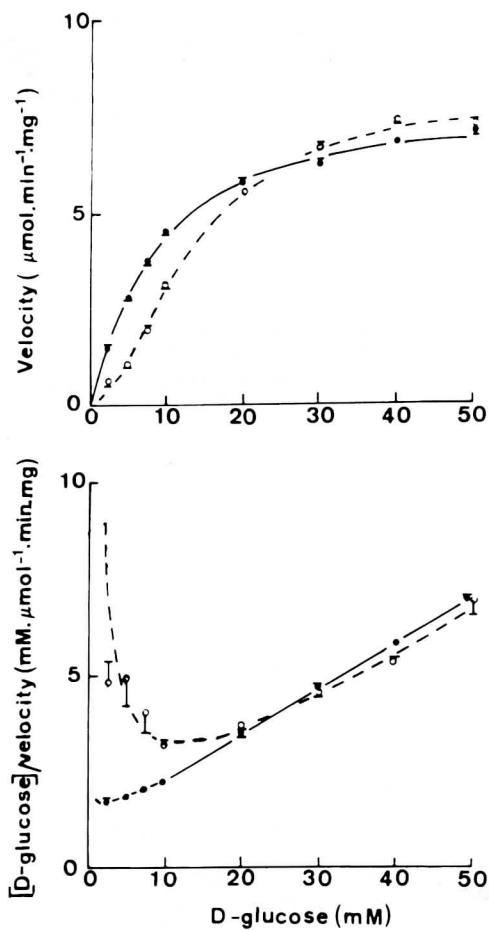
The anomeric specificity of liver and B-cell glucokinase was first examined over 10 min incubation at 20–22°C.



**FIG. 2.** Cartesian representation (top) and Hanes-Woolf plot (bottom) for the phosphorylation of equilibrated D-glucose by either liver (left) or B-cell (right) glucokinase. Mean values ( $\pm \text{SEM}$ ) are derived from three individual experiments in each case. The inset in each panel illustrates the results obtained in the low range of D-glucose concentrations up to 10 mM. The curves were calculated from the mean experimental values at each hexose concentration (see equation under Materials and Methods).

In the case of liver glucokinase, the results illustrated in Fig. 3 indicate that the reaction velocity was higher with  $\alpha$ -D-glucose than with  $\beta$ -D-glucose in the low range of hexose concentrations up to at least 10 mM, while the opposite situation prevailed at high hexose concentrations in the 30 to 50 mM range. The paired  $\alpha/\beta$  ratio in reaction velocity indeed averaged  $209.6 \pm 17.5\%$  ( $n = 16$ ;  $P < 0.001$ ) in the 2.5–10.0 mM range of hexose concentrations and  $95.1 \pm 1.8\%$  ( $n = 12$ ;  $P < 0.025$ ) in the 30–50 mM range.

Both the Cartesian and Hanes-Woolf plot revealed another anomeric difference in that the phenomenon of positive cooperativity at low hexose concentrations was much more obvious in the case of  $\beta$ -D-glucose than in the case of  $\alpha$ -D-glucose. Incidentally, even at the lowest concentration of  $\beta$ -D-glucose (2.5 mM), the reaction velocity in the calculated mean curve (Fig. 3) remained



**FIG. 3.** Cartesian representation (top) and Hanes-Woolf plot (bottom) for the phosphorylation of either  $\alpha$ -D-glucose (closed circles and solid line) or  $\beta$ -D-glucose (open circles and dashed line) by liver glucokinase. Mean values ( $\pm$ SEM) are derived from four individual experiments in each case. The curves were calculated from the mean experimental values at each hexose concentration.

within the 95% confidence limits of the experimental measurements.

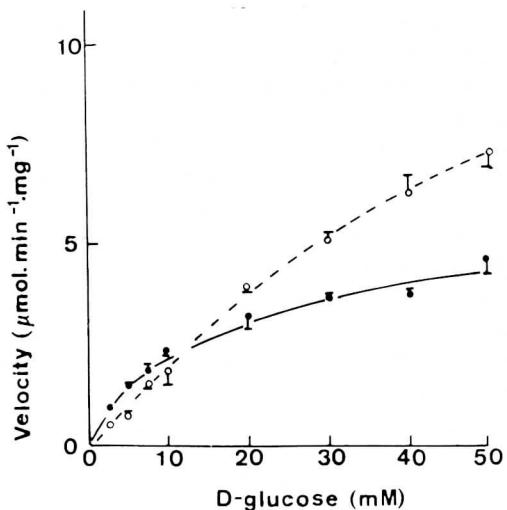
Analysis of each individual experiment indicated that the maximal velocity for  $\alpha$ -D-glucose averaged  $8.03 \pm 0.25 \mu\text{mol min}^{-1} \text{mg}^{-1}$  and the paired  $\alpha/\beta$  ratio for such a  $V_{\max}$   $94.5 \pm 6.9\%$  ( $n = 4$  in both cases). The Hill number averaged  $1.17 \pm 0.07$  ( $P < 0.07$  versus unity) and  $1.88 \pm 0.28$  ( $P < 0.025$  versus unity) in the case of  $\alpha$ - and  $\beta$ -D-glucose, respectively. It was indeed significantly higher ( $P < 0.025$ ) with the  $\beta$ -anomer than the  $\alpha$ -anomer. Last, the  $K_m$  for  $\alpha$ - and  $\beta$ -D-glucose averaged  $8.70 \pm 0.64$  and  $14.23 \pm 2.61$  mM, respectively ( $n = 4$  in both cases;  $P < 0.05$ ).

The situation found with the B-cell enzyme was essentially comparable to that just described for the liver enzyme. First, in the range of D-glucose concentration between 2.5 and 10.0 mM, the phosphorylation of  $\alpha$ -D-

glucose exceeded that of  $\beta$ -D-glucose, with a mean paired  $\alpha/\beta$  ratio of  $153.1 \pm 15.6\%$  ( $n = 16$ ;  $P < 0.001$ ). Inversely, in the 30 to 50 mM range, the phosphorylation of  $\alpha$ -D-glucose was lower than that of the  $\beta$ -anomer, with a mean paired  $\alpha/\beta$  ratio of  $63.8 \pm 2.8\%$  ( $n = 12$ ;  $P < 0.001$ ). Second, the sigmoidal pattern in the Cartesian representation was again quite obvious in the case of  $\beta$ -D-glucose, but not so in the case of  $\alpha$ -D-glucose (Fig. 4). As a matter of fact, in the curves illustrated in Fig. 4, the  $\alpha/\beta$  ratios in  $V_{\max}$ , Hill number, and  $K_m$  amounted to 56.4, 61.1, and 78.9%, respectively. It should be stressed, however, that the paired  $\alpha/\beta$  ratio in reaction velocity in the low range of hexose concentrations was significantly lower ( $P < 0.025$ ) in the case of the islet than liver enzyme. Likewise, in the high range of hexose concentrations (30 to 50 mM), the  $\alpha/\beta$  paired ratio in reaction velocity was significantly lower ( $P < 0.001$ ) with the islet than liver enzyme.

#### Interference of the Glucokinase Regulatory Protein

Whether in the case of liver or B-cell glucokinase, the relative extent of the inhibitory action of the glucokinase regulatory protein (together with 0.2 mM D-fructose 6-phosphate) upon D-glucose phosphorylation was little affected by the length of incubation (6 to 30 min). For instance, in the presence of 0.4–0.5  $\mu\text{g}/\text{ml}$  of enzymatic protein and at 30°C, the phosphorylation rate of 10 mM equilibrated D-glucose in the presence/absence of the glucokinase regulatory protein (5 U/ml, together with 0.2 mM D-fructose 6-phosphate) yielded after



**FIG. 4.** Cartesian representation for the phosphorylation of either  $\alpha$ -D-glucose (closed circles and solid line) or  $\beta$ -D-glucose (open circles and dashed line) by B-cell glucokinase. Mean values ( $\pm$ SEM) are derived from four individual experiments in each case. The curves were calculated from the mean experimental values at each hexose concentration.

6–10 and 30 min incubation, respectively, ratios of  $51.0 \pm 7.9$  and  $56.5 \pm 1.5\%$  ( $n = 2$ –4).

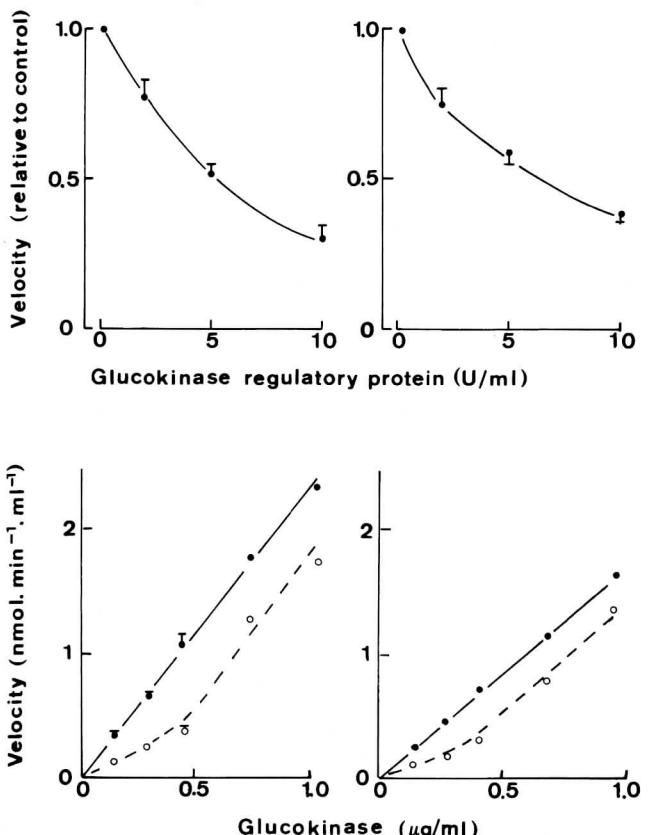
In the absence of the glucokinase regulatory protein, D-fructose 6-phosphate (0.2 mM) failed to decrease the rate of D-glucose phosphorylation, whether by liver or B-cell glucokinase. For instance, over 30 min incubation at 30°C, the phosphorylation of 10 mM equilibrated D-glucose by liver and B-cell glucokinase averaged, in the presence of 0.2 mM D-fructose 6-phosphate, respectively  $108.4 \pm 5.2$  and  $112.9 \pm 3.4\%$  ( $n = 6$  in both cases) of the paired control value (no D-fructose 6-phosphate).

As illustrated in Fig. 5 (top), at fixed concentrations of either liver or B-cell glucokinase (0.14–0.15 µg/ml), the relative extent of the inhibitory action of the regulatory protein (together with 0.2 mM D-fructose 6-phosphate) progressively increased as its concentration was raised from 2 to 10 U/ml. At fixed concentrations of the regulatory protein (5 U/ml) and D-fructose 6-phosphate (0.2 mM), the relative extent of the inhibition of D-glucose phosphorylation decreased ( $P < 0.001$ ) from  $59.2 \pm 0.9\%$  ( $n = 12$ ) to  $24.5 \pm 3.3\%$  ( $n = 4$ ) as the concentration of liver or B-cell glucokinase was increased from 0.14–0.44 to 0.70–1.03 µg/ml (Fig. 5, bottom).

In order to assess the possible influence of the glucokinase regulatory protein upon the anomeric specificity of liver and B-cell glucokinases, the rate of α- and β-D-glucose phosphorylation was measured over 6 min incubation at 30°C in the absence or presence of the glucokinase regulatory protein (together with 0.2 mM D-fructose 6-phosphate). The concentration of the regulatory protein (5 U/ml) was selected to decrease the reaction velocity by about half at a low concentration of the hexose (4.5 mM).

The control experiments (no glucokinase regulatory protein) conducted with liver glucokinase yielded results comparable to those obtained over 10 min incubation at 20–22°C. Thus, the  $\alpha/\beta$  paired ratio in reaction velocity exceeded unity at a low concentration (4.5 mM) of the anomers ( $183.4 \pm 23.3\%$ ;  $n = 5$ ;  $P < 0.01$ ), while the opposite situation ( $\alpha/\beta$  paired ratio in reaction velocity,  $79.0 \pm 5.0\%$ ;  $n = 5$ ;  $P < 0.025$ ) prevailed at a 36.0 mM concentration of the hexose. Likewise, the phenomenon of positive cooperativity was quite obvious in the case of β-D-glucose, but not α-D-glucose (Fig. 6). The  $\alpha/\beta$  ratio for the Hill number represented 59.8%.

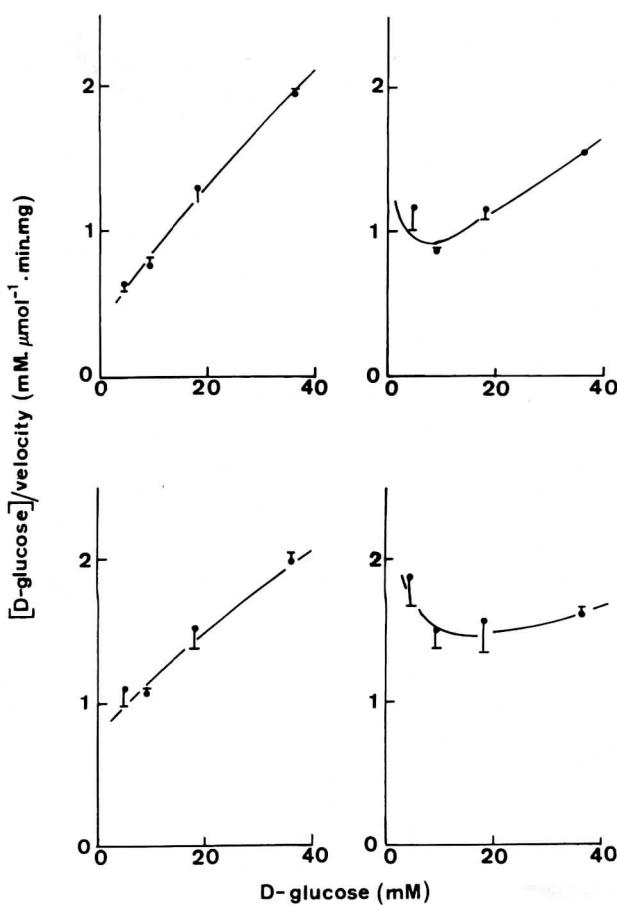
The paired ratio for reaction velocity in the presence/absence of the glucokinase regulatory protein progressively increased from  $60.5 \pm 2.8$  to  $93.6 \pm 3.0\%$  ( $n = 10$  in both cases;  $P < 0.001$ ) as the concentration of D-glucose anomers was raised from 4.5 to 36.0 mM. Except at the lowest hexose concentration (4.5 mM), such a paired ratio was higher ( $P < 0.05$  or less), however, in



**FIG. 5.** Phosphorylation of equilibrated D-glucose (10 mM) over 30 min incubation at 30°C in the presence of liver (left) or B-cell (right) glucokinase. (Top) Effects of increasing concentrations of the glucokinase regulatory protein (together with 0.2 mM D-fructose 6-phosphate) at a fixed concentration of glucokinase (0.14–0.15 µg/ml). Mean values ( $\pm$ SEM) refer to six individual experiments in each case, the reaction velocity being expressed relative to the paired control value (no regulatory protein). Such a control value averaged  $0.38 \pm 0.04$  and  $0.20 \pm 0.02$  nmol min<sup>-1</sup> per ml of incubation medium in the case of liver and B-cell glucokinase, respectively. (Bottom) Effects of increasing concentrations of glucokinase in the absence (closed circles and solid line) or presence (open circles and dashed line) of a fixed concentration of its regulatory protein (5 U/ml, together with 0.2 mM D-fructose 6-phosphate). Mean values ( $\pm$ SEM, when in excess of the circle) are derived from three individual experiments in each case.

the case of α-D-glucose than β-D-glucose (Fig. 7), indicating that the phosphorylation of the α-anomer was more resistant than that of the β-anomer to the inhibitory action of D-fructose 6-phosphate, as mediated by the glucokinase regulatory protein.

In the presence of the regulatory protein (together with D-fructose 6-phosphate), the  $\alpha/\beta$  ratio in reaction velocity remained significantly higher ( $P < 0.005$ ) than unity at low hexose concentrations (4.5 to 9.0 mM) and slightly lower ( $P < 0.07$ ) than unity at a much higher hexose concentration (36.0 mM), averaging, respectively,  $160.8 \pm 16.3\%$  ( $n = 10$ ) and  $90.6 \pm 3.9\%$  ( $n = 5$ ).



**FIG. 6.** Hanes-Woolf plot for the phosphorylation of  $\alpha$ -D-glucose (left) and  $\beta$ -D-glucose (right) by liver glucokinase tested in the absence (top) or presence (bottom) of its regulatory protein (5 U/ml, together with 0.2 mM D-fructose 6-phosphate). Mean values ( $\pm$ SEM) are derived from five individual experiments in each case. The curves were calculated from the mean experimental values at each hexose concentration.

Under these conditions, the phenomenon of positive cooperativity was again more pronounced in the case of  $\beta$ -D-glucose than  $\alpha$ -D-glucose (Fig. 6), with an  $\alpha/\beta$  ratio for the Hill number of 73.5%.

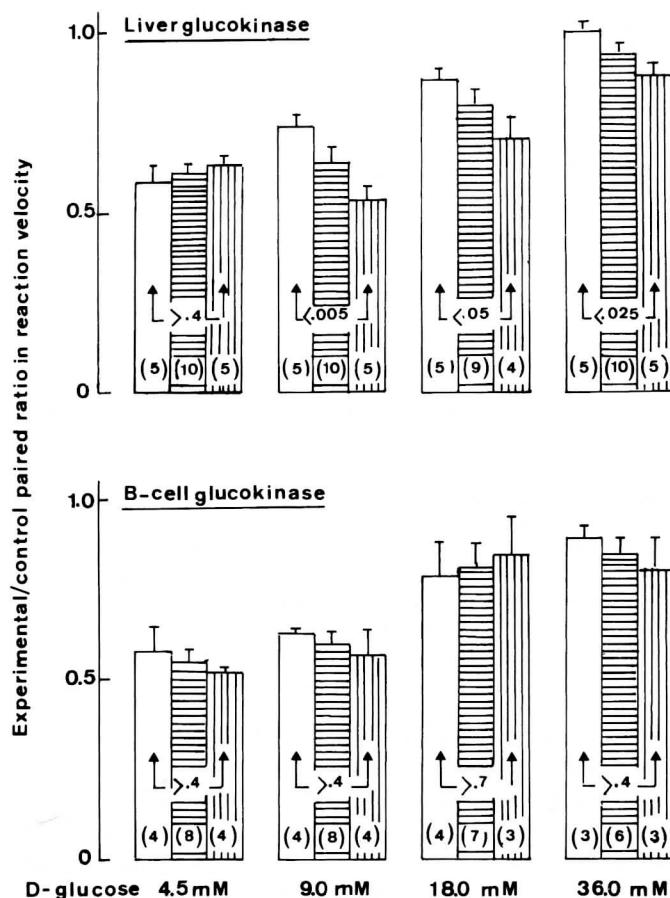
A comparable, albeit not identical, situation was found in the case of the B-cell glucokinase.

First, the results recorded in the absence of the glucokinase regulatory protein confirmed those obtained at a lower temperature. Thus, the  $\alpha/\beta$  ratio in reaction velocity significantly exceeded unity ( $145.6 \pm 23.2\%$ ;  $n = 8$ ;  $P < 0.05$ ) at low hexose concentrations (4.5 and 9.0 mM), while such was no more the case at 18.0 or 36.0 mM (Fig. 8).

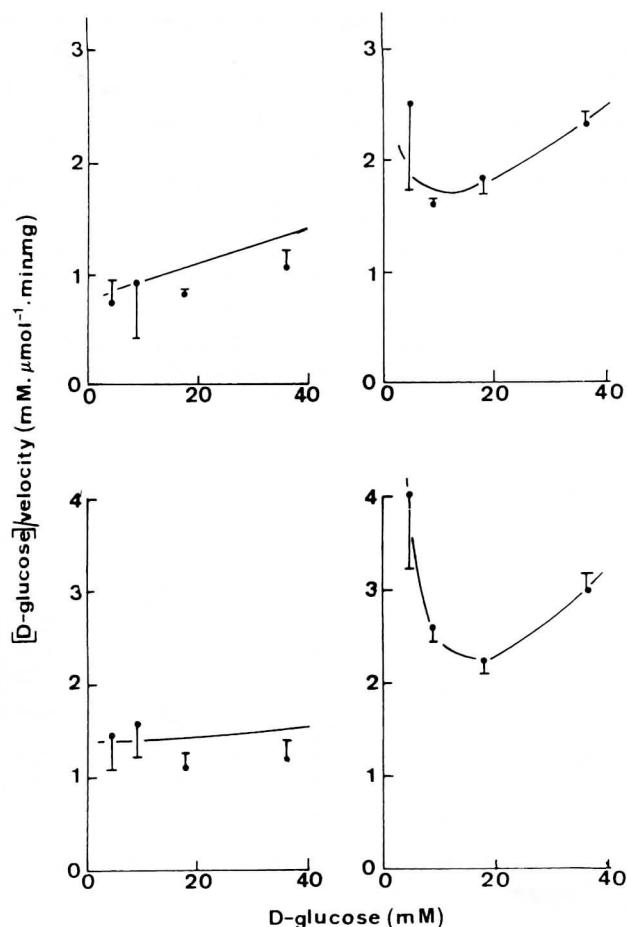
Second, the paired ratio in reaction velocity in the presence/absence of the glucokinase regulatory protein (together with D-fructose 6-phosphate) increased ( $P < 0.001$ ) from  $54.8 \pm 3.5\%$  ( $n = 8$ ) to  $84.8 \pm 4.8\%$  ( $n = 6$ )

as the concentration of the glucose anomers was raised from 4.5 to 36.0 mM.

Third, as judged by such a paired ratio in reaction velocity, the glucokinase regulatory protein failed to affect significantly the anomeric specificity of B-cell glucokinase (Fig. 7). Indeed, the ratios obtained with  $\alpha$ -D-glucose averaged  $106.1 \pm 5.2\%$  ( $n = 15$ ;  $P > 0.35$ ) of the mean corresponding values found, within the same experiments and at the same hexose concentrations with  $\beta$ -D-glucose ( $100.0 \pm 6.0\%$ ;  $n = 14$ ). In the presence of the glucokinase regulatory protein (together with D-fructose 6-phosphate), the  $\alpha/\beta$  ratio in



**FIG. 7.** Paired ratio for the phosphorylation of D-glucose anomers, at increasing hexose concentrations, by either liver (top) or B-cell (bottom) glucokinase in the presence/absence of the glucokinase regulatory protein (together with D-fructose 6-phosphate). Mean values ( $\pm$ SEM) refer to the data obtained with either  $\alpha$ -D-glucose (open columns) or  $\beta$ -D-glucose (vertically hatched columns), the middle horizontally hatched columns corresponding to the pooled measurements made with the two anomers. Also shown are the number of individual determinations (in parentheses at the bottom of each column), the significance ( $P$  values) of differences between  $\alpha$ - and  $\beta$ -D-glucose, and the four hexose concentrations tested in these experiments.



**FIG. 8.** Hanes-Woolf plot for the phosphorylation of  $\alpha$ -D-glucose (left) and  $\beta$ -D-glucose (right) by B-cell glucokinase tested in the absence (top) or presence (bottom) of its regulatory protein (5 U/ml, together with 0.2 mM D-fructose 6-phosphate). Mean values ( $\pm$ SEM) are derived from three individual experiments in each case, with only two of them referring to both anomers.

reaction velocity indeed remained significantly higher than unity ( $161.8 \pm 29.9\%$ ;  $n = 8$ ;  $P < 0.05$ ) at low hexose concentrations (4.5 and 9.0 mM), whereas such was no more the case at 18.0 or 36.0 mM. For instance, in the presence of 4.5 mM  $\alpha$ - and  $\beta$ -D-glucose, respectively, the data illustrated in Fig. 8 corresponded to reaction velocities of  $3.4 \pm 1.2$  and  $1.2 \pm 0.2 \mu\text{mol min}^{-1} \text{mg}^{-1}$ , as compared to control values (no glucokinase regulatory protein) of  $5.2 \pm 1.1$  and  $2.1 \pm 0.5 \mu\text{mol min}^{-1} \text{mg}^{-1}$ .

Last, in both the absence and the presence of the glucokinase regulatory protein (together with D-fructose 6-phosphate), the Hill number derived from the mean experimental curves were again close to unity (0.96 and 1.01) in the case of  $\alpha$ -D-glucose, but higher than unity (1.40 and 1.93) in the case of  $\beta$ -D-glucose.

## DISCUSSION

The present results confirm two major features of the anomeric specificity of glucokinase, namely the higher affinity and lower maximal velocity found with  $\alpha$ - as compared to  $\beta$ -D-glucose (Table I). Previous investigations of this matter were conducted in either liver and islet homogenates or with the enzyme purified from cell extracts, whereas the wild-type recombinant forms of human liver and B-cell glucokinase were used in this work.

Two new findings, however, emerge from the present experiments.

First, the phenomenon of positive cooperativity typically found at low D-glucose concentrations was quite obvious with  $\beta$ -D-glucose, while being much less evident with  $\alpha$ -D-glucose. This contrasting behavior was documented with both liver and B-cell glucokinase, at two temperatures (20–22 and 30°C), and in both the absence or the presence of the glucokinase regulatory protein.

Second, while confirming that the inhibitory action of D-fructose 6-phosphate upon D-glucose phosphorylation, as mediated at the intervention of the glucokinase regulatory protein, fades out at high concentrations of either D-glucose or glucokinase relative to that of its regulatory protein (31, 34–36), the present experiments indicate that, in relative terms, the phosphorylation of  $\alpha$ -D-glucose is more resistant than that of  $\beta$ -D-glucose to such an inhibitory action. Such was the case at three of the four tested concentrations of the D-glucose anomers in the case of liver glucokinase. A comparable trend was observed, also at three out of four D-glucose concentrations, in the case of the B-cell glucokinase. In the latter case, however, such an anomeric difference failed to achieve statistical significance.

The possible relevance of these two new findings to a better understanding of the molecular determinants for the interaction between glucokinase and both its substrate and regulatory protein, e.g., in the phenomenon of positive cooperativity, should now be considered by the experts in such a field (37–39).

**TABLE I**  
Kinetics of  $\alpha$ - and  $\beta$ -D-Glucose Phosphorylation  
by Human Liver and B-cell Glucokinase

Glucokinase regulatory protein	Absent	Present
Maximal velocity	$\alpha < \beta$	$\alpha < \beta$
$K_m$	$\alpha < \beta$	$\alpha < \beta$
Hill number	$\alpha < \beta$	$\alpha < \beta$
Inhibition by regulatory protein	NA	$\alpha \leq \beta$

Note. NA, not applicable.

Meanwhile, the implications of the present work should not be overlooked. To begin with, they suggest, at first glance, that the participation of glucokinase to the phosphorylation of D-glucose in insulin-producing cells could account, in part at least, for the higher insulinotropic capacity of  $\alpha$ - than  $\beta$ -D-glucose at hexose concentrations not in excess of about 12 mM. Second, however, they clearly indicate that the intrinsic properties of glucokinase cannot account for the finding that no anomeric difference in the B-cell secretory response to D-glucose is anymore detected at much higher concentration of D-glucose (40, 41). Last, taking into account the modulation of glucokinase activity by its regulatory protein in intact cells, they suggest that, at least in hepatocytes, the higher rate of  $\beta$ -D-glucose, rather than  $\alpha$ -D-glucose, phosphorylation at high concentrations of these anomers, as expected from the higher maximal velocity for phosphorylation of the former anomer, could well be masked by the greater resistance of  $\alpha$ -D-glucose phosphorylation to the inhibitory action of the glucokinase regulatory protein.

In conclusion, therefore, the present work further illustrates the sophistication of the kinetics for D-glucose phosphorylation by glucokinase, especially when considering that a mixture of  $\alpha$ - and  $\beta$ -D-glucose represents its physiological substrate (42). In this respect, the most salient finding in this study, namely the vastly different Hill number for each of these anomers, should now be integrated in the scheme previously proposed to account for the respective contribution of  $\alpha$ - and  $\beta$ -D-glucose to the overall rate of D-glucose phosphorylation by glucokinase when the hexose is present at anomeric equilibrium (43). This is most relevant to the regulation of D-glucose metabolism in hepatocytes and B-cells since each of the two anomers of D-glucose 6-phosphate may be preferentially channelled into distinct pathways (44, 45).

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