Mannose Phosphorylation by Glucokinase from Liver and Transplantable Insulinoma

Cooperativity and Discrimination of Anomers

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SUMMARY

Glucokinase from rat liver or transplantable, radiation-induced insulinomas was partially purified by ion exchange chromatography using DEAE-Cibacron Blue F3GA agarose. Phosphorylation of α,β-D-mannose by glucokinase occurred with cooperative rate dependence on mannose concentration (n_H: 1.50). Half-maximal phosphorylation rate occurred at 14 mM α,β-D-mannose. The α- and β-anomers of mannose were phosphorylated with sigmoidal kinetics (n_H: 1.57 and 1.42, respectively). The affinity of glucokinase for α-D-mannose is higher than for β-D-mannose ($S_{0.5}$: 12 mM versus 19 mM). The maximum phosphorylation rate is slightly higher, about 10%, with β-D-mannose than with α-D-mannose. Islet glucokinase has previously been shown to be chromatographically and kinetically identical to glucokinase from insulinoma and liver; therefore, evidence that glucokinase from these two tissues phosphorylates mannose with cooperative rate dependence and differentiates mannose anomers supports the glucokinase-glucose sensor hypothesis. DIABETES *32*:1146–1151, December 1983.

Glucokinase (ATP: D-glucose 6-phosphotransferase, EC 2.7.1.2) has been suggested to be the rate-limiting step determining steady-state glycolytic flux in pancreatic β-cells under conditions of increasing glucose concentration.[1,2] Evidence for this hypothesis is substantial. Glucose usage by intact rat pancreatic islets is equal to glucokinase activity of islet homogenates in a variety of physiologic states and at different glucose concentrations.[2–4] Also, treatment of islets with specific glucokinase inhibitors diminishes islet glucose usage.[5–7] Glucokinase purified from islets shows glucose-dependent positive cooperativity similar to the sigmoidicity of glucose usage by the predominant low-affinity component of islet hexose usage.[2,3,8] Also, glucokinase purified from transplantable insulinomas and liver has a higher affinity for α-D-glucose than its β-anomer,[9] a finding similar to the potency of the glucose anomers as insulin secretagogues[10–12] and as substrates for islet glycolysis.[13]

Mannose is the only hexose able to mimic fully the effects of glucose on islets.[14,15] Mannose is metabolized by rat islets with equal effectiveness as glucose, albeit at higher concentrations, i.e., with half-maximal usage occurring at 21 mM mannose compared with 11 mM glucose.[3] Mannose stimulation of insulin secretion is similarly shifted, occurring at higher concentrations than those required for stimulation by glucose with half-maximally effective concentrations at 15 and 8 mM, respectively.[16] Sigmoidal dependence on mannose concentration occurs for both mannose-stimulated insulin secretion[16] and mannose usage. Reanalysis of data previously published[3] indicates that the predominant low-affinity component of islet mannose usage has sigmoidal rate dependence on mannose concentration (n_H: 1.5). Mannose anomers differ in potency both as stimulants of insulin secretion and as substrates for islet hexose usage with the α-anomer being more potent than the β-anomer.[17,18]

Glucokinase in homogenates of islets or purified from islets or liver phosphorylates mannose, although with a higher Michaelis-Menten constant than that for glucose.[3,5,8,15] It is likely, therefore, that the rate-limiting step for mannose usage by islets also occurs at the glucokinase step. Glucokinase would determine the kinetic properties for usage of both hexoses by islets under steady-state conditions and, thereby, the capacity of β-cells to generate trigger substance(s) initiating insulin secretion in response to increased glucose or mannose. Little information is available, however, about the kinetic behavior of glucokinase with mannose as substrate. For example, it has yet to be reported whether glucokinase phosphorylates mannose with cooperative rate dependence or discriminates between mannose anomers, although both characteristics are essential to the concept

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that glucokinase serves as the β-cell glucose sensor.[19] It is the purpose of this article to report the kinetic properties of glucokinase with pure anomers of mannose, especially at mannose concentrations producing submaximal (graded) stimulation of insulin secretion.

METHODS

Glucokinase was prepared from rat liver or a transplantable, radiation-induced insulinoma previously described.[20] Livers were removed from fed male Wistar rats (Hilltop Laboratory Animals, Scottdale, Pennsylvania), 200–300 g body weight, anesthetized with sodium pentobarbital. Radiation-induced insulinomas were grown subcutaneously by serial transplantation in NEDH rats (New England Deaconess Hospital, Boston, Massachusetts) from a tumor line donated by Dr. William L. Chick (University of Massachusetts, Worcester, Massachusetts). Insulinomas from three rats were removed under pentobarbital anesthesia and pooled for each experiment. Tissues were homogenized in a Kontes teflon-glass tissue grinder with 2 vol of homogenization buffer (50 mM Hepes, pH 7.8, 120 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol), and then centrifuged at 105,000 × *g* as previously described.[21] The supernatant was diluted with 2.5 vol buffer containing 50 mM Hepes, pH 7.8, 15 mM $MgCl_2$, 1 mM EDTA, and 1 mM dithiothreitol, and then applied to a column of DEAE-Cibacron Blue F3GA agarose (Biorad, Richmond, California). The column, 1.5 × 4.0 cm in size, was pre-equilibrated with the starting buffer of 50 mM Hepes, pH 7.8, 11 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol. The column was eluted with a linear gradient of KCl to a concentration of 200 mM over 16 h at a flow rate of 3.5 ml/h.

Glucose 6-phosphotransferase activity in chromatogram fractions was determined at 0.5 and 100 mM D-glucose by a fluorometric enzymatic method described previously.[8,21] Glucokinase activity was determined as the difference in phosphorylation at high and low glucose concentrations. Glucokinase activity is expressed as units whereby 1 unit equals phosphorylation of 1 μmol glucose · min^{-1} at 30°C. The protein content of chromatogram fractions was determined using fluorescamine.[22]

Phosphorylation of mannose was determined by a fluorometric assay method. The reaction was conducted in 100 μl of buffer (50 mM Hepes, pH 7.6, 100 mM KCl, 1 mM dithiothreitol, 0.05% bovine serum albumin, and 4.3 mM ATP). The $MgCl_2$ concentration was 1–1.5 mM in excess of ATP after allowing for the EDTA content of added eluate. Reactions were conducted at 24°C for 3 min with glucokinase prepared from liver or 2 min with glucokinase prepared from insulinomas. The reaction was terminated by addition of 10 μl of 0.75 N HCl. Assay tubes were incubated at 90°C for 3 min, and then the pH was adjusted by addition of 10 μl 0.8 M Hepes, pH 7.6, containing 0.75 N NaOH. Glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* (0.25 μg), phosphoglucose isomerase from yeast (0.4 μg), phosphomannose isomerase from yeast (2.0 μg), and NAD^+ (0.05 μmol) were added to assay tubes in a volume of 10 μl and the samples incubated for 30 min at 30°C. The indicator reaction was terminated by addition of 1 ml of 0.5 M sodium bicarbonate buffer, pH 9.4. All auxiliary enzymes were obtained as ammonium sulfate precipitates from Boehringer Mannheim (Indianapolis, Indiana). Before use the ammonium sulfate was removed by centrifugation and the enzyme dissolved in 50 mM Tris buffer, pH 8.1, containing 0.05% bovine serum albumin. The indicator reaction had a half-time of 1.5 min. NADH specific fluorescence was proportional to the volume of the glucokinase-enriched fraction added and the incubation time. Nonspecific fluorescence occurring in the absence of added glucokinase or glucokinase incubated without addition of ATP was equal to 0.5 nmol NADH.

The α- and β-anomers of D-mannose were a gift from Dr. Atsushi Niki (Aichigakuin University, Nagoya, Japan). Stock solutions of anomerically pure mannose were prepared at a concentration of 0.3 M by dissolving 54 mg mannose in 1 ml assay reagent (temperature = 24°C). The solution was mixed for 30 s, then the reactions were initiated by addition of aliquots of the stock solution to assay tubes containing assay buffer and the glucokinase-enriched fraction pre-equilibrated to 24°C. Each anomer was tested twice with each enzyme preparation using freshly prepared stock solutions. Mannose anomers were tested alternately and were added to assay tubes in order of both increasing and decreasing concentration to eliminate the time between dissolving the anomeric sugar and initiation of the reaction as a factor. Mutarotated solutions of mannose were prepared by incubating stock solutions of anomerically pure mannose for 2 h at 24°C. Mannose from both anomeric batches produced NADH with a stoichiometric ratio of 1 when incubated with excess yeast hexokinase in combination with the auxiliary enzymes.

Kinetic analysis of glucokinase was performed using Hanes-Woolf and Hill plots.[23] Hill coefficients (n_H) were determined from Hill plots at $v = 0.5\ V_{max}$ using least-squares linear regression. The mannose concentration producing half-maximal velocity ($S_{0.5}$) was determined from the Hill plot. Apparent kinetic coefficients were determined from Hanes-Woolf plots as described by Storer and Cornish-Bowden[24] using the range 16–120 mM mannose where the plot was linear. Sample statistics were determined for the means and their standard errors from four preparations of glucokinase from liver and two preparations from insulinomas. Statistical comparisons of combined data from experiments with liver and insulinoma glucokinase were made using the Mann-Whitney U test.[25]

RESULTS

Glucokinase was partially purified by chromatography on DEAE-Cibacron Blue F3GA agarose. Peak glucokinase fractions prepared from liver had specific activities averaging 1.2 U/mg protein and those prepared from insulinomas had average specific activities of 84 mU/mg protein. Hexokinase contamination, determined as phosphorylation of 0.5 mM glucose, was 1.4 ± 0.1% for glucokinase prepared from liver and 3.0 ± 0.3% for glucokinase prepared from insulinomas. N-acetylglucosamine kinase has been shown previously to be absent from glucokinase prepared by this method.[9]

Phosphorylation of mannose by glucokinase from liver or insulinoma occurs with cooperative rate dependence on mannose concentration (Figure 1; Table 1). Hanes-Woolf plots are nonlinear at mannose concentrations less than 15 mM, indicating activity deviating markedly from the Michae-

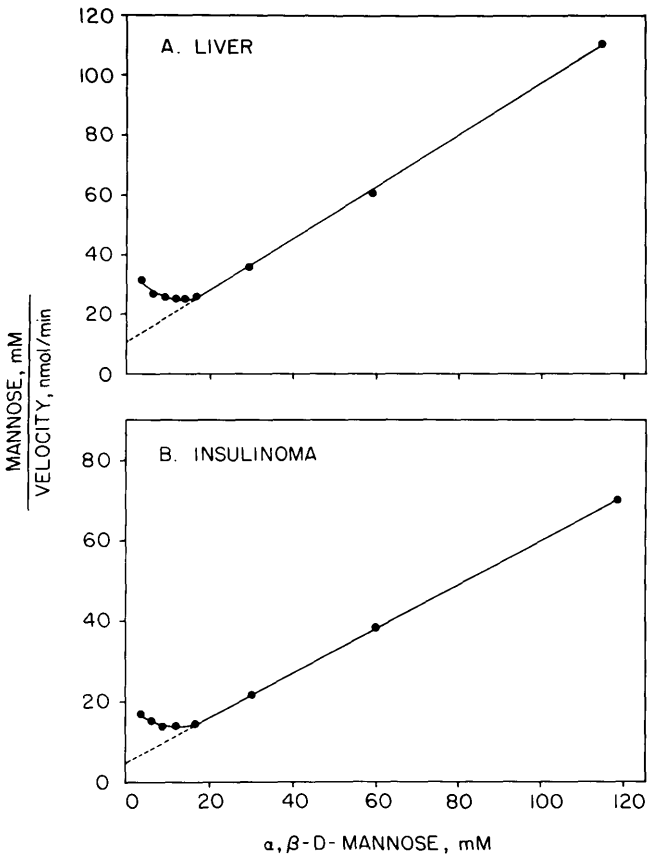

FIGURE 1. Hanes-Woolf plots for phosphorylation of mutarotated mannose by glucokinase prepared from liver (A) or insulinoma (B). Data from typical experiments are shown.

lis-Menten equation. Hill coefficients > 1 are observed with mutarotated (α,β) mannose as well as with its α- and β-anomers. Glucokinase from either tissue phosphorylates α,β-mannose with a Hill coefficient of 1.50. Slightly lower Hill coefficients are observed using β-mannose as substrate (n_H: 1.42) and slightly higher values are observed using α-mannose (n_H: 1.57; α versus β, $P < 0.001$). The affinity of glucokinase for the α-anomer of mannose is greater than that for β-mannose ($P < 0.001$). Half-maximal saturation of glucokinase by mannose occurs at 11–13 mM with α-mannose as substrate (Table 1). By comparison, the $S_{0.5}$ value for β-mannose is 17–20 mM and an intermediate value of 13–15 mM is observed with α,β-mannose. The Michaelis-Menten constants determined from the linear portion of Hanes-Woolf plots show a similar ordered sequence with α-mannose < α,β-mannose < β-mannose for glucokinase preparations from liver or insulinomas (Table 1).

Comparison of the relative velocities for phosphorylation of mannose anomers by glucokinase shows that α-mannose phosphorylation exceeds β-mannose phosphorylation at concentrations of 30 mM or less (Figures 2 and 3). The phosphorylation of α,β-mannose is intermediate between that observed for the individual anomers (data not shown). At high mannose concentrations phosphorylation of β-mannose by glucokinase exceeds the rate with α-mannose. Accordingly, the maximum velocity computed from the linear portion of Hanes-Woolf plots is 9–11% higher for glucokinase with β-mannose as substrate compared with α-mannose. Mutarotated (α,β) mannose solutions prepared from anomerically pure mannose batches were phosphorylated equally well by liver glucokinase (Figure 3).

DISCUSSION

Glucokinase prepared from rat liver or transplantable insulinomas discriminates between the two anomers of mannose. The α-anomeric configuration is phosphorylated with a higher affinity, but a lesser maximum velocity, than is β-D-mannose. The result is greater phosphorylation of α-D-mannose than its β-anomer at concentrations less than 30–60 mM. A concentration-dependent preference of glucokinase for α-D-glucose at low concentrations has also been observed; however, the α- and β-anomers of glucose are phosphorylated equally at 15–20 mM. The lower equivalence point for phosphorylation of α- and β-anomers of glucose is approximately proportionate to the higher affinity of glucokinase for glucose compared with mannose.[3,5]

The α-anomers of glucose or mannose are more effective than their β-anomers at modulating numerous processes in islets, i.e., insulin secretion,[10–12] glycolytic rate,[13,18] ion fluxes,[13,26] cAMP accumulation,[27] and proinsulin synthesis.[28] Several of these cellular processes have been tested at more than one hexose concentration. These studies indicate that the greater effectiveness of the α-anomers of glucose or mannose, relative to their β-anomers, declines as hexose concentrations are increased. In fact, the anomeric preference of islets for α-D-glucose and α-D-mannose occurs only within a narrow window of concentrations. The preferential stimulation of insulin secretion[11] or islet cAMP accumulation[27] by α-D-glucose is lost at glucose concentrations above 16 mM. The anomers of mannose stimulate insulin secretion equally at a concentration of 27 mM.[17] The preferential phosphorylation of glucose[9] and mannose by glucokinase shows dependence on hexose concentrations similar to that for discrimination of anomers by intact islets. Notably, the

TABLE 1
Kinetic constants for phosphorylation of mannose by glucokinase*

	Liver glucokinase (N = 4)			Insulinoma glucokinase (N = 2)			Combined data (N = 6)			
Mannose anomer	α	α, β	β	α	α, β	β	α	α, β	β	P value (α vs. β)
$S_{0.5}$ (mM)	12.9 ± 0.6	14.9 ± 1.1	20.2 ± 0.7	10.9 (10.8, 11.0)	13.1 (12.8, 13.3)	16.6 (15.9, 17.2)	12.2 ± 0.54	14.3 ± 0.79	19.0 ± 0.89	<0.001
K_m apparent (mM)	9.1 ± 1.0	11.3 ± 1.6	19.0 ± 1.3	6.1 (5.6, 6.5)	9.3 (8.8, 9.7)	12.8 (11.4, 14.2)	8.1 ± 0.91	10.6 ± 1.08	16.9 ± 1.58	<0.001
n_H	1.57 ± 0.04	1.50 ± 0.06	1.41 ± 0.04	1.57 (1.53, 1.61)	1.50 (1.42, 1.58)	1.45 (1.40, 1.49)	1.57 ± 0.03	1.50 ± 0.04	1.42 ± 0.03	<0.001

*Data from experiments with insulinoma glucokinase are shown as means and individual values are shown in parentheses. Data from experiments with liver glucokinase or the combination of data from liver and insulinoma experiments are shown as means ± SEM.

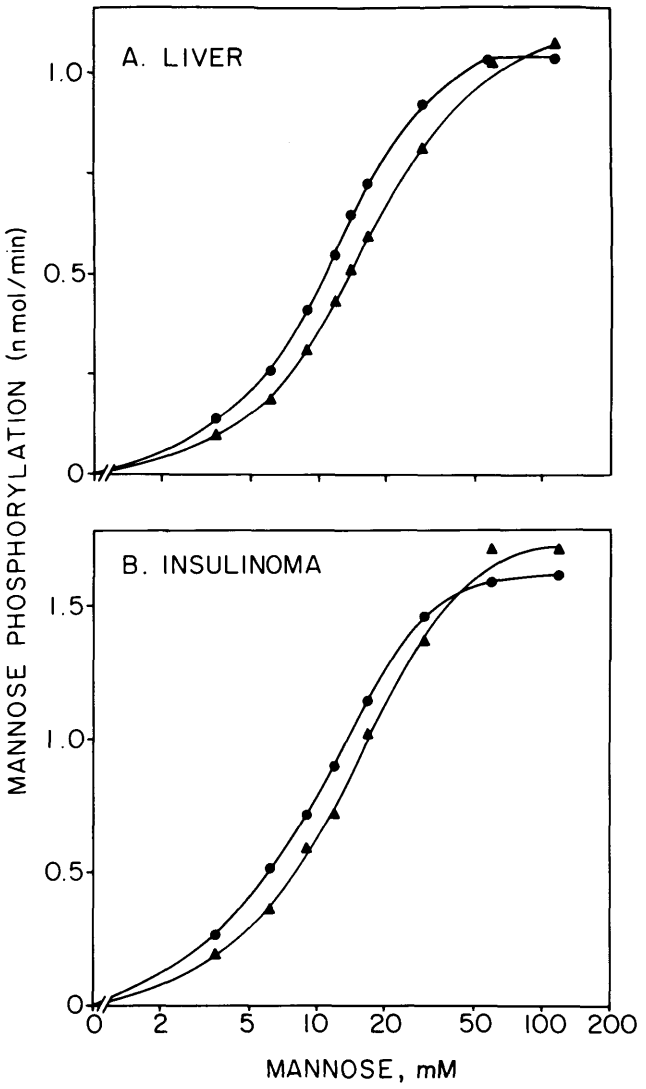

FIGURE 2. Phosphorylation of α-D-mannose (●) or β-D-mannose (▲) by glucokinase prepared from liver (A) or insulinoma (B). Data are from typical experiments shown in Figure 1. Assays were performed for 3 min using 1.3 μg of liver eluate protein or for 2 min using 30 μg of insulinoma eluate protein. Note that the abscissa is a logarithmic scale.

concentrations where phosphorylation rates of α- and β-anomers of glucose or mannose become equal are approximately the concentrations where cellular discrimination of anomers is lost.

Phosphorylation of mannose by glucokinase occurs with cooperative rate dependence on mannose concentration using either mutarotated or anomerically pure mannose. Small differences in Hill coefficients are observed with α-D-mannose and β-D-mannose as substrate for glucokinase. It is unclear, however, whether differences in sigmoidicity occur with the two anomers or if this variation reflects the difficulty in determining the V_{max} of enzymes with nonlinear kinetic plots. Positive cooperativity with glucose as substrate is characteristic of glucokinase and has been observed with preparations purified from rat liver,[9,24] islets of Langerhans,[8] and transplantable insulinoma.[9,21]

The affinity of partially purified glucokinase for mutarotated mannose is greater than that reported for mannose phosphorylation by homogenates of rat islets.[3,15] Mannose phosphorylation in islet homogenates occurs with apparent Km values of 24 or 31 mM compared with 11 mM, which we observe using partially purified glucokinase. The apparent Km values for glucose phosphorylation by crude preparations of glucokinase, e.g., homogenates, are also higher than those observed with purified glucokinase.[24] For example, glucokinase purified from pancreatic islets has an apparent Km for glucose of 5 mM compared with 10 mM in homogenates of islets.[3,8] The source of this variation is unknown. It has been argued that the higher K_m values for crude glucokinase preparations result from improper interpretation of kinetic plots.[24] It is also possible, however, that the enzyme is somehow altered during its purification, e.g., by the removal of a regulatory factor. K_m and $S_{0.5}$ values observed in this study are uniformly lower, albeit slightly, for mannose phosphorylation by glucokinase prepared from insulinomas compared with glucokinase prepared from liver. Similar observations were made for glucose phosphorylation.[9] The lower kinetic constants may be the result of small amounts of hexokinase contaminating glucokinase preparations from insulinomas. Hexokinase contamination could not affect the pattern of anomeric preference observed, however; hexo-

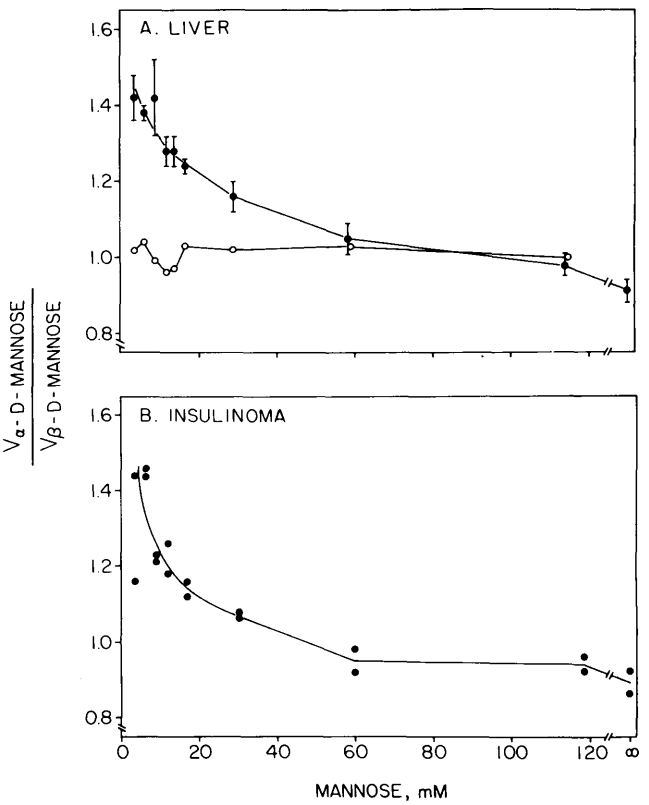

FIGURE 3. Relative phosphorylation rates for α-D-mannose and β-D-mannose (●) by glucokinase prepared from liver (A) or insulinoma (B). Data are shown as means and their standard errors for four liver glucokinase preparations and as individual values for two insulinoma glucokinase preparations. The ratios of computed V_{max} values are shown (∞). The average ratio for phosphorylation of mutarotated solutions of mannose prepared from pure anomers is shown for a preparation of liver glucokinase tested with three solutions, each prepared from the two anomers (○).

kinase has a low K_m for mannose; therefore, it would be saturated at even the lowest mannose concentration tested.[3] Additionally, with glucose as substrate hexokinase is reported to lack anomeric specificity[29] or to have only a slightly higher V_{max} (10–20%) with α-D-glucose.[30]

The finding of preferential activity of glucokinase with the α-anomers of glucose and mannose suggests that this enzyme is the site generating anomeric specificity in islets by controlling the phosphorylation rates of these sugars. Other explanations for the anomeric discrimination of hexoses by islets have been advanced; namely, that differentiation of anomers occurs at phosphoglucose isomerase[31] or phosphoglucomutase.[18] Anomeric discrimination by phosphoglucose isomerase is unlikely although this enzyme is known to be specific for α-glucose 6-phosphate.[32] The catalytic activity of phosphoglucose isomerase in islets is much higher than that of glucokinase or phosphofructokinase.[3,33] Since this enzyme lacks allosteric regulation, this high activity makes it an unlikely regulatory site. Additionally, phosphoglucose isomerase is 20-fold more active with α-glucose 6-phosphate than the β-anomer[32] rather than the more limited, concentration-dependent discrimination of glucose anomers evident in islets. Finally, isomerization of mannose 6-phosphate, in contrast to glucose 6-phosphate, occurs by a β-anomer specific isomerase[34] although the anomeric discrimination of these hexoses by islets appears similar except for a lesser affinity for mannose.[3] Phosphoglucomutase also appears unlikely to be the site of anomeric discrimination by islets. Phosphoglucomutase is known to have α-anomeric specificity for its substrate.[35] It is suggested to stimulate glycolysis in islets by activation of phosphofructokinase with α-glucose 1,6-diphosphate or α-mannose 1, 6-diphosphate.[18] However, it is unclear whether this enzyme or a specific glucose 1, 6-diphosphate synthetase[36] controls formation of hexose 1,6-diphosphates under physiologic conditions. Also, mannose phosphates are poor substrates for phosphoglucomutase when compared with glucose phosphates.[37] Synthesis of another phosphofructokinase activator, fructose 2, 6-diphosphate, is by a β-anomer specific reaction,[38] suggesting that anomeric discrimination in the synthesis of glucose 1, 6-diphosphate, if it occurs, may be offset at phosphofructokinase by the effects of fructose 2, 6-diphosphate synthesis.

An anomeric discrimination step in islets that is yet to be explained is the greater effectiveness of α-D-glucose than β-D-glucose in inhibiting alloxan toxicity at the β-cell.[39] Glucokinase may be the discriminator for this effect as well. Glucokinase, by allowing greater metabolism of α-D-glucose, may control generation of a metabolite or metabolic product that blocks alloxan toxicity, either by an effect on alloxan or its molecular site of attack.[40]

Glucokinase is suggested to function as the glucose sensor of the β-cell.[1,2] Glucokinase phosphorylates glucose with cooperative rate dependence and anomeric discrimination[8,9] similar to the properties of glucose-stimulated insulin secretion.[11] Evidence that mannose is handled by glucokinase in a manner similar to glucose but with lower affinity paralleling the potency of mannose as a stimulant of insulin secretion[17] and islet glycolysis[3] identifies glucokinase as the anomeric discrimination step in glucose and mannose metabolism by pancreatic islets.

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