Analysis of the Cooperativity of Human β-Cell Glucokinase through the Stimulatory Effect of Glucose on Fructose Phosphorylation*

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Using overexpressed Escherichia coli sorbitol-6-phosphate dehydrogenase to monitor fructose 6-phosphate formation, we found that the stimulation of fructose phosphorylation by glucose was reduced in two human β-cell glucokinase mutants with a low Hill coefficient or when the activity of wild type glucokinase was decreased by replacing ATP with poorer nucleotide substrates. Mutation of two other residues, neighboring glucose-binding residues in the catalytic site, also reduced the affinity for glucose as a stimulator of fructose phosphorylation. Among a series of glucose analogs, only 3, all substrates of glucokinase, stimulated fructose phosphorylation; other analogs were either inactive or inhibited glucokinase. Glucose increased the apparent affinity for inhibitors that are glucose analogs but not for the glucokinase regulatory protein or palmitoyl-CoA. These data indicate that the stimulatory effect of glucose on fructose phosphorylation reflects the positive cooperativity for glucose and is mediated by binding of glucose to the catalytic site. They support models involving the existence of two slowly interconverting conformations of glucokinase that differ through their affinity for glucose and for glucose analogs. We show by computer simulation that such a model can account for the kinetic properties of glucokinase, including the differential ability of mannoheptulose and N-acetylglucosamine to suppress cooperativity (Agius, L., and Stubbs, M. (2000) Biochem. J. 346, 413–421).

Glucokinase (hexokinase D or IV), an enzyme playing the role of a glucose sensor in β-cells of pancreatic islets and in liver (reviewed in Refs. 1 and 2), displays a sigmoidal saturation curve for its hexose substrate (3–5). This property, which sensitizes the enzyme to changes in the blood glucose level, cannot be accounted for by classical models of cooperativity (6, 7), because glucokinase is a monomer (8). Sigmoidal behavior due to random, nonequilibrium addition of the two substrates, glucose and ATP-Mg (9), can be excluded on the basis that there is no inhibition by ATP (10). Models that better account for the kinetic behavior of glucokinase assume the existence of two different conformations with different affinities for glucose that interconvert slowly (11, 12), thus allowing glucose to increase the proportion of the conformation with higher affinity. These kinetic models are supported (a) by the observation that cooperativity is lost when the rate of the reaction is slow, due to the use of a poor nucleotide substrate (13) or to the presence of a low ATP concentration and an inhibitory ADP concentration (11); (b) by the identification of slow kinetic transients (14); and (c) by the demonstration of slow conformational changes by fluorescence spectroscopy (15).

The possibility that the cooperativity is due to the existence of two binding sites for glucose, with regulatory and catalytic function, respectively, is usually ruled out (16) on the basis of experiments showing that glucose protects glucokinase against inactivation by a substrate analog, N-(N-bromoacetyl)-6-aminoheptulose glucosamine, with an hyperbolic instead of sigmoidal dependence on concentration (17). However, Agius and Stubbs (18) recently observed that mannoheptulose, a competitive inhibitor of glucokinase, is more effective to decrease the Hill coefficient of this enzyme than other inhibitors such as N-acetylglucosamine or 5-thioglucose. This led them to postulate that mannoheptulose inhibits glucokinase by binding to a putative allosteric site for glucose.

Fructose is known to be a poor substrate for glucokinase, displaying no (12, 19) or positive (14, 20) cooperativity for this enzyme. Remarkably, fructose phosphorylation was shown to be markedly stimulated by glucose and, to a lesser extent, by mannose (19). The stimulation of fructose phosphorylation by glucose and its analogs could therefore be a simple way of probing the specificity of a putative allosteric site.

In the present work we have further characterized the effect of glucose and its analogs on the phosphorylation of fructose by wild type glucokinase and by point mutants that have a reduced cooperativity or affinity for glucose. As the use of [14C]fructose in a radiochemical assay of glucokinase is hampered by the low affinity of the enzyme for this substrate, we have developed an enzymatic assay involving sorbitol-6-phosphate dehydrogenase, which allows the specific measurement of fructose 6-phosphate without interference by glucose 6-phosphate. Our results indicate that the stimulation exerted by glucose on the phosphorylation of fructose is mediated by binding of glucose to the catalytic site.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were from Sigma or Merck. D-Fructose was from Acros. The recombinant Xenopus regulatory protein of glucokinase was purified up to the DEAE-Sepharose step (21). The recombinant human islet glucokinase was purified as described previously (22). The preparation, purification, and characterization of human β-cell glucokinase mutants have been described by Veiga-da-Cunha et al. (23) for N166R, D158A, and V203A/D158A and by Moukil et al. (22) for Y214A and C230S.

Preparation of Escherichia coli Sorbitol-6-phosphate Dehydrogenase—The open reading frame of E. coli sorbitol-6-phosphate dehydrogenase (24) was amplified by polymerase chain reaction using genomic DNA from strain JM109 as a template and oligonucleotide primers with
the following sequences: upstream primer, 5'-gggtagatagctagcgggtatg-3'; downstream primer, 5'-ggctgtactaactacagctcgcagccc-3'. The upstream primer incorporates the starting codon (bold) in the NdeI restriction site (underlined), whereas the downstream primer incorporates a BamHI restriction site (underlined) which flanks the termination codon (bold). The ~0.8-kilobase pair amplified fragment was inserted in pET3a, and the resulting plasmid was used to overexpress sorbitol-6-phosphate dehydrogenase in B21pLysS (25). Expression was carried out in 1 liter of ZB medium at 19 °C for 40 h in the presence of 0.4 mM isopropylthiogalactoside. The cells were pelleted by centrifugation, resuspended in 45 ml of homogenization buffer (25 mM Heps, pH 7.1, 5 mM EDTA, 1 mM dithiothreitol, 0.4 mM leupeptin, 10 µg/ml antipain, 0.5 mM phenylmethylsulfonyl fluoride), and extracted in a French pressure cell. The extract was centrifuged at 15,000 × g for 30 min, and an ammonium sulfate fraction (15–30% of saturation) was prepared from the resulting supernatant. This fraction was resuspended in 2 ml of Buffer A (20 mM Heps, pH 7.1, 1 mM dithiothreitol) and chromatographed on a 1.6 × 13-cm DEAE-Sepharose equilibrated in Buffer A supplemented with 10 mM NaCl; the column was washed with the same buffer, and protein was eluted with a linear NaCl gradient (10–500 mM in 2 × 50 ml of Buffer A). About 5000 units of >95% homogeneous sorbitol-6-phosphate dehydrogenase could be obtained in this way. The enzyme had a specific activity of ~200 µmol/min/mg. It was stored at 4 °C as a precipitate in 70% ammonium sulfate.

Enzyme Assays—In the course of its purification, sorbitol-6-phosphate dehydrogenase was assayed at 30 °C through the change in A$_{340}$ in a reaction mixture containing 50 mM Heps, pH 7.1, 2 mM fructose 6-phosphate, and 0.15 mM NADH. Glucokinase was assayed spectrophotometrically at 30 °C in 1 ml of assay mixtures containing, unless otherwise indicated, 25 mM Heps, 2 mM ATP-Mg$_2$, 5 mM MgCl$_2$, 25 mM KCl, 1 mM dithiothreitol, and the indicated concentrations of substrates and effectors. Fructose 6-phosphate production was measured in the presence of 0.15 mM NADH and 2 units of sorbitol-6-phosphate dehydrogenase; glucose 6-phosphate production, in the presence of 0.5 mM NAD$^+$ and 10 µg/ml Leuconostoc mesenteroides glucose-6-phosphate dehydrogenase; and ADP formation, with 0.15 mM NADH, 0.25 mM phosphoenolpyruvate, 10 µg/ml pyruvate kinase, and 10 µg/ml lactate dehydrogenase. Results shown are means of at least three determinations ± S.E.

Other Methods—Protein concentration was measured according to Bradford (26) using bovine serum albumin as a standard. Modelization—To simplify the writing of equations derived from the model shown in Fig. 7, the concentrations of E, EG, E', and E'G are called “A,” “B,” “C,” and “D,” and the concentration of glucose is termed “G.” Kinetic constants are symbolized by letters (a-h and m). Note that the product (a.g.d.f) is necessarily equal to (b.h.c.e). A system of four equations with 4 unknowns can be written as follows.

\[
A + B + C + D = 1 \
(c + g + f)C = (d + m + n)D + eA \
(d + m + h)D = cGC + gB \
(b + g)B = aGA + hD
\]

In Equation 1, the sum is actually equal to a constant, which, for the sake of simplicity, is assumed to be equal to 1. Equations 2–4 are derived from the consideration that, under steady state conditions, C, D, and B remain constant.

A fifth equation allows one to calculate the rate of product formation.

\[
v = mD
\]

Values are assigned to the kinetic constants (see Fig. 7). The system of Equations 1–4 was solved by matrix calculus, and \( v \) was calculated for discrete concentrations of glucose using Microsoft Excel, Version 1998. Hill coefficients were calculated for substrate concentrations corresponding to ~25% of \( V_{max} \).

RESULTS

Purification of Sorbitol-6-phosphate Dehydrogenase and Its Use in the Assay of Glucokinase—To monitor specifically the formation of fructose 6-phosphate without interference of glucose 6-phosphate, we decided to use sorbitol-6-phosphate dehydrogenase. This bacterial enzyme catalyzes the reversible NADH-dependent conversion of fructose 6-phosphate to sorbitol 6-phosphate (24). The open reading frame encoding \( E. coli \) sorbitol-6-phosphate dehydrogenase (24) was inserted into a pET3a expression vector (25) and used to overexpress the enzyme. An activity of ~40 µmol/min/mg protein was observed in extracts of cells induced with isopropyl-1-thio-\( \beta \)-galactopyranoside as compared with 0.25 µmol/min/mg protein in cells that did not harbor the plasmid.

The enzyme was purified by chromatography on Sephacryl S-200 and DEAE-Sepharose. SDS polyacrylamide gel electrophoresis indicated that the preparation contained one single band with the expected size (26 kDa). The preparation was free from enzymes such as hexokinase and phosphoglucose isomerase, which could potentially interfere in the glucokinase assay. The \( K_m \) for fructose 6-phosphate was 0.38 mM and that for NADH was 28 µM. Using this enzyme, we determined that, at pH 7.0, the thermodynamic equilibrium of the reaction [NAD$^+$]/[sorbitol 6-phosphate]/[NADH]/[fructose 6-phosphate] amounted to 252 ± 17.

In the presence of this enzyme and of NADH, glucokinase could be assayed with fructose as a substrate through the decrease in A$_{340}$. Similar rates were observed with this assay as with others in which the formation of fructose 6-phosphate is monitored through the reduction of NAD$^+$ to NADH in the presence of phosphoglucose isomerase and glucose-6-phosphate dehydrogenase or in which the formation of ADP is measured through the oxidation of NADH in the presence of phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase (not shown).

Saturation Curve for Fructose and Effect of Glucose—Fig. 1 shows that human islet glucokinase displayed a positive cooperativity for fructose with an \( S_{0.5} \) of 580 mM and a Hill coefficient of about 1.5. Glucose suppressed this cooperativity and, at low concentrations, stimulated the enzyme, with \( S_{0.5} \) decreasing to about 170 mM in the presence of 4 mM glucose. At more elevated concentrations, glucose exerted competitive inhibition.

As some authors (12, 19) but not others (14, 20) reported a hyperbolic saturation curve for fructose, we also studied the fructose saturation curve under the conditions described by
Cardenas et al. (12) but found sigmoidal kinetics similar to those shown in Fig. 1. We checked also enzymatically (27) that the preparation of fructose that we used was not detectably contaminated by glucose (<0.01%). We therefore have no explanation for this discrepancy.

The effect of glucose on fructose phosphorylation was further investigated in the experiment shown in Fig. 2 in which the rates of both fructose and glucose phosphorylation have been recorded. Fructose phosphorylation was stimulated by a factor of up to about 6 by 5 mM glucose (Fig. 2A). By contrast, glucose phosphorylation was not stimulated by fructose, but rather inhibited, particularly at the highest concentrations of glucose used (Fig. 2B). Remarkably, the increment in the rate of fructose phosphorylation induced by glucose amounted in some cases to much more than the rate of glucose phosphorylation. Thus at 200 mM fructose, the increment of fructose phosphorylation induced by 1 mM glucose represented ~18 μmol/min/mg as compared with a rate of glucose phosphorylation of ~1.5 μmol/min/mg.

Suppression of the Stimulatory Effect of Glucose with Poor Phosphoryl Donors—It was previously shown that the cooperativity for glucose is lost when ATP is substituted by a poor phosphoryl donor such as ITP (13) or when the concentration of ATP is low, most particularly in the presence of ADP, which acts as a competitive inhibitor versus ATP (11). Fig. 3 shows that the stimulation of the phosphorylation of 20 mM fructose by 5 mM glucose is about 6-fold in the presence of 0.4 to 5 mM ATP and that it is less than 2-fold in the presence of nucleotides that are poor substrates (ITP, CTP, GTP, and UTP) or when the enzyme is measured in the presence of a low concentration of ATP and an inhibitory concentration of ADP. There is a clear hyperbolic relationship between the rate of fructose phosphorylation in the absence of glucose and the relative stimulation exerted by glucose.

Glucose Analogs Acting as Stimulators or Inhibitors of Fructose Phosphorylation—Among a series of analogs that we tested, only three could stimulate fructose phosphorylation, mannose, 2-deoxyglucose, and glucosamine (Fig. 4). These compounds are all substrates for glucokinase, and their capacity to stimulate fructose phosphorylation (Fig. 4A) correlated with their ability to be substrate (Fig. 4B), mannose, and 2-deoxyglucose displaying, respectively, about 2- and 5-fold higher S0.5 values with Hill coefficients of 1.6 and 1.1. As expected, these substrates acted as inhibitors of fructose phosphorylation at more elevated concentrations.

Other glucose analogs did not act as stimulators but only as inhibitors of fructose phosphorylation (Fig. 5 and not shown). These were either poor substrates of the enzyme (2-fluoro-2-deoxyglucose (Km = 50 mM, Vmax = 70% of the Vmax with glucose) and mannoheptulose (Km = 4 mM, Vmax = 0.2%, IC50 = 9 mM at 100 mM fructose) or pure inhibitors (5-thioglucose; IC50 = 2 mM), N-acetylglucosamine (IC50 = 2 mM), N-acetylmannosamine (IC50 = 60 mM), N-benzylxyllosamine (IC50 = 0.85 mM), and d-xylene (IC50 > 600 mM). The effect of some of these inhibitors was also tested in the presence of 5 mM glucose, which stimulated the enzyme about 4-fold. When activities were expressed relative to the activity measured in the absence of inhibitor, it appears that the apparent affinity for the inhibitors that are structural analogs of glucose was increased in the presence of glucose, whereas the inhibition exerted by other competitive inhibitors (Xenopus regulatory protein (21) and acetyl-CoAs (28, 29)) was decreased in the presence of glucose (Fig. 5).

Other compounds had no effect on the phosphorylation of fructose either in the presence or in the absence of glucose. These include (with the maximal concentration tested shown in parentheses) 1,5-anhydro-D-sorbitol (50 mM), α-methyl-α-glucoside (300 mM), 1,5-anhydro-D-mannitol (50 mM), d-allose (50 mM), d-galactose (250 mM), L-sorbose (600 mM), 6-deoxy-D-glucose (10 mM), and d-glucuronamide (100 mM) (not shown).

Studies on Glucokinase Mutants—To further investigate whether the stimulation exerted by glucose was linked to the positive cooperativity for this substrate, we investigated this effect in two point mutants (N166R and Y214A) that have been produced by substituting a glucokinase-specific residue with a residue in an equivalent position in hexokinases with low Km values. These mutants have a reduced Hill coefficient and an increased affinity for glucose without marked change in Vmax (23, 22). The saturation curves for fructose were almost hyper-
bolic (Hill coefficients ≤ 1.2), and the $S_{0.5}$ values equated 150 and 120 mM, respectively (not shown). As shown in Fig. 6A, glucose exerted a modest, maximally 1.6-fold stimulatory effect in these mutants as compared with the control (up to 4-fold).

We have also investigated the effect of glucose on fructose phosphorylation in two glucokinase mutants in which a residue that is next to the glucose-binding residue Asn-204 or Asn-231 had been substituted (30). These mutants are V203A (which is combined with the incidental mutation D158A) and C230S. In each case, fructose phosphorylation was tested at a concentration corresponding to ~15% of the $V_{\text{max}}$ value observed with glucose as substrate. As shown in Fig. 6B, these two mutations increased the concentration of glucose required to half-maximally stimulate fructose phosphorylation by 8- and 2-fold, respectively, whereas the $S_{0.5}$ values for glucose were increased by 12- and 2-fold (23, 22), respectively, compared with the appropriate controls.

**Simulation of a Slow Transition Model—**Because the results obtained did not indicate the existence of a second binding site for glucose, we decided to check whether a slow transition model could account for the kinetic properties of glucokinase, including the cooperativity for glucose and for fructose, the effect of glucose on fructose phosphorylation, the effect of mutations such as Y214A that reduce the Hill coefficient while increasing the apparent affinity for glucose, and the better stimulation of fructose phosphorylation by glucose reflects the positive cooperativity for this substrate.

**Lack of Evidence for an Allosteric Glucose-binding Site—**The results presented in this work extend the previous observations of Scruel et al. (19) that glucose stimulates the phosphorylation of fructose. We show in addition in this paper that this effect is largely reduced in two mutants (N166R and Y214A) that have a reduced cooperativity for glucose. Furthermore, the stimulation is also decreased when the activity of the wild type enzyme is reduced by replacing ATP by poorer phosphoryl donors, or when the ATP concentration is reduced, most particularly in the presence of ADP. Such a situation is known to decrease the Hill coefficient of the glucose saturation curve to ~1.0 (11, 13). These observations indicate that the stimulation of fructose phosphorylation by glucose reflects the positive cooperativity for this substrate.

Therefore, if a potential allosteric glucose-binding site existed in glucokinase as suggested by Agius andStubbs (18), stimulation of fructose phosphorylation would be a good means to characterize it. In the present work, two pieces of evidence indicate, however, that such a site does not exist. A first one is the fact that two mutants that have a reduced affinity for glucose as a substrate, because of mutation of residues in the catalytic site next to glucose binding residues Asn-204 and Asn-231, also have a reduced affinity for glucose as a stimulus of fructose phosphorylation. Mutations affecting residues that directly interact with glucose (Asn-204, Asp-205, Glu-256, and Glu-290) are not informative in this respect, because they lead to proteins that have markedly decreased affinity for glucose and for other hexose substrates and that do not display sigmoidal kinetics (31).

**DISCUSSION**

The set of parameters shown in Fig. 7 predicted an $S_{0.5}$ value of 8.4 mM for glucose and of 460 mM for fructose, with Hill coefficients of ~1.7 and 1.4, respectively, close to the measured values. The effect of a mutation such as Y214A on the glucose saturation curve could be easily simulated by increasing the E/E ratio at equilibrium by a factor of 100. This decreased the $S_{0.5}$ value for glucose to 1.5 mM and the Hill coefficient to 1.16 (as compared with measured values of 1.3 mM and 1.2, respectively; see Ref. 22).

The model also simulated the activation exerted by glucose on the phosphorylation of fructose. As observed experimentally, the increase in the rate of fructose phosphorylation was under some conditions “more than stoichiometric” with respect to the rate of glucose phosphorylation, and glucose phosphorylation was not detectably stimulated by fructose (Fig. 8). As expected, loss of cooperativity for glucose was observed when the rate of the catalytic step was reduced (e.g. a Hill coefficient of 1.2 was obtained when kinetic constant m was decreased from 100 to 3 s$^{-1}$; not shown).

The model could also account for the differential behavior of $N$-acetylglucosamine and mannoheptulose, if one assumed that the difference between the affinities of the two states for $N$-acetylglucosamine was lower (by a factor of 100 in the simulation shown in Fig. 7, the E' form having still 37.5-fold more affinity for $N$-acetylglucosamine than the E form) than for glucose, whereas it was the same for mannoheptulose as for glucose. Fig. 9 shows a plot of the Hill coefficient versus the $S_{0.5}$ values for glucose in the presence of increasing concentrations of the two inhibitors. The curves corresponding to the values obtained by simulation are close to the experimental values reported by Agius and Stubbs (18).
the phosphorylation of fructose. If glucokinase had an independent (allosteric) binding site for glucose that would favor the binding of glucose to the catalytic site, one would expect the existence of glucose analogs able to stimulate the phosphorylation of fructose without being substrate. However, no such compound was found among analogs modified on C1 to C6.

Furthermore, the order of potency (glucose, mannose, 2-deoxyglucose) in the stimulation of fructose paralleled the capacity of these compounds to act as substrates. The same was true for glucosamine, which at low concentrations, was intermedi-
Nature of the Slow Conformational Change: a Hypothesis—Because glucokinase is a monomer and because there is no evidence for a second binding site for glucose, the explanation for the cooperativity for glucose must be kinetic. Because explanations such as a random order of addition of substrate (9) or the recycling of an ADP-enzyme complex (32) do not apply to glucokinase (10), the best models of cooperativity for glucokinase are those in which the enzyme undergoes a slow conformational change between two states with different affinities for glucose (11, 12). The stimulatory effect of glucose on the phosphorylation of fructose (see Ref. 19 and this work), mannose (19), and 2-deoxyglucose1 indicate that the state that has a higher affinity for glucose also has a higher affinity for other substrates. As shown in this paper, it also has a higher affinity for glucose analogs that act as inhibitors, although not for other competitive inhibitors such as palmitoyl-CoA or the regulatory protein of glucokinase.

The nature of this conformational change is still elusive as no crystal structure of glucokinase is yet available, but the localization of mutations that decrease cooperativity in the three-dimensional model of glucokinase derived from the structure of yeast hexokinase B (30) may be instructive in this respect. Mutations that decrease cooperativity while considerably decreasing $k_{cat}$ values or the affinity for glucose are not informative in this respect, because their effect is most likely due to the slowing down of the catalytic cycle, similar to the effect of replacing ATP by poor phosphoryl donors in the wild type enzyme. However, two mutations (N166R and Y214A) decrease cooperativity while increasing the affinity for glucose and barely changing $V_{max}$ values. Interestingly, they involve residues that are in the hinge region, at some distance from the catalytic site, or at the interface between the two domains (22). Because of the location of these residues, we postulate that, in addition to the classical open and closed states common to all hexokinas (33, 34), glucokinase would also exist in a “super-open” state (Fig. 7). Because of the larger distance between the glucose binding residues present in the larger and smaller domains, this conformation would have a lower affinity for glucose and for its analogs than the open conformation. For an as yet unknown reason, this super-open form would only slowly equilibrate with the open conformation. By binding to the super-open conformation, glucose would facilitate partial closure of the catalytic cleft to the open state. Binding of ATP-Mg, complete closure of the catalytic cleft, and the phosphoryl transfer reaction would then ensue. Reopening of the catalytic cleft (to the open state) would be rapidly followed by extrusion of the products of the reaction, leaving the free enzyme in its high affinity state. The enzyme would then be more prone to catalyze the phosphorylation of any substrate (and to be inhibited by substrate analogs). If no substrate comes in time, the enzyme would “relax” to the super-open (low affinity) state. However, if sufficient substrate is present, the enzyme “recruited” by glucose to the open (high affinity) state can undergo several additional cycles with another substrate such as fructose before relaxing to the super-open state, explaining the more than stoichiometric stimulation of fructose phosphorylation that we observed.

Functionally, this model is similar to the one proposed by Storer and Cornish-Bowden (11) and differs from the slow transition model of Cardenas et al. (12) in that catalysis can only occur with the high affinity state. Testing of the model by mathematical simulations indicates that it can account for the sigmoidal behavior of glucokinase with both glucose and fructose as substrates, for the effect of mutations that decrease cooperativity, and for the stimulation of fructose phosphorylation by glucose. It can also account for the differential behavior of N-acetylglucosamine and mannohexulose on the kinetics of glucokinase if one admits that the difference between the af-
finities of the two states is larger in the case of mannoheptulose than in the case of N-acetylglucosamine. This difference may be because of the presence of a bulkier substituent on C2 than on the corresponding carbon in mannoheptulose.

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