Biochemical Characterization of MODY2 Glucokinase Variants V62M and G72R Reveals Reduced Enzymatic Activities Relative to Wild Type[†]

Erik C. Ralph and Shaoxian Sun*

Department of Biochemical Pharmacology, La Jolla Laboratories, Pfizer Global Research and Development, San Diego, California 92121

Received January 6, 2009; Revised Manuscript Received January 30, 2009

ABSTRACT: The glucokinase V62M and G72R mutations are naturally occurring and known to associate with hyperglycemia in humans. Structurally, V62 and G72 residues are located in close proximity to the allosteric site where hypoglycemia-linked activating mutations are clustered. To address the mechanism by which these variants alter the physiological phenotype, we characterized the biochemical and biophysical properties of the enzymes. Recombinant proteins were purified without affinity tags, and their steady-state kinetics and glucose binding affinities were determined. Both enzymes showed reduced rates of turnover (k_{cat}) and reduced glucose affinity (i.e., increased $K_{0.5}$ and K_D values). Their thermal stability did not largely differ from that of wild-type glucokinase. However, V62M and G72R lost the stabilizing protein interactions with glucokinase regulatory protein, which may contribute to lower activity *in vivo*. Both mutants were subject to activation by small molecule activators. In conclusion, the decreased enzyme activities of V62M and G72R observed in this study are consistent with the hyperglycemic phenotype.

Glucokinase (GK)[1] is one of the human hexokinases that catalyzes the phosphorylation of glucose to glucose 6-phosphate in the first step of glycolysis. Expressed in the liver, pancreas, brain, and gut, GK plays a key role in maintaining glucose homeostasis through regulation of glucose-dependent insulin secretion in pancreatic β cells and glucose uptake and storage in the liver (*1*). GK is distinguished from other hexokinases by its relatively low glucose affinity ($K_{0.5}$ 7 mM) in the range of blood-glucose levels and its cooperative kinetics (Hill coefficient 1.8). These properties allow for GK to rapidly respond to changes in glucose concentrations under physiological conditions and thereby function as a glucose sensor (*2*). The biochemical mechanism behind these kinetic features has been investigated and is closely linked to the dynamic nature of the protein structure. The protein crystal structures provided by Kamata et al. (*3*) show that GK has two substantially different structural conformations: a glucose-bound conformation which entails a well-defined active site and an inactive apo conformation which has a poorly defined glucose binding site. The transition from the inactive to the active enzyme conformation is driven by glucose binding, thereby yielding the observed kinetic cooperativity (*4*).

Structural studies of GK have also revealed the presence of an allosteric site where small molecule activators bind, approximately 20 Å away from the active site (*3, 5, 6*). Interestingly, this allosteric site is present only in the glucose-bound conformation of GK (*3*). GK activators (GKAs) shift the conformational equilibrium of GK from the inactive to active form by selectively binding to the enzyme in its glucose-bound form, thereby increasing the observed enzyme activity (*7*). GKAs have been shown to stimulate insulin secretion in β cells and glucose uptake in hepatocytes and to lower glucose levels in diabetic animal models (see refs *8*−*11* for review). Because of their effectiveness in modulating glucose concentrations *in vitro* and *in vivo*, GKAs have emerged as a promising antidiabetic agent, and their therapeutic efficacy is being evaluated in clinical trials.

The central role played by GK in maintaining glucose homeostasis is underscored by the demonstrated relationship between mutations in the GK gene and metabolic diseases. GK mutations with loss of function cause hyperglycemia in maturity onset diabetes of the young (MODY) subtype 2. Approximately 200 GK mutations were reported in MODY2 and represent as high as 60% of MODY cases (*12*). While not all MODY2 mutations have been characterized at the protein level, the available data suggest that the associated hyperglycemic phenotype is often the result of decreased enzyme catalytic activities in mutants (i.e., reduced k_{cat} or increased glucose $K_{0.5}$ values) or of a significant reduction in protein thermal stability such as seen with the E300K variant (*13*−*16*). On the other hand, GK mutations with gain of function are linked to hypoglycemia (*17*−*20*).

However, two exceptions in MODY2 mutations, V62M and G72R as recombinant glutathione *S*-transferase fusion proteins were previously reported to have kinetic parameters of activation with reduced glucose $K_{0.5}$ values of 5 mM (*21, 22*). Both mutations have mildly decreased thermal stability compared to the wild type and are insensitive to synthetic small molecule activators. Interestingly, both V62 and G72 are located in close proximity to the allosteric site

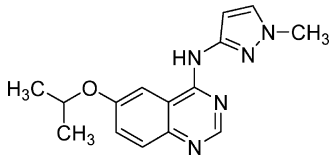
[†] This work was supported by the Pfizer Global Research and Development La Jolla Postdoctoral Program.

* Address correspondence to this author. Phone: 858-526-4922. Fax: 858-526-4240. E-mail: shaoxian.sun@pfizer.com.

[1] Abbreviations: GK, glucokinase; GKRP, glucokinase regulatory protein; BME, β-mercaptoethanol; DTT, dithiothreitol; GKA, glucokinase activator; SEC, size-exclusion chromatography; WT, wild type; DSC, differential scanning calorimetry; PK, pyruvate kinase; LDH, lactate dehydrogenase.

10.1021/bi900020n CCC: $40.75
Published on Web 02/02/2009

Scheme 1: (6-Isopropoxyquinazolin-4-yl)-(1-methyl-1*H*-pyrazol-3-yl)amine



where five naturally occurring activating mutations (T65I, W99R, Y214C, V455M, and A456V) are clustered, all of which have increased glucose affinities (*23*). The observation of MODY2 mutants with increased enzyme activity invokes a complex mechanism to explain the associated hyperglycemia phenotype, such as a defect of GK regulation by a putative endogenous activator (*21, 22*).

In this study, we characterized the kinetic and biochemical properties of V62M and G72R in order to address the mechanism by which these variants affect glucose homeostasis. The mutants were purified as nontagged proteins and characterized in detail, including steady-state enzyme kinetics, glucose binding, thermal stability, and allosteric regulation by hepatic glucokinase regulatory protein (GKRP) and GKAs. The results suggest that both variants are less catalytically active than the wild-type enzyme and are subject to activation by small molecules. The reduced enzyme activity and loss of stabilizing protein interaction with GKRP may account for the hyperglycemic phenotype associated with these variants.

EXPERIMENTAL PROCEDURES

Materials. Custom oligonucleotides were obtained from Integrated DNA Technologies. Glucose, HEPES, pyruvate kinase/lactate dehydrogenase, NADH, phosphoenolpyruvate, dithiothreitol (DTT), β-mercaptoethanol (BME), and ATP were purchased from Sigma. The small molecule allosteric activator was previously reported as GKA1 (*7*), and its chemical structure is shown in Scheme 1. Data analysis was performed using GraphPad Prism (GraphPad, Inc.), with the exception of the differential scanning calorimetry (DSC) data which used ORIGIN software provided with the calorimeter.

Expression and Purification of Recombinant Proteins. Expression and purification of human GKRP with a C-terminal FLAG tag was previously described (*4, 23*). The cloning of human β-cell GK has also been previously described (*24*). Mutations were introduced with the QuickChange site-directed mutagenesis kit (Stratagene). The sequences of oligonucleotides used to modify GK are shown in Supporting Information Table 1. Constructs for nontagged proteins were expressed from a pET43 vector (Novagen). A pGEX-4T vector (Amersham Biosciences) containing an inactive thrombin site (GS → HN substitution at the P1/P1′ position) was used for expression of N-terminal GST-tagged constructs. The G72R construct containing an N-terminal 6×His tag was expressed in pET15b (Novagen). All constructs were expressed from *Escherichia coli* BL21(DE3) cells. Cells were grown at 37 °C in TB-complete media to an OD of approximately 2. The shaker temperature was then lowered to 20 °C, and expression was induced with 200 μM IPTG. Cells were harvested after approximately 16 h. Nontagged GK WT and V62M were purified via sequential hydrophobic, anion-exchange, and size exclusion chromatographies (SEC). First, clarified cell lysates in 100 mg mL^{-1} ammonium sulfate, 25 mM HEPES, 100 mM KCl, and 5 mM DTT, pH 7.5, were loaded onto a phenyl-Sepharose fast-flow column and eluted with a 5 column volume gradient from 100 to 0 mg mL^{-1} ammonium sulfate. Excess salt was removed via dialysis or dilution. The protein was then loaded onto a Q-Sepharose column equilibrated with 25 mM HEPES, 100 mM KCl, and 5 mM DTT, pH 7.5, and eluted with a 50 column volume gradient of 160−300 mM KCl. Protein was further purified by SEC as previously described for His-tagged GK (*4*). Nontagged G72R was not retained on the hydrophobic interaction column and was partially purified by the anion-exchange and SEC described above. The concentration of G72R was determined from the absorbance at 280 nm after correcting for the percentage of peak area of G72R measured by analytical SEC. This concentration was used to calculate the specific enzyme activity. N-Terminal GST-tagged proteins were purified using a glutathione−Sepharose 4B column (GE Healthcare) and subsequent SEC. His-tagged G72R GK was purified by nickel affinity and anion-exchange chromatographies, as previously described for His-tagged GK (*4*). Protein purity was analyzed by SDS−PAGE, and molecular masses were confirmed by Q-TOF mass spectrometry. Protein concentrations were determined by the Bradford method (*25*) and by UV spectroscopy at 280 nm using extinction coefficients of 33.6 au mM^{-1} cm^{-1} for nontagged or His-tagged enzymes and 76.8 au mM^{-1} cm^{-1} for GST-tagged enzymes. The extinction coefficients were calculated based on the amino acid sequences using the ExPASY proteomics server from the Swiss Institute of Bioinformatics (*26, 27*).[2] Excellent agreement was found between the two methods.

Analytical Size Exclusion Chromatography. The oligomeric state of enzymes was evaluated using a TSK-Gel G3000SW analytical size exclusion column (7.5 mm i.d. × 30 cm; Tosoh Bioscience). Samples were run at 1 mL min^{-1} in 25 mM HEPES, 100 mM KCl, and 5 mM DTT, pH 7.2, using a Hitachi D7000 HPLC, and the elution was monitored for absorbance at 280 nm, as previously described (*7*). Protein molecular masses were calculated based on the elution time in reference to protein standards (Bio-Rad). To monitor the GK-GKRP complex formation, GK proteins (4 μM) and GKRP (2 μM) were incubated at room temperature for 20 min in 25 mM HEPES, 100 mM KCl, 50 μM fructose 6-phosphate, and 5 mM DTT, pH 7.2, before injection to the HPLC column (*7*).

Steady-State Kinetics. GK activity was monitored using the pyruvate kinase/lactate dehydrogenase (PK/LDH) coupled enzyme system as previously described (*4, 7*) with the following changes. Assays were run at 30 or 37 °C in 100 mM Tris and 125 mM KCl, pH 7.4, in the presence of 14 mM BME or 5 mM DTT. Buffers were prepared from 4× stocks which had been titrated to pH 7.4 at the desired temperature. Utilization of the PK/LDH assay instead of the glucose-6-phosphate dehydrogenase assay as the GK-coupled enzyme system has the advantages of maintaining a constant ATP concentration in the reaction by recycling ADP to ATP. This avoids the accumulation of ADP, a noncompetitive inhibitor against ATP (*28*).

[2] http://www.expasy.ch/tools/protparam.html.

When the ATP concentration was varied, assays contained a saturating concentration of 100 mM glucose, and $MgCl_2$ was kept in 1 mM excess of the ATP concentration. The K_m values for ATP were determined by fitting the data to the Michaelis−Menten equation. For experiments where the glucose concentration was varied, the assay buffer contained a saturating concentration of 10 mM ATP and 12 mM $MgCl_2$. The data were fit to the Hill equation (eq 1), where v is the reaction rate normalized to the enzyme concentration, [Glc] is the glucose concentration, $K_{0.5}$ is the glucose concentration at half-maximal activity, and h is the Hill coefficient which indicates the level of glucose cooperativity.

$$v = \frac{k_{cat}[Glc]^h}{K_{0.5}^h + [Glc]^h} \quad (1)$$

The kinetic parameters determined using the Michaelis−Menten and Hill equations were then used to calculate cellular glucokinase phosphorylating activity via eq 2. This equation is comprised of the Hill equation (eq 1) and a correction factor for the ATP K_m value (K_{ATP}) relative to a cellular ATP concentration of 2.5 mM. For a given GK enzyme, relative activity is presented as a ratio of the WT activity.

$$\text{GK phosphorylating activity} = \frac{k_{cat}[Glc]^h}{K_{0.5}^h + [Glc]^h}\left(\frac{2.5}{K_{ATP} + 2.5}\right) \quad (2)$$

Apparent glucose K_D values were determined by monitoring the increase in protein tryptophan fluorescence upon glucose binding and fitting the observed fluorescence to a Langmuir isotherm with a nonzero intercept, as previously described (*4*). Buffer conditions were 100 mM Tris, 125 mM KCl, and 14 mM BME or 5 mM DTT, pH 7.4, consistent with the assay conditions for the steady-state kinetic studies.

Thermal Stability. The thermal stability of the various GK constructs was assessed by two approaches. In the first approach, the transition temperature T_m of protein unfolding was measured via differential scanning calorimetry. The experiment was performed in the presence and absence of 100 mM glucose as previously described (*4*) with the exception that 14 mM BME replaced TCEP as the reducing agent; enzymes maintained higher activity in the presence of BME than with TCEP (data not shown). Data were fit to a non-two-state model with a cubic baseline correction using ORIGIN software provided with the instrument. In the second approach, the kinetic thermal stability was tested when enzymes were incubated at 50 °C for various time periods. The 50 °C was close to the GK transition temperature, at which the enzyme becomes sensitive to the thermal inactivation. Therefore, the loss of enzymatic activity can be easily monitored on a short time scale (within 1 h). Enzyme was first diluted to 5 μM in a buffer containing 50 mM HEPES, 25 mM KCl, and 14 mM BME, pH 8, at room temperature. The enzyme solution (100 μL) was then placed in a 0.2 mL PCR tube and heated to 50 °C using a thermocycler with a heated lid. No attempt was made to correct for temperature effects on the solution pH. Aliquots of 3 or 4 μL samples were taken at various time points and "quenched" by dilution into 100 μL of cold (4−10 °C) buffer.

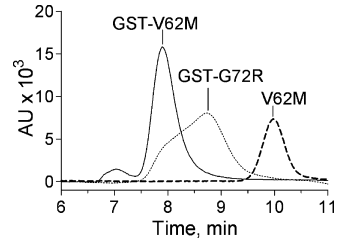


FIGURE 1: Elution profiles of GST-V62M, GST-G72R, and nontagged V62M by analytical size exclusion chromatography. GST-V62M (solid line) is shown predominately as a tetramer with a small percentage of aggregates. GST-G72R (dotted line) has a broad elution peak indicating a mixture of tetramer and dimer. Nontagged V62M (dashed line) has an elution time of 10 min, corresponding to a molecular mass of a monomer (52 kDa).

To minimize evaporation, adhesive foil was used to cover the samples between time points. After all time points were collected, the samples were analyzed simultaneously for kinetic activity in the presence of 100 mM glucose as described above. The enzyme activity was plotted as a function of the incubation time at 50 °C and fit to a single exponential decay. The resulting rate constant (k) was converted to a half-life time ($\tau_{1/2}$) via the relationship $\tau_{1/2} = \ln(2)/k$. Protein concentrations (5 μM) and glucose concentrations (0 or 100 mM) were matched for all enzyme samples, as either parameter may affect the observed stability.

Allosteric Regulation. Regulation of GK activity by GKRP and small molecule activators was measured as previously described (*7, 23*). Briefly, EC_{50} values were determined by plotting the observed enzymatic activity (v_{obs}) as a function of the GKA or GKRP concentration [L] and fitting the data to eq 3. In this equation, v_0 is the rate obtained in the absence of allosteric ligand, v_m is the rate at saturating ligand concentration, and EC_{50} is the ligand concentration which yields one-half the maximal activation or inhibition. When included, GKRP was dialyzed into the assay buffer prior to titrations. GKA stock solutions were prepared in DMSO. The final concentration of DMSO in the assays was 2% (v/v) or less and had no effect on the GK activity.

$$v_{obs} = \frac{v_0 + v_m\left(\frac{[L]}{EC_{50}}\right)}{1 + \frac{[L]}{EC_{50}}} \quad (3)$$

RESULTS

Protein Purification and Characterization. WT, V62M, and G72R recombinant full-length human β-cell GK were overexpressed in *E. coli* and purified as nontagged proteins. Both WT and V62M were purified to near homogeneity. G72R was purified to ~50% with the main contaminant being a slightly lower molecular mass protein lacking GK activity. Alternatively, His-tagged G72R was obtained with high purity (>95%) and used as a substitute for nontagged G72R. V62M and G72R were also produced as GST-fusion proteins with high purity.

Purified enzymes were analyzed for their oligomeric state via analytical SEC. Nontagged WT, V62M, and His-tagged proteins eluted as a single protein peak corresponding to a molecular mass of GK monomers (52 kDa). A representative elution profile of V62M is shown in Figure 1. In contrast,

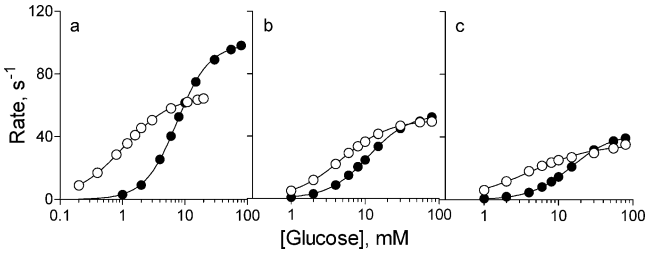


FIGURE 2: Representative kinetic data for GK WT (a), V62M (b) and G72R (c) in the presence (○) or absence (●) of 200 μM GKA. The assay was run at 37 °C as described in the Experimental Procedures.

both GST-tagged variants eluted as high molecular mass proteins suggesting oligomerization (Figure 1). GST-V62M showed one major peak corresponding to a molecular mass of 340 kDa, consistent with a tetramer (predicted mass 315 kDa). A small percentage (<10%) of higher order aggregates was also observed. The GST-G72R displayed a broad elution peak which can be broken into two peaks with estimated molecular masses of 330 and 188 kDa, indicating a mixture of tetramer and dimer.

Steady-State Kinetic Characterization. The steady-state enzyme kinetics and glucose equilibrium binding affinity of GK WT, V62M, and G72R were determined at 30 and 37 °C. Representative data are shown in Figure 2, and the kinetic parameters are summarized in Table 1. Both V62M and G72R show reduced k_{cat} values and slightly increased $K_{0.5}$ values relative to the WT enzyme. These differences were more pronounced at 37 °C. Relative to WT, the phosphorylation activities (eq 2) at 5 mM glucose for V62M and G72R are 0.7 and 0.2 at 30 °C and 0.4 and 0.1 at 37 °C, respectively. These kinetic measurements suggest that both V62M and G72R are catalytically less active than WT GK. Despite the different oligomeric states, GST-V62M (tetramer) and GST-G72R (mixture of dimer and tetramer) showed similar steady-state kinetics to the nontagged or His-tagged proteins (Supporting Information Table 2).

Glucose binding to GK yields a significant increase in protein fluorescence (*29*) which was used for the determination of glucose binding equilibria. The tryptophan fluorescence change upon glucose binding is 1.6−1.8-fold for WT, while for V62M and N-His G72R, the changes are smaller with 1.3−1.4-fold and 1.2−1.3-fold, respectively. The observed glucose dissociation constants ($K_{D,Glc}$) were increased for both variant enzymes, consistent with the increases in steady-state $K_{0.5}$ values (Table 1).

Thermal Stability. The thermal stability of the proteins was first assessed by measuring the unfolding transition temperature T_m using differential scanning calorimetry. As shown in Table 2, the transition temperatures for the WT enzyme are 48.4 and 51.6 °C in the absence and presence of saturating glucose (100 mM), respectively, and are in good agreement with values previously determined for the His-tagged enzyme (*4, 7*). The significant increase in the T_m value suggests protein stabilization by glucose for WT. The GK variants had very similar transition temperatures to WT in the apo form, indicating that neither the V62M nor the G72R substitutions significantly destabilize the folded protein structure. However, neither variant showed stabilization by glucose, a clear distinction between these variants and WT. Similar results were seen when the thermal stability was determined utilizing kinetic measurements. In this method, the kinetic thermal stability was described by a half-life of activity after the enzyme was incubated at 50 °C for various time periods (Supporting Information Figure 1). All three proteins showed a similar stability in the apo form of the enzyme, with a half-life of 5−7 min (Table 2). Again, whereas glucose binding significantly stabilized the WT enzyme, it had no apparent effect on the stability of the variant proteins. Together, the observed lack of stabilization by glucose binding as demonstrated with both the DSC and kinetic assessment and the less apparent fluorescence changes upon glucose binding may reflect significant structural differences in the glucose-bound forms of these enzymes relative to the WT. These observations highlight the dynamic nature of GK in conformational flexibility, consistent with the recent report by Molnes et al. (*30*).

Allosteric Regulation. In hepatocytes, GK is negatively regulated by GKRP by formation of an inactive heterodimeric complex (*31, 32*). GKRP is also found to have an additional role of stabilizing GK protein from degradation (*33, 34*). The inhibition of glucokinase activities by GKRP was measured *in vitro* in the presence of 5 mM glucose at 37 °C. The results show that GKRP is a potent inhibitor for WT with an EC_{50} of 40 nM, while V62M and G72R enzymes are no longer inhibited by GKRP at a concentration as high as 1 μM (Supporting Information Figure 2). The lack of kinetic regulation by GKRP could be due to a loss of physical interaction with the variant GK enzymes. Alternatively, GKRP may still interact with the variants and protect them from degradation without inhibiting activity. To discriminate between these two possibilities, the protein−protein interactions between GKRP and GK proteins were monitored in the absence of glucose using analytical SEC. Figure 3 shows a protein peak at 8.8 min as expected for the GK-GKRP complex with the WT enzyme, while no complex formation was observed for V62M or G72R, suggesting either amino acid substitution disrupts protein interactions with GKRP.

GK is also subject to allosteric modulation by small molecule activators. The effects of GKA on the enzyme kinetics of GK WT, V62M, and G72R were studied in the absence and presence of 200 μM GKA (Figure 2), and results are summarized in Table 3. The GKA lowers the $K_{0.5}$ and Hill coefficient for V62M and G72R, following the same trend as the WT, although the k_{cat} values are unaffected. At 5 mM glucose, the potency of the activator is reduced for the variant enzymes with EC_{50} values of 22 ± 2 μM for V62M and 8.8 ± 0.9 μM for G72R vs 0.86 ± 0.10 μM for WT.

DISCUSSION

To better understand the mechanism by which GK variants V62M and G72R associate with a hyperglycemic phenotype, we purified and characterized the recombinant enzymes as nontagged proteins. Steady-state kinetic parameters were determined under near physiological conditions by using pH 7.4 buffer at 37 °C. For V62M, the glucose $K_{0.5}$ value is increased to 10.8 mM compared to 7.2 mM with WT. A similar increase in the $K_{D,Glc}$ value was also observed, indicating a weaker binding of glucose to V62M. In addition, the k_{cat} value is decreased by 40%. The net results of the effects on $K_{0.5}$ and k_{cat} yield a significant reduction in the

Table 1: Steady-State Kinetic Parameters and Glucose K_D Values for WT and Variant GK[a]

temp (°C)		k_{cat} (s^{-1})	h	$K_{0.5,Glc}$ (mM)	$K_{m,ATP}$ (mM)	$K_{D,Glc}$ (mM)	relative activity[b]
30	WT	74 ± 3	1.8 ± 0.1	7.6 ± 0.3	0.70 ± 0.08	4.0 ± 0.3	1.0
	V62M	54 ± 5	1.5 ± 0.1	8.2 ± 0.4	0.82 ± 0.06	5.9 ± 0.8	0.7
	G72R[c]	24 ± 2	1.6 ± 0.1	10 ± 1	1.1 ± 0.1	10 ± 3	0.2
37	WT	101 ± 13	1.7 ± 0.1	7.2 ± 0.5	0.77 ± 0.10	3.6 ± 0.2	1.0
	V62M	61 ± 7	1.6 ± 0.1	10.8 ± 1.4	0.85 ± 0.11	6.8 ± 0.5	0.4
	G72R[c]	34 ± 4	1.7 ± 0.3	14 ± 1	1.1 ± 0.1	11 ± 3	0.1

[a] Results are the average of two or more independent determinations. [b] Relative activity was calculated at 5 mM glucose using eq 2, and values are normalized relative to the WT value. [c] Kinetic parameters for the nontagged G72R construct were determined with partially purified protein. The k_{cat} value was calculated based on the G72R concentration as described in the text.

Table 2: Transition Temperatures and Half-Life Values for GK WT, V62M, and G72R[a]

	no glucose		100 mM glucose	
	T_m (°C)	$\tau_{1/2}$ (min)	T_m (°C)	$\tau_{1/2}$ (min)
WT	48.4 ± 0.2	6.4 ± 0.1	51.6 ± 0.3	15 ± 2
V62M	48.1 ± 0.2	7.3 ± 0.3	49.3 ± 0.1	7.1 ± 1.1
G72R[b]	49.2 ± 0.4	4.9 ± 0.4	49.2 ± 0.1	4.8 ± 0.7

[a] Transition temperatures were determined by DSC, and half-life values were determined from measuring catalytic activity after incubation at 50 °C for various time periods. Transition temperatures show the value and fitting errors from a single determination. $\tau_{1/2}$ values are the average of two independent half-life determinations. [b] Values were determined using His-G72R.

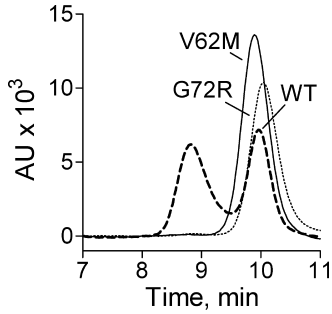


FIGURE 3: Loss of GKRP regulation for V62M and G72R as evidenced by protein interaction analyses. GK proteins WT, V62M, and His-tagged G72R were incubated with GKRP and monitored for the formation of a GK-GKRP complex by analytical SEC. Both the V62M (solid line) and His-tagged G72R (dotted line) failed to show GKRP complex formation, while the WT enzyme (dashed line) shows the heterodimer eluting at 8.8 min and the GK and GKRP monomers coeluting at 9.9 min.

Table 3: Steady-State Kinetic Parameters at 37 °C in the Presence and Absence of 200 μM GKA[a]

	k_{cat} (s^{-1})		h		$K_{0.5}$ (mM)	
	−GKA	+GKA	−GKA	+GKA	−GKA	+GKA
WT	101 ± 13	64 ± 5	1.7 ± 0.1	1.1 ± 0.1	7.2 ± 0.5	1.1 ± 0.1
V62M	61 ± 7	57 ± 10	1.6 ± 0.1	1.3 ± 0.1	11 ± 1	4.6 ± 0.8
G72R[b]	30 ± 5	28 ± 7	1.5 ± 0.1	1.2 ± 0.2	18 ± 1	3.4 ± 0.8

[a] The results shown are the average of two or more independent determinations. [b] Values were determined using His-G72R.

enzyme activity. For example, at a normal fasting blood-glucose level of 5 mM, the V62M phosphorylation activity is only 40% compared to WT. For G72R, the 7 mM increase in $K_{0.5}$ and $K_{D,Glc}$ values coupled with the 70% reduction in k_{cat} yields a 90% loss of relative activity at 5 mM glucose. The reduced catalytic activities observed for V62M and for G72R are consistent with the hyperglycemic phenotype associated with MODY2 mutations.

Decreased activities of V62M and G72R were also observed in a cellular measurement from a previous study of overexpressed GFP-tagged GK proteins (*35*). Clarified lysates from MIN6 cells expressing WT, V62M, or G72R enzymes were analyzed for catalytic activity at 50 mM glucose, and the activity was normalized to the enzyme concentration, as determined by immunoreactivity. V62M and G72R were shown to have 40% and 15% activity relative to WT, respectively. These values are in excellent agreement with our relative activity values at 37 °C (Table 1).

Previous characterizations of V62M and G72R (*21, 22*) reported a mild decrease in the steady-state glucose $K_{0.5}$ values relative to WT. One main procedural difference between the previous and the current report is the utilization of GST-tagged vs nontagged enzymes. Our results show that inclusion of the GST moiety induces oligomerization of GK to a tetramer (GST-V62M) or a mixture of tetramer and dimer (GST-GR) as opposed to the monomeric nontagged and His-tagged GK proteins. It is possible that oligomerization of GST-fusion proteins is caused by the dimeric nature of GST. GK is a highly dynamic enzyme with substantial conformational changes induced by substrate binding. The formation of dimeric and tetrameric GST-fusion proteins could potentially affect the biochemical properties for the recombinant GK proteins. It may be worth noting that the GST tag used in this study is not identical to the GST tag used in the previous study, due to removal of the proteolytic cleavage site. In addition, oligomerization of the GST fusion proteins may depend on the expression and purification conditions. Therefore, the oligomeric states of the GST-V62M and GST-G72R in the previous studies and the potential effects on the observed kinetics may differ from the current study. Another procedural difference is the temperature at which the kinetics parameters were analyzed. Previous characterizations of these enzymes were done at 30 °C (*21, 22*). In this study, the k_{cat} and Hill values at 30 °C are in reasonable agreement with previous reports, while the glucose $K_{0.5}$ values remain increased relative to WT, although to a lesser extent compared to the values determined at 37 °C. Overall, the decrease in catalytic activity of V62M and G72R relative to WT GK is more significant at 37 °C than at 30 °C.

In this study, the phosphorylating activity of GK proteins (eq 2) at 5 mM glucose is utilized to characterize the GK variants as inactivating or activating enzymes. This is different from the activity index ((*15*), also referred to as the glucokinase index) commonly used for characterization of GK variants. Compared to eq 2, the activity index described by eq 4 does not account for the glucose concentration, leaving the first term as $k_{cat}/K_{0.5}^{h}$. This term appears similar to the noncooperative expression of catalytic efficiency, k_{cat}/K_m, although its meaning is less well defined.

Because the activity index does not contain the $[Glc]^h$ term, it is reversely proportional to the effects of $K_{0.5}$ raised to the power of the Hill exponential and, therefore, highly sensitive to small changes in either parameter. Equation 2 includes the glucose concentration in the GK activity calculation. Thus, the contributions from each kinetic parameter are put into a context relevant to the glucose levels. The different mathematical treatments of kinetic data can lead to different conclusions for GK variants. For example, GST-V62M and GST-G72R were previously reported with activity index values normalized to WT of 3.2 and 1.7, respectively, suggesting significantly greater catalytic activity (*21, 22*). However, using the same kinetic parameters, the relative phosphorylating activity of GST-V62M is 1.3 at 5 mM glucose, suggesting only modest activation (Supporting Information Table 3). More dramatically, the relative activity for GST-G72R is 0.7, consistent with the G72R substitution causing inactivation at physiological glucose concentrations.

$$\text{activity index} = \left(\frac{k_{cat}}{K_{0.5}^{h}}\right)\left(\frac{2.5}{K_{ATP} + 2.5}\right) \quad (4)$$

In addition to enzyme kinetic measurements, the protein thermal stability was assessed to address the possibility that a loss of stability may exacerbate the inactivating effects. This phenomenon has been demonstrated for the MODY2 variant E300K (*13*−*16*). Using nontagged WT, V62M, and His-tagged G72R, we observed similar thermal stability in the apo form, while only the WT enzyme showed stabilization upon glucose binding. The lack of stabilization by glucose in the variants may suggest a slightly decreased stability at cellular glucose concentrations.

Loss of GKRP interaction yields reduced GK levels and activity in the liver, as shown for both WT enzyme in GKRP knockout studies (*33, 34*) and kinetically activating variant A456V, which has an approximate 100-fold reduction in GKRP binding affinity (*36*). The current study confirms a lack of GKRP regulation for V62M and G72R and shows this is due to the loss of direct GKRP interaction. The GKRP interaction is critical in maintaining GK protein levels in the liver; thus the loss of GKRP interaction may lead to lower GK activity *in vivo*. GKRP also has an additional role of inhibiting GK activity, which may counterbalance the protein stabilization effect. Interestingly, decreased GKRP interactions have been observed for hyperglycemia variants as well as hypoglycemia variants, indicating that alteration in GKRP regulation is not always a key factor in determining the outcome of clinical phenotype.

In this study, both V62M and G72R were subject to activation by the small molecule activator as evidenced by reduced glucose $K_{0.5}$ values and Hill coefficients, which are the defining characteristics for GKAs with WT. It is reasonable to propose that the mechanism of activation for the variants is the same as that of the WT enzyme. The activator used in this study has little effect on k_{cat} for both GK variants, while it decreases the k_{cat} for the WT. The different effects on k_{cat} have been observed among various activators with the WT (*7*). Further studies are required to address the mechanistic aspect of this observation. The potency of GKA at 5 mM glucose is significantly decreased by 30-fold for V62M and 10-fold for G72R, suggesting a perturbation of the allosteric site by the single amino acid substitution. Possible explanations for the reduced potencies can be postulated from GK structural information. The cocrystal structure of the GK/glucose/activator complex shows that the V62 side chain is directly involved in GKA binding by forming hydrophobic interactions with the activator (*3*). Using structural models, Gloyn et al. (*22*) showed that the V62M substitution blocked the preferred ligand binding position for a GKA, thereby explaining the reduced potency. As for G72, it is located at the end of connecting region I, which is a flexible loop surrounding the allosteric site (*3*). Mutations at this position may affect the structural flexibility or positioning of this loop and thus block the activator's access to the allosteric site. Importantly, the kinetic activation presented in this study suggests that MODY2 patients may respond to GKA antidiabetic therapy, although the efficacy will likely differ among different GK mutations.

The discovery of synthetic GKAs raises an interesting possibility regarding the existence of a physiologically endogenous activator that binds at the allosteric site but has yet to be identified. This possibility was highlighted in the previous studies that observed increased enzyme kinetics of MODY2 variants V62M and G72R and their resistance to GKAs (*21, 22, 35*). In light of that evidence, the loss of regulation by an endogenous activator had been proposed as a favorable explanation for the associated MODY2 phenotype. Although the current study does not exclude the possibility of an endogenous GK activator, the decreased catalytic activities of V62M and G72R presented here may be sufficient to explain the hyperglycemic phenotype.

In summary, the current study sought to examine the biochemical mechanism by which GK variants V62M and G72R associate with the MODY2 phenotype. Upon purifying and characterizing the enzymes in the nontagged form, we conclude that both V62M and G72R have reduced catalytic activities due to both weaker glucose binding and lower k_{cat} values. The loss of function in both mutants is consistent with their physiological phenotype. In addition, we show that the diminished GKRP regulation is due to the loss of direct protein interactions, which may also contribute to the reduced enzyme activity since GKRP no longer protects GK from degradation. Although the explanation for discrepancy between the current and previous conclusions is unclear, differences in protein construction (i.e., a GST tag vs no tag) and assay temperature may contribute. This study supports the conclusion that MODY2 clinical phenotype results from a loss of GK activity either through kinetic inactivation, as seen here, or through loss of protein stability.

ACKNOWLEDGMENT

We extend special thanks to Vladi Heredia for contributions to the initiation of this work, to Mike Gehring for advice regarding the preparation of the pGEX constructs, and to John Barker and Fannie Chau for expert help in protein purification. We also thank Steve Grant for critical reading of the manuscript and insightful discussions.

SUPPORTING INFORMATION AVAILABLE

One figure showing the representative data of GK WT for half-life determination, tables of oligonucleotides used for

site-directed mutagenesis, steady-state kinetic parameters for GK WT and variants, and relative activity and activity index. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Matschinsky, F. M., Glaser, B., and Magnuson, M. A. (1998) Pancreatic beta-cell glucokinase: closing the gap between theoretical concepts and experimental realities. *Diabetes 47*, 307–315.
2. Matschinsky, F. M., and Davis, E. A. (1998) The distinction between "glucose setpoint", "glucose threshold" and "glucose sensor" is critical for understanding the role of the pancreatic b-cell in glucose homeostasis, in *Molecular and Cell Biology of Type 2 Diabetes and Its Complications* (Belfiore, F., Lorenzi, M., Molinatti, G. M., and Porta, M., Eds.) pp 14−29, Karger, Basel.
3. Kamata, K., Mitsuya, M., Nishimura, T., Eiki, J., and Nagata, Y. (2004) Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. *Structure 12*, 429–438.
4. Heredia, V. V., Thomson, J., Nettleton, D., and Sun, S. (2006) Glucose-induced conformational changes in glucokinase mediate allosteric regulation: transient kinetic analysis. *Biochemistry 45*, 7553–7562.
5. Grimsby, J., Sarabu, R., Corbett, W. L., Haynes, N. E., Bizzarro, F. T., Coffey, J. W., Guertin, K. R., Hilliard, D. W., Kester, R. F., Mahaney, P. E., Marcus, L., Qi, L., Spence, C. L., Tengi, J., Magnuson, M. A., Chu, C. A., Dvorozniak, M. T., Matschinsky, F. M., and Grippo, J. F. (2003) Allosteric activators of glucokinase: potential role in diabetes therapy. *Science 301*, 370–373.
6. Efanov, A. M., Barrett, D. G., Brenner, M. B., Briggs, S. L., Delaunois, A., Durbin, J. D., Giese, U., Guo, H., Radloff, M., Gil, G. S., Sewing, S., Wang, Y., Weichert, A., Zaliani, A., and Gromada, J. (2005) A novel glucokinase activator modulates pancreatic islet and hepatocyte function. *Endocrinology 146*, 3696–3701.
7. Ralph, E. C., Thomson, J., Almaden, J., and Sun, S. (2008) Glucose modulation of glucokinase activation by small molecules. *Biochemistry 47*, 5028–5036.
8. Guertin, K. R., and Grimsby, J. (2006) Small molecule glucokinase activators as glucose lowering agents: a new paradigm for diabetes therapy. *Curr. Med. Chem. 13*, 1839–1843.
9. Sarabu, R., and Grimsby, J. (2005) Targeting glucokinase activation for the treatment of type 2 diabetes—a status review. *Curr. Opin. Drug Discov. Dev. 8*, 631–637.
10. Leighton, B., Atkinson, A., and Coghlan, M. P. (2005) Small molecule glucokinase activators as novel anti-diabetic agents. *Biochem. Soc. Trans. 33*, 371–374.
11. Printz, R. L., and Granner, D. K. (2005) Tweaking the glucose sensor: adjusting glucokinase activity with activator compounds. *Endocrinology 146*, 3693–3695.
12. Velho, G., Froguel, P., Gloyn, A., and Hattersley, A. (2004) Maturity onset diabetes of the young type, in *Glucokinase and Glycemic Disease: From Basics to Novel Therapeutics* (Matschinsky, F. M., and Magnuson, M. A., Eds.) Karger, Basel.
13. Liang, Y., Kesavan, P., Wang, L. Q., Niswender, K., Tanizawa, Y., Permutt, M. A., Magnuson, M. A., and Matschinsky, F. M. (1995) Variable effects of maturity-onset-diabetes-of-youth (MODY)-associated glucokinase mutations on substrate interactions and stability of the enzyme. *Biochem. J. 309*, 167–173.
14. Kesavan, P., Wang, L., Davis, E., Cuesta, A., Sweet, I., Niswender, K., Magnuson, M. A., and Matschinsky, F. M. (1997) Structural instability of mutant beta-cell glucokinase: implications for the molecular pathogenesis of maturity-onset diabetes of the young (type-2). *Biochem. J. 322*, 57–63.
15. Davis, E. A., Cuesta-Munoz, A., Raoul, M., Buettger, C., Sweet, I., Moates, M., Magnuson, M. A., and Matschinsky, F. M. (1999) Mutants of glucokinase cause hypoglycaemia- and hyperglycaemia syndromes and their analysis illuminates fundamental quantitative concepts of glucose homeostasis. *Diabetologia 42*, 1175–1186.
16. Burke, C. V., Buettger, C. W., Davis, E. A., McClane, S. J., Matschinsky, F. M., and Raper, S. E. (1999) Cell-biological assessment of human glucokinase mutants causing maturity-onset diabetes of the young type 2 (MODY-2) or glucokinase-linked hyperinsulinaemia (GK-HI). *Biochem. J. 342*, 345–352.
17. Christesen, H. B., Jacobsen, B. B., Odili, S., Buettger, C., Cuesta-Munoz, A., Hansen, T., Brusgaard, K., Massa, O., Magnuson, M. A., Shiota, C., Matschinsky, F. M., and Barbetti, F. (2002) The second activating glucokinase mutation (A456V): implications for glucose homeostasis and diabetes therapy. *Diabetes 51*, 1240–1246.
18. Cuesta-Munoz, A. L., Huopio, H., Otonkoski, T., Gomez-Zumaquero, J. M., Nanto-Salonen, K., Rahier, J., Lopez-Enriquez, S., Garcia-Gimeno, M. A., Sanz, P., Soriguer, F. C., and Laakso, M. (2004) Severe persistent hyperinsulinemic hypoglycemia due to a de novo glucokinase mutation. *Diabetes 53*, 2164–2168.
19. Glaser, B., Kesavan, P., Heyman, M., Davis, E., Cuesta, A., Buchs, A., Stanley, C. A., Thornton, P. S., Permutt, M. A., Matschinsky, F. M., and Herold, K. C. (1998) Familial hyperinsulinism caused by an activating glucokinase mutation. *N. Engl. J. Med. 338*, 226–230.
20. Gloyn, A. L., Noordam, K., Willemsen, M. A., Ellard, S., Lam, W. W., Campbell, I. W., Midgley, P., Shiota, C., Buettger, C., Magnuson, M. A., Matschinsky, F. M., and Hattersley, A. T. (2003) Insights into the biochemical and genetic basis of glucokinase activation from naturally occurring hypoglycemia mutations. *Diabetes 52*, 2433–2440.
21. Sagen, J. V., Odili, S., Bjorkhaug, L., Zelent, D., Buettger, C., Kwagh, J., Stanley, C., Dahl-Jorgensen, K., de Beaufort, C., Bell, G. I., Han, Y., Grimsby, J., Taub, R., Molven, A., Sovik, O., Njolstad, P. R., and Matschinsky, F. M. (2006) From clinicogenetic studies of maturity-onset diabetes of the young to unraveling complex mechanisms of glucokinase regulation. *Diabetes 55*, 1713–1722.
22. Gloyn, A. L., Odili, S., Zelent, D., Buettger, C., Castleden, H. A., Steele, A. M., Stride, A., Shiota, C., Magnuson, M. A., Lorini, R., d'Annunzio, G., Stanley, C. A., Kwagh, J., van Schaftingen, E., Veiga-da-Cunha, M., Barbetti, F., Dunten, P., Han, Y., Grimsby, J., Taub, R., Ellard, S., Hattersley, A. T., and Matschinsky, F. M. (2005) Insights into the structure and regulation of glucokinase from a novel mutation (V62M), which causes maturity-onset diabetes of the young. *J. Biol. Chem. 280*, 14105–14113.
23. Heredia, V. V., Carlson, T. J., Garcia, E., and Sun, S. (2006) Biochemical basis of glucokinase activation and the regulation by glucokinase regulatory protein in naturally occurring mutations. *J. Biol. Chem. 281*, 40201–40207.
24. Nishi, S., Stoffel, M., Xiang, K., Shows, T. B., Bell, G. I., and Takeda, J. (1992) Human pancreatic beta-cell glucokinase: cDNA sequence and localization of the polymorphic gene to chromosome 7, band p 13. *Diabetologia 35*, 743–747.
25. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem. 72*, 248–254.
26. Gasteiger, E., Gattiker, A., Hoogland, C., Ivany, I., Appel, R. D., and Bairoch, A. (2003) ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res. 31*, 3784–3788.
27. Gasteiger, E., Hoogland, C., Gattiker, A., Duvaus, S., Wilkins, M. R., Appel, R. D., and Bairoch, A. (2005) Protein identification and analysis tools on the ExPASy server, in *The Proteomics Protocols Handbook* (Walker, J. M., Ed.) pp 571−607, Humana Press, Totowa, NJ.
28. Monasterio, O., and Cardenas, M. L. (2003) Kinetic studies of rat liver hexokinase D ("glucokinase") in non-co-operative conditions show an ordered mechanism with MgADP as the last product to be released. *Biochem. J. 371*, 29–38.
29. Lin, S. X., and Neet, K. E. (1990) Demonstration of a slow conformational change in liver glucokinase by fluorescence spectroscopy. *J. Biol. Chem. 265*, 9670–9675.
30. Molnes, J., Bjorkhaug, L., Sovik, O., Njolstad, P. R., and Flatmark, T. (2008) Catalytic activation of human glucokinase by substrate binding: residue contacts involved in the binding of D-glucose to the super-open form and conformational transitions. *FEBS J. 275*, 2467–2481.
31. van Schaftingen, E., and Veiga da Cunha, M. (2004) Discovery and role of glucokinase regulatory protein, in *Glucokinase and Glycemic Disease: From Basics to Novel Therapeutics* (Matschinsky, F. M., and Magnuson, M. A., Eds.) pp 193−207, Karger, Basel.
32. Baltrusch, S., and Tiedge, M. (2006) Glucokinase regulatory network in pancreatic B-cells and liver. *Diabetes 55*, S55−S64.
33. Farrelly, D., Brown, K. S., Tieman, A., Ren, J., Lira, S. A., Hagan, D., Gregg, R., Mookhtiar, K. A., and Hariharan, N. (1999) Mice mutant for glucokinase regulatory protein exhibit decreased liver glucokinase: a sequestration mechanism in metabolic regulation. *Proc. Natl. Acad. Sci. U.S.A. 96*, 14511–14516.

34. Grimsby, J., Coffey, J. W., Dvorozniak, M. T., Magram, J., Li, G., Matschinsky, F. M., Shiota, C., Kaur, S., Magnuson, M. A., and Grippo, J. F. (2000) Characterization of glucokinase regulatory protein-deficient mice. *J. Biol. Chem. 275*, 7826–7831.
35. Arden, C., Trainer, A., de la Iglesia, N., Scougall, K. T., Gloyn, A. L., Lange, A. J., Shaw, J. A., Matschinsky, F. M., and Agius, L. (2007) Cell biology assessment of glucokinase mutations V62M and G72R in pancreatic beta-cells: evidence for cellular instability of catalytic activity. *Diabetes 56*, 1773–1782.
36. Pino, M. F., Kim, K. A., Shelton, K. D., Lindner, J., Odili, S., Li, C., Collins, H. W., Shiota, M., Matschinsky, F. M., and Magnuson, M. A. (2007) Glucokinase thermolability and hepatic regulatory protein binding are essential factors for predicting the blood glucose phenotype of missense mutations. *J. Biol. Chem. 282*, 13906–13916.

BI900020N