Variable effects of maturity-onset-diabetes-of-youth (MODY)-associated glucokinase mutations on substrate interactions and stability of the enzyme

Yin LIANG,* Prabakaran KESAVAN,* Li-qun WANG,* Kevin NISWENDER,† Yukio TANIZAWA,‡ M. Alan PERMUTT,‡ Mark A. MAGNUSON† and Franz M. MATSCHINSKY*§

*Department of Biochemistry and Biophysics and the Diabetes Research Center, University of Pennsylvania, Philadelphia, PA 19104,

†Department of Molecular Physiology and Biophysics, Vanderbilt University Medical School, Nashville, TN 37232, ‡Metabolism Division, Washington University School of Medicine, St. Louis, MO 63110, U.S.A.

Mutations in the human glucokinase (GK) gene are thought to cause maturity-onset diabetes of youth (MODY) by leading to the production of enzymes with reduced catalytic activities and increased glucose K_m values. However, in some cases the diabetic phenotype is more severe than might be predicted from these apparent kinetic effects alone. To determine whether these mutations might also affect other characteristics of the enzyme, nine MODY-associated mutants were expressed as fusion proteins with *Schistosoma japonicum* glutathione S-transferase (GST) and compared with three wild-type human GK isoforms that were also expressed in the same manner. Three GST–GK isoforms (liver 1, liver 2 and islet) were kinetically indistinguishable from each other and from purified rat liver GK. Noteworthy is a glucose-induced fit effect for the interaction of trinitrophenyl (TNP)-ATP with GST–GK, whereby glucose significantly

increased the affinity of TNP-ATP binding to GST–GK without changing the stoichiometry of binding. The nine MODY-associated mutations studied either showed diminished catalytic activity, substrate affinities, allosteric regulation, or stability of the fusion enzyme. We conclude that: (1) Gly²⁶¹ and Lys⁴¹⁴ are important for ATP binding; (2) Val²⁰³ may be essential for a glucose-induced fit effect; and (3) the stability of fusion protein may be significantly reduced when Glu³⁰⁰ is replaced by Lys. These results suggest that, in addition to effects on the K_m and V_{max} of GK, a decrease in the ATP-binding affinity or stability of the mutated enzyme may also contribute to a reduction of GK activity in individuals with GK-MODY. In the B-cell this would have the effect of blunting glucose-stimulated insulin release, thereby contributing to the diabetic phenotype.

INTRODUCTION

Glucokinase (GK) is expressed in liver, pancreatic B-cells and certain rare neuroendocrine cells of the brain and gut [1–4]. The GK gene contains alternate tissue-specific promoters and produces isoforms that are differentially regulated [5–7]. This high- K_m hexokinase is now widely accepted as playing a crucial role in glucose metabolism by the pancreatic B-cell, where it is implicated in the mechanism of glucose-stimulated insulin release [2,8,9], although the functional importance of the enzyme in other sites of expression is either less well, or not at all, established. In humans, mutations in the GK gene are a cause of early-onset non-insulin-dependent diabetes mellitus ('NIDDM') or maturity-onset diabetes of youth (MODY). MODY-associated GK mutations are reported to decrease the V_{max} and increase the glucose K_m of GK, thus providing strong support for the notion that a decrease in GK activity in tissues that express the enzyme contributes to the diabetic phenotype [10–12].

Over 25 GK mutations in individuals with MODY have been reported [13–15]. Studies of the effects of these mutations on the kinetics of the enzyme have shown both variable reductions in the catalytic activity and increases in the glucose K_m . The mutations occur most frequently in exons 5–8, which are thought, on the basis of structural similarities with yeast hexokinase, the only hexokinase in which the X-ray crystal structure has been determined [16], to encode the glucose-binding pocket of the enzyme. No effects on the affinity of ATP binding to GK have been reported, nor have the effects of these mutations on other aspects of the behaviour of the enzyme that may affect its activity

in cells, such as its stability or co-operative behaviour. However, these aspects of GK function may be important, since the catalytic activity and glucose K_m of certain MODY mutants are not as severely affected as might be suggested from the clinical phenotype of the affected individuals [9,13–15].

In the present study, we expressed both wild-type and mutant human GK isoforms as a fusion protein with GST (GST–GK) to explore further structure–function aspects of the enzyme. This approach, which has the advantage of rapidly providing pure recombinant protein, produces fusion proteins with kinetic characteristics indistinguishable from authentic rat liver GK. Studies of the wild-type and mutant GST–GK proteins have led to the identification of effects of mutations on the interaction of ATP with GK and on the stability of the enzyme. These results suggest that the use of GST–GK fusions is a convenient and valid means of characterizing the kinetics of GK-MODY mutations and may point to additional explanations for reductions in GK activity in affected individuals which were not revealed by initial surveys. Both advances provide us an opportunity to extend our understanding of wild-type and mutant human GK, and the mechanisms by which gene mutations cause diabetes.

MATERIALS AND METHODS

Generation of recombinant wild-type and mutant GST–GK

DNA fragments of ≈ 1.4 kb containing the coding sequences of three human GK isoforms and *Bam*HI and *Eco*RI sites at the ends were prepared by linker-primer PCR of the human liver and

Abbreviations used: GK, glucokinase; LGK, liver GK; IGK, islet GK; MODY, maturity-onset diabetes of youth; TNP-, trinitrophenyl-; GST, glutathione S-transferase; DTT, dithiothreitol.

§ To whom correspondence should be addressed.

islet GK cDNAs. The primers used were: liver GK (LGK)1-5' (gtggatccacatgcccagaccaagatcc) + GK-3' (ctgaattcactggcccagcat-acag), LGK2-5' (ccggatccagatggcgatggatgtcaca) + GK-3', islet GK (IGK)-5' (tgggatccagatgctggacgacagag) + GK-3' for human liver GK1, human liver GK2 and human islet GK respectively. The PCR products were ligated into the *Bam*HI and *Eco*RI sites of pGEX-3X (Pharmacia, Piscataway, NJ, U.S.A.), and the coding sequences were confirmed by DNA sequencing. MODY-associated mutations were introduced into the human islet GK cDNA by the method of Kunkel [17], except for Lys⁷⁰, which was made using PCR mutagenesis. All mutating oligonucleotides were 21 bases in length, with the single altered base at position 11. After mutagenesis and DNA sequence confirmation in pEMBL19, the *Eco*RI-*Bam*HI cDNA fragments were cloned into pGEX-3X for expression as a fusion with a 26 kDa *S. japonicum* GST C-terminal protein fragment. At the completion of the study, each mutation was re-confirmed by DNA sequencing of the mutated bases in the pGEX-3X vector. GST-GK was produced in *Escherichia coli* grown overnight at 22 °C in Luria-Bertani medium/ampicillin/isopropyl thiogalactoside medium, then purified from crude *E. coli* extracts to near-homogeneity by a single-step affinity-chromatography procedure using GSH-agarose (Sigma, St. Louis, MO, U.S.A.) [18]. Briefly, 1 litre of bacterial culture was pelleted and resuspended in 10 ml of 0.1 M PBS containing 5 mM dithiothreitol (DTT). Cells were lysed on ice by sonication for 1–2 min after adding Triton X-100 to a concentration of 1%. The supernatant of the lysate was mixed with 50% GSH-agarose beads (sulphur-linked; Sigma), which were collected by brief centrifugation and washed with the same buffer. Fusion protein was eluted with 50 mM Tris/HCl (pH 8.0)/200 mM KCl/10 mM GST/5 mM DTT. A Coomassie Blue binding assay (Bio-Rad) was used to quantify the GST-GK protein. The yields of GST-GK proteins were 13 ± 2, 20 ± 3 and 16 ± 2 mg/litre of culture for LGK1, LGK2 and IGK respectively. Purified GST-GK preparations were stored at -80 °C until used.

Kinetic parameters of GST-GK

Catalytic assays were performed with an NADP⁺-coupled spectrophotometric assay using glucose-6-phosphate dehydrogenase [19]. The assay reagents included 100 mM Tris, pH 7.40, 6 mM MgCl₂, 14 mM 2-mercaptoethanol, 0.1% BSA, 150 mM KCl, 0.4 mM NADP⁺, 0.2 unit/ml glucose-6-phosphate dehydrogenase and substrate concentrations as specified. A total of 18 glucose concentrations (0.1–100 mM) with 5 mM ATP were used to determine the $V_{\max}$ and glucose K_m of glucokinase, and nine concentrations of ATP (0.02–5 mM) with 100 mM glucose were chosen for ATP K_m determinations. The Hanes-Woolf plot was utilized to calculate the glucokinase $V_{\max}$ as well as the K_m values for glucose and for ATP [20]. The Hill plot was applied to estimate the Hill coefficient (h) of glucokinase towards glucose or towards ATP. The specific velocity ($v/V_{\max}$) at the inflection point of the sigmoidal curve was calculated by the equation [21]:

$$v/V_{\max} = (h-1)/2h$$

The glucose concentration at the inflection point was calculated using the following equation:

$$v/V_{\max} = [S]^h / K' + [S]^h$$

where

$$K' = [S_{0.5}]^h$$

The effect of 20% glycerol and that of different inhibitors on GK activity was examined by adding them at suitable concentrations

(ranging from 0.1 to 50 mM) to the reaction buffer containing 3.13, 6.25, 12.5 and 25 mM glucose. The inhibitor constant, K_i , was determined by Cornish-Bowden [22] and Dixon [23] methods. The hysteretic behaviour of GK was studied by measuring initial and steady-state velocities of GK using a method described by Neet et al. [24]. The initial velocity (V_i) and the steady-state velocity (V_{ss}) (in terms of $\Delta A/\text{min}$) were calculated using linear regression of the 'enzyme mechanism' software for the Beckman model DU 640 UV spectrophotometer. The effect of ATP on the GK glucose K_m was investigated by measuring GK activity in a reaction mixture containing 0.05–5 mM ATP for 5 min and the initial velocity was calculated as described above. GK stability was tested by determining GK activity after incubating GK for 0, 5, 10, 20, and 30 min in a water bath at different temperatures as described in the Results section.

Interaction of GST-GK with trinitrophenyl (TNP)-nucleotides

The interaction of nucleotides with GK was monitored fluorometrically in a 600 μl quartz cuvette (Hellma Cells, New York, NY, U.S.A.) containing, in a volume of 200 μl , 10 mM Tris/HCl buffer, pH 7.4, 0.5 mM MgCl₂, different glucose concentrations (as indicated in the Results section) and 10 μM of either GST-GK (wild-type or mutant) or yeast hexokinase. TNP-nucleotides (TNP-ATP, -ADP, and -AMP; Molecular Probes, Eugene, OR, U.S.A.) at concentrations from 2 to 20 μM were added to the cuvette, and fluorescence was monitored by a computer-controlled Hitachi F-4500 fluorescence spectrophotometer set at an excitation wavelength of 410 nm and an emission wavelength of 520 nm. The fluorescence of protein or of TNP-ATP alone was subtracted from the total relative fluorescence. Unlabelled nucleotides (ATP, ADP and AMP; Boehringer-Mannheim, Indianapolis, IN, U.S.A.) were used in some experiments for displacement of the fluorescent ligand. *E. coli*-expressed GST was also purified and used as a control in these binding studies. The stoichiometry and K_d for the interaction of TNP-nucleotides with GK were calculated using the formula:

$$K_d/(1-\theta) = ([\text{TNP-nucleotide}]_{\text{total}}/\theta) - (\eta \cdot [\text{protein}])$$

where $[\text{TNP-nucleotide}]_{\text{total}}$ and $[\text{protein}]$ refer to the total concentrations of nucleotides and enzyme respectively [25]. The fractional saturation (θ) was estimated using the method described by Stinson and Holbrook [26], and a correction factor [27] was applied to the fluorescence data at each TNP-nucleotide concentration greater than 10 μM to limit the inner-filter effect.

RESULTS

Both wild-type and mutant human GK isoforms were expressed as a fusion protein with *S. japonicum* GST in *E. coli* and purified in a single step to near-homogeneity by affinity chromatography. The GST-human GK fusion proteins (Figure 1) had apparent molecular masses of 75 kDa and appeared to be virtually pure after the purification procedure.

Kinetic characteristics of wild-type GST-GK isoforms

In a spectrophotometric assay the glucose-dependency curves of the three human GK isoforms showed a typical sigmoidal shape in the Michaelis-Menten plot comparable with observations made with purified rat liver or islet glucokinase [20,28]. Correspondingly, the Hanes-Woolf plot ($[S]$ versus $[S]/v$) exhibited a characteristic hook at glucose concentrations lower than 5 mM (results not shown). When GST-GK activity was determined with 20% glycerol in the reaction mixture, the $V_{\max}$ was reduced

Figure 1 Purification of human recombinant wild-type GST-GK isoforms from *E. coli*

The results of SDS/PAGE of crude extracts from isopropyl thiogalactoside-induced (+ IPTG) and non-induced (— IPTG) *E. coli* cultures and of the preparations after a single-step purification of the three GST-GK isoforms (hLGK1, hLGK2 and hIGK) are shown alongside molecular-mass (*M*) standards (STD).

Table 1 Kinetic constants of recombinant human GST-GK fusion protein

The activity of GK was determined with an NADP⁺-coupled spectrophotometric assay using glucose-6-phosphate dehydrogenase. A total of 18 glucose concentrations (0.1–100 mM) with 5 mM ATP were used to determine the V_{max} and glucose K_m of glucokinase, and nine concentrations of ATP (0.02–5 mM) with 100 mM glucose were chosen for ATP K_m determinations. Results were obtained from two or three experiments using enzyme produced in two expressions. A unit of enzyme activity is defined as that amount of enzyme catalysing the phosphorylation of 1 μ mol of glucose/min at 30 °C.

GK isoform	V_{max} (units./mg of GST-GK)	Glucose K_m (mM)	h	ATP K_m (mM)	h	Glucose concn. at inflection point (mM)
0% Glycerol in the reaction buffer						
LGK1	48.2	11.4	1.5	0.24	1.2	4.0
LGK2	53.5	8.1	1.6	0.24	1.2	3.1
IGK	49.4	7.7	1.5	0.49	1.1	2.7
20% Glycerol in the reaction buffer						
LGK1	24.4	3.8	1.8	0.08	1.0	2.0
LGK2	28.3	5.2	1.5	0.10	1.0	1.7
IGK	29.4	4.0	1.7	0.38	1.0	1.7

to about 50 % of control and the glucose K_m was decreased more than 50 %, with no significant change in h . The ATP K_m and h of these glucokinase isoforms are summarized in Table 1, which shows both the kinetic data from these studies and the glucose concentration at the calculated inflection point. The apparent K_i values of four inhibitory hexoses for GST-GK were determined by Dixon and Cornish-Bowden plots (Table 2). We also tested the corresponding K_i values of mannoheptulose in the presence of 20 % glycerol. They were 0.65, 0.55 and 0.50 mM for GST-LGK1, GST-LGK2, and GST-IGK respectively. The four

Table 2 Apparent K_i values for GK-inhibitory sugars

The effect of different inhibitors of GK activity was examined by adding different inhibitors at suitable concentrations (ranging from 0.1 to 50 mM) to the reaction buffer containing 3.13, 6.25, 12.5 and 25 mM glucose. The inhibitor constant, K_i , was determined by Cornish-Bowden [20] and Dixon [21] methods.

Inhibitory sugar	GK isoform ...	K_i (mM)		
		LGK1	LGK2	IGK
<i>N</i> -Acetylglucosamine		0.25	0.21	0.20
2-Deoxyglucose		19.50	19.75	22.00
D-Glucosamine		2.50	2.60	1.70
Mannoheptulose		2.50	2.40	2.25

Table 3 Hysteretic behaviour of GST-GK isoforms in 30% glycerol

GST-GK isoforms were kept at 4 °C for 16 h and the enzyme activity was measured in reagent with 1 mM glucose. The velocity expressed by dA/min was calculated using linear regression of the enzyme mechanism software for the Beckman DU 640 spectrophotometer.

GK isoform	Storage conditions	Specific velocity (dA/min per μ g of protein)		
		V_i	V_{ss}	V_i/V_{ss}
LGK1	+ 50 mM Glucose	0.0465	0.0349	1.33
	0 mM Glucose	0.0290	0.0358	0.81
LGK2	+ 50 mM Glucose	0.0526	0.0370	1.42
	0 mM Glucose	0.0327	0.0383	0.86
IGK	+ 50 mM Glucose	0.0652	0.0538	1.21
	0 mM Glucose	0.0395	0.0461	0.86

chemicals we tested here inhibited the GST-GK isoforms in a competitively inhibitory manner.

The initial and steady-state velocities of GST-GK stored at 0 or 50 mM glucose were determined to assess whether the GST-GK fusion proteins retained the characteristic hysteretic behaviour. When GST-GK isoforms were stored at –80 °C and used for assay immediately after thawing, no significant difference between the V_i and V_{ss} was observed. However, the hysteretic behaviour became manifest when these preparations were kept at 4 °C for 16 h or longer after thawing. GST-GK stored in 50 mM glucose showed an initial burst of activity which decreased to a lower steady-state velocity over a short period, while GST-GK stored without glucose started with a slower initial rate which accelerated during the assay period of 10 min. V_{ss} was indistinguishable for these two preparations. Table 3 shows the V_i , V_{ss} and the ratios of GST-GK isoforms stored in the absence or presence of glucose. Similar behaviour was observed for all three isoforms.

Interactions of TNP-ATP with GST-GK and yeast hexokinase

Interactions of TNP-nucleotides with the wild-type GST-GK isoforms were investigated using a fluorometric assay. Yeast hexokinase was also used in this study on the basis of two considerations. First, the amino acid sequence of this related enzyme has 31 % identity with human glucokinase, and secondly, the interaction of ATP with yeast hexokinase has been well

Figure 2 Interaction of TNP-ATP with GST-IGK

Fluorescence measurements were carried out as described in the Materials and methods section. The concentration of GST-IGK was held constant at 10 μ M, and TNP-ATP was added at the indicated concentrations. The relative-fluorescence-enhancement values shown here were obtained by subtracting the fluorescence arising from TNP-ATP or protein alone. (a) Illustrates the interaction of TNP-ATP with GST-IGK in the absence or presence of different nucleotides. (b) Shows the concentration-dependency of the glucose effect on the ATP binding to GST-IGK. Results with 20 and 50 mM glucose (G) were omitted for reasons of clarity. Data represent the mean values from three experiments.

characterized. Enhanced fluorescence was observed when the TNP-nucleotides were bound to GST-GK, owing to changes in the quantum yield. This interaction was specific, since it was decreased by ATP in a concentration-dependent manner (Figure 2a). To rule out interference of the GST protein component in the GST-GK fusions, GST produced from *E. coli* bearing the pGEX-3X vector without a cDNA insert was used to test its interaction with TNP-nucleotides, and no fluorescence enhancement was observed (results not shown). Glucose was able to regulate the interaction of TNP-ATP with GK or with yeast hexokinase in a dose-dependent manner (Figure 2b and Table 4). The K_d of GST-GK or of yeast hexokinase binding with TNP-

ATP was significantly decreased when 100 or 1 mM glucose was added to the assay mixture. This glucose effect on the interaction of TNP-ATP with GK was similar for hLGK1, hLGK2 and hIGK. The stoichiometric analysis showed that 1 mM glucose increased the stoichiometry of yeast hexokinase binding to TNP-ATP. This was not the case with these three GST-GK isoforms, even in buffer containing 100 mM glucose (Table 4). GST-GK and yeast hexokinase bound TNP-ADP and TNP-AMP with lower affinity ($\text{AMP} \leq \text{ADP} < \text{ATP}$). This was also demonstrated in replacement experiments. The interaction of TNP-ATP with either GST-GK or hexokinase was partially prevented by addition of nucleotides, with the following order of affinity: $\text{ATP} > \text{ADP} \geq \text{AMP}$. The K_d values of both enzymes for binding of TNP-ADP or TNP-AMP were not statistically different in the presence or absence of glucose.

Effect of MODY mutations on the islet GK isoform

On the basis of an understanding of the behaviour of wild-type GST-GK, we next examined the effect of gene mutation on GST-islet GK (IGK) activity. Nine mutants were prepared in the IGK isoform, expressed as a fusion with GST, and purified in the same manner as for the wild-type isoforms. The kinetic characteristics of these mutants, i.e., V_{max} , glucose K_m and h , ATP K_m and h , were then determined. The nine mutants gave different results. The V_{max} of all mutants was variably reduced, and mutants T228M (i.e. Thr²²⁸ $\rightarrow$ Met; one-letter code), G261R and L309P showed a decrease in catalytic activity to a barely detectable rate; G175R, V182M, V203A and G261R showed an increased glucose K_m ; G175R, V203A, G261R and K414E had diminished ATP affinity, and G175R and G261R showed an h value of close to 1.0 for glucose (Table 5).

ATP binding to GST-IGK was examined further in three mutants (V203A, G261R, and K414E) that had at least a 4-fold-higher-than-normal ATP K_m . The stoichiometry of ATP binding to GST-IGK was not significantly changed by mutation of Val²⁰³ or Lys⁴¹⁴ (1.0 and 0.6 mol/mol of enzyme respectively), but was significantly reduced in the G261R mutant (0.2 mol/mol of enzyme, $P < 0.01$). The K_d of ATP binding to these mutants and the glucose-ATP interactions were differently affected (Figure 3a). With glucose absent, the K_d of V203A was similar to that of wild-type GST-IGK (2.6 and 2.4 μ M respectively), while it was increased about 2-fold in G261R and K414E (6.2 and

Table 4 Interaction of recombinant human GK and yeast hexokinase with TNP-nucleotides

The interaction of TNP-nucleotides with GK was determined as described in the Materials and methods section. A 10 μ M concentration of enzyme was used in each assay. TNP-nucleotides (TNP-ATP, -ADP, and -AMP) ranged from 2 to 20 μ M, and fluorescence was monitored by a computer-controlled Hitachi F-4500 fluorescence spectrophotometer set at an excitation wavelength of 410 nm and an emission wavelength of 520 nm. Results are means $\pm$ S.E.M. for three experiments; the glucose concentration was 1 mM for hexokinase, and 100 mM for GK. Statistical significance: * $P < 0.05$, ** $P < 0.01$.

	Dissociation constant (K_d , μ M)			Stoichiometry (mol/mol of enzyme)		
	TNP-ATP	TNP-ADP	TNP-AMP	TNP-ATP	TNP-ADP	TNP-AMP
In glucose-free buffer						
HK	3.30 $\pm$ 0.14	2.22 $\pm$ 0.27	2.92 $\pm$ 0.38	0.24 $\pm$ 0.03	0.20 $\pm$ 0.10	0.23 $\pm$ 0.05
LGK1	2.29 $\pm$ 0.42	1.79 $\pm$ 0.10	2.58 $\pm$ 0.26	0.70 $\pm$ 0.09	0.64 $\pm$ 0.07	0.72 $\pm$ 0.06
LGK2	1.69 $\pm$ 0.13	2.05 $\pm$ 0.04	2.46 $\pm$ 0.30	0.81 $\pm$ 0.17	0.99 $\pm$ 0.03	0.83 $\pm$ 0.04
IGK	2.19 $\pm$ 0.05	2.61 $\pm$ 0.08	2.78 $\pm$ 0.43	0.83 $\pm$ 0.10	0.92 $\pm$ 0.08	0.86 $\pm$ 0.12
In buffer with glucose						
HK	0.94 $\pm$ 0.02**	1.58 $\pm$ 0.05	2.44 $\pm$ 0.45	0.60 $\pm$ 0.03**	0.47 $\pm$ 0.05	0.34 $\pm$ 0.07
LGK1	0.81 $\pm$ 0.07*	1.48 $\pm$ 0.25	2.43 $\pm$ 0.06	0.77 $\pm$ 0.07	0.78 $\pm$ 0.12	0.59 $\pm$ 0.13
LGK2	1.03 $\pm$ 0.05*	2.25 $\pm$ 0.34	1.54 $\pm$ 0.07	0.72 $\pm$ 0.05	0.77 $\pm$ 0.06	0.66 $\pm$ 0.03
IGK	1.14 $\pm$ 0.03**	1.52 $\pm$ 0.07	2.00 $\pm$ 0.29	0.82 $\pm$ 0.05	0.81 $\pm$ 0.10	0.69 $\pm$ 0.03

Table 5 Properties of wild-type and mutant forms of recombinant human islet GST-GK

The activity of GK was determined with an automated NADP⁺-coupled spectrophotometric assay using glucose-6-phosphate dehydrogenase. Ten glucose concentrations (0.2–100 mM) with 5 mM ATP were used to determine the $V_{\max}$ and glucose K_m of glucokinase, and nine concentrations of ATP (0.02–5 mM) with 100 mM glucose were chosen for ATP K_m determinations. Results were obtained from two to four expressions. A unit of activity is defined as that amount of enzyme catalysing the phosphorylation of 1 μ mol of glucose/min at 30 °C.

GST-GK	Activity (units/mg of GST-GK)	Glucose K_m (mM)	h	ATP K_m (mM)
Wild-type	53.5 $\pm$ 1.9	5.9 $\pm$ 0.9	1.4 $\pm$ 0.1	0.4 $\pm$ 0.03
Mutants				
E70K	35.8 $\pm$ 0.4	8.3 $\pm$ 1.2	1.5 $\pm$ 0.1	0.35 $\pm$ 0.03
G175R	12.8 $\pm$ 0.4	30.8 $\pm$ 3.3	1.1 $\pm$ 0.04	0.74 $\pm$ 0.2
V182M	13.4 $\pm$ 2.0	37.4 $\pm$ 2.8	1.3 $\pm$ 0.03	0.20 $\pm$ 0.02
V203A	11.4 $\pm$ 3.6	122.5 $\pm$ 9.0	1.5 $\pm$ 0.1	1.63 $\pm$ 0.5
T228M	0.04, 0.01	14.3, 4.4	1.4, 1.1	0.5, 0.7
G261R	2.8 $\pm$ 0.5	143.5 $\pm$ 19.3	1.0 $\pm$ 0.03	4.38 $\pm$ 1.5
E300K	28.6 $\pm$ 2.4	4.7 $\pm$ 0.3	1.3 $\pm$ 0.2	0.55 $\pm$ 0.1
L309P	1.0, 0.5	23.8, 32.4	1.4, 1.3	1.0, 0.8
K414E	8.4, 7.7	28.9, 16.9	1.2, 1.4	1.4, 2.8

Figure 3 Effects of three MODY mutations on the interaction of ATP with GST-GK

(a) The affinity of ATP binding to GK. The interaction of TNP-ATP with wild-type GST-IGK and with mutants V203A, G261R and K414E was monitored fluorometrically as described in the Materials and methods section. Enzyme (10 μ M) was added to buffer containing 0, 10, 50 and 100 mM glucose, and 2–20 μ M of TNP-ATP was used in this assay. Results represent the mean value from three experiments. (* P < 0.05; ** P < 0.01, compared with wild-type GST-IGK at the same glucose concentration). (b) The effect of ATP on the glucose K_m . The glucose K_m of wild-type GST-IGK, mutants V203A and K414E were determined spectrometrically as described in the Materials and methods section. The ATP concentration ranged from 0.5 to 5 mM. Results represent the percentage change in the glucose K_m values compared with the glucose K_m values with 5 mM ATP, which were 7.7, 122.8 and 22.9 mM respectively, for GST-IGK, V203A and K414E.

4.5 μ M respectively). However, the affinities of ATP in the K414E and G261R mutants were still regulated by glucose. This was not the case for the V203A mutant. The influence of an increased ATP K_m on the K_m for glucose was studied in mutants V203A and K414E at different ATP concentrations (0.05–5 mM). ATP affected the glucose K_m for wild-type GST-IGK and the

Figure 4 Effect of temperature on the stability of wild-type GST-IGK and mutant E300K

(a) Enzymes were kept in storage buffer [50 mM Tris/HCl (pH 8.0)/10 mM GSH/50 mM glucose/5 mM DTT/200 mM KCl/30% glycerol], and incubated in a water bath at 30, 40, 50, 55 and 60 °C for 0 or 30 min and the GK activity was then determined spectrometrically as described in the Materials and methods section. (b) Enzymes were first incubated at 55 °C for 0, 5, 10, 20 and 30 min and were then used for activity determination. At zero time the values for the $V_{\max}$ of wild-type GST-IGK (●) and E300K (○) were 59.3, and 30.2 units of GST-GK/mg respectively. Relative activity values are shown as means $\pm$ S.E.M. from three or four experiments. * P < 0.05; ** P < 0.01; mutant E300K versus wild-type GST-IGK.

K414E mutant similarly, while in the V203A mutant ATP was less effective (Figure 3b).

The stability of wild-type GST-IGK and mutant E300K were tested as a function of temperature (Figure 4a). Wild-type GST-IGK, incubated at 50 °C for 30 min in the stock solution, retained its activity. When the temperature was increased to 55 °C, the enzyme activity fell gradually with a $t_{1/2}$ of 43.6 min. However, the activity of E300K significantly decreased at 50 °C, and its $V_{\max}$ decreased even faster at 55 °C, with a $t_{1/2}$ of 5.5 min (Figure 4b), which is only 13% of that of wild-type GST-IGK (P < 0.05), indicating that this particular gene mutation reduced enzyme stability. No change in the glucose K_m and h were observed for the wild-type GST-IGK or the E300K mutant as a result of this heat treatment.

DISCUSSION

A strategy whereby GK was expressed as a fusion protein with GST was used to study the kinetic characteristics of the three human wild-type isoforms and nine MODY-associated mutants. Attachment of the GST moiety to the N-terminus of GK permitted us to purify a fusion protein in a single-step procedure. The GST-GK preparations obtained were homogeneous and, after correcting mass differences, had a specific activity equivalent to that of recombinant human LGK and IGK prepared by a different procedure [10]. To further test whether the GST-GK fusion protein was similar to the wild-type enzyme, a variety of kinetic characteristics and the hysteretic behaviour of the fusion proteins were determined. The results showed that wild-type

GST-GK isoforms have similar K_m values and h numbers for glucose and for ATP compared with those of purified rat liver GK [24]. The hysteretic behaviour of GK, which is presumably an expression of its co-operativity, is demonstrated by the slow transitions of GST-GK activity in 30% glycerol [24]. The determination of these slow transitions depended on glucose in the enzyme storage buffer. When GST-GK was kept at 4 °C for more than 16 h in the presence or absence of 50 mM glucose, the high V_i of the enzyme stored in 50 mM glucose decayed and the low V_i of the enzyme stored without glucose accelerated. However, the V_{ss} values of the two preparations were equal. Glycerol markedly influenced the behaviour of GST-GK by increasing substrate affinities and unmasking the hysteretic nature of this enzyme. Thus the attachment of the 26 kDa C-terminus of GST appears to have no measurable effect on these characteristic kinetic features of GK. These findings suggest that the N-terminus of GK may not be crucial for the three-dimensional structure, since the addition of GST sequences appeared inconsequential. The similar kinetic behaviour of the three human GK isoforms themselves, as discussed below, also supports this notion. The possibility of modifying the N-terminus, for instance for biochemical or pharmacological studies or for attachment of GK to a solid matrix, may be a useful feature of the enzyme.

Human and rat GK show 97% amino acid identity; however, humans have two distinct hepatic isoforms, of which GK-L2 appears to be a major one [29], whereas there is only a single functional hepatic isoform in rats. The structural differences of 15 N-terminal amino acid sequences among the different human GK isoforms, including two liver isoforms and an islet isoform, and of enzymes from different species, do not seem to affect enzyme function as tested here. The glucose K_m of GST-GK varied between 7.7 and 11.4 mM, values comparable with those obtained from purified rat liver GK or recombinant rat IGK [24,30]. This range is also indistinguishable from that shown by a recombinant human GK expressed in *E. coli* without the GST fusion protein [10]. Using the glucose K_m and h value, we calculated an inflection point of 2.7–4.0 mM glucose for the three isoforms. For IGK this value coincides with the threshold of the pancreatic B-cell for glucose stimulation. With an inflection point of about 2.7 mM and a glucose K_m of ≈ 10 mM, IGK is capable of efficiently regulating islet glucose metabolism by B-cells following changes in the blood glucose concentration in the physiological range. These results further support the notion that IGK is an ideal glucose sensor in the pancreatic B-cells of humans. From this it can be extrapolated that even subtle changes in the glucose K_m , h and thus the inflection point due to physiological modification or pathological events could readily alter the kinetics of GK and, in turn, modify the response of B-cells to glucose.

The characteristics of ATP binding to GK, which are clearly important for GK catalysis, may also be affected in the MODY mutants. Using fluorimetric methods, we have monitored the interaction of TNP-ATP with GST-GK. This method allowed us to directly study ligand binding independently of the catalytic process. The results demonstrate that the interaction of ATP with GST-GK is manifested in three phenomena: stoichiometry of ATP binding, ATP binding affinity and regulation of affinity by glucose. Stoichiometry data showed that ATP binds to GST-GK with a 1:1 molar ratio. The apparent K_d value for the binding of TNP-ATP to glucokinase is much lower than that of ATP, a finding consistent with the observation of the interaction of TNP-ATP with hexokinase [31]. The possible explanation might be that the chromophore of TNP-ATP increases binding of the nucleotide [32]. The affinity of ATP to GST-GK was significantly increased by glucose. This glucose regulation was

dose-dependent, with a half-maximal effect apparent at around 10 mM, similar to the glucose K_m of GK itself. The characteristics of ATP binding to GST-GK were different from those of yeast hexokinase in two aspects: (1) in the absence of glucose, the stoichiometry of hexokinase was 0.2 mol/mol, while it was 0.8 mol/mol in the case of GST-GK; (2) glucose only increased the affinity of ATP to GST-GK, but had no effect on the stoichiometry of ATP binding, contrasting with the situation in hexokinase. MODY-associated mutations appear to be able to change the stoichiometry, affinity or glucose-induced fit effect of ATP binding independent of each other. The G261R mutant showed significantly reduced stoichiometry and affinity of ATP binding. Additionally, its affinity to glucose and the catalytic activity were also reduced, indicating that the residue Gly²⁶¹ is crucial for GK catalysis. The residue Lys⁴¹⁴ corresponds to Thr⁴²² in the yeast hexokinase B, which is known to be an essential component of the ATP-binding domain of hexokinase [33]. Here we found that the K414E mutant has a 4-fold increase of the ATP K_m and a significantly increased K_d of ATP binding to GK. However, this particular mutation did not change the stoichiometry of ATP binding, and binding remained regulatable by glucose. This phenomenon suggests that the glucose-regulatory domain may be distinct from the ATP-binding domain. This postulation is supported by the analysis of the V203A mutant, which affects a residue located outside the putative ATP-binding domain but close to the glucose-binding site [16]. V203A showed decreased affinity to both glucose and ATP. However, in the absence of glucose, the K_d and stoichiometry of ATP binding to this mutant were similar to those of wild-type. Increasing the glucose concentration in the reaction buffer, even to 300 mM, did not increase its ATP affinity. It is thus possible that the increased ATP K_m is actually due to the elimination of the induced fit effect of glucose. This result indicates that the glucose regulatory effect on ATP binding to GK is essential for enzyme function. Mutation at Val²⁰³ also interfered with glucose binding to GK, which may not be surprising. Previously, analysis of the hexokinase crystal structure suggests that ATP bound to the enzyme might induce a secondary conformational change facilitating the catalytic function [34]. Since varying ATP concentration changes the glucose K_m of GK [24], it is not unreasonable to suggest that ATP might regulate glucokinase physiologically. Supporting this interpretation is the effect of the mutation at Val²⁰³, which altered the effect of ATP on glucose K_m of GST-GK.

Enzyme stability is another crucial issue in the structure-function relationships of GK. Changes of protein stability rather than activity have been observed in mutations that cause cystic fibrosis [35]. Even though the mutant E300K shows close-to-normal catalytic activity, its stability is significantly reduced when exposed to high temperature. The amino acid residue Glu³⁰⁰ is thought to form a salt bridge with Arg³⁰³, and changing Glu to Lys could break this salt bridge and thus reduce the stability of GK [11]. The thermal stability of this GK mutant appeared to be decreased *in vitro*. This reduction of thermal stability in mutant GK may explain, in part at least, the phenotype of MODY patients carrying this mutation. Additional studies are needed to explore fully the possibility that alterations of enzyme stability are pathophysiologically significant.

GK is a monomer and shows co-operative binding for glucose with an h value of 1.5, although the molecular mechanism of this co-operativity remains under investigation. Here we found that the mutants G175R and G261R showed an h value of close to 1.0, indicating that the co-operativity of GK was lost. In these two mutants, glycine, an uncharged amino acid residue, is replaced by arginine, a basic amino acid. Therefore, Gly may serve important functions in the allosteric regulation of GK. This

speculation is supported by the observation that mutating ADP glucose pyrophosphorylase at Gly³³⁶ with Asp eliminates the allosteric regulation of this enzyme, whereas replacement of this residue with the neutral residue Asn does not change the *h* value (C. R. Meyeer, personal communication).

In conclusion, these studies point to a greater complexity in the effects of MODY-associated GK mutations than has been suggested previously by the studies of Gidh-Jain et al.[10] and Takeda et al. [11]. First, we observed variations in the ATP *K_m* that were generally larger than have been reported. Secondly, we observed dramatically more activity than reported for both the V203A and E70K mutations, but much less activity for K414E. Thirdly, our determinations of the glucose *K_m* values for the G261R and L309P mutants resulted in values over 10-fold higher. While the basis for these differences remains to be established, methodological differences are likely to be involved. It should also be recognized that the determination of an accurate glucose or ATP *K_m* value is particularly difficult with the low-activity GK mutants. In this regard, the attachment of the GST moiety to GK, which enables the recombinant enzyme to be more quickly purified, may enable unstable mutants to be obtained before their activity is lost by more lengthy purification schemes. Such a difference may account for the greater activity observed for V203A and E70K. On the other hand, effects of the extra N-terminal sequence cannot be fully excluded, even though it had no noticeable effects on the kinetics of the wild-type isoforms. However, we think it unlikely that the attachment of this sequence can account for all the differences observed. Indeed, it is even possible that the GST moiety may have served to unmask alterations in ATP binding that are not as easily detected in enzyme preparations lacking this addition at the N-terminus. In any case, further studies of the effects of other mutations on the mechanisms of GK catalysis are warranted which can be aided by using the GST-GK fusion protein strategy.

These studies were supported by the American Diabetes Association, the Juvenile Foundation International and the University of Pennsylvania Research Foundation grants to Y.L., National Institutes of Health grants DK22122 and DK19525 to F.M.M., DK42612 and DK42502 to M.A.M. and DK16746 to M.A.P.

REFERENCES

- Weinhouse, S. (1976) *Curr. Top. Cell. Regul.* **11**, 1–50
- lynedjian, P. B. (1993) *Biochem. J.* **293**, 1–13
- Matschinsky, F. M. and Ellerman, J. E. (1968) *J. Biol. Chem.* **243**, 2730–2736
- Jetton, T. L., Liang, Y., Pettepther, C. C., Zimmerman, E. C., Cox, F. G., Horvath, K. et al. (1994) *J. Biol. Chem.* **269**, 3641–3654
- Bedoya, F. J., Matschinsky, F. M., Shimizu, T., O'Neil, J. J. and Appel, M. C. (1986) *J. Biol. Chem.* **261**, 10760–10764
- Magnuson, M. A. and Shelton, K. D. (1989) *J. Biol. Chem.* **264**, 15936–15942
- Liang, Y., Najafi, H., Smith, R. M., Zimmerman, E. C., Magnuson, M. A., Tal, M. et al. (1992) *Diabetes* **41**, 92–806
- Printz, R. L., Magnuson, M. A. and Granner, D. K. (1993) *Annu. Rev. Nutr.* **13**, 463–496
- Matschinsky, F. M., Liang, Y., Kesavan, P., Wang, L., Froguel, P., Velho, G. et al. (1993) *J. Clin. Invest.* **92**, 2092–2098
- Gidh-Jain, M., Takeda, J., Xu, L. Z., Lange, A. J., Vionnet, N., Stoffel, M. et al. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 1932–1936
- Takeda, J., Gidhjain, M., Xu, L. Z., Froguel, P., Velho, G., Vaxillaire, M. et al. (1993) *J. Biol. Chem.* **268**, 15200–15204
- Stoffel, M., Froguel, P., Takeda, J., Zouali, H., Vionnet, N., Nishi, S. et al. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7698–7702
- Froguel, P., Zouali, H., Vionnet, N., Velho, G., Vaxillaire, M., Sun, F. et al. (1993) *N. Engl. J. Med.* **328**, 697–702
- Bell, G. I., Froguel, P., Nishi, S., Pilkis, S. J., Stoffel, M., Takeda, J. et al. (1993) *Trends Endocrinol. Metab.* **4**, 86–90
- Katagiri, H., Asano, T., Ishihara, H., Inukai, K., Anai, M., Miyazaki, J. et al. (1992) *Lancet* **340**, 316–317
- Sturis, J., Kurland, I. J., Byrne, M. M., Mosekilde, E., Froguel, P., Pilkis, S. J. et al. (1994) *Diabetes* **43**, 18–723
- Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 488–492
- Smith, D. B. (1989) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, K., Kingston, R. E. et al., eds.), pp. 16.7.1–8, Greene Publishing Associates and Wiley Interscience, New York
- Pilkis, S. J. (1975) in *Methods in Enzymology*, (Wood, W. A., ed.), pp. 31–44, Academic Press, New York and London
- Storer, A. C. and Cornish-Bowden, A. (1976) *Biochem. J.* **159**, 7–14
- Segel, I. H. (1975) in *Enzyme Kinetics* (Segel, I. H., ed.), pp. 346–464, John Wiley and Sons, New York
- Cornish-Bowden, A. (1973) *Biochem. J.* **137**, 43–144
- Dixon, M. (1953) *Biochem. J.* **55**, 170–171
- Neet, K. E., Keenan, R. P. and Tippet, P. S. (1990) *Biochemistry* **29**, 770–777
- Garboczi, D. N., Thomas, P. J. and Pedersen, P. L. (1990) *J. Biol. Chem.* **265**, 14632–14637
- Stinson, R. A. and Holbrook, J. J. (1973) *Biochem. J.* **131**, 719–728
- Ward, L. D. (1985) *Methods Enzymol.* **117**, 400–414
- Meglasson, M. D., Burch, P. T., Berner, D. K., Najafi, H., Vogin, A. P. and Matschinsky, F. M. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 85–89
- Tanizawa, Y., Koranyi, L. I., Welling, C. M. and Permutt, M. A. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 294–297
- Liang, Y., Jetton, T. L., Zimmerman, E. C., Najafi, H., Matschinsky, F. M. and Magnuson, M. A. (1991) *J. Biol. Chem.* **266**, 6999–7007
- Arora, K. K., Shenbagamurthi, P., Fanciulli, M. and Pedersen, P. L. (1990) *J. Biol. Chem.* **265**, 324–328
- Moczydlowski, E. G. and Fortes, P. A. G. (1981) *J. Biol. Chem.* **256**, 2346–2356
- Bork, P., Sander, C. and Valencia, A. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7290–7294
- Shoham, M. and Steitz, T. A. (1980) *J. Mol. Biol.* **140**, 1–14
- Thomas, P. J., Shenbagamurthi, P., Sondek, J., Hulihan, J. M. and Pedersen, P. L. (1992) *J. Biol. Chem.* **267**, 5727–5730