Structure–function analysis of the $\alpha 5$ and the $\alpha 13$ helices of human glucokinase: Description of two novel activating mutations

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Abstract

It was recently described that the $\alpha 5$ and the $\alpha 13$ helices of human pancreatic glucokinase play a major role in the allosteric regulation of the enzyme. In order to understand the structural importance of these helices, we have performed site-directed mutagenesis to generate glucokinase derivatives with altered residues. We have analyzed the kinetic parameters of these mutated forms and compared them with wild-type and previously defined activating mutations in these helices (A456V and Y214C). We found two new activating mutations, A460R and Y215A, which increase the affinity of the enzyme for glucose. Our results suggest that substitutions in the $\alpha 5$ or the $\alpha 13$ helices that favor the closed, active conformation of the enzyme, either by improving the interaction with surrounding residues or by improving the flexibility of the region defined by these two helices, enhance the affinity of the enzyme for glucose, and therefore its performance as a glucose phosphorylating enzyme.

Keywords: glucokinase; activating mutation; GKAs; glucose signaling; enzyme kinetics

Glucokinase B (GKb, hexokinase IV) is the first enzyme of the glycolytic pathway in pancreatic β -cells. It phosphorylates the glucose that enters these cells through the GLUT2 glucose transporter to produce Glu-6P, which is then used to obtain the energy necessary for cell viability. The kinetic properties of GLUT2 (high capacity,

low-affinity glucose transporter) and GKb (low affinity for glucose and absence of allosteric regulation by Glu-6P) define GKb as the key regulator of glucose consumption. For this reason, several authors have considered GKb as the “glucose sensor” of pancreatic β -cells (Matschinsky et al. 1993, 1998; Matschinsky 1996, 2002; Zelent et al. 2005). In addition, a positive correlation between the functionality of GKb and the ability of pancreatic β -cells to respond to increased levels of glucose in blood by enhancing insulin secretion and production has been described. In this way, GKb participates in maintaining the blood glucose concentration around 5 mM. Inactivating mutations in one or both alleles of glucokinase gene (*GCK*) leads to an enzyme with low activity, which increases the threshold

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Abbreviations: GST, glutathione-S-transferase; IPTG, isopropyl- β -D-thiogalactoside; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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for glucose stimulated insulin secretion (GSIS) from its physiological setting of 5 mM. These type of mutations are associated with a mild form of diabetes mellitus in young people, known as maturity-onset diabetes of the young type 2 (MODY2), or with severe permanent neonatal diabetes mellitus (PNDM). On the other hand, mutations in the *GCK* gene that increase the affinity of the enzyme for glucose decrease the threshold for GSIS, and therefore trigger insulin secretion at lower concentrations of glucose in blood. These mutations are associated with both mild and severe forms of persistent hyperinsulinaemic hypoglycaemia in infancy (PHHI-GK) (for reviews, see Matschinsky 2002, Gloyn 2003, and Zelent et al. 2005).

The recently defined crystal structure of human GlkB (Kamata et al. 2004) indicates that this protein has a large and a small domain, separated by a deep cleft. Glucose binds to the interdomain cleft, composed of residues of the large domain (Glu256 and Glu290), the small domain (Thr168 and Lys169), and the connecting region I (Asn204 and Asp205). Upon binding to substrates (glucose and ATP), GlkB undergoes a conformational change that brings the large and the small domain physically closer, resulting in a closed, active conformation. The $\alpha 13$ helix, within the small domain, plays a crucial role in the conformational change that occurs between the active and inactive forms of the enzyme (Kamata et al. 2004). It was proposed that modifications in the $\alpha 13$ helix that make the closed conformation more stable should render enzymes with higher glucose affinity. Moreover, it was suggested that activating mutations within the $\alpha 13$ helix, such as V455M (Glaser et al. 1998) and A456V (Christesen et al. 2002), seemed to inhibit the release of this helix after glucose consumption, perhaps as a result of their bulkier side chains (Kamata et al. 2004). Other activating mutations, such as Y214A (Moukil et al. 2000) and Y214C (Cuesta-Munoz et al. 2004) could affect the flexibility of the region involved in the conformational change maintaining the enzyme in the closed, active form (Kamata et al. 2004), thus explaining the severe clinical phenotype presented by the patient with the glucokinase-activating mutation Y214C recently identified (Cuesta-Munoz et al. 2004).

To study the structure–function relationship of the domains involved in the conformational change of GlkB, we have constructed different mutants in residues located in the $\alpha 5$ and the $\alpha 13$ helices of the enzyme and tested the kinetic parameters of the resulting forms. We have found that substitutions that favor the closed, active conformation, either by improving the interaction with surrounding residues or by improving the flexibility of the region defined by these two helices, enhance the affinity of the enzyme for glucose, and therefore its performance as a glucose phosphorylating enzyme.

Results

Structure–function analysis of the $\alpha 13$ helix

In order to perform a structure–function analysis of the glucokinase $\alpha 13$ helix, we constructed different mutants in this helix and tested the kinetic parameters of the resulting forms. Modifications were chosen according to the amino acid sequence present in the same helix of human hexokinase I, a high-affinity glucose phosphorylating enzyme (Wilson 2003). As observed in Figure 1, the $\alpha 13$ helices of glucokinase and hexokinase I display a high degree of homology; however, the similarity between both sequences is not maintained in the C-terminal part of the helices. For this reason, we modified the following residues in GlkB sequence: C457V, K458R, K459L, and A460R (Table 1). We then compared the kinetic parameters of the mutated forms with those of the wild type and the activating mutation A456V (Christesen et al. 2002), a modification previously reported to produce a high-affinity glucose phosphorylating glucokinase. Since in regular conditions of glucose homeostasis the concentration of glucose in human blood is around 5 mM and there are saturating concentrations of ATP, we assumed that the activity of the enzyme at 5 mM glucose and saturating concentrations of ATP (5 mM) would be a better parameter than $V_{\max}$ to correlate the activity of the enzyme with its performance under physiological conditions. As shown in Figure 2A and Table 2, the K458R mutation resulted in an enzyme with lower glucose affinity ($S_{0.5}$, 12.28 mM) and lower ATP affinity (K_m , 0.81 mM) than the wild type ($S_{0.5}$, 7.52 mM; K_m , 0.41 mM, respectively). The activity of the K458R mutant at 5 mM glucose was also lower than the wild type (3.6 units/mg vs. 5.9 units/mg; Fig. 2C). The lower affinity for glucose and ATP and the lower activity at 5 mM glucose suggested that this mutated form had a poorer performance as a glucose phosphorylating enzyme.

$\alpha 13$ helix			
		VVRLR	
GlkB 443	EGSGRGAALVSAVACKKA	460	
HxkI 895	DGSGKGAALITAVGVRLR	912	
	****:*****:*** :		
$\alpha 5$ helix			
	G CA		
GlkB 204	NDTVATMISCYYE	216	
HxkI 656	NDTVGTMTTCAYE	668	
	****.***:*** **		

Figure 1. Alignment of the $\alpha 5$ and the $\alpha 13$ helices of human GlkB and hexokinase I. Amino acid sequences corresponding to the $\alpha 5$ and $\alpha 13$ helices of human GlkB (Swiss-Prot accession no. P35557) and hexokinase I (Swiss-Prot accession no. P19367) were aligned using the CLUSTALW program. Mutated residues were on top of the GlkB sequence. Numbers indicate the beginning and the end of each helix in each sequence.

Figure 2. Glucose-dependent activity of mutated forms of glucokinase. (A) Mutated forms in the $\alpha 13$ helix. (B) Mutated forms in the $\alpha 5$ helix. (C) Specific activity of mutated forms of glucokinase at 5 mM glucose and saturating concentrations of ATP (5 mM). A representative experiment of at least three different kinetic determinations is shown.

The C457V and K459L mutated enzymes showed a slightly higher glucose affinity ($S_{0.5}$, 5.90 mM and 7.03 mM, respectively) than the wild type (Fig. 2A; Table 2), although a slightly lower affinity for ATP (K_m , 0.65 mM and 0.60 mM, respectively). The activity of the enzymes at 5 mM glucose was in both cases higher than the wild type (C457V, 9.1 units/mg; K459L, 12.8 units/mg) (Fig. 2C), suggesting a better performance as glucose phosphorylating enzymes.

In the case of the A460R mutant, it displayed a very high affinity for glucose ($S_{0.5}$, 1.94 mM), similar to the one found in the previously reported (Christesen et al. 2002) activating mutation A456V ($S_{0.5}$, 1.81 mM) (Fig. 2A; Table 2), although the affinity for ATP was slightly lower than the wild type (K_m , 0.55 mM). The activity of the mutated enzyme at 5 mM glucose was also higher than

the wild type (18.9 units/mg) (Fig. 2C) and similar to the activity found in the A456V mutant (20.6 units/mg) (Fig. 2C). These results indicate that A460R is a novel activating mutation of GlkB.

Structure–function analysis of the $\alpha 5$ helix

As mentioned above, the crystal structure of GlkB indicated that the $\alpha 13$ helix is involved in a major conformational change between the closed and open forms of the enzyme, requiring the breakage and reformation of numerous interactions among the layers (Kamata et al. 2004). Since the $\alpha 5$ helix is close to the $\alpha 13$ helix in this movement, we wondered whether modifications in residues of the $\alpha 5$ helix could affect the transition movement of the $\alpha 13$ helix and, therefore, modify the glucose affinity of the enzyme. Following a similar approach as above (Fig. 1), we constructed mutant A208G and compared its kinetic parameters with Y214C, a recently identified activating mutation of GlkB located in $\alpha 5$ helix (Cuesta-Munoz et al. 2004). In addition, we constructed mutant Y215A, due to its close similarity to Y214C. As shown in Figure 2B and Table 2, the A208G mutant displayed a slightly higher glucose affinity ($S_{0.5}$, 5.66 mM) and similar ATP affinity (K_m , 0.43 mM) to the wild type. The activity of the enzyme at 5 mM glucose was also moderately higher than the wild type (A208G, 7.2 units/mg), suggesting a slightly better performance as glucose phosphorylating enzyme. However, the kinetic parameters of the Y215A mutant ($S_{0.5}$, 2.20 mM; specific activity at 5 mM glucose, 15.5 units/mg) (Fig. 2; Table 2) were in the range of those of the activating mutations A456V and A460R described above, although the affinity for ATP was slightly lower than the wild type (K_m , 0.65 mM). Therefore, Y215A could be considered as another novel activating mutation of GlkB. Finally, the activating mutation Y214C exhibited a high affinity for glucose and ATP ($S_{0.5}$, 1.87 mM; K_m , 0.29 mM) and the highest activity at 5 mM glucose (31.7 units/mg) among all the mutants tested (Fig. 2; Table 2).

Discussion

It was recently suggested that GlkB may exist in three structural conformations that define two catalytic cycles. One cycle involves the transition from a closed, active form to an intermediate open active form and then to a super-open inactive form (slow catalytic cycle). The other proposed cycle involves only a transition from the closed active form to the open active form (fast catalytic cycle). The ratio between these two catalytic cycles is responsible for the characteristic sigmoidal response to glucose of this enzyme. In the case of the slow catalytic cycle, the enzyme shows high kinetic

Table 1. Oligonucleotides used in the study

Name oligo	Sequence
GlkB-A208G-1	5'-GCAATGGTGAATGACACGGTGGGCACGATGATCTCCTGCTACTAC-3'
GlkB-A208G-2	5'-GTAGTAGCAGGAGATCATCGT <u>GCC</u> CACCGTGTCATTACCATTCG-3'
GlkB-Y214C-1	5'-GTGGCCACGATGATCTCCTGCTGCTACGAAGACCATCAGTGCGAGG-3'
GlkB-Y214C-2	5'-CCTCGCACTGATGGTCTTCGTAGCAGCAGGAGATCATCGTGGCCAC-3'
GlkB-Y215A-1	5'-GCCACGATGATCTCCTGCTACGCGAAGACCATCAGTGCGAGGTC-3'
GlkB-Y215A-2	5'-GACCTCGCACTGATGGTCTTCGGCGTAGCAGGAGATCATCGTGGC-3'
GlkB-A456V-1	5'-GCGGCCCTGGTCTCGGCGGTGGTCTGTAAGAAGGCCGTATGCTG-3'
GlkB-A456V-2	5'-CAGCATAACAGGCCTTCTTACAGACACCGCCGAGACCAGGGCCGC-3'
GlkB-C457V-1	5'-GCCCTGGTCTCGGCGGTGGCCGTTAAGAAGGCCGTATGCTGGGC-3'
GlkB-C457V-2	5'-GCCCAGCATAACAGGCCTTCTTAAACGGCCACCGCCGAGACCAGGGC-3'
GlkB-K458R-1	5'-CTGGTCTCGGCGGTGGCCCTGTCCGAAGGCCCTGTATGCTGGGCCAG-3'
GlkB-K458R-2	5'-CTGGCCCAGCATAACAGGCCTTCCGACAGGCCACCGCCGAGACCAG-3'
GlkB-K459L-1	5'-GTCTCGGCGGTGGCCCTGTAAGCTTGCCCTGTATGCTGGGCCAGTGA-3'
GlkB-K459L-2	5'-TCACTGGCCCAGCATAACAGGCAGCTTACAGGCCACCGCCGAGAC-3'
GlkB-A460R-1	5'-GTCTCGGCGGTGGCCCTGTAAGAAGCGCTGTATGCTGGGCCAGTGA-3'
GlkB-A460R-2	5'-TCACTGGCCCAGCATAACAGCGCTTCTTACAGGCCACCGCCGAGAC-3'

Mutated codons are underlined.

cooperativity (high Hill number) and low affinity for glucose, whereas in the fast catalytic cycle the enzyme shows no cooperativity (low Hill number) and has high affinity for glucose (Kamata et al. 2004) (Fig. 3A). Our results are consistent with this hypothesis, since all the activating mutations we have studied (A456V, A460R, Y214C, and Y215A) produced enzymes with low cooperativity (low Hill number) and high glucose affinity (Table 2). Our results also suggest that in these mutants the fast catalytic cycle is much more important than the slow catalytic cycle and that all these mutations favor the closed, active conformation of the enzyme.

In this work, we present evidence that modifications in either the $\alpha 5$ or the $\alpha 13$ helices of human glucokinase can dramatically affect the performance of the protein as a glucose phosphorylating enzyme. To understand these results we have evaluated the impact of the mutations at the structural level by introducing these mod-

ifications into the closed active and super-open inactive GlkB structure models (Figs. 3,4).

We describe that a K458R mutation lowers both glucose and ATP affinity, rendering an enzyme with poor glucose phosphorylating capacity. As seen in Figure 3, in both closed and super-open structures, the K458 residue is exposed to the solvent, but in the super-open form, the Lys residue forms a salt bridge with Glu216. The K458R change could reinforce this interaction, stabilizing the super-open inactive conformation of the enzyme. This interpretation would explain the observed reduction in glucose and ATP affinity.

We also describe three mutations that produce enzymes with a slightly higher activity (C457V, K459L, and A208G). We have evaluated these mutations using the model-based approach described above. As shown in Figure 3, the transition from the super-open to the closed conformation produces a change in the solvent exposure

Table 2. Kinetic parameters of mutated forms of glucokinase

Mutant	S0.5 (mM)	Hill number	$V_{\max}$ (units/mg)	K_m ATP (mM)
Wild type	7.52 $\pm$ 0.31	1.65 $\pm$ 0.04	19.9 $\pm$ 2.0	0.41 $\pm$ 0.03
A456V	1.81 $\pm$ 0.12	1.25 $\pm$ 0.20	32.4 $\pm$ 1.7	0.28 $\pm$ 0.02
C457V	5.90 $\pm$ 0.92	1.15 $\pm$ 0.22	22.9 $\pm$ 3.2	0.65 $\pm$ 0.06
K458R	12.28 $\pm$ 1.10	1.34 $\pm$ 0.11	21.8 $\pm$ 0.6	0.81 $\pm$ 0.15
K459L	7.03 $\pm$ 0.32	1.65 $\pm$ 0.19	33.4 $\pm$ 2.0	0.60 $\pm$ 0.04
A460R	1.94 $\pm$ 0.31	1.10 $\pm$ 0.15	23.3 $\pm$ 3.2	0.55 $\pm$ 0.03
A208G	5.66 $\pm$ 0.61	1.38 $\pm$ 0.23	17.0 $\pm$ 2.0	0.43 $\pm$ 0.08
Y215A	2.20 $\pm$ 0.19	1.33 $\pm$ 0.09	20.4 $\pm$ 2.3	0.65 $\pm$ 0.02
Y214C	1.87 $\pm$ 0.30	1.32 $\pm$ 0.15	41.6 $\pm$ 2.1	0.29 $\pm$ 0.02

Glucose-dependent activity (S0.5, Hill number, $V_{\max}$) and ATP-dependent activity (K_m) parameters were determined in purified GST-Gl kB fusion proteins. Values are the mean of at least three different purifications for each mutant $\pm$ standard deviation.

Figure 3. Effects of the A208G, C457V, K459L, and K458R modifications on the structure model of GlkB. (A) Closed (left) and super-open (right) conformations of wild-type glucokinase (Kamata et al. 2004). Large and small domains are colored in dark and in light gray, respectively. The $\alpha 5$ helix is colored in blue, the $\alpha 13$ helix is in yellow, and the connecting region I is in green. Residues that are modified are indicated as red ($\alpha 13$ helix) or purple ($\alpha 5$ helix) spheres. The position of glucose (cyan) in the closed conformation is also indicated. (B,C) Impact of described mutations in the closed (B) and super-open (C) conformations of glucokinase. The modified and interacting residue side chains are displayed as ball-and-stick structures with carbon atoms colored in orange and in pale green, respectively. Nitrogen atoms are colored in blue, sulfur in violet, and oxygen in red. The hydrogen bonds and salt bridges are denoted with dotted black lines. A rich area in hydrophobic residues (hydrophobic patch) is defined by a hatched line.

of the side chain of the residues located in the $\alpha 13$ -helix. The C457 side chain is completely exposed to the solvent in the super-open form, but is relocated to the boundary of a hydrophobic patch in the closed, active enzyme. The C457V change increases the side chain hydrophobicity, favoring interactions in the closed conformation of the enzyme. This effect is accentuated in the previously described A456V activating mutation (Christesen et al. 2002), located one residue before, where the new Val residue side chain is completely oriented toward the hydrophobic patch in the closed active form (not shown). On the contrary, the K459 side chain is pointed toward the $\beta 4$ strand in the super-open conformation, and is totally exposed to the solvent in the closed conformation. The K459L mutation is difficult to evaluate because the new residue, rigid and highly hydrophobic, does not seem to properly accommodate in either of the two conformations: The closed form would be unfavorable because of the exposure of the new Leu side chain to the solvent, but in the super-open form this residue would have steric problems with the $\beta 4$ strand. Finally, the A208 residue is located in the $\alpha 5$ helix and the mutation to a Gly residue may increase the flexibility of the helix. The higher flexibility of this key helix may promote the transition between the super-open and closed forms, decreasing the Hill number, although with a minimum repercussion on the kinetic enzyme parameters.

In addition, we describe two novel activating mutations of GlkB, A460R, and Y215A, affecting the $\alpha 13$ and the $\alpha 5$ helices, respectively. In the case of A460R, the new positive charge of the Arg side chain is well positioned to interact with Asp158 and Asp160, when the enzyme adopts the closed, active form (Fig. 4A shows an Arg rotamer forming two salt bridges with Asp158 and Asp160). In addition, the new Arg residue could also interact with Asp198, mimicking the interaction of the corresponding Arg residue in hexokinase I (Arg912) with Asp650 (Asp198 in GlkB; data not shown). These new interactions would lock the protein into the closed conformation, blocking the transition to the super-open form and preventing the enzyme from entering the slow catalytic cycle. This suggestion is consistent with our observation that the mutated enzyme presents a very low cooperativity (low Hill number) and a high affinity for glucose (Table 2). In the case of the Y215A, the substitution of Tyr by an Ala residue would eliminate a hydrogen bond between the former Tyr residue side chain and the Gly72 residue main chain in the super-open conformation (Fig. 4B). Furthermore, in the super-open conformation, Tyr215 is accommodated in a hydrophobic pocket formed by Trp99, Val101, Ile211, Leu451, and Val455 (not shown). As a result of the Y215A mutation, a reduction in these hydrophobic interactions is expected. The loss of the hydrogen bond and the reduction in the hydrophobic

Figure 4. Effects of the A460R and Y215A modifications on the structure model of GlkB. (A) Impact of the A460R mutation in the closed conformation of glucokinase. (B) Impact of the Y215A mutation in the super-open conformation of glucokinase. Similar drawing code as in legend of Figure 3.

interactions might favor the transition from the super-open to the closed, active form of the enzyme. In addition, the Tyr215 residue is located in the way of the transition movement that follows the $\alpha 13$ helix during the conformational change, so the replacement of the Tyr bulky side chain by the small one of the new Ala residue would favor the acquisition of the closed active conformation (Fig. 4B). A similar explanation could be applied for the activating Y214C mutation.

The two new mutations we describe (A460R and Y215A) adds to the group of activating GlkB mutations described so far: T65I, W99R, Y214C, V455M, and A456V (Glaser et al. 1998; Christesen et al. 2002; Gloyn et al. 2003; Cuesta-Munoz et al. 2004). Surprisingly, all the activating mutations are clustered in a defined area about 20 Å away from the glucose binding site, and therefore could represent an allosteric activator site that may be responsive to an endogenous activator still to be discovered (Grimsby et al. 2003; Kamata et al. 2004; Zelent et al. 2005). This fact has prompted the development of drugs that act as allosteric activators of GlkB (GKAs), that can be used as potential anti-diabetic drugs (Grimsby et al.

2003; Brocklehurst et al. 2004; Kamata et al. 2004). In one case, the drug contact points have been identified: V62, R63, M210, I211, Y214, Y215, M235, V452, and V455 (Kamata et al. 2004). It is worth noting that two naturally occurring activating mutations (Y214C and V455M) and one artificial mutation described in this work (Y215A) are GKAs contact sites. Moreover, the other naturally occurring activating mutations (T65I, W99R, and A456V) and the other artificial mutation described in this work (A460R) are located within or close to the activator site.

In conclusion, we demonstrate that substitutions in the $\alpha 5$ and the $\alpha 13$ helices which favor the interaction with surrounding residues in the closed, active conformation or eliminate those in the super-open, inactive conformation, and substitutions in the $\alpha 5$ helix that improve the flexibility of the region defined by these two helices, displace the super-open/closed equilibrium toward the acquisition of a closed, active conformation. These modifications improve the affinity of the enzyme toward its substrate and, therefore, its performance as a glucose phosphorylating enzyme.

Materials and methods

Site-directed mutagenesis

All the mutants used in this study were obtained using the QuikChange site-directed mutagenesis kit from Stratagene. Plasmid pUC-GlbK (Mayordomo and Sanz 2001), containing wild-type human pancreatic glucokinase gene, was used as the template in the PCR reactions using the oligonucleotides described in Table 1. Mutants were sequenced to confirm the absence of undesired mutations. Plasmids containing the corresponding mutations were digested with EcoRI and SalI, and the resulting fragments subcloned into plasmid pGEX6P-1 (Amersham Biosciences) to allow its expression in *Escherichia coli* as a glutathionyl S-transferase (GST) fusion protein.

Purification of GST-fusion proteins

Purification of the mutated GST-GlbK fusion proteins was carried out as described by Zhang et al. (1992), with some modifications. *E. coli* transformants harboring the different GST-fusions were grown in 50 mL of LB + ampicillin (50 µg/mL) at 30°C until the absorbance at 600 nm reached a value of around 0.5. IPTG was then added to a concentration of 1 mM and cultures were grown at 30°C for 2 h. Cells were harvested and resuspended in 20 mL of sonication buffer (100 mM Triethanolamine-HCl [pH 7.6], 10 mM MgCl₂, 2 mM DTT, 200 mM KCl, 10% glycerol, 0.1% Triton X-100, 2 mM EDTA, 2 mM PMSF, and complete protease inhibitor cocktail) (Roche). Cells were disrupted by sonication and the fusion proteins purified by passing the extracts through 1-mL bed volume of glutathione-sepharose columns (Amersham Biosciences). GST-GlbK fusion proteins were eluted from the column with 5 mM glutathione in sonication buffer. Samples were stored at -80°C. Purity of the recombinant GST-GlbK proteins was routinely screened by SDS-PAGE. At least three different purifications were carried out for each mutant.

Kinetic determinations

To determine the kinetic parameters of the recombinant GST-GlKB proteins, the standard glucokinase assay (Liang et al. 1995) was performed using a range of substrate concentrations. To determine the affinity for glucose the assay contained 5 mM ATP and a concentration of glucose ranging from 0.5 mM to 100 mM (11 different dilutions). Because of the cooperative nature of the GST-GlKB enzymes, data for glucose-dependent activity were fitted to the Hill equation to determine the $S_{0.5}$ (mM), V_{max} (units/mg protein) and Hill coefficient (h). One unit of glucokinase is defined as the amount of enzyme that is able to phosphorylate 1 μ mol of glucose per minute, at 30°C under the assay conditions. To determine the affinity for ATP the assay contained 100 mM glucose and a concentration of ATP ranging from 0.1 to 5 mM (11 different dilutions). Data for ATP-dependent activity was fitted to the Michaelis-Menten equation to determine the K_m (mM) (Hofstee plots). It has been previously described that the addition of an N-terminal GST tail to GlKB has no effects on enzyme kinetics (Liang et al. 1995).

Prediction of structural effects of glucokinase mutations

Crystal coordinates from the closed active (1V4S) and super-open inactive (1V4T) conformation of GlKB (Kamata et al. 2004) were visualized and mutations evaluated using O program (Jones and Kjeldgaard 1997). Figures 3 and 4 were generated with MolScript (Kraulis 1991) and Render 3D (Merritt and Bacon 1997) programs using the original or O-program modified PDBs.

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