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Crystal structure of E339K mutated human glucokinase reveals changes in the ATP binding site

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ABSTRACT

Human glucokinase (GK) plays an important role in glucose homeostasis. An E339K mutation in GK was recently found to be associated with hyperglycemia. It showed lower enzyme activity and impaired protein stability compared to the wild-type enzyme. Here, we present the crystal structure of E339K GK in complex with glucose. This mutation results in a conformational change of His416, spatially interfering with adenosine-triphosphate (ATP) binding. Furthermore, Ser411 at the ATP binding site is phosphorylated and then hydrogen bonded with Thr82, physically blocking the ATP binding. These findings provide structural basis for the reduced activity of this mutant.

1. Introduction

Human glucokinase (GK) (adenosine-triphosphate (ATP): D-glucose 6-phosphotransferase, EC 2.7.1.2) is a 52 kD enzyme that can phosphorylate glucose to glucose-6-phosphate. It is mainly expressed in liver, pancreas, gut and brain [1,2]. Also known as hexokinase IV or D, GK is a member of the hexokinase family that catalyzes the first step in glycolysis, playing a crucial role in glucose homeostasis. However, GK exhibits sigmoidal kinetics instead of the Michaelis–Menten kinetics of non-allosteric hexokinases [3]. It is also different from non-allosteric hexokinases in its lack of product inhibition and relatively lower glucose affinity. In pancreatic β-cells, GK functions as a glucose sensor [4]. In liver, it regulates glucose uptake and storage [5]. Given its key role in glucose homeostasis, mutations of the *GK* gene have profound influence on its functions in human cells. To date, about 600 mutations have been reported for the *GK* gene, including non-sense, missense, and frameshift mutations [6,7]. A large number of GK mutations are inactivating mutations associated with maturity-onset diabetes of the young, type2 (MODY2). MODY2 is a form of diabetes mellitus caused by mutations in an autosomal dominant gene. A number of GK mutations, such as T51I, W99R, Y214C, V455M and A456V, are activating mutations that result in persistent hyperinsulinemic hypoglycemia of infancy (PHHI) [8–11].

In 2004, Katama and colleagues determined the crystal structures of human GK in both active and inactive forms [12]. Their results showed that GK has a small domain and a large domain separated by a deep cleft and undergoes a large conformational change by the rotation of the small domain induced by glucose binding. Their structure provides a general explanation of how the enzyme activity of GK can be affected by some of the mutations, especially those located in the glucose and ATP binding pockets and those involved in the conformation transition. Recently, a new inactivating mutation E339K was found by Shen et al. in a Chinese family with hyperglycemia [13]. This E339K mutation, however, is situated far from the ATP binding site. To understand how this mutation affects GK activity, we determined the crystal structure of E339K mutated GK. This first mutated GK crystal structure will shed light on how the mutation causes the enzyme kinetic alterations and will provide a better understanding on how the enzyme works.

2. Materials and methods

2.1. Protein expression and purification

The E339K GK, with an N-terminal MGHHHHHHENLYFQGM tag and corresponding amino acid residues 12–465, was cloned into

Abbreviations: GK, glucokinase; AMPPNP, adenylyl-imidodiphosphate; SEP, phosphorylated serine; ATP, adenosine-triphosphate; IPTG, isopropyl-β-D-thiogalactoside; DTT, dithiothreitol; TCEP, tris (2-carboxyethyl) phosphine; G6PDH, glucose-6-phosphate dehydrogenase; NADP+, nicotinamide adenine dinucleotide phosphate

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pET-21a using *Nde*I and *Xho*I cloning sites and verified by sequencing. *Escherichia coli* strain BL21 (DE3) was transformed with the constructed plasmid. The cells were cultured at 37 °C in 1 l of LB medium containing 100 μg/ml of ampicillin until OD_{600} reached a value of 0.7–1.0. The temperature was lowered to 20 °C and expression was induced by an addition of 0.1 mM isopropyl-β-D-thiogalactoside. After cultivating for 16 h, cells were harvested by centrifugation. All subsequent handling was at 4 °C. The pellets were resuspended in 50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol, 2 mM dithiothreitol (DTT), 50 mM imidazole and lysed by sonication. After centrifugation (30 min at 12,000×*g*), the supernatant was applied onto a Ni-NTA column (Qiagen). The target protein was eluted with 250 mM imidazole and loaded onto a Resource Q column (GE Healthcare) pre-equilibrated with 50 mM Tris pH 8.0, 150 mM NaCl, 10% glycerol and 2 mM DTT. Protein was eluted with a linear NaCl gradient (150–400 mM). Fractions containing GK were concentrated and loaded on a Superdex 200 column (GE Healthcare) pre-equilibrated with 20 mM Tris pH 8.0, 50 mM NaCl, and 5 mM tris (2-carboxyethyl) phosphine. Wild-type GK used in the following enzyme assay was prepared similarly.

2.2. Crystallization, data collection, and processing

For crystallization trials, glucose was added to the protein solution at a final concentration of 50 mM. Crystals of E339K GK were grown at 20 °C using the sitting drop vapor diffusion technique. The best crystals were obtained within two weeks by mixing equal volumes of protein solution (5 mg/ml) and reservoir solution containing 25% (w/v) PEG3350, 0.1 M HEPES pH 7.3, 0.2 M ammonium sulfate. For cryo data collection, crystals were sequentially immersed in mother liquors containing an additional 10% and 20% (v/v) glycerol as cryoprotectant. Data were collected at the Shanghai Synchrotron Radiation Facility, using beamline BL17U1 (λ = 0.9793 Å) at 100 K with a MX-225 CCD (Marresearch, Germany). The data were processed with MOSFLM [14] and SCALA from the CCP4 suite [15]. The structure was solved by molecular replacement using MOLREP in CCP4 and wild-type GK structure (PDB entry 1V4S) as the search model. Interactive model building and refinement were carried out using Coot [16] and PHENIX [17]. The final model was evaluated with MolProbity [18]. The coordinates and structure factors have been deposited in the Protein Data Bank under the accession code 3QIC. The data collection and structure refinement statistics are listed in Table 1. All structure figures were created using PyMOL [19].

2.3. Enzyme assay

Both wild-type and E339K mutant GK activities were measured using glucose-6-phosphate dehydrogenase (G6PDH)/nicotinamide adenine dinucleotide phosphate ($NADP^+$) coupled enzyme assay with some modification [20]. The assay buffer contained 50 mM HEPES pH 7.5, 50 mM KCl, 2 mM DTT, 0.1% BSA, 5 mM $MgCl_2$, 0.5 mM $NADP^+$ and 2.5 units/ml G6PDH. To determine glucose and ATP-related kinetic parameters, glucose was varied (0–100 mM) with a fixed saturating concentration of 5 mM ATP and then ATP was also varied (0–5 mM) with a fixed saturating concentration of 100 mM glucose. Assays were performed in a 96-well microplate with a final volume of 100 μL per well at 30 °C by monitoring the absorbance at 340 nm. The kinetic parameters were calculated as described previously [20,21].

2.4. Phosphorylation site analysis by mass spectrometry

Protein samples were prepared by SDS–PAGE and sent to Center of Technical Service for Life Sciences, University of Science and Technology of China, Hefei, China. Sample processing, data searching and analysis were all performed there.

Data-dependent MS/MS was performed. Electrospray spectra were acquired on a Thermo-Finnigan LTQ linear quadrupole ion trap mass spectrometer. The samples were digested by trypsin. Experimental results for the LTQ data were acquired using a C18 Reversed Phased Capillary Column. The mobile phase consisted of mobile A (0.1% formic acid in ddH_2O) and mobile B (100% acetonitrile). All spectra were obtained in positive ionization mode. Sample analyses were performed using data-dependent/dynamic exclusion with a repeat count of one to maximize coverage. All data files were searched against the human database.

Table 1
Data collection and refinement statistics

Data collection parameters	
Space group	$P4_32_12$
Cell dimensions (Å)	$a = b = 80.74$, $c = 178.41$
Resolution range (Å) (highest shell)	41.19–2.20 (2.32–2.20)
No. of observations	184 277
No. of unique reflections	30 831
Completeness (overall/outer shell) (%)	99.9/100.0
I/σ_I (overall/outer shell)	13.2/3.4
R_{merge} (%)	7.7(54.4)
Refinement parameters	
R_{work}/R_{free} (%)	21.3/25.2
No. of non-hydrogen atoms	3611
No. of water molecules	49
R.m.s deviations from ideal geometry	
Bond lengths (Å)	0.008
Bond angles (°)	1.097
Avg. B-factor	45.0
Ramachandran plot (%)	
Favored region	97.6
Allowed region	2.4

3. Results

3.1. Overall structure of E339K GK

The X-ray crystal structure of the E339K GK (glucose binding form) was determined at a resolution of 2.2 Å by molecular replacement. The crystal belongs to space group $P4_32_12$ with one molecule in the asymmetric unit. The refined structure of E339K GK consists of 453 residues, from Tyr7 to Lys459. The overall structure of the E339K GK is similar to the active form of wild-type GK (PDB entry 1V4S) with a root mean square deviation (r.m.s.d.) of 1.1 Å between Cα atoms of the two structures. Superimposing the mutant GK structure to the wild-type GK structure with only the large domain reveals a slight movement of the small domain (Fig. 1A). R.m.s.d. for the large and the small domain between the two structures is 1.0 and 1.6 Å, respectively. Significant differences are observed around the mutated residue.

The electron density map for K339 and surrounding residues is shown in Fig. 2A. The E339K mutation was also confirmed by mass spectrometry (data not shown). Surprisingly, the electron density map clearly showed extra density around the OG atom of Ser411 (Fig. 2B). It can be modeled very well as a phosphate (PO_3) covalently linked to residue Ser411. Mass spectrometry data confirmed the phosphorylation at Ser411. One phosphate was found in the peptide from residues 404–414 and was located in Ser411 (Fig. 3). No phosphorylation at Ser411 was found in the wild-type GK (data not shown).

3.2. Structural comparison of E339K with wild-type GK

In wild-type structure, Glu339 is located in the loop between helix α9 (residues 331–338) and helix α10 (residues 345–355).