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Structural Basis for Allosteric Regulation of the Monomeric Allosteric Enzyme Human Glucokinase

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Summary

Glucokinase is a monomeric enzyme that displays a low affinity for glucose and a sigmoidal saturation curve for its substrate, two properties that are important for its playing the role of a glucose sensor in pancreas and liver. The molecular basis for these two properties is not well understood. Herein we report the crystal structures of glucokinase in its active and inactive forms, which demonstrate that global conformational change, including domain reorganization, is induced by glucose binding. This suggests that the positive cooperativity of monomeric glucokinase obeys the "mnemonical mechanism" rather than the well-known concerted model. These structures also revealed an allosteric site through which small molecules may modulate the kinetic properties of the enzyme. This finding provided the mechanistic basis for activation of glucokinase as a potential therapeutic approach for treating type 2 diabetes mellitus.

Introduction

Glucokinase (hexokinase IV or D) is a cytoplasmic enzyme that phosphorylates glucose and triggers glucose utilization and metabolism. Glucokinase expressed in liver and pancreas is thought to be a component of the glucose sensor controlling plasma glucose levels; glucose-mediated activation of the enzyme in pancreatic β cells ultimately stimulates insulin secretion, while that in liver enhances hepatic glucose uptake and glycogen synthesis (Van Schaftingen et al., 1994; Matschinsky et al., 1998). Both of these effects in turn reduce plasma glucose levels. The critical role of glucokinase in glucose homeostasis is indicated by enzyme variants observed in human subjects. Glucokinase variants with decreased activity are associated with maturity-onset diabetes of the young type 2 (MODY-2) (Vionnet et al., 1992), whereas variants with increased activity result in mild hypoglycemia and increased glucose tolerance (Glaser et al., 1998; Christesen et al., 2002).

The role of glucokinase as a glucose sensor is due its allosteric properties. The activity of glucokinase exhibits a sigmoidal glucose dependency instead of the Michaelis-Menten hyperbolic dependency of nonallosteric enzymes (Storer and Cornish-Bowden, 1976). The inflexion point on the sigmoidal glucose saturation curve is at 8 mM, which is comparable to normal plasma glucose levels, thus guaranteeing optimal responsiveness at physiological glucose levels. However, the molecular mechanism of this glucose sensor has yet to be elucidated, although allosteric kinetics have been extensively studied and structural analyses have been performed in several other allosteric enzymes (Kantrowitz and Lipscomb, 1988; Barford and Johnson, 1989; Schirmer and Evans, 1990; Iwata et al., 1994; Mattevi et al., 1996; MacRae et al., 2002). For these oligomeric enzymes, the active sites on several subunits are activated and inactivated in a concerted manner (Monod et al., 1965), but such a mechanism does not appear to be employed by glucokinase, because glucokinase is monomeric enzyme with a single active site (Cardenas et al., 1978; Connolly and Trayer, 1979). Several potential mechanisms for cooperativity of glucokinase have been proposed (Cornish-Bowden and Cardenas, 1987), but none has been proven, due to the lack of a crystal structure for glucokinase, although the various conformations of yeast hexokinase and human hexokinase I have been determined (Anderson et al., 1978; Bennett and Steitz, 1978; Aleshin et al., 1998a, 1998b, 2000; Rosano et al., 1999). The crystal structures of yeast hexokinase indicated that binding of glucose induced the conformational change from an open form to a closed form. Similar conformational change was observed in crystal structures of hexokinase I, which is a glucokinase isozyme and exhibits a high degree of sequence homology (54.4%). However, this structural information gave no insight into the positive cooperativity of glucokinase, because yeast hexokinase and human hexokinase I do not exhibit sigmoidal dependency for glucose (Katzen and Schimke, 1965; Magnani et al., 1992). In this report, we successfully analyzed the crystal structures of human glucokinase in both its active and inactive forms. Substrate binding to the active site induced a global alteration in enzyme conformation, which can account for the allosteric properties of monomeric glucokinase. These data provide new insight into the structural mechanisms by which glucokinase is able to function as a glucose sensor.

Glucokinase plays an important role in the regulation of glucose metabolism and thus represents a novel molecular target for drug development in type 2 diabetes (Matschinsky, 2002; Grimsby et al., 2003). We recently identified small molecule synthetic activators that activated glucokinase and decreased blood glucose levels in several murine models of diabetes. The crystal structures clearly showed that one of these activators binds to an allosteric site in the active form of glucokinase and allosterically activates the enzyme. The data from the current study should provide valuable information for the development of novel glucokinase activators as a new treatment for diabetes.

Results and Discussion

Structure Determination of Human Glucokinase

In order to elucidate the molecular mechanism of the allosteric regulation of glucokinase, we analyzed the

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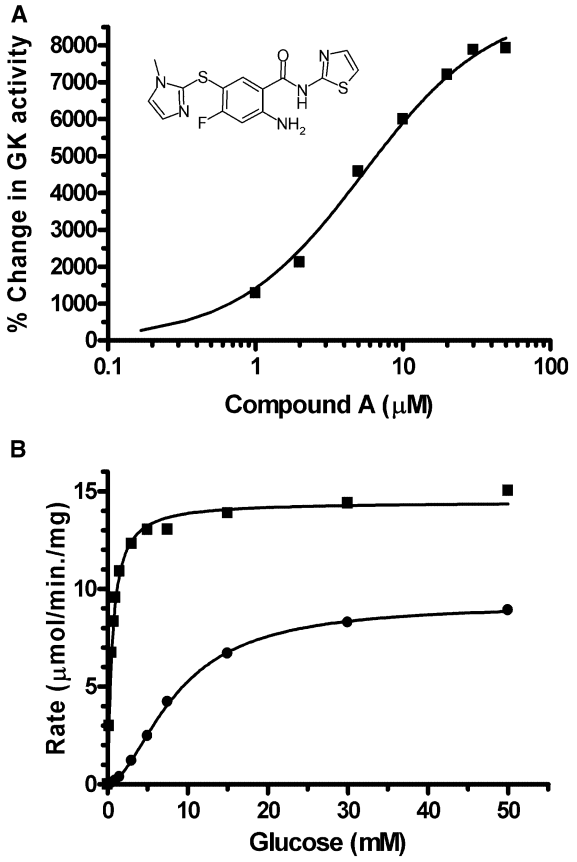

Figure 1. Synthetic Activator of Glucokinase

(A) Chemical formula of compound A, *N*-Thiazol-2-yl-2-amino-4-fluoro-5-(1-methylimidazol-2-yl)thiobenzamide (inset), and the effect of compound A on glucokinase enzymatic activity at 0.5 mM glucose concentration.

(B) Rate versus glucose concentration plot in the absence (solid circle) and presence of compound A (solid square, 30 μM). The data were fit to Hill equation. The graph shows actual data points (symbols) and the best-fit line.

crystal structures of human glucokinase in both its active and inactive forms. For crystallization, we deleted the 11 or 15 N-terminal residues of hepatic glucokinase in order to reduce structural flexibility. Hepatic glucokinase differs from pancreatic β cell glucokinase in the 15 N-terminal residues as a result of the alternative splicing of its mRNA, but both forms possess similar enzymatic properties (Tanizawa et al., 1991; Nishi et al., 1992). The deletion mutants glucokinase GK(Δ1-11) and GK(Δ1-15) showed similar values for $S_{0.5}$ (concentration of glucose at which glucokinase shows the half activity of V_{max}), V_{max}, and Hill coefficient as wild-type glucokinase.

To determine the active conformation of glucokinase, we cocrystallized GK(Δ1-11) with glucose and a synthetic activator, compound A (Figure 1A). Compound A is a potent activator with equal activity toward deletion mutants and wild-type glucokinase. This compound changes the shape of the glucose saturation curve from sigmoidal to a Michaelis-Menten hyperbolic curve and increases V_{max} (Figure 1B). In the presence of 30 μM of compound A, the Hill coefficient was decreased from 1.78 to 1.11, and the $S_{0.5}$ value for glucose was decreased from 8 mM to 0.6 mM. At the same time, V_{max} was increased by ~1.6-fold. To determine the inactive conformation of glucokinase, we used GK(Δ1-15) due to its superior production of crystals. The crystal structures of GK(Δ1-11) and GK(Δ1-15) were analyzed at resolutions of 2.3 Å and 3.4 Å, respectively (Table 1).

Active Conformation of Glucokinase

The crystal structure of GK(Δ1-11) in complex with glucose and compound A reveals a palm shape (Figure 2A). The polypeptide chain is composed of 448 amino acid residues that are distinctly folded into two domains of unequal size; the large and small domains. These are separated by a deep cleft, which forms the active site for phosphorylation, and an α anomer of the glucose molecule was observed to be bound to this active site in GK(Δ1-11). The electron density of compound A was identified in an allosteric site at the interface between the two domains.

The folding pattern observed in crystal structure of GK(Δ1-11) is identical to that of hexokinase I (Aleshin et al., 1998a, 1998b, 2000; Rosano et al., 1999). Hexokinase I is an isozyme of glucokinase and is expressed mainly in the brain. The molecular weight of hexokinase I is approximately twice that of glucokinase and is probably the result of gene duplication. Although the N-terminal and C-terminal halves of hexokinase I show 49.9% and 54.4% sequence identity with glucokinase, respectively, the enzymatic activity of hexokinase I does not exhibit a sigmoidal glucose response, and this is also true of hexokinase II and III (Katzen and Schimke, 1965; Magnani et al., 1992). The crystal structures of hexokinase I indicate that binding of glucose to the center cleft induces a domain rotation from an open form to a closed form in the same manner as is observed with yeast hexokinase (Bennett and Steitz, 1978). When compared with hexokinase I, the spatial relationship between the large and small domains of GK(Δ1-11) complexed with glucose and compound A corresponds to the closed form. As shown in Figure 3A, GK(Δ1-11) and the C-terminal half of the closed form of hexokinase I show very similar structures in which the root-mean-squared difference of Cα atoms of the two structures is 1.09 Å.

The glucose binding site is located in the interdomain cleft and is composed of residues of the large domain (Glu256 and Glu290), the small domain (Thr168 and Lys169), and connecting region II (Asn204 and Asp205) (Figure 4A). In comparison with the glucose binding site of hexokinase I, we were unable to identify any structural differences that might account for the low affinity for glucose.

Although the closed form of glucokinase is almost identical to that of hexokinase I, a substantial difference is seen in the region between Ser64 and Gly72 of GK(Δ1-11) (Figure 3A). This region corresponds to connecting region I and represents a flexible structure that is exposed to the solvent (Figure 4B). In contrast, the corresponding region of hexokinase I forms a rigid structure. The allosteric site is located near this glucokinase-specific structure and is surrounded by connecting region

Table 1. Data Collection Parameters and Refinement Statistics

Crystal	Complex form	Free form
Source	PF BL6B	Spring-8 BL32B2
Wavelength	1.000 Å	1.000 Å
Resolution (Å)	2.3 (2.38–2.3)	3.4 (3.58–3.4)
Total no. of observations	135,203	78,693
No. of unique observations	27,916	12,839
Completeness (%)	98.5 (96.1)	100.0 (100.0)
R_{sym} [a] (%)	3.3 (12.8)	8.0 (21.4)
$<I>/<\sigma(I)>$	31.3 (4.2)	6.8 (3.3)
Refinement statistics[b]		
Resolution (Å)	50–2.3	50–3.4
No. of reflections in refinement[c]	26,981	12,436
No. of protein atoms	3691	3326
No. of atoms in ligands	35	0
No. of solvent atoms	149 H_2O, 1 Na^+	7 H_2O, 2 SO_4^{2-}, 1 Na^+
$R_{working}$ [d] (%)	23.2	23.8
R_{free} [d](%)	27.4	30.6
Average B factor (Å) ^{2}		
Protein	47.5	39.2
Solvent	41.4	39.3
Glucose	30.2	—
Activator	45.8	—
Root-mean-squared deviations		
Bond lengths (Å)	0.006	0.010
Bond angles (°)	1.16	1.23

[a] R_{sym} is the unweighted R values on I of symmetry-related reflections ($\Sigma_{hkl} |I_{hkl} - <I_{hkl}>| / \Sigma_{hkl} I_{hkl}$).
[b] Residues in the disordered region were eliminated from the model: 155–179 of the free form.
[c] Only reflections with $|F_{obs}| > 2\sigma$ were used in the refinement.
[d] $R_{working} = \Sigma_{hkl} |F_{obshkl} - F_{calchkl}| / \Sigma_{hkl} |F_{obshkl}|$, for all reflections $>2\sigma$. R_{free} is calculated from reflections not used in model refinement.

I, the large domain (β1 strand and α5 helix), and the small domain (α13 helix) (Figure 3B). Compound A makes hydrophobic interactions with Val452 and Val455 on the α13 helix of the small domain. At the same time, compound A interacts with residues in the large domain, hydrogen bonding with Arg63 and Tyr215 and making hydrophobic interactions with Val62, Met210, Ile211, Tyr214, and Met235 (Figure 4B). The molecular details of compound A binding provide us with useful information for designing the next generation of activators with improved properties, although the activation mechanism of compound A is still not clear based solely on the structure of the active conformation because the allosteric site is approximately 20 Å away from the active site.

Inactive Conformation of Glucokinase

Free form GK(Δ1-15) showed a very different overall conformation (Figure 2B). The structure of the large domain was almost the same as that of complexed GK(Δ1-11), in which the root-mean-squared difference of Cα atoms is 0.93 Å. However, the orientation and organization of the small domain differed substantially. The main part of the small domain was rotated about 99° when compared to the closed form. Because the spatial relationship between the two domains is also different from that of open form hexokinase I, we referred to the structure of free form glucokinase as the "super-open form." Surprisingly, the organization of the small domain was also different in the super-open form. As shown in Figure 2A, the α13 helix, which is the C-terminal region of glucokinase, is part of the small domain in the closed form, but it was released from the small domain and lies between the two domains in the super-open form. As a result, the interface between the large and small domains also differs from that of the closed form. In the closed form, glucose binds to the bottom of the deep interdomain cleft, and one rim of the glucose binding pocket is formed by residues 157–179 of the small domain (Figure 4A). However, due to domain rotation and disorder of residues 157–179, the glucose binding site is exposed to the solvent in the super-open form (Figure 4C). Since several important residues for enzymatic activity are missing from this site, the super-open form of glucokinase must be inactive, even if glucose was able to bind to the imperfect active site with low affinity. The side chain of Asp205 in connecting region II is oriented in the opposite direction when compared to the closed form, and interaction between Asp205 and glucose may trigger the conformational change to the closed form.

The structure of the allosteric site is also changed (Figure 4D). Connecting region I shows a rigid turn structure instead of the loose structure seen in the closed form, and the α13 helix is released from the small domain. Thus, the binding site of compound A is completely absent from the super-open form.

Conformational Change between Active and Inactive Forms

The two crystal structures indicated that glucokinase undergoes a much larger conformational change than hexokinase I during the catalytic cycle, although the active conformations (closed form) are almost identical. We analyzed the conformational change of glucokinase by comparing it with that of hexokinase I (Figure 5). In the closed form, small domains of glucokinase and

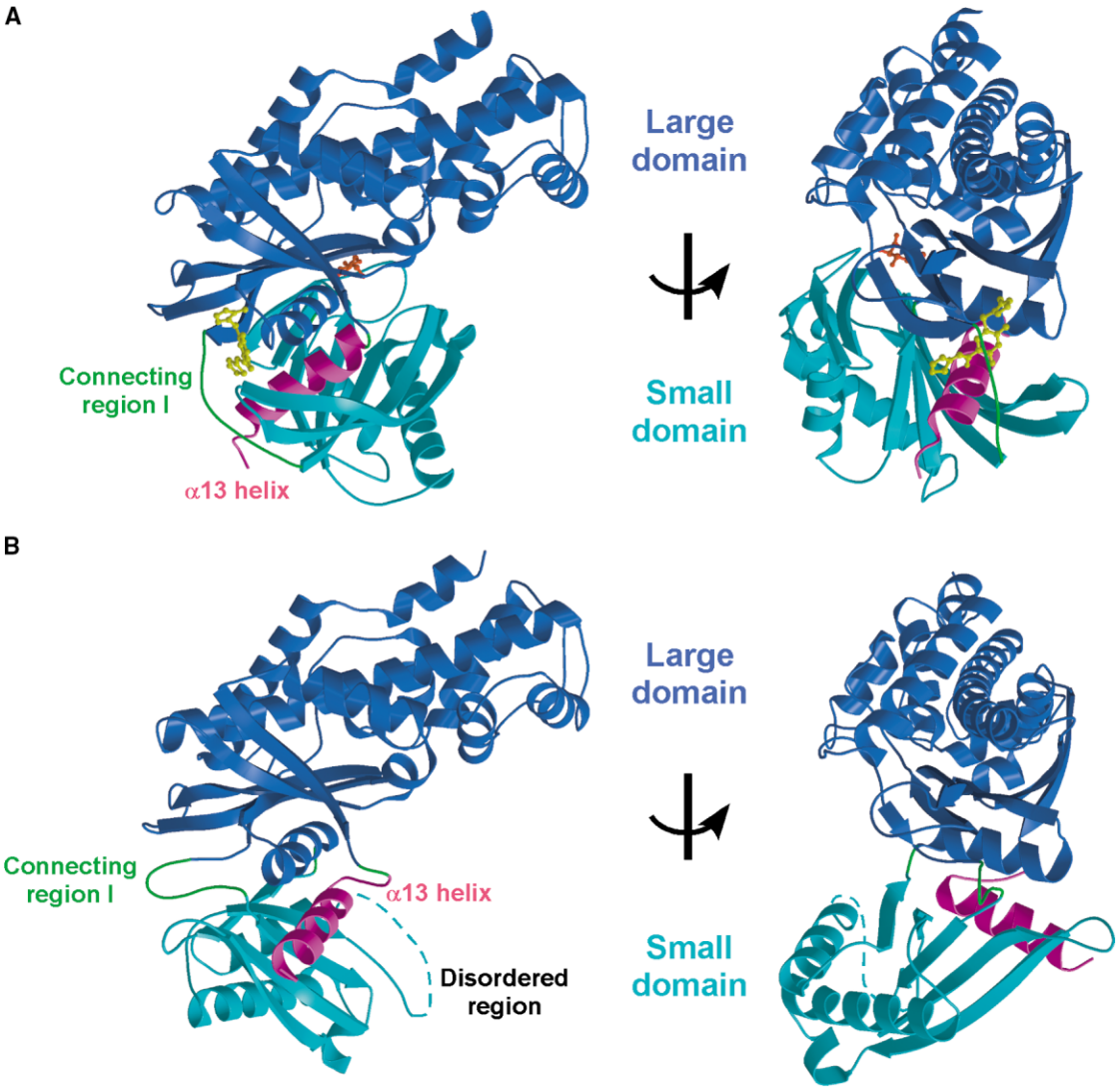

Figure 2. Overall Structures of Glucokinase

(A) Ribbon drawing of the GK(Δ1-11) structure complexed with glucose (red, ball-and-stick model) and compound A (yellow, ball-and-stick model). The spatial relationship of the large domain (blue) and the small domain (cyan) exhibited a closed form. The two domains are connected by connecting regions I–III (green). The α13 helix (magenta) is included in the small domain of the closed form.

(B) Ribbon drawing of free form GK(Δ1-15). The spatial relationship of two domains was designated the super-open form. The α13 helix is released from the small domain in the super-open form. Figures were prepared with MOLSCRIPT (Kraulis, 1991) and Raster3D (Merritt and Murphy, 1994).

hexokinase I show the same three-layer architectures (Gerstein et al., 1994). The α13 helix is in the inner layer, which forms the domain interface with the α5 helix of the large domain. During the conformational change of hexokinase I, the three layers of the small domain rotate about 12° as a rigid body, with connecting regions I–III acting a hinge (Aleshin et al., 1998a). In contrast, while the core region of the middle and outer layers of glucokinase rotate by 99° as a rigid body, the inner layer of glucokinase moves in a different direction by an approximately 70° rotation. As a result, the α13 helix of the inner layer is completely released from the small domain, although the three-layer architecture is conserved in the super-open form. During this large conformational change, the α13 helix moves from a perpendicular to a parallel orientation relative to α5 helix of the large domain while the middle layer of the β sheet rotates by approximately 120° relative to the α13 helix, if the movements of both layers are combined. These sliding movements require the breakage and reformation of numerous interactions among the layers, which is not necessary for the hinge movement observed in the closed-open conformational change of hexokinase I. Therefore, the transition between closed and super-open forms takes longer than the closed-open conformational change, due to the larger energy barrier that likely exists between the two conformations, even if the super-open form is more stable in the absence of glucose.

Structural differences in connecting region I between

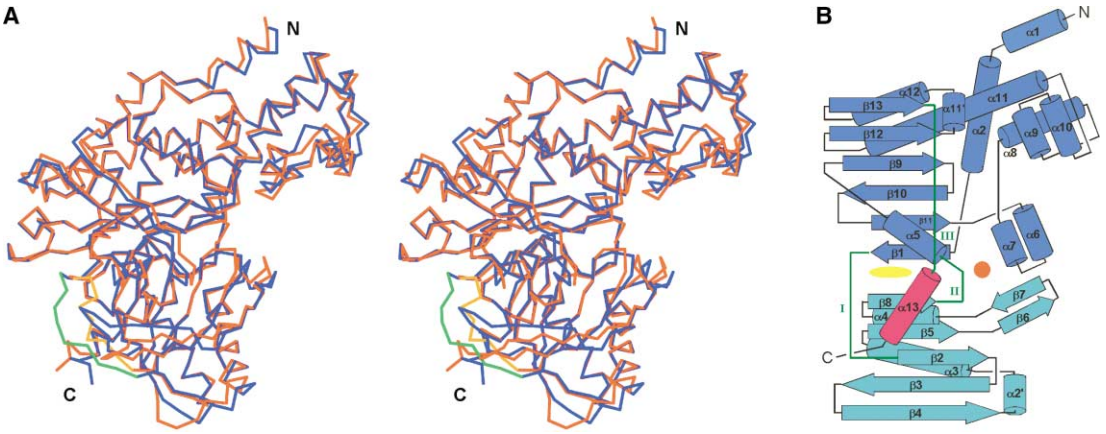

Figure 3. Active Conformation of Glucokinase

(A) Stereo view of superposed Cα models of complex form glucokinase (blue and green) and the C-terminal half of closed form hexokinase I (Aleshin et al., 2000) (red and orange). The structures of connecting region I are different for the two enzymes (green and orange).

(B) Schematic drawing of domain composition and secondary structure of GK (Δ1-11) in complex with glucose and compound A. Glucokinase is composed of the large domain (blue) and the small domain (cyan and magenta). Connecting regions I–III (green) connect the two domains. The C-terminal region of glucokinase forms the α13 helix (magenta) and is included in the small domain of GK(Δ1-11). The glucose binding site (red circle) and an allosteric site (yellow oval) are positioned between two domains.

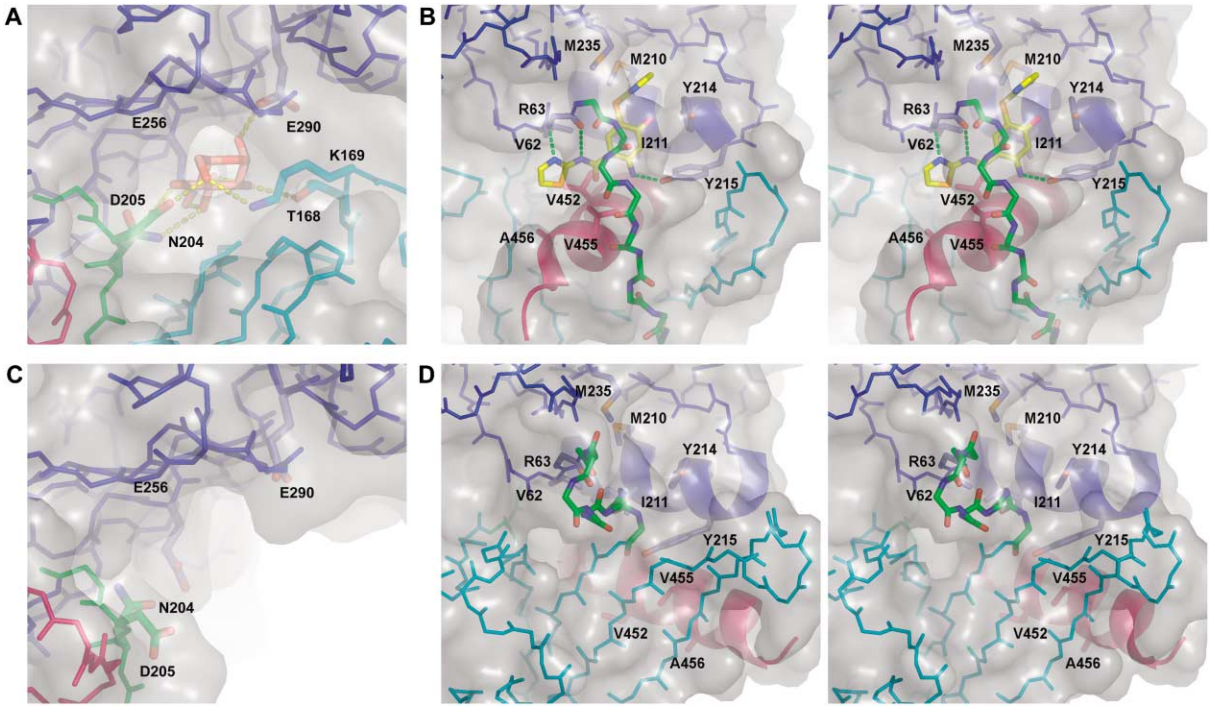

Figure 4. Structures of the Active Site and the Allosteric Site

(A) Glucose binding site of closed form glucokinase. Glucose binds to the bottom of the deep cleft between the large domain (blue) and the small domain (cyan). E256, E290 (blue stick model) of the large domain, T168, K169 (cyan stick model) of the small domain, and N204, D205 (green stick model) of connecting region II (green) form hydrogen bonds with glucose (pink stick model).

(B) Stereo view of the allosteric site in closed form glucokinase. The allosteric site is located below connecting region I (green stick model). Compound A (yellow stick model) forms hydrogen bonds with R63 and Y215 (blue stick model) and hydrophobically interacts with M210, Y214 (blue stick model) of the α5 helix (blue ribbon model) and V452, V455 (magenta stick model) of the α13 helix (magenta ribbon model).

(C) Glucose binding site of super-open form glucokinase. The active site is exposed to solvent. Color coding is as described for Figure 4A.

(D) Stereo view of the allosteric site in super-open form glucokinase. Due to the conformational change of connecting region I and the α13 helix, compound A is unable to bind. Color coding is as described for Figure 4B.

The figure was prepared using the PyMOL Molecular Graphics System (DeLano Scientific LLC, San Carlos, CA; http://www.pymol.org).

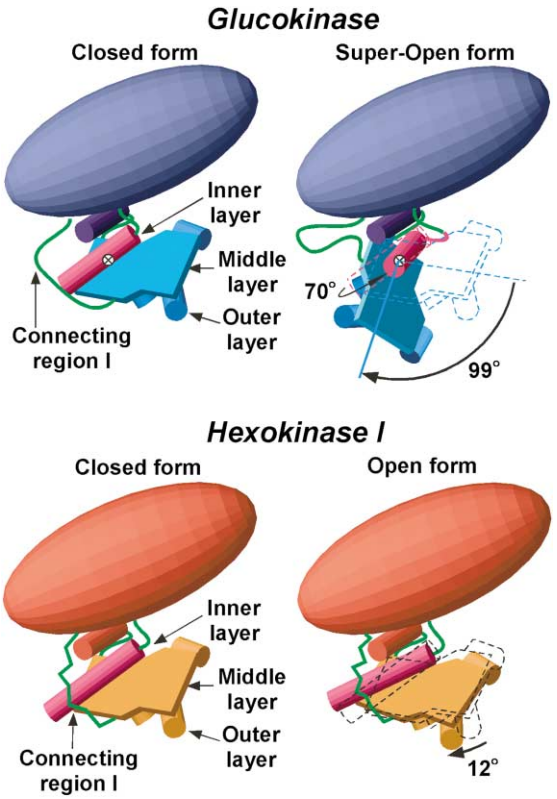

Figure 5. Schematic Drawing of Conformational Changes in Glucokinase and Hexokinase I

The small domains of glucokinase and hexokinase I exhibited three-layer architecture; the inner layer of the α13 helix (magenta cylinder), the middle layer of the β sheet (cyan plate or orange plate), and the outer layer of the α2′, α3, and α4 helices (cyan cylinders or orange cylinders). During the closed-super-open conformational changes of glucokinase, the core region of the middle and outer layers rotates 99° around an axis perpendicular to the plane indicated by the "X." The inner layer moves in a different direction from the other two layers due to the loose connecting region I. During the closed-open conformational change of hexokinase I, the three layers of the small domain rotate 12° as a rigid body due to the rigidity of connecting region I, which tightly holds the α13 helix.

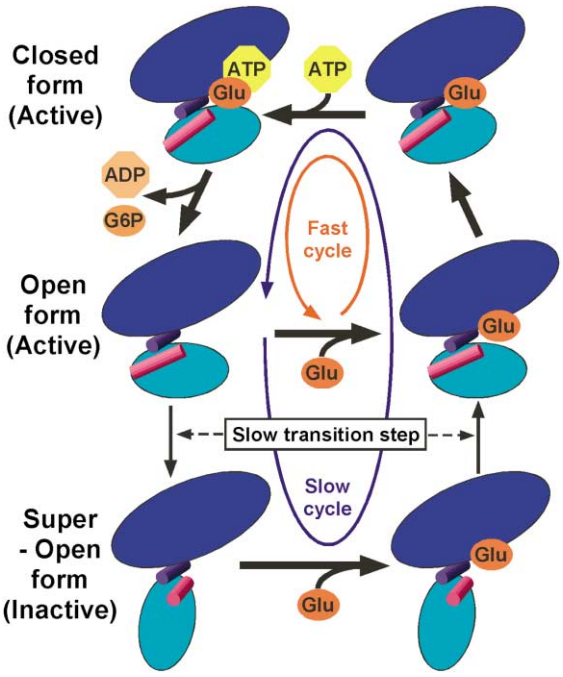

Figure 6. Kinetic Model of Glucokinase

Glucokinase appears to exist in three conformations and to have two catalytic cycles. The ratio of two catalytic cycles is responsible for the sigmoidal response to glucose. Although ordered process in the slow cycle of glucokinase was indicated by isotope-exchange experiment, the addition of substrates in the fast cycle may be random.

glucokinase and hexokinase I explain why much larger domain motion occurs in glucokinase. As shown in Figure 4B, residues 64–72 of the closed glucokinase exhibit a loose structure that is exposed to the solvent. Below this region, there is a large space in which a synthetic activator may bind. The corresponding region of hexokinase I forms a rigid structure that interacts with the α5 and α13 helices, and there is no space for compound A to bind. During the conformational change in hexokinase I, residues in connecting region I change their dihedral angles but keep their tightly packed structure. This rigid structure in connecting region I restricts the sliding movement of the α13 helix and prevents the helix release observed in glucokinase. In contrast, the α13 helix of glucokinase is easily released from the small domain because of the loose structure of connecting region I, thus facilitating a larger rotation of the small domain.

Although we have only succeeded in crystallizing the closed form and the super-open form, glucokinase may also exist in an intermediate state, an open form similar to that of hexokinase I, as indicated by the high degree of sequence homology and its structural similarity with closed form hexokinase I. In the open form, the center cleft would be sufficiently open to release glucose 6-phosphate and to take in a new glucose molecule, while the α13 helix would maintain similar interactions with the α5 helix and the middle layer of the small domain as in the closed form.

Mechanism of Positive Cooperativity

As described in previous sections, the crystal structures of glucokinase clearly show a large conformational change between the active form and the inactive form. In addition, an intermediate open form is also expected based on analysis of domain movement. Based on this assumption and the structural data, we have devised a kinetic model of glucokinase that accounts for the sigmoidal nature of its enzymatic activity with plasma glucose level.

As shown in Figure 6, glucokinase may exist in three conformations, and the conformational changes between the open form and the super-open form are slower steps than the closed-open conformational change observed during glucose phosphorylation. Unless glucose is present, the majority of glucokinase remains in the thermodynamically favorable super-open form. When glucose binds to the super-open form, glucokinase undergoes a conformational change to the open form. This

change may be triggered by interaction between Asp205 and glucose and, as indicated by the crystal structures, may take longer than the enzymatic reaction itself. After the slow transition to the open form, the enzymatic reaction is then carried out by changing to the closed form in the presence of ATP. After the reaction is completed, glucokinase returns to its open form in order to release glucose 6-phosphate and ADP. Because the open form slowly equilibrates with the super-open form, a large proportion of the glucokinase stays in the open form for some time. If glucose binds to the open form during this period, glucokinase re-enters the catalytic cycle. If this does not happen, glucokinase returns to the super-open form. In other words, glucokinase has two catalytic cycles: a "slow cycle" and a "fast cycle." The ratio of the two cycles is determined by the concentration of glucose and the length of time that glucokinase remains in the open form. At low glucose concentrations, glucokinase mainly uses the slow cycle with its low-affinity structure, because the super-open form is more stable than other forms in the absence of glucose. When glucose concentrations are sufficiently high, glucokinase uses the fast cycle with its high-affinity structure. This shift of the major catalytic cycle explains the low affinity and the sigmoidal saturation curve for glucose that glucokinase exhibits, as well as its ability to regulate blood glucose levels in vivo.

Among several proposed models for positive cooperativity of a monomeric enzyme, the "mnemonical mechanism" is consistent with our model (Storer and Cornish-Bowden, 1977). The mnemonical mechanism is based on the concept that an enzyme "memorizes" the active conformation after the catalytic reaction and subsequently "forgets" it some time afterwards. Isotope exchange studies supported this mechanism and indicated that glucokinase follows a predominantly ordered mechanism, with glucose binding first and glucose 6-phosphate released last (Gregoriou et al., 1981). Moukil and colleagues postulated the existence of the super-open form and showed that the mnemonical mechanism simulated the kinetic behavior of mutants and wild-type glucokinase very well (Moukil and Van Schaftingen, 2001). In addition, several experiments demonstrated the existence of the slow transition step during the catalytic cycle of glucokinase (Lin and Neet, 1990; Neet et al., 1990). Comparison with hexokinase I also provides supporting evidence for the structural basis of cooperativity. Hexokinase I shows a Michaelis-Menten hyperbolic saturation curve for glucose, in spite of the very similar active structures. Because hexokinase I exists only in the closed form or the open form and does not exist in the super-open form due to differences in the structure of connecting region I, hexokinase I uses the fast catalytic cycle at any glucose concentration. The lack of a slow transition step results in a Michaelis-Menten hyperbolic saturation curve for glucose.

Mechanism of Activation by Synthetic Activator and Mutagenesis

Allosteric activators for glucokinase as potential drugs for type 2 diabetes have been pursued in recent years. Allosteric activators change the shape of the glucose saturation curve from sigmoidal to a Michaelis-Menten hyperbolic curve and also increase V_{max}. From the crystal structure of GK(Δ1-11) in complex with glucose and compound A, we identified an allosteric site and determined the binding mode of a synthetic allosteric activator. Taken together with the kinetic model proposed in the previous section, this structural information is indicative of the activation mechanism of compound A. It is apparent that binding of compound A to the allosteric site sterically inhibits the release of the α13 helix from the small domain. As result, glucokinase is unable to change to the super-open conformation and can exist only in the open or closed forms even at low concentrations of glucose, as is observed for hexokinase I. As discussed above, the slow transition step between the inactive and active forms is critical for the regulatory role of this monomeric cooperative enzyme; glucokinase exhibits a Michaelis-Menten saturation curve in the presence of compound A. Under this condition, glucokinase shows high affinity for glucose in the same range of other hexokinases that is consistent with very similar active conformations. The increase V_{max} is more difficult to explain due to the lack of a crystal structure for the open form. However, we speculate that the stabilization of the open form by compound A results in increased V_{max} values.

Our theory of allosteric regulation also explains the activated kinetic properties of several glucokinase mutants. Two such mutants cause a metabolic disease, persistent hyperinsulinemic hypoglycemia in infant (Glaser et al., 1998; Christesen et al., 2002). Patients have a heterozygous mutation substituting methionine for valine at residue 455 (V455M) or substituting valine for alanine at residue 456 (A456V). In addition, the synthetic glucokinase mutant Y214A, in which tyrosine 214 is substituted with alanine, which is the corresponding residue in hexokinase I, exhibited activated kinetic properties (Moukil et al., 2000). Kinetic analysis of these three mutants shows substantially decreased $S_{0.5}$ values (3.2, 2.5, and 1.3 mM, respectively) from the 8 mM of wild-type glucokinase (Christesen et al., 2002; Davis et al., 1999). These residues are included in the binding site of compound A (Figure 4B), and these mutations seem to inhibit of the release of the α13 helix either as a result of their bulkier side chains (V455M and A456V) or by decreasing the flexibility of connecting region I (Y214A).

Regulation of Hepatic Glucokinase by Glucokinase Regulatory Protein

The activity of hepatic glucokinase is also regulated by glucokinase regulatory protein (GKRP). Several experiments suggest that glucokinase is localized in the hepatocyte nucleus as an inactive complex with GKRP at low plasma glucose levels, but is dissociated from the complex at higher glucose levels and then is translocated into the cytoplasm (Shiota et al., 1999; De la Iglesia et al., 2000). GKRP would likely be an allosteric inhibitor of glucokinase that specifically binds to the super-open form of glucokinase. Mutation studies in *Xenopus* glucokinase showed that two regions of glucokinase may be included in the binding site of GKRP (Veiga-da-Cunha et al., 1996). In the closed form, these two sites (Glu51$\sim$Glu52

and His141∼Leu144) are separated by approximately 40 Å, and the no-effect region (Val154 Asp158) is positioned between these two sites. In contrast, the two regions are much closer together (approximately 20 Å) in the super-open form of glucokinase. This supports the idea that GKRP specifically binds to the super-open form of glucokinase. We are currently attempting to determine the structure of the glucokinase-GKRP complex in order to identify the interactions between glucokinase and GKRP.

In summary, the crystal structures of glucokinase revealed the structural basis of the glucose sensor, which is an essential component for glucose homeostasis. Furthermore, human glucokinase is the first monomeric allosteric enzyme of which the regulation mechanism is clearly explained by structural information. This finding indicates that the mnemonical mechanism is also important for metabolic regulation of allosteric enzymes as well as the well-known concerted model. With regard to potential applications, the allosteric site of glucokinase suggests the possibility that small synthetic activators may act as new drugs to treat type 2 diabetes, although endogenous allosteric activators have yet to be identified.

Experimental Procedures

Construction of Human Hepatic Glucokinase Deletion Mutants

The deletion mutants GK(Δ1-11) and GK(Δ1-15) were constructed by polymerase chain reaction (PCR)-based site-directed mutagenesis. To insert a HindIII recognition site and initiation codon sequence just before Ala12 or Val16, the primer 5′-GTCACAAGGAGCCAG**AAG CTTATG**GCCTTGACTCTGGTAG-3′ or 5′-CCAGGCCCAGACAGCC **AAGCTTATG**GTAGAGCAGATCC-3′ was used with 5′-GAAGCCC CACGACATTGTTCCCTTCTGC-3′ during PCR. From the PCR products, 1.2 kb fragments were excised using the newly introduced HindIII site and a ClaI site and was used to replace the nonmutated sequences in a pFLAG-CTC expression vector containing the human wild-type liver glucokinase gene between the HindIII and EcoRI sites. Mutations were confirmed by sequencing the inserted DNA fragments.

Expression and Purification of Mutant Glucokinase

E. coli strain DH5α, transformed with mutant pFLAG-CTC vectors, was grown in LB medium at 37°C to an A_{600} of 0.8, and the temperature was then decreased to 25°C, and IPTG added to a final concentration of 0.4 mM. At 16–24 hr after induction, the cells were harvested and resuspended in buffer A (50 mM potassium phosphate buffer [pH 7.5], 50 mM NaCl, 2 mM dithiothreitol, 0.5 mM AEBSF; Merck KgaA) and a proteinase inhibitor mixture (Roche Diagnostics). Cells were lysed using a sonicator and centrifuged, after which the supernatant was dialysed against buffer A at a temperature of 4°C. The dialysed solution was applied to a HiTrap Q column (Amersham Biosciences) and eluted using a KCl gradient from 50 to 400 mM. Pooled fractions of mutant glucokinase were purified on Glucosamine-Sepharose and Mono Q (Amersham Biosciences) columns, as described previously (Miwa et al., 1990; Takeda et al., 1993). Further purification was performed by Superdex 200 gel filtration column (Amersham Biosciences) with buffer B (50 mM Tris-HCl [pH 7.2], 50 mM NaCl, 2 mM dithiothreitol).

Enzyme Assays

Enzymatic activity of glucokinase was assayed spectrophotometrically at 30°C in a total volume of 100 μl including 25 mM HEPES-NaOH (pH 7.2), 1 mM ATP-Mg, 1 mM $MgCl_2$, 1 mM dithiothreitol, and the indicated concentrations of glucose and compound A. Glucose 6-phosphate production was measured in the presence of 0.5 mM Thio-NAD^+ and 2 units of glucose 6-phosphate dehydrogenase.

Crystallization of Mutant Glucokinase

Crystals of glucokinase were grown by the hanging drop method. Glucokinase was dialyzed against 20 mM Tris-HCl, 50 mM NaCl, 5 mM Tris (2-carboxyethyl) phosphine hydrochloride solution (pH 7.5) and concentrated to 10 mg/ml. For crystallization of the complex form, glucose and compound A were added to the protein solution so that final concentrations were 20 mM and 300 μM, respectively. A volume of 1.5–3.0 μl of the resulting solution was combined with an equal volume of a precipitant solution containing 28%–30% PEG 1500 and 0.1 M HEPES-NaOH (pH 6.6). The drops were equilibrated against 1.0 ml of the precipitant solution. Prismatic crystals of length 0.5–0.8 mm and width 0.2–0.3 mm grew in a week. The crystals belonged to the $P6_522$ space group (a = b = 79.86 Å, c = 322.22 Å) with a monomer in an asymmetric unit.

Crystals of the free form were obtained by using a precipitant solution containing 1.5–1.6 M ammonium sulfate, 50 mM sodium chloride, and 0.1 M Bicine-NaOH (pH 8.7). Rod shaped crystals of length 0.5–0.8 mm and width 0.03–0.07 mm grew in a week. The crystals belonged to the $P6_522$ space group (a = b = 103.18 Å, c = 281.02 Å) with a monomer in an asymmetric unit.

Data Collection, Structure Determination, and Refinement

X-ray diffraction data of the complex form at 100 K were collected at BL6B beam line of the Photon Factory in KEK and processed with DENZO and SCALEPACK software (Otwinowski and Minor, 1997). X-ray diffraction data of the free form at 100 K were collected at the BL32B2 beam line in Spring-8 and processed with MOSFLM (Leslie, 1998) and SCALA software (Evans, 1993; CCP4, 1994). Measurement parameters and derived data are given in Table 1. Structures were solved by the molecular replacement method using the AMORE program (Navaza, 1994) of the CCP4 package (CCP4, 1994). For structure determination of the complex form, the structure of the C-terminal domain of human hexokinase I (Protein Data Bank identification: 1CZA; Aleshin et al., 2000) was used as the model. For structure determination of the free form, the large domain and the small domain of complex form glucokinase were separately used as models. Model building, electron density map calculation, and model refinement were performed with CNX (Brunger et al., 1998) (Accelrys Inc.) and O (Jones et al., 1991) programs. Crystal structures of the complex form and the free form were refined to an R value of 23.2% (R_{free} = 27.4%) and an R value of 23.8% (R_{free} = 30.6%) with good stereochemistry (Table 1).

Supplemental Data

Supplemental data, including the synthetic method of compound A, is available at: http://www.structure.org/cgi/content/full/12/3/429/DC1.

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Accession Numbers

The coordinates have been deposited in the Protein Data Bank under accession code 1V4S for the complex form and 1V4T for the free form.