Structure/Function Studies of Human β-Cell Glucokinase

ENZYMATIC PROPERTIES OF A SEQUENCE POLYMORPHISM, MUTATIONS ASSOCIATED WITH DIABETES, AND OTHER SITE-DIRECTED MUTANTS*

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Glucokinase plays a key role in the regulation of glucose metabolism in insulin-secreting pancreatic β-cells and in the liver. Recent studies have shown that mutations in this enzyme can lead to the development of a form of non-insulin-dependent diabetes mellitus that is characterized by an autosomal dominant mode of inheritance and onset during childhood. Here, we report the catalytic properties of five additional missense mutations associated with diabetes (Glu70 → Lys, Ser131 → Pro, Ala156 → Thr, Thr257 → Arg and Lys414 → Glu), one polymorphism present in both normal and diabetic subjects (Asp4 → Asn), and three site-directed mutations (Glu177 → Lys, Glu256 → Ala, and Lys414 → Ala). The Thr257 → Arg mutation generated an enzyme that had an activity that was less than 0.5% of that for native human β-cell glucokinase. By contrast, the Glu70 → Lys, Ser131 → Pro, Ala156 → Thr, and Lys414 → Glu mutations had a Vₘₙₐₓ that was 20-100% of normal but a Kₘ for glucose that was 8-14-fold greater than the native enzyme. There was no effect of the Asp4 → Asn polymorphism or the Glu177 → Lys substitution on glucokinase activity. The Lys414 → Ala substitution had no effect on Vₘₐₓ but increased the Kₘ for glucose 2-fold and the Glu256 → Ala substitution caused a ~200-fold decrease in Vₘₐₓ. These studies have led to the identification of additional residues involved in glucokinase catalysis and substrate binding.

Glucokinase (ATP:α-D-hexose 6-phosphotransferase, EC 2.7.1.2.) or hexokinase type IV catalyzes the phosphorylation of glucose, the first rate-limiting reaction in glycolysis (1-3). It is expressed in the pancreatic β-cells and liver and is readily distinguished from other hexokinases in mammalian cells by its smaller size (50 kDa), an affinity for glucose (Kₘ = 8 mM) that is lower than other hexokinase isozymes and is in the physiological range of plasma glucose levels, and a relative lack of inhibition by glucose 6-phosphate. These properties ensure a gradient for glucose entry into the hepatocyte and β-cells, especially following a meal when plasma glucose levels are elevated (3).

Recent studies have shown that mutations in glucokinase can lead to the development of an autosomal dominant form of NIDDM¹ that has an onset in childhood (4-10). Clinical studies suggest that the threshold for glucose-stimulated insulin secretion in subjects with glucokinase mutations is increased, implying that the mutations affect glucose sensing by the pancreatic β-cells (11). Twenty-three different mutations have been described that are associated with diabetes, of which 16 are missense mutations, four are nonsense mutations, and three are splicing mutations (Fig. 1). We have previously reported the effects of 11 missense mutations on the enzymatic properties of human β-cell glucokinase, and all are associated with a decrease in Vₘₐₓ and/or in affinity for glucose (7). These mutations were in regions of the protein encoded by exons 5–8, and molecular modeling predicts that they are in the active site cleft or surface loops leading into this cleft as well as in a region of the smaller of the two globular domains of the glucokinase molecule that is believed to undergo a substrate-induced conformation change on glucose binding (12–15). Here, we describe the catalytic properties of five additional missense mutations associated with glucokinase-deficient diabetes (Glu70 → Lys, Ser131 → Pro, Ala156 → Thr, Thr257 → Arg and Lys414 → Glu) that are encoded by exons 2, 4, 5, 7, and 9, respectively; one polymorphism (Asp4 → Asn) that is encoded by exon 1a and thus is present only in the β-cell form of glucokinase, and which is not associated with diabetes, and three site-directed mutants (Glu177 → Lys, Glu256 → Ala and Lys414 → Ala). The results of these studies define additional key residues involved in glucokinase activity.

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¹ The abbreviations used are: NIDDM, non-insulin-dependent diabetes mellitus; MODY, maturity-onset diabetes of the young; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis; FPLC, fast pressure liquid chromatography.
Experimental Procedures

Materials—Mono Q-Sepharose Fast Flow and Sepharose were from Pharmacia LKB Biotechnology Inc. Glucose-6-phosphate dehydrogenase and glucosamine were from Boehringer Mannheim. Glucosamine-Sepharose was prepared as described (18).

Construction of pET Expression Plasmids for Human β-Cell Glucokinase—A 2.6-kb human pancreatic β-cell glucokinase cDNA clone, p-GK-20 (16), was used to generate the construct pEbgk-WT. An NdeI site was generated at the 5' end using polymerase chain reaction with an oligonucleotide that has a 1-base pair mismatch (GGC TGG TGT GCA TAT GCT GGA CGA CAG). The insert in the pET3a expression construct included the protein coding region of the cDNA as well as the 3'-untranslated region.

Site-directed Mutagenesis of Human β-Cell Glucokinase—In vitro mutagenesis of human β-cell glucokinase was carried out using the Applied Biosystems in Vitro Mutagenesis System (Promega, Madison, WI). Mutations in the native β-cell glucokinase expression construct were generated by replacing appropriate restriction fragments with the corresponding fragment containing the mutation from the mutagenesis vector. A SacII-BamHI restriction fragment was used for mutations Glu70→Lys and Ser313→Pro. Glu177→Lys, Ala188→Thr, Glu256→Ala, Trp257→Arg, Lys414→Ala, and Lys414→Gly were generated by using the Clal-BamHI sites. An NdeI-BamHI restriction fragment from the mutagenesis vector was used to replace the corresponding fragment from the native β-cell construct to generate the Asp4→Asn mutant. All mutations were confirmed by DNA sequencing.

Results and Discussion

Expression in E. coli and Purification of Native and Mutant Forms of Human β-Cell Glucokinase—Native and mutant human glucokinase were expressed in Escherichia coli using the pET expression system essentially as described previously (7, 17, 18). Glucokinase was purified 20-fold from extracts of E. coli in four steps, including (NH4)2SO4 fractionation. Equal amounts of protein were applied to the gel. The single-letter code for amino acids is used. Glucokinase has a molecular size of 50 kDa and is indicated. Molecular size markers are shown at right.
We generated the Lys414 + mutation to test whether the substitution of Lys414 per se was responsible for the abnormal activity as was the site-directed mutant Glu256 + to Ala. This result indicates the importance of Glu256 for enzymatic activity. We generated the Lys414 + to Ala mutant to test whether or not the substitution of Lys414 per se was responsible for the abnormal activity of the Lys414 + to Glu mutation or if the decreased affinity of this enzyme for glucose was due to the introduction of a negatively charged amino acid. The Lys414 + to Ala mutant exhibited a small although significant increase in K_m for glucose with no change in V_max or in K_m for ATP, suggesting that the introduction of a negatively charged amino acid at this site was primarily responsible for the decreased affinity for glucose of the Lys414 + to Glu mutation. The mutations Gly175 + to Arg, Val182 + to Met, and Ala188 + to Thr are all associated with a modest reduction in V_max and an increase in K_m for glucose. To assess the effect of other mutations in this region of the glucokinase molecule on its enzymatic activity, we generated the Glu177 + to Lys mutation. This mutant enzyme had normal activity, implying that not all residues in this region of the molecule are critical for catalysis and/or substrate binding.

**Table I**

Purification of native human β-cell glucokinase expressed in E. coli

| Step | Total units | Specific activity | Purification fold | Yield % |
|------|-------------|-------------------|------------------|--------|
| Crude extract | 822 | 0.9 | 1 | 100 |
| (NH_4)_2SO_4 | 850 | 3.6 | 4 | 103 |
| Glucosamine | 822 | 45.0 | 50 | 78 |
| FPLC Mono Q | 630 | 94.0 | 104 | 52 |

One liter of the BL21 (DE3) pLys S strain harboring pEhGK in LB medium was induced at 22 °C for 24 h. Cells were pelleted and lysed by freeze-thaw three times. The crude extract was centrifuged at 10,000 × g for 30 min and glucokinase precipitated with 40–65% (NH_4)_2SO_4. The pellet was dissolved in and dialyzed against 50 mM KPO_4, 50 mM KCl, 1 mM MgCl_2, 0.1 mM EDTA, 1 mM DTT, and 1 mM PMSF, and then loaded onto a glucosamine-Sepharose column, washed with 80 mM KCl and eluted with 80 mM KCl and 0.5 mM glucose. The enzyme was then applied to a Mono Q-Sepharose column and eluted with a linear gradient of KC1 (100–500 mM). The enzyme assay was done by coupling NADH formation as described (17, 18).

We also examined the effects of three site-directed mutations on glucokinase activity (Table II). Glu256 + to Lys mutation identified in a subject with diabetes (7) was inactive as was the site-directed mutant Glu256 + to Ala. This result indicates the importance of Glu256 for enzymatic activity. We generated the Lys414 + to Ala mutant to test whether or not the substitution of Lys414 per se was responsible for the abnormal activity of the Lys414 + to Glu mutation or if the decreased affinity of this enzyme for glucose was due to the introduction of a negatively charged amino acid. The Lys414 + to Ala mutant exhibited a small although significant increase in K_m for glucose with no change in V_max or in K_m for ATP, suggesting that the introduction of a negatively charged amino acid at this site was primarily responsible for the decreased affinity for glucose of the Lys414 + to Glu mutation. The mutations Gly175 + to Arg, Val182 + to Met, and Ala188 + to Thr are all associated with a modest reduction in V_max and an increase in K_m for glucose. To assess the effect of other mutations in this region of the glucokinase molecule on its enzymatic activity, we generated the Glu177 + to Lys mutation. This mutant enzyme had normal activity, implying that not all residues in this region of the molecule are critical for catalysis and/or substrate binding.
Native and mutant forms of human β-cell glucokinase were expressed in BL21 (DE3) pLysS cells. Glucokinase activity was determined as described previously (17, 18). The values shown are the mean ± S.E. of at least four separate determinations.

| Enzyme                      | Level of expression (soluble, active enzyme) | $V_{\text{max}}$ | $K_m$ (Glucose) | $K_m$ (ATP) |
|-----------------------------|---------------------------------------------|------------------|-----------------|--------------|
| Mutations associated        |                                             |                  |                 |              |
| Native β-cell               | 20                                          | $93 ± 8$         | $8.0 ± 2$       | $0.20 ± 0.1$ |
| Glu<sup>70</sup> → Lys<sup>70</sup> | 18                                          | $23 ± 4$         | $62.0 ± 16^c$   | $0.20 ± 0.02$|
| Ser<sup>131</sup> → Pro<sup>131</sup> | 17                                          | $46 ± 5^c$       | $110.0 ± 22^c$  | $0.04 ± 0.02^c$|
| Ala<sup>188</sup> → Thr<sup>188</sup> | 8                                           | $43 ± 5^c$       | $72.0 ± 8^c$    | $0.08 ± 0.02$|
| Trp<sup>257</sup> → Arg<sup>257</sup> | 10                                          | ≤0.5<sup>a</sup> | $5.0 ± 2$       | $0.16 ± 0.04$|
| Lys<sup>414</sup> → Glu<sup>414</sup> | 20                                          | $94 ± 10$        | $80.3 ± 0.005^b$| $0.07 ± 0.03$|
| Polymorphism                |                                             |                  |                 |              |
| Asp<sup>a</sup> → Asn<sup>a</sup> | 25                                          | $94 ± 11$        | $6.0 ± 0.07$    | $0.18 ± 0.02$|
| Site-directed mutations     |                                             |                  |                 |              |
| Glu<sup>77</sup> → Lys<sup>77</sup> | 20                                          | $95 ± 12$        | $5.2 ± 1.5$     | $0.17 ± 0.02$|
| Glu<sup>256</sup> → Ala<sup>256</sup> | 15                                          | ≤0.5<sup>a</sup> | $4.0 ± 2.1$     | $0.15 ± 0.08$|
| Lys<sup>414</sup> → Ala<sup>414</sup> | 15                                          | $94 ± 13$        | $18.0 ± 7.8^c$  | $0.23 ± 0.05$|

<sup>a</sup> $V_{\text{max}}$ values differ significantly from the native β-cell enzyme.

<sup>b</sup> $K_m$ values differ significantly from the native β-cell enzyme.

<sup>c</sup> Residue is located in the cleft near glucose.

**Table II**

Enzymatic properties of native and mutant forms of human β-cell glucokinase

Fig. 4. Model for human glucokinase. A ribbon drawing of the α-carbon backbone of glucokinase, based on yeast hexokinase B structure in open conformation, is shown. Those amino acids in which missense mutations have been identified are indicated. The ones reported in this paper include Glu<sup>70</sup> → Lys, Ser<sup>131</sup> → Pro, Ala<sup>188</sup> → Thr, Trp<sup>257</sup> → Arg, and Lys<sup>414</sup> → Glu. The glucose molecule is shown in green.

Specificity for glucose as measured by $K_m$ for glucose. However, since the glucose-induced conformational change and cleft closure are essential for catalysis (14), it is likely that the $K_m$ for glucose reflects a large amount of nonproductive glucose binding. If the glucose-induced conformational change is compromised, a decrease in $V_{\text{max}}$ would follow. Consistent with these notions, all the mutants bound to a glucosamine column.

Based on analogy to the heat shock protein, actin, and sugar kinase structures (22–24), Lys<sup>414</sup>, which corresponds to Thr<sup>222</sup> in the yeast hexokinase B structure, is in a region that has been predicted to represent a common ATP binding domain. In this common domain the phosphate tail of ATP is bound by residues on two β-hairpins, one from each of two subdomains, and by other segments. However, there is at present no experimental evidence for hexokinase to relate the binding of ATP to any specific residues in this domain. Lys<sup>414</sup> is actually located in a region postulated to bind the adenosine ring, and this residue is not conserved in this family of ATP-binding proteins (24). The Lys<sup>414</sup> → Glu mutation resulted in a 10-fold increase in $K_m$ for glucose but with no change in $K_m$ for ATP or maximal velocity (Table II), and the Lys<sup>414</sup> → Ala mutation showed a much smaller increase in $K_m$ for glucose, implying that the introduction of a negatively charged residue at this site was primarily responsible for the decreased affinity for glucose. We previously studied the effects of mutation of Thr<sup>228</sup>, another residue predicted to be involved in ATP binding (5), on glucokinase activity (7). The Thr<sup>228</sup> → Met mutation has a greatly decreased $V_{\text{max}}$ but no change in affinity for ATP. The results from mutational analysis of both Thr<sup>228</sup> and Lys<sup>414</sup> suggest that they do not play a critical role in ATP binding in glucokinase. Both Lys<sup>414</sup> and Thr<sup>228</sup> are, however, directed toward the active site (Fig. 4), and it is thus not surprising that their substitution affects enzyme activity. Alternatively, the analogous residues in yeast hexokinase B may bind ATP but have another function in glucokinase. Final resolution of this question will require the x-ray crystal structure of glucokinase.

Two other mutations affect residues that could be important for maintaining the correct conformation of the molecule. Ala<sup>188</sup> corresponds to Gln<sup>189</sup> in yeast hexokinase α-helix 4, which has loops at either end that connect to active site residues Lys<sup>189</sup> and Asp<sup>205</sup>. Asp<sup>205</sup> has been shown to act as a base catalyst in the phosphorylation of glucose (18). Since Ala<sup>188</sup> is partly internal, the larger Thr residue may cause a small distortion of the structure that possibly could be transmitted to the active site residues Lys<sup>189</sup> and Asp<sup>205</sup> and thereby affect catalysis. The presence of Thr at position 188 also provides the oxygens of residues 184 and 185. This might preferentially stabilize one conformation in this region and thus be unfavorable for the conformation change that occurs on glucose binding. Consistent with these predictions the Ala<sup>188</sup> → Thr mutation resulted in a decrease in the affinity for glucose and also caused a 50% reduction in $V_{\text{max}}$.

We previously described the enzymatic properties of two other missense mutations in the region of Ala<sup>188</sup>, Gly<sup>175</sup> → Arg and Val<sup>182</sup> → Met (7). To assess the possible effects of other
mutations in the region of residues 175–188 on enzymatic activity, we mutated Glu177 to Lys. This mutation had no effect on glucokinase activity. The model for the structure of glucokinase predicts that Glu177 is a surface residue and substitution with Lys would not be expected to create any steric or electrostatic problems, a prediction consistent with the experimental results.

Ser131 is located in α-helix 3 in the smaller of the two domains and is far from the active site. The mutation to Pro is predicted to disrupt the α-helix. Glucokinase residue 131 is adjacent to Phe155, which connects with β-strand 8 to the base catalyst Asp340. Therefore changes at residues 131 can easily be transmitted to the active site. This could be the basis for the large change in $K_m$ for glucose (14-fold) and the decrease in $V_{max}$ observed in this mutation.

The conserved Glu177 lies close to the conserved basic residues Lys468 and Lys496 in the yeast hexokinase B structure. Mutation of Glu177 to Lys would be expected to be electrostatically unfavorable due to the other nearby positively charged residues. Furthermore, Glu177 forms a hydrogen bond interaction with the amide of residue 67, stabilizing the turn and β-strand 2; this interaction would not be possible for Lys. This region is predicted to participate in the substrate-induced conformational change that results in cleft closure so that the destabilizing Glu177 to Lys mutation is probably responsible for the significant changes in enzyme activity observed (Table II). However, the precise molecular mechanism involved in the increased $K_m$ for glucose exhibited by the Glu177 to Lys mutation is not clear, but may reflect the necessity for high glucose concentrations to affect the conformational change/cleft closure.

The kinetic properties of the Asp4 → Asn mutant were unchanged from the native enzyme. This finding is not surprising since Asp4 is at the NH2 terminus far from the active site in the region of the hexokinase crystal structure that is not visible and presumed to be disordered and flexible (12–15). The lack of effect of this mutation on enzyme activity is consistent with the fact that it is found in both affected and unaffected members of a family with glucokinase-deficient disorder, but did not segregate with the diabetic phenotype (8).

CONCLUSIONS

The recent demonstration that mutations in glucokinase can contribute to the development of NIDDM has provided new insight into the etiology of this genetically heterogeneous disorder (4–10). It has also led to the identification of amino acid residues that are important for glucokinase activity. We have previously proposed that mutations in glucokinase cause an autosomal dominant disorder of glucose metabolism by a gene dosage mechanism since splicing, nonsense, and missense mutations have been identified in patients with diabetes (4–8). Moreover, since glucokinase is a monomer, it is unlikely that mutations have a dominant-negative effect on enzymatic activity. In support of this hypothesis, no effect on $V_{max}$ or on $K_m$ for glucose was observed when an equal amount of native glucokinase was incubated with a “dead” mutant (Try369 → Arg) or with Ser131 → Pro (data not shown). The decreased cellular levels of glucokinase activity in pancreatic β-cells are predicted to alter glucose sensing by these cells and thereby increase the threshold for glucose-induced insulin secretion. The altered enzymatic activity of the missense mutations associated with glucokinase-deficient diabetes described in this report is consistent with this hypothesis. However, it is also possible that some mutations may affect glucose sensing at the level of the glucose transporter (5) and/or association with the glucokinase regulatory protein (26). Finally, the characterization of the enzymatic properties of these mutations provides an opportunity to examine structure/function relationships in glucokinase. A model for human glucokinase based on the structure of yeast hexokinase has been particularly useful in this regard (5, 7).

It is noteworthy that all 16 observed missense mutations associated with the diabetic phenotype are located in regions of conserved sequence in the two enzymes. The strong correlation between the observed effects of changes in amino acid sequence on enzymatic activity and those predicted from structural considerations indicates the utility of this model for considering the effects of amino acid replacements on structure/function relationships.

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