Study of the Regulatory Properties of Glucokinase by Site-Directed Mutagenesis

Conversion of Glucokinase to an Enzyme With High Affinity for Glucose

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To identify the amino acids involved in the specific regulatory properties of glucokinase, and particularly its low affinity for glucose, mutants of the human islet enzyme have been prepared, in which glucokinase-specific residues have been replaced. Two mutations increased the affinity for glucose by twofold (K296M) and sixfold (Y214A), the latter also decreasing the Hill coefficient from 1.75 to 1.2 with minimal change in the affinity for ATP. Combining these two mutations with N166R resulted in a 50-fold decrease in the half-saturating substrate concentration ($S_{0.5}$) value, which became then comparable to the K_m of hexokinase II. The location of N166, Y214, and K296 in the three-dimensional structure of glucokinase suggests that these mutations act by favoring closure of the catalytic cleft. As a rule, mutations changed the affinity for glucose and for the competitive inhibitor mannoheptulose (MH) in parallel, whereas they barely affected the affinity for *N*-acetylglucosamine (NAG). These and other results suggest that NAG and MH bind to the same site but to different conformations of glucokinase. A small reduction in the affinity for the regulatory protein was observed with mutations of residues on the smaller domain and in the hinge region, confirming the bipartite nature of the binding site for the regulatory protein. The K296M mutant was found to have a threefold decreased affinity for palmitoyl CoA; this effect was additive to that previously observed for the E279Q mutant, indicating that the binding site for long-chain acyl CoAs is located on the upper face of the larger domain. *Diabetes* 49:195–201, 2000

There is now overwhelming evidence that glucokinase, the enzyme that phosphorylates glucose in the liver and in pancreatic islets, plays a critical role as a glucose-sensing device (1). This evidence, originally derived from studies on the control of glucose metabolism in the liver and in islets (2), has now received strong support with the discovery that a loss of glucokinase activity, due to either mutations in the glucokinase gene in humans (3–5) or gene knock-out in animal models (6,7), results in diabetes. Another strong piece of evidence in favor of glucokinase being the glucose sensor in β-cells is the finding that an autosomal-dominant form of hyperinsulinemia is linked to a mutation of glucokinase that increases the affinity of this enzyme for glucose by about threefold (8).

The role played by glucokinase as a glucose sensor is due to its specific regulatory properties, mainly a low affinity for glucose, with a sigmoidal saturation curve for this substrate, and a lack of inhibition by physiological concentrations of glucose-6-phosphate (G-6-P) (9–11). These properties are in sharp contrast with those of homologous enzymes such as the mammalian low-K_m hexokinases. In addition, glucokinase has, compared with other hexokinases, the unique property of being inhibited by a regulatory protein and by long-chain acyl CoAs. Kinetic evidence indicates that these two types of inhibitors bind to a site distinct from the catalytic site despite the fact that their action is competitive with glucose (12). The properties mentioned above appear to be shared by all animal glucokinases (13,14), suggesting that the amino acid residues participating in these regulatory functions have been conserved.

The cDNA encoding *Xenopus* liver glucokinase has been cloned and shown to encode a protein with 78% amino acid identity with mammalian glucokinases (15). Glucokinase-specific residues have then been mutated in the *Xenopus* enzyme and replaced by their counterparts in the C-half of rat hexokinase I, an enzyme that does not share the regulatory properties of glucokinase. This approach led to the identification of two sets of residues involved in the binding of the regulatory protein, one at the tip of the smaller domain and another close to the hinge region (15) according to the glucokinase model developed by St. Charles et al. (16). Confirmation of this localization came from other experiments per-

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G-6-P, glucose-6-phosphate; I_{50}, 50% inhibition; MH, mannoheptulose; NAG, *N*-acetylglucosamine; PCR, polymerase chain reaction, $S_{0.5}$, half-saturating substrate concentration.

TABLE 1
Primers used for preparation of the mutants

Mutant	Primers	Diagnostic restriction site
K161Q	5′-cagggcatccttctcaactggaccaaggcc-3′ 5′-atcgatgtcttcgtgcctcacaggaaagga-3′	+ *Sau*3AI
N180D, G183S (G)	5′-gtgtcgcttctgcgagacgctatcaaacgg-3′ 5′-gacatcgttcccttctgctcctgaggcctt-3′	
A208G	5′-acgatgatctcctgctactacgaagaccat-3′ 5′-gcccaccgtgtcattcaccattgccaccac-3′	– *Bal*I
Y214A	5′-gcgtacgaagaccatcagtgcgagg-3′ 5′-gcaggagatcatcgtggccaccg-3′	+ *Rsa*I
C230S	5′-tcgaatgcctgctacatggaggagatgc- 3′ 5′-gcccgtgcccacgatcatgccgac-3′	+ *Taq*I
L271T, E272D, R275K (H)	5′-gacaagctagtggacgagagctctgcaaac-3′ 5′-atagtccgtcaggaactcgtccagctcgcc-3′	+ *Mae*I
Y289F, G294S (I)	5′-ctcataagtggcaagtacatgggcgagctg-3′ 5′-cttctcgaacagctgctgaccggggtttgc-3′	+ *Taq*I
K296M	5′-atgtacatgggcgagctggtgcgg-3′ 5′-gccacctatgagcttctcatacag-3′	+ *NspH*I
L304N, R308D, V310T, E312K, N313G (J)	5′-acggacaaaggcctgctcttccacggg-3′ 5′-gaggtcgagcagcacattccgcaccagctc-3′	+ *Taq*I

Mutated nucleotides are underlined. The diagnostic restriction sites are also shown.

formed on human islet glucokinase where it was found that all mutations that decreased the affinity of glucokinase for its regulatory protein clustered in the hinge region and nearby in the larger and the smaller domains (17).

The goal of the present work was to extend these mutagenesis studies, using the same rationale as described above (15), to identify additional residues involved in the regulatory properties of glucokinase.

RESEARCH DESIGN AND METHODS

Materials. The source of materials was as previously reported (15,17). The regulatory protein was purified from rat liver up to the hydroxylapatite step (18) and was freed of inorganic phosphate by gel filtration on Sephadex G-25.

Preparation of the mutant glucokinases. Mutants of human islet glucokinase were constructed by polymerase chain reaction (PCR)-based site-directed mutagenesis using back-to-back primers and *Pwo*-polymerase (15). Primers used to introduce the mutations are shown in Table 1. The PCR reaction was performed using as a template a pBluescript plasmid with an insert encoding the "true" wild-type human islet glucokinase, i.e., one in which codon 158 has been reverted to an aspartate-encoding codon (17). After PCR amplification, the linear plasmids were recircularized and cloned into *Escherichia coli* JM 109. Mutated plasmids were selected by restriction analysis and sequenced with M13F and M13R primers. *Sac*II/*Sac*I (nucleotides 126–837 bp) or *Sac*I/*Bam*HI (nucleotides 838–1764 bp) fragments containing the mutated sequences were excised from the plasmids and used to replace the nonmutated sequences in pET-HIGK, a pET3a expression vector (19) containing the human wild-type islet glucokinase. Mutant N166R-Y214A was constructed in the same manner, using as a template pBS-HIGK-N166R. Mutant E279Q was constructed by "repairing" the mutation D158A in pET-HIGK-E279Q-D158A (17). Mutants N166R-Y214A-K296M and E279Q-K296M were constructed by inserting a *Sac*II/*Sac*I fragment containing the N166R-Y214A or E279Q mutation in pET-HIGK-K296M. These cDNAs were subsequently used to transform *E. coli* BL21 (DE3) pLysS (19), and the recombinant proteins were expressed and purified as described elsewhere (15) by a combination of ammonium sulfate precipitation and chromatography on Sephadex G-25 and on DEAE-Sepharose. Fractions with the highest specific activities were then chromatographed on a 1.6 × 13 cm Q-Sepharose column equilibrated with 50 mmol/l phosphate, pH 8, 1 mmol/l dithiothreitol, 150 mmol/l KCl, and 1 μg/ml antipain. The column was washed with 100 ml of the same buffer and eluted with a linear KCl gradient (150–500 mmol/l in 200 ml of this buffer). The glucokinase proteins were ~70–80% pure, as judged by SDS-PAGE and densitometric analysis performed with the NIH Image program (developed at the U.S. National Institutes of Health in Bethesda, MD, and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Glucokinase assays. Glucokinase was assayed at 30°C by a G-6-P dehydrogenase–coupled assay in an assay mixture (1 ml) containing, unless otherwise indicated, 25 mmol/l HEPES, pH 7.1, 25 mmol/l KCl, 2.5 mmol/l $MgCl_2$, 0.6 mmol/l NAD^+, 1 mmol/l ATP-Mg, the indicated glucose concentrations, and 10 μg/ml *Leuconostoc mesenteroides* G-6-P dehydrogenase. The inhibition by palmitoyl CoA was tested with no excess of Mg^{2+} over ATP, to avoid partial inhibition (20) possibly due to the lowering of the critical micelle concentration. The effects of the regulatory protein and the phosphorylation of substrates other than glucose were tested with the pyruvate kinase/lactate dehydrogenase–coupled assay previously described (18). The concentration of glucokinase used to study the effect of the regulatory protein was kept low (<160 μg/l) in order to avoid overestimation of the inhibitor constant (K_i) (18). The sensitivity of the wild-type and mutant glucokinases to inhibition by competitive inhibitors, and the K_m for ATP-Mg were determined at a glucose concentration close to the half-saturating substrate concentration ($S_{0.5}$) value. Both the $S_{0.5}$ values and Hill coefficient were derived from Hill plots. Concentrations of inhibitors causing 50% inhibition (I_{50}) were derived from Dixon plots (21).

Other methods. Protein concentration was measured according to Bradford (22) using bovine serum albumin as a standard.

RESULTS

Effect of mutations on the affinity of glucokinase for glucose. A series of residues conserved among glucokinases but not in low-K_m mammalian hexokinases were mutated to the corresponding residues in the carboxyl-half of rat hexokinase I or II. One additional mutant (E279Q) contained a human mutation that had been previously studied in the context of the "pseudo wild-type" (D158A) and was reinvestigated here in the context of the "true" wild-type enzyme, because of its effect on the affinity for long-chain acyl CoAs (17). Mutants combining some of these mutations were also prepared. All proteins were produced in *E. coli* and purified. A summary of the kinetic properties of these enzymes is shown in Table 2.

The mutations affected differently the affinity of glucokinase for the substrate glucose. On the one hand, this affinity was decreased in mutants K161Q, C230S, "G", "H," and "J" by a factor of 1.3–2.2 and in mutant E279Q by a factor of ~5. In the latter case, the decrease in affinity was comparable to that previously observed in the context of the "pseudo wild-type" ($S_{0.5}$ = 18.4 mmol/l for the double mutant E279Q-D158A compared with 3.3 mmol/l for the D158A mutant) (17). On the other hand, the affinity for glucose was increased in mutant

TABLE 2
Kinetic properties of the glucokinase mutants

Enzyme	$S_{0.5}$ (mmol/l)	h	V_{max} (U/mg)	K_m ATP (mmol/l)	I_{50} palmitoyl CoA (μmol/l)	I_{50} regulator (U/ml)	I_{50} NAG (mmol/l)	I_{50} MH (mmol/l)
Wild-type	8	1.75	85	0.10	1.9	2.1	0.24	1.6
K161Q*	11.7	1.72	60	0.12	2.1	4.1	0.25	2.2
N180D, G183S (G)	16	1.65	60	0.11	2	5.2	0.24	2.6
A208G	8.4	1.58	82	0.10	1.8	6.1	0.27	2.0
Y214A	1.3	1.20	142	0.20	2.1	2.6	0.20	0.9
C230S*	14.7	1.66	90	0.37	1.9	2	0.31	2.6
L271T, E272D, R275K (H)	10.8	1.68	45	0.14	2.2	2.1	0.22	1.8
Y289F, G294S (I)	6.9	1.70	75	0.09	2.6	2.3	0.25	1.3
L304N, R308D,V310T, E312K, N313G (J)	17	1.60	80	0.09	1.7	2.1	0.29	2.4
K296M	4.7	1.60	45	0.13	5.3	2.2	0.25	0.6
E279Q†	43.3	1.48	30	0.24	6.2	3.8	2.12	8.2
E279Q-K296M	18.4	1.50	22	0.31	11.7	4.7	2.34	3.6
N166R-Y214A	0.5	1.23	100	0.12	2.2	6.2	0.43	0.8
N166R-Y214A-K296M	0.12	1.15	62	0.10	6	6.9	0.46	0.5

All measurements have been performed in triplicate. For the sake of clarity, SE values are not shown. They are equal to 2–12% of the value to which they refer. Values differing from the control by more than twofold or for the Hill coefficient by more than 1.4-fold are in bold. *Residues K161 and C230 have been replaced by the amino acid in equivalent position in the COOH-terminus of rat hexokinase II instead of rat hexokinase I to avoid the introduction of a cysteine residue in the case of K161 or of a bulkier residue (threonine) than the native one in the case of C230; †mutation found in maturity-onset diabetes of the young patients.

K296M by ~1.8-fold whether in the context of the wild-type enzyme or in the presence of the E279Q mutation. The K296M mutation was also found to decrease the $S_{0.5}$ value of *Xenopus* glucokinase from 2.3 to 0.7 mmol/l (results not shown). A remarkable observation was that mutant Y214A had an about sixfold lower $S_{0.5}$ value compared with the wild-type enzyme and had lost most of its cooperative behavior with respect to glucose, with a Hill coefficient of 1.2 instead of 1.75 for the control enzyme (Table 2, Fig. 1). Hill coefficients of other mutants were either not or only barely affected.

The replacement of the glucokinase-specific residue N166 by arginine was previously shown to cause an about twofold decrease in the $S_{0.5}$ value of glucokinase as well as a decrease of the Hill coefficient to 1.3 (17). We decided to test the effect of combinations of this mutation with the two mutations that were now found to increase the affinity for glucose. As shown in Table 2 and Fig. 1, combination of N166R and Y214A further reduced the $S_{0.5}$ value to 0.5 mmol/l, whereas the triple mutant (N166R-Y214A-K296M) had an $S_{0.5}$ value of 0.12 mmol/l. Both proteins still showed a low degree of cooperativity with a Hill coefficient of 1.2. The triple mutant was also found to have an increased affinity for mannose (K_m = 0.12 mmol/l vs. an $S_{0.5}$ of 18 mmol/l for the wild-type), 2-deoxyglucose (K_m = 0.45 mmol/l vs. an $S_{0.5}$ of 55 mmol/l) and fructose (K_m = 4 mmol/l vs. an $S_{0.5}$ 550 mmol/l). For these three substrates, the Hill coefficient of the triple mutant was close to 1.

Effect on K_m for ATP and on the V_{max}. Table 2 also shows that most of the K_m values for ATP-Mg were unchanged compared with the control enzyme except for a twofold increase in mutants Y214A and E279Q and a fourfold increase in mutant C230S. The V_{max} value of the control enzyme (85 U/mg protein) is comparable to that previously reported by other authors (23,24). Except for the E279Q mutants, the effect of the mutations on the V_{max} values was less than twofold. Of interest is the fact that the value for the triple mutant was only marginally decreased.

Effect on the affinity for competitive inhibitors. *N*-acetylglucosamine (NAG), mannoheptulose (MH), the regulatory protein, and palmitoyl CoA act on glucokinase as competitive inhibitors with respect to glucose (12,25–27). Because several of the mutant proteins that we produced had a modified affinity for glucose, it was important to test the inhibitory effect of these compounds at equivalent concentrations of glucose. The wild-type and mutant forms of glucokinase were therefore tested at a concentration of substrate corresponding to their $S_{0.5}$ value. The value obtained under the latter condition is expected to reflect the affinity for the effector independently of changes in the affinity for glucose.

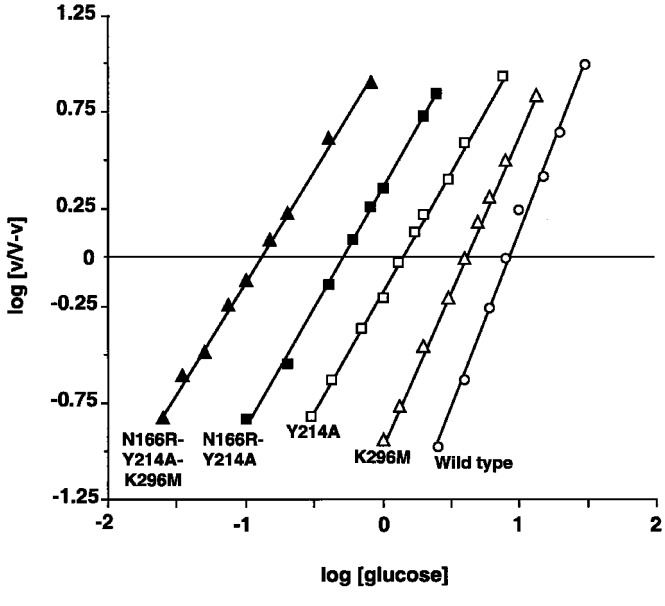

FIG. 1. Effect of some mutations on the Hill plot for glucose.

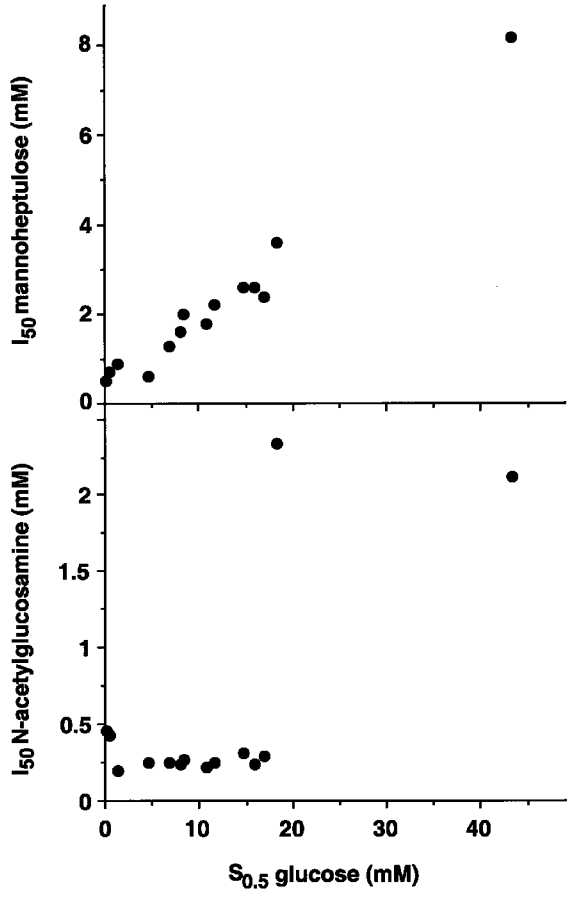

FIG. 2. Plot of I_{50} values for NAG and MH as a function of the $S_{0.5}$ for glucose. I_{50} values were determined at the glucose concentration corresponding to $S_{0.5}$ of each mutant. Each point represents one of the mutant proteins shown in Table 2.

Most mutants were half-maximally inhibited by concentrations of NAG that did not differ from the control value by more than 40%, except for the E279Q mutant, which showed an ~10-fold decrease in its affinity for this inhibitor. By contrast, the affinity for MH was found to be affected in several mutants, for most of them in parallel with the changes in the affinity for glucose (Fig. 2). Because these results could indicate that NAG and MH bind to different sites, we tested the effect of a combination of these two inhibitors on the activity of wild-type glucokinase. As shown in Fig. 3, a plot of the reciprocal of the velocity as a function of the NAG concentration gave, for different concentrations of MH, a family of parallel lines, indicating that the two inhibitors do not bind simultaneously to the enzyme (28), most likely because they compete for one single binding site.

The mutants were unaffected in their affinity for the regulatory protein, with the exception of three of them (K161Q, mutant "G," and A208G), which had a moderately reduced affinity for the regulatory protein. Remarkably, the "hexokinase-like" triple mutant was still sensitive to the inhibition exerted by the regulatory protein.

The E279Q mutation was previously shown to cause a decrease in the affinity for acyl CoA while made in the context of the "pseudo wild-type" (D158A). We now show that this mutation also caused a decrease in the affinity for

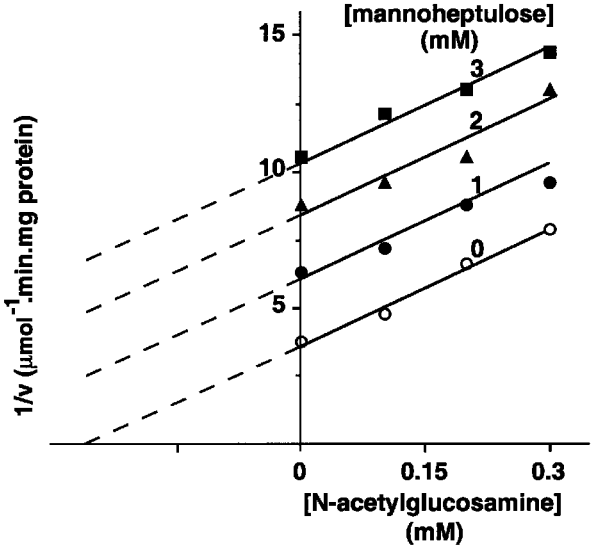

FIG. 3. Inhibition of wild-type glucokinase by combinations of MH and NAG. The wild-type enzyme was assayed at 5 mmol/l glucose with the indicated concentrations of inhibitors. Results are presented as Dixon plots.

palmitoyl CoA in the context of the true wild-type enzyme. In addition we show that the K296M mutation also caused a twofold decrease in the affinity for palmitoyl CoA, and that the effect of this mutation was additive to that of the E279Q mutation (Fig. 4).

Mammalian low-K_m hexokinases are characterized by a high affinity for their substrate glucose and by their sensitivity to inhibition by micromolar concentrations of G-6-P. Because we had produced an enzyme with a K_m comparable to that of hexokinase II (29), we tested the sensitivity of the triple mutant. In the presence of 0.1 mmol/l ATP and a glucose concentration corresponding to the $S_{0.5}$ value, half-maximal inhibition was observed with 14 mmol/l G-6-P, compared with 20 mmol/l for the wild-type enzyme.

DISCUSSION

Residues implicated in the low affinity of glucokinase for glucose. One of the characteristics of glucokinase is its low affinity for glucose, the importance of which is critical for its role as a glucose sensor. As a result of this and of a previous study (15), a total of more than 35 amino acids that are conserved in glucokinases have been replaced by their counterparts in the low-K_m enzymes, hexokinase I or II. Two of these replacements were found to increase the affinity for glucose, whereas most others were ineffective or slightly decreased this affinity. The most dramatic effect was observed with the Y214A mutation, which caused a sixfold increase in the affinity for glucose, whereas the K296M mutation caused a 1.8-fold increase in affinity. The Y214A mutation also caused a loss of cooperativity, which is further discussed below.

To propose a mechanism for these effects, it is interesting to localize the residues in the three-dimensional structure of glucokinase modeled by St. Charles et al. (16) on the crystal structure of the yeast hexokinase B. Although glucokinase shares only 33% identity with this enzyme, we believe that the model is appropriate to discuss at least the approximate position of the mutated residues.

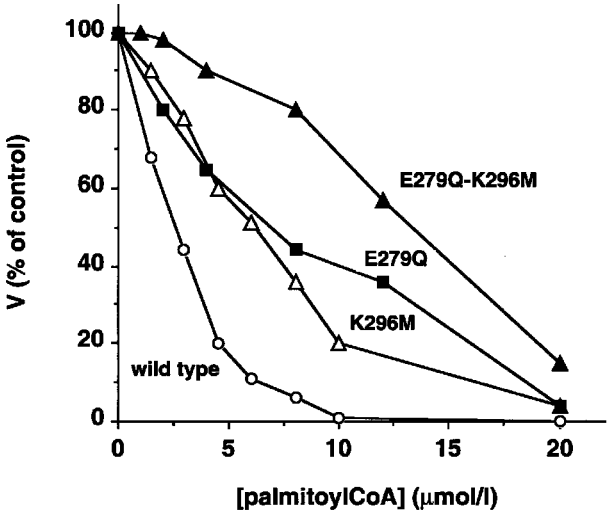

FIG. 4. Sensitivity of wild-type and mutant glucokinases to palmitoyl CoA. Enzymes were measured with a concentration of glucose corresponding to their $S_{0.5}$ value (see Table 2).

Our hypothesis is that these mutations increase the affinity for glucose by favoring closure of the catalytic cleft. Such an increase in affinity can be easily accounted for by the fact that residues that participate in the binding of glucose are present both in the smaller domain (S151, N204, and D205) and in the larger domain (N231, E256, and E290) (16). K296 is a residue on the upper face of the larger domain (Fig. 5*A* and *B*) that is conserved among glucokinases and is replaced by methionine in the two halves of hexokinases I and II (15). Its replacement by a noncharged residue could possibly strengthen hydrophobic interaction with K169, an extremely conserved residue present in the smaller domain, and therefore facilitate closure of the catalytic cleft. Inspection of the structure of brain hexokinase I indicates that the residues in equivalent positions (K621 and M748 in the C-half, equivalent to K169 and M296, respectively, in glucokinase) lie ~9 Å apart in the open conformation (30) but are in close contact in the closed conformation (31) through the extremity of the side chain of the methionine residues and the α- and β-carbon of the lysine residue. Interestingly, the K296M mutation does not change the affinity of glucokinase for NAG, which, as discussed below,

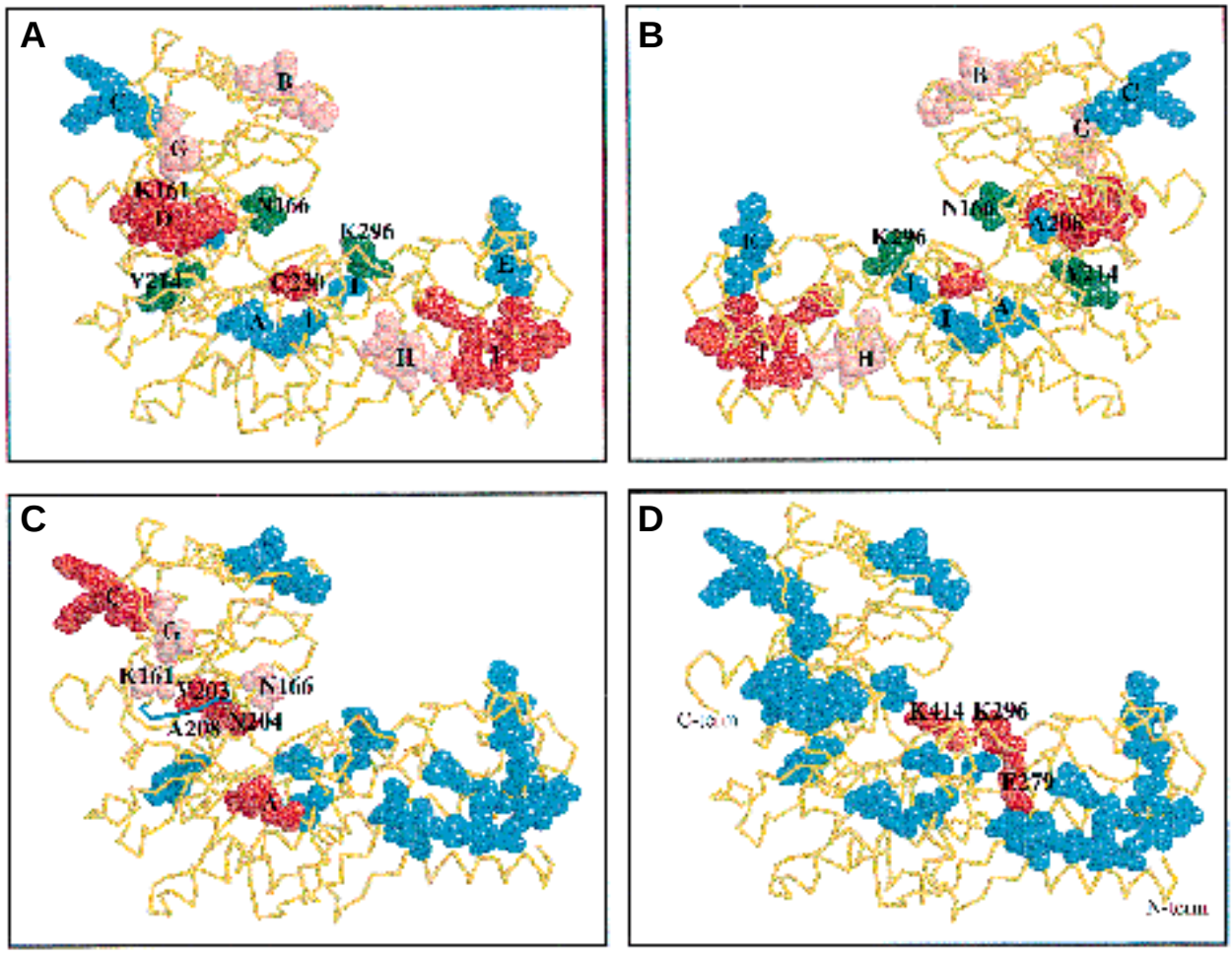

FIG. 5. Three-dimensional model of glucokinase showing the residues involved in determining the affinity for glucose (*A* and *B*), the regulatory protein (*C*), and palmitoyl CoA (*D*). This model is based on the crystal structure of yeast hexokinase B (16). The larger domain is horizontally oriented at the bottom of each panel. Mutations that *decrease* the affinity for a ligand are indicated in red, or in pink for effects less than twofold for glucose and less than fivefold for the regulatory protein. Those that *increase* the affinity are indicated in green and those that *do not affect* the affinity are indicated in cyan. The figure includes the residues mutated in this paper, those corresponding to mutants A–E of Veiga-da-Cunha et al. (15) as well as V203 and N204 in *C* (17). Mutants A–E are defined as follows: mutant A, E51S-E52K; mutant B, A114I-T116Q-M121E-Y125H; mutant C, H141G-K142P-K143H-L144M; mutant D, V154C-R155H-H156Q-E157T-D158N; and mutant E, T348V-L349R-R353Q (15).

binds to the open conformation of glucokinase. The location of Y214 at the back of the hinge region (Fig. 5) indicates that it could also play a role in the conformational movements. The closure of the catalytic cleft in hexokinase appears to be a movement of two essentially rigid bodies corresponding to the smaller and the larger domains (30). These movements are caused by reorientation of three peptide segments, one of which corresponds (residues 63–68 in glucokinase) to a region that contacts the side chain of Y214 and a second one (residues 204–209 in glucokinase) that is five residues NH_2-terminal to Y214 in the protein sequence. The presence of a tyrosine at position 214 in glucokinase instead of a much smaller residue (alanine or glycine) in animal hexokinases could favor the open state of the enzyme. Interestingly, all other mutations reported to increase the affinity for glucose concern residues that are either in the hinge region (D158) (17) (V455) (8) or on the face of the smaller domain that is opposite to the larger domain (N166), suggesting that they could act through the same mechanism.

The fact that by combining three mutations, glucokinase gets an affinity for glucose that is similar to that of low-K_m hexokinases indicates that the difference in affinity for glucose between glucokinase and low-K_m hexokinases results from the replacement of a limited number of residues. The absence of any parallel increase in the affinity for G-6-P agrees with kinetic (32) and structural (31) studies indicating that this inhibitor binds to the ATP-binding site and not to the glucose-binding site.

Binding of NAG and MH to different conformations of glucokinase. Many other replacements of glucokinase-specific residues by "hexokinase residues" cause a modest decrease in the affinity for glucose while not changing the affinity for NAG. These mutations concern amino acids that are on the smaller domain, the hinge region, and the larger domain of glucokinase, sometimes relatively distant from the catalytic site. Remarkably, the affinity for MH is modified almost in parallel with the affinity for glucose. Xu et al. (33) had found similarly that the K56A mutation reduced the affinity for glucose and MH to the same extent while not affecting the affinity for NAG. These observations could indicate that MH and *NAG* bind to different sites. However, studies with a combination of these two inhibitors indicated that they do not bind simultaneously to glucokinase (this study). Furthermore, mutations of the glucokinase residues that directly interact with glucose markedly affect the affinity for both inhibitors (33). A more likely interpretation is therefore that NAG and MH bind to the same site, i.e., the catalytic site, but in different conformations of the enzyme, either open (NAG) or closed (MH). Accordingly, acylglucosamine derivatives bind to the open conformation of yeast hexokinase and are not substrate because the acyl group borne by C2 prevents closure of the catalytic cleft (34). MH, on the contrary, is a substrate, although a poor one, of glucokinase (with a V_{max} equal to 0.2% of that observed with glucose and a K_m of ~4 mmol/l; M.A.M., unpublished observations), which indicates that it can induce closure of the catalytic cleft. The binding of NAG and MH to different conformations of glucokinase is also consistent with the observation that the first one acts noncompetitively, and the latter competitively, with respect to the regulatory protein (35). This indicates that the regulatory protein binds to the open conformation of glucokinase.

The E279Q mutation, which is found in maturity-onset diabetes of the young–2, is one example of a mutation that decreases the affinities for glucose (36), MH, and NAG in parallel (this study). We hypothesize that in this case, the mutation acts not by altering the equilibrium between the open and closed states of glucokinase but by modifying somewhat the structure of the catalytic site. E279 is indeed a residue on a loop between E256 and E290, two residues that interact directly with glucose (16). Its replacement by an uncharged residue could possibly cause displacement of the side chain of either of the two glucose-binding residues.

Origin of the positive cooperativity for glucose. It has been known for more than 20 years that the saturation curve of glucokinase for glucose is sigmoidal (37,38). This is an important property of glucokinase because it sensitizes this "glucose-sensing" enzyme to its substrate. Because glucokinase is a monomeric enzyme whether in the absence or presence of its substrates (39), classical models of cooperativity, such as the symmetrical model of Monod et al. (40) or the sequential model of Koshland et al. (41) are not applicable. Random nonequilibrium addition of substrates can give rise, under some conditions, to cooperativity for one of the substrates (42), but this explanation can be excluded on the basis that there is no significant inhibition by ATP (43). Therefore, other models have been proposed in which the cooperativity is based on the existence of two different conformations with different affinities for glucose that interconvert slowly (44).

Two mutations are most interesting with respect to cooperativity, the Y214A mutation (this study) and the N166R mutation (17). Because in both cases the decrease in cooperativity is accompanied by an increase in the affinity for glucose, it is likely that they act by favoring the conformation of glucokinase that has the highest affinity for glucose.

Binding site for the regulatory protein. Previous studies (15,17) have shown that residues implicated in the binding of the regulatory protein are located on the smaller domain and in the hinge region (indicated in red in Fig. 5*C*). Residues that we now identify as playing a role in the binding of the regulatory protein are located between these two regions, except for A208, which is buried in the hinge region. However, the effect of the mutations studied here is less important (1.5- to 2.5-fold) than those reported previously (40-fold for mutant A; 770-fold for mutant C; 15-fold for V203A; and 10-fold for N204Q). These considerations indicate that the binding site for the regulatory protein may consist of two regions that are well separated on the surface of glucokinase and are in the appropriate position (relative to each other) to bind the regulatory protein, only when glucokinase is in its open conformation. We speculate that these two regions are recognized by two different domains of the regulatory protein and that the distance between these two domains is affected by the conformational movements induced by fructose-6-phosphate and fructose-1-phosphate. These two ligands bind to the regulatory protein and affect in opposite manner its ability to bind and inhibit glucokinase (18).

Binding site for palmitoyl CoA. Changes in the affinity for palmitoyl CoA that have been observed until now concern residues K296, E279, and K414 (17, this study), all of which are on the upper face of the larger domain (Fig. 5*D*). The fact that two of the mutations also affect the affinity for ATP indicates that acyl CoAs may bind to the ATP-binding site possibly because of their partial structural homology with this substrate. This is in agreement with the notion that the inhi-

bition by acyl CoAs is competitive with respect to ATP (20) but contradicts a previous conclusion that acyl CoAs would bind to an allosteric site (45). This conclusion was based on the fact that the inhibition was partial and reached a plateau at concentrations of inhibitor calculated to be below the critical micelle concentration. One should, however, be cautious in interpreting such values, since the critical micelle concentration may be modified by small changes in the pH or in the concentrations of ions. The fact that the inhibition by palmitoyl CoA is competitive with respect to glucose but that it is additive with the inhibition by NAG (12) suggests that the competition with glucose is mediated by preventing closure of the catalytic cleft.

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