Biochemical characterization of novel glucokinase mutations isolated from Spanish maturity onset diabetes of the young (MODY2) patients.

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ABSTRACT

MODY2 (mature onset diabetes of the young, type2) is associated with mutations in the GCK gene that result in impaired glucokinase activity. Although more than 200 inactivating GCK mutations have been reported, only less than 20% of these mutations have been functionally characterized. In this work we describe the biochemical characterization of six missense glucokinase mutations associated to MODY2 from Spanish patients, namely Y61S, V182L, C233R, E265K, A379V and K420E. All these mutations produced enzymes that presented reduced enzymatic activity in various degrees, from a mild affectation (K420E) to a more severe effect (C233R). The severity of the mutation correlated with the importance of the structural changes introduced by the mutations. For example, the C233R affected a critical residue of the active centre of the enzyme and rendered a protein with undetectable enzymatic activity. These data add new information on the structure-function relationship of human glucokinase.

Key words: MODY2, glucokinase, inactivating mutation, enzyme kinetics.

INTRODUCTION

Glucokinase B (GlkB, hexokinase IV) is the first enzyme of the glycolytic pathway in pancreatic β-cells. It phosphorylates the glucose that enters these cells through the GLUT2 glucose transporter to produce Glu-6P, which is then used to obtain the energy necessary for cell viability. The kinetic properties of GLUT2 (high capacity, low-affinity glucose transporter) and GlkB (low affinity for glucose and absence of allosteric regulation by Glu-6P) define GlkB as the key regulator of glucose consumption. For this reason, several authors have considered GlkB as the "glucose sensor" of pancreatic β-cells [(Matschinsky et al. 1993), (Matschinsky 1996), (Matschinsky et al. 1998), (Matschinsky 2002), (Zelent et al. 2005)]. In addition, a positive correlation between the functionality of GlkB and the ability of pancreatic β-cells to respond to increased levels of glucose in blood by enhancing insulin production and secretion has been described. In this way, GlkB participates in maintaining the blood glucose concentration around 5 mM. Mutations in glucokinase gene (GCK) that increase the affinity of the enzyme for glucose decrease the threshold for glucose stimulated insulin secretion (GSIS) and therefore trigger insulin secretion at lower concentrations of glucose in blood. These mutations are associated with both, mild and severe forms of persistent hyperinsulinaemic hypoglycaemia in infancy (PHHI-GK). On the other hand, inactivating mutations in one or both alleles of glucokinase gene leads to an enzyme with low activity which will increase the threshold for glucose stimulated insulin secretion (GSIS) from its physiological setting of 5 mM. These type of mutations are associated with a mild form of diabetes mellitus in young people, known as maturity onset diabetes of the young type 2 (MODY2), or with severe permanent neonatal

diabetes mellitus (PNDM) [see (Matschinsky 2002), (Zelent et al. 2005) and (Gloyn 2003), for review].

MODY2 is a form of diabetes mellitus characterized by an onset that usually appears before 25 years of age and abnormal β-cell function and insulin secretion (Velho et al. 2004). The MODY2 phenotype is characterized by a mild, non-progressive form of hyperglycemia present from birth, often asymptomatic and only detected later in life. MODY2 patients are usually treated with diet alone and rarely develop diabetic-associated complications (Velho et al. 2004). Nearly 200 mutations have been already identified as inactivating GCK mutations. They are almost evenly distributed along the 10 exons that comprise the GCK gene and include missense, nonsense and frameshift mutations produced by deletions or insertions. However, only less than 20% of these mutations have been functionally characterized [(Gloyn 2003), (Velho et al. 2004), (Gloyn et al. 2004)]. Despite considerable differences in the effect of the mutations on the *in vitro* activity of the enzyme, it is generally accepted that the MODY2 phenotype is quite homogeneous [(Miller et al. 1999), (Stride et al. 2002)].

The crystal structure of human GlkB has been recently characterized (Kamata et al. 2004). These results indicate that the protein has a large and a small domain, separated by a deep cleft. Glucose binds to the interdomain cleft, composed of residues of the large domain (Glu256 and Glu290), the small domain (Thr168 and Lys169) and the connecting region I (Asn204 and Asp205). Upon binding to substrates (glucose and ATP), GlkB undergoes a conformational change that brings the large and the small domain physically closer, resulting in a closed, active conformation. This structural model has

helped in the understanding of the abnormal biochemistry of different GCK missense mutations [(Pedelini et al. 2005), (Galan et al. 2006)].

In this study we report the biochemical characterization of six GCK missense mutations found in Spanish MODY2 families (Estalella et al. 2007) and evaluated the impact of the mutation at the structural level by introducing these modifications into the closed active and super-open inactive GlkB structure models described recently (Kamata et al. 2004).

MATERIALS AND METHODS

1.- Subjects

A total of 21 subjects from 9 families (Estalella et al. 2007) with one of the following six GCK mutations (Y61S, V182L, C233R, E265K, A379V and K420E) were included in this study. These mutations were selected among the different mutations identified in the previous study (Estalella et al. 2007) because they were novel missense mutations (except E265K) that were representative of different areas of the molecular structure of glucokinase. All the patients met the classical MODY2 diagnostic criteria, presenting a defect in glucose homeostasis, absence of autoimmunity markers and an autosomal dominant mode of inheritance (Velho and Froguel 1997). The human ethic committee of Cruces hospital approved the study and all analyses were performed after informed consent was obtained from individuals or their parents.

2.- Site directed mutagenesis

All the mutants used in this study were obtained as described in (Pedelini et al. 2005) using the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA) and the oligonucletides described in Table 1.

3.- Purification of GST-fusion proteins and kinetic determinations

Purification of the mutated GST-GlkB fusion proteins was carried out as described in (Pedelini et al. 2005). The kinetic parameters of the recombinant GST-GlkB proteins were also measured as described in (Pedelini et al. 2005).

4.- Prediction of structural effects of glucokinase mutations.

Crystal coordinates from the closed active (1V4S) and super-open inactive (1V4T) conformation of GlkB (Kamata et al. 2004) were visualized and mutations evaluated using O program (Jones and Kjeldgaard 1997). Figure 2 was generated with MolScript (Kraulis 1991) and Render 3D (Merritt and Bacon 1997) programs using the original or O-program modified PDBs.

RESULTS AND DISCUSSION

Kinetic parameters of novel GCK missense mutations

Selected missense mutations from Spanish MODY2 families (Y61S, V182L, C233R, E265K, A379V and K420E; see Table 2) were introduced in GCK cDNA by site-directed mutagenesis, using appropriated oligonucleotides. Mutated cDNAs were subcloned into plasmid pGEX6P-1 to produce GST-GlkB proteins that were purified from bacteria. We then compared the kinetic parameters of the mutated forms with those of the wild type glucokinase (Table 2 and Fig. 1A). Since in regular conditions of glucose homeostasis the

concentration of glucose in human blood is around 5 mM and there are saturating concentrations of ATP, we assumed that the activity of the enzyme at 5 mM glucose and saturating concentrations of ATP (5 mM) is a good parameter to correlate the activity of the enzyme with its performance under physiological conditions (Pedelini et al. 2005) (Fig. 1B). All the six missense mutations presented reduced enzymatic activity, in various degrees, from a mild affectation to a more severe effect. The K420E mutation was the milder; it had a lower glucose affinity (S0.5: 11.6 mM) in comparison to wild type (S0.5: 7.6 mM). Interestingly, the activity of the mutated form at 5 mM glucose was almost half of wild type, suggesting a poor performance as a glucose phosphorylating enzyme under physiological conditions (Fig. 1B).

The E265K and A379V mutations had affected both Vmax (37.9 and 28.7 Units/mg, respectively) and S0.5 (11.5 mM and 13.7 mM, respectively). In addition, A379V had also a lower ATP affinity (Km 0.69 mM). The activity of the E265K mutated form at 5 mM glucose was also half of the wild type, whereas the activity of the A379V mutated form was even poorer (Fig. 1B).

Mutations V182L and Y61S affected severely glucose affinity (S0.5: 28.8 mM and 39.2 mM, respectively) and the Vmax of the mutated forms (16.7 and 7.2 Units/mg, respectively). Both mutations increased the ATP affinity (KmATP: 0.16 and 0.21 mM, respectively). The activity of the mutated forms at 5 mM glucose was very poor (Fig 1B), suggesting that these enzymes had a very poor performance as glucose phosphorylating enzymes under physiological conditions.

Finally, when we assayed the activity of the C233R mutant we were unable to detect any activity, suggesting that this mutation had abolished the glucose phosphorylating activity of the enzyme.

Prediction of structural effects of glucokinase mutations.

To understand the changes in the kinetic parameters of the mutated enzymes, we evaluated the impact of the mutation at the structural level by introducing these modifications into the closed active and super-open inactive GlkB structure models described recently (Kamata et al. 2004). Fig. 2 shows the effects of the mutations on the closed active conformation of the enzyme.

The K420 residue (Fig. 2F) is located in the α12 helix of the large domain and it is exposed to the solvent in both the closed and the super-open conformations. In the K420E mutation there is a change in the charge of the side chain (from the positive Lys to the negative Glu residue) and perhaps this may affect its interaction with other negatively charged residues in the surrounding such as E421 and E440. The E265 residue (Fig. 2D) is located also in the large domain and exposed to the solvent. The E265K mutation would affect putative interactions with residues of the α2 helix, such as E46 and R40. In has been recently characterized a closely related mutation G264S (Massa et al. 2001) which also has minor effects on the kinetics of the enzyme (S0.5: 9.76 mM; KmATP: 0.48 mM) (Gloyn et al. 2004). Since at the structural level the modifications introduced by all these three mutations (K420E, E265K and G264S) are minor, perhaps this is the reason why the kinetic properties of the mutated enzymes are not greatly affected. Recently, the characterization of the E265K mutation was described. The authors suggested that although the

impact of the mutation was minor at the level of the kinetics of the enzyme, it strongly affected protein stability, suggesting a possible structural level defect of this mutant protein (Galan et al. 2006).

The A379 residue (Fig. 2E) is located in the α11 helix of the large domain, at the back of the ATP binding site. Since this residue is very close to residues of the α12 helix (such as F419, R422 and F423), the increase in size of the side chain of the new valine residue in the A379V mutation would force a reorganization of this protein region. For this reason, the kinetic properties of the mutated enzyme should be more affected. It is worth to point out that a closely related mutation A378V has been recently described as a glucokinase mutation found in a case of permanent neonatal diabetes (Njolstad et al. 2003). The A378V is buried in the large domain hydrophobic core, and directly oriented towards the ATP binding site, showing the kinetics of the mutated enzyme a severe defect (S0.5: 584 mM; KmATP: 8.08 mM) (Gloyn et al. 2004). Both modest Ala to Val mutations reflect the importance of the correct disposition of the α11 helix, although the relative orientation of the residue has a different impact on the structure of the protein and consequently on the kinetics of the enzyme.

The V182 residue (Fig. 2B) is located in the α4 helix base of the small domain, following a catalytic loop that is involved in glucose binding site formation. In the super-open conformation, the glucose molecule is absent, and consequently this loop is disorganized. The transition between super-open and closed conformations involves a ~90º displacement of the α4 helix, pivoting over its base (where V182 localizes), in order to accommodate the catalytic loop. The V182L mutation introduces a residue with bigger side-chain and

higher hydrophobicity that might disturb an area packed with the side chains of residues D160 and K161 (in the loop) and M202. In this way, it would destabilize the catalytic loop disposition in the closed conformation of the enzyme. Furthermore, the change V182L might interfere in the transition between the different catalytic conformations. For this reason the mutated protein should have lower Vmax and glucose affinity, and higher Hill number than wild type. The substitution of Val by a Met residue (V182M) has also been reported (Costa et al. 2000). In this case, the mutated enzyme also displays lower Vmax and lower glucose affinity (S0.5: 83.9 mM) than wild type (Gloyn et al. 2004). On the contrary, both V182L and V182M mutations displayed a higher ATP affinity (KmATP 0.16 and 0.10 mM, respectively).

The Y61 residue (Fig. 2A) is located in the connexion between small and large domains. In the closed conformation, the bulky hydrophobic Tyr side chain may make hydrophobic interactions with surrounding residues, such as R63, V244 and M251, and a hydrogen bridge bond with E157 main chain oxygen, stabilizing the closed conformation of the enzyme. In the Y61S mutation, the big hydrophobic Tyr residue is changed to a small polar Ser residue that does not longer maintain these interactions, destabilizing the closed conformation of the enzyme. This would result in a decrease in the Vmax and the glucose affinity of the mutated enzyme. In a closely related mutation V62A (Njolstad et al. 1998), the kinetic parameters were similarly affected (S0.5: 27.4 mM; KmATP: 0.20 mM) (Gloyn et al. 2004), suggesting that this area may play an important role in maintaining the closed conformation of the enzyme. Interestingly, a V62M mutation shows higher glucose affinity (S0.5: 4.88 mM), although the mutation was isolated from patients suffering hyperglycemia (Gloyn et al. 2005).

Finally, the C233 residue (Fig. 2C) is located in the β10 sheet that forms part of the hydrophobic interface between small and large domains in the base of the active centre of the enzyme (glucose binding site). In the C233R mutation, the new Arg residue has a huge side-chain in comparison with the small original Cys. To accommodate the new side-chain, an important reorganization in the surrounding area must occur, destabilizing the active centre, and consequently, affecting severely the activity of the enzyme.

Phenotype/Genotype relationship

The clinical characteristics of the patients carrying the corresponding GCK mutations are shown in Table 3. To determine whether the degree of biochemical *in vitro* defect in glucokinase activity was related to phenotype severity, mutations were ordered according to the magnitude of the change in enzymatic activity. However, no differences were observed among patients regarding clinical characteristics. Average fasting plasma glucose (FPG) (6.95 ± 0.8 mmol/l) was found in all the cases and was similar to previously reported values (Stride et al. 2002). Although most of the MODY2 patients required only diet for control, three of them were treated with oral hypoglycaemic agents (OHA) or insulin, and these patients carried severe changes in the in vitro glucokinase activity. However, as there were other patients carrying the same mutation that only required diet for control, we can not establish a clear relationship between the severity of the biochemical defect and the actual diagnosis and treatment.

In summary, our data confirmed that MODY2-associated glucokinase mutations can be due to modifications in conserved residues of the active site, as in the case of C233R, to modifications that distort the structure of the protein, as in the case of A379V, V182L and Y61S, or to modifications in residues of the periphery of the molecule that may disrupt interactions with other residues of the protein or with other interacting proteins, as in the case of K420E and E265K, as previously suggested ((Miller et al. 1999), (Velho and Froguel 1997)). These data add new information on the structure-function relationship of human glucokinase.

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Table 1: Oligonucleotides used in this study. The modified codon is underlined.

Name	Sequence
Y61S-1	5′-CAGTGTGAAGATGCTGCCCACCTCCGTGCGCTCCACCCCAGAAGG-3´
Y61S-2	5´-CCTTCTGGGGTGGAGCGCACGGAGGTGGGCAGCATCTTCACACTG-3´
V182L-1	5´-GGAGCAGAAGGGAACAATGTCTTGGGGCTTCTGCGAGACGCTATC-3´
V182L-2	5´-GATAGCGTCTCGCAGAAGCCCCAAGACATTGTTCCCTTCTGCTCC-3´
C233R-1	5´-GGCACGGGCTGCAATGCCCGCTACATGGAGGAGATGCAGAATGTG-3´
C233R-2	5´-CACATTCTGCATCTCCTCCATGTAGCGGGCATTGCAGCCCGTGCC-3´
E265K-1	5´-GCCTTCGGGGACTCCGGCAAGCTGGACGAGTTCCTGCTGGAGTATG-3´
E265K-2	5´-CATACTCCAGCAGGAACTCGTCCAGCTTGCCGGAGTCCCCGAAGGC-3´
A379V-1	5´-GAGAGCGTGTCTACGCGCGCTGTGCACATGTGCTCGGCGGGGCTGG-3´
A379V-2	5´-CCAGCCCCGCCGAGCACATGTGCACAGCGCGCGTAGACACGCTCTC-3´
K420E-1	5´-GTACAAGCTGCACCCCAGCTTCGAGGAGCGGTTCCATGCCAGCGTG-3´
K420E-2	5´-CACGCTGGCATGGAACCGCTCCTCGAAGCTGGGGTGCAGCTTGTAC-3´

Table 2: Kinetic parameters of mutated forms of glucokinase. Glucose-dependent activity (Vmax, S0.5, Hill number) and ATP-dependent activity (Km) parameters were determined in purified GST-GlkB fusion proteins. Values are the mean of at least two different kinetic determinations for each purification procedure for each mutant ± standard deviation. The nucleotide change and the exon location for each mutant is also indicated. N.d. not detectable.

Nucleotide change	Exon	Amino acid change	Vmax (Units/mg)	S0.5 (mM)	Hill number	Km ATP (mM)
		Wild type	44.1 ± 2.0	7.6± 0.3	1.42 ± 0.04	0.45 ± 0.03
TAC>TCC	2	Y61S	7.2 ± 0.6	39.2 ± 0.3	1.50 ± 0.17	0.21 ± 0.01
GTG>TTG	5	V182L	16.7 ± 0.1	28.8 ± 2.7	1.61 ± 0.05	0.16 ± 0.03
TGC>CGC	7	C233R	n.d.	n.d.	n.d.	n.d.
GAG>AAG	7	E265K	37.9 ± 3.2	11.5 ± 0.5	1.37 ± 0.07	0.40 ± 0.07
GCG>GTG	9	A379V	28.7 ± 7.5	13.7 ± 0.1	1.45 ± 0.02	0.69 ± 0.13
AAG>GAG	10	K420E	41.3 ± 5.7	11.6 ± 0.3	1.47 ± 0.04	0.41 ± 0.05

Table 3: Clinical characteristics of subjects with the different MODY2 mutations ordered by decreasing *in vitro* enzymatic activity. (IGT: impaired glucose tolerance; OHA: oral hypoglycemic agents; FPG: fasting plasma glucose; 2-h OGTT: plasma glucose 2 hours after an oral glucose tolerance test).

Mutation	Age	Years since diagnosis	FPG (mmol/l)	2-h OGTT (mmol/l)	HbA_{1c} (%)	Diagnosis	Actual treatment
K420E	10	5	6.4	10.2	6.4	IGT	diet
K420E	14	2	6.7	10.1	6.0	IGT	diet
K420E	68	-	6.8	10.5	-	IGT	diet
K420E	44	29	6.0	10.9	-	IGT	diet
E265K	25	17	7.2	-	7.2	Diabetes	diet
E265K	36	21	6.6	8.1		IGT	diet
E265K	39	20	7.8	-	6.5	Diabetes	diet
E265K	42	11	7.8	-	6.6	Diabetes	diet
A379V	39	9	7.4	12.1	-	Diabetes	diet
A379V	39	16	5.7	12.5	5.7	Diabetes	diet
A379V	68	12	7.6	-	-	Diabetes	diet
V182L	10	7	7.5	-	5.6	Diabetes	diet
V182L	11	3	7.3	-		Diabetes	diet
V182L	21	11	7.1	9.5	6.8	Diabetes	diet
V182L	49	31	7.2	-	6,2	Diabetes	OHA
Y61S	27	23	7.5	11.4	5.7	Diabetes	diet
Y61S	45	10	7.8	-	5.8	Diabetes	diet
C233R	28	17	5.6	9.3	-	IGT	diet
C233R	30	17	6.2	10.9	-	IGT	diet
C233R	54	30	-	-	5.1	Diabetes	OHA
C233R	62	17	-	-	-	Diabetes	insulin

Figure legends

Fig. 1: Enzymatic functionality of the mutated forms of glucokinase. A) Glucose-dependent activity of mutated forms of glucokinase. B) Specific activity of mutated forms of glucokinase at 5 mM glucose and saturating concentrations of ATP (5 mM) at 30ºC. A representative experiment of at least two different kinetic determinations for each purification procedure is shown in each case.

Fig. 2: Effects of the mutations on the structure model of glucokinase. (A-F) The different mutations were studied at the structural level by introducing these modifications into the closed active GlkB structure model. Wild type residues are coloured in green, whereas the mutated residues are coloured in magenta. Interacting residues are coloured in orange. In some panels, the position of the glucose (Gluc) or the glucokinase activator (Act) molecules is also indicated.

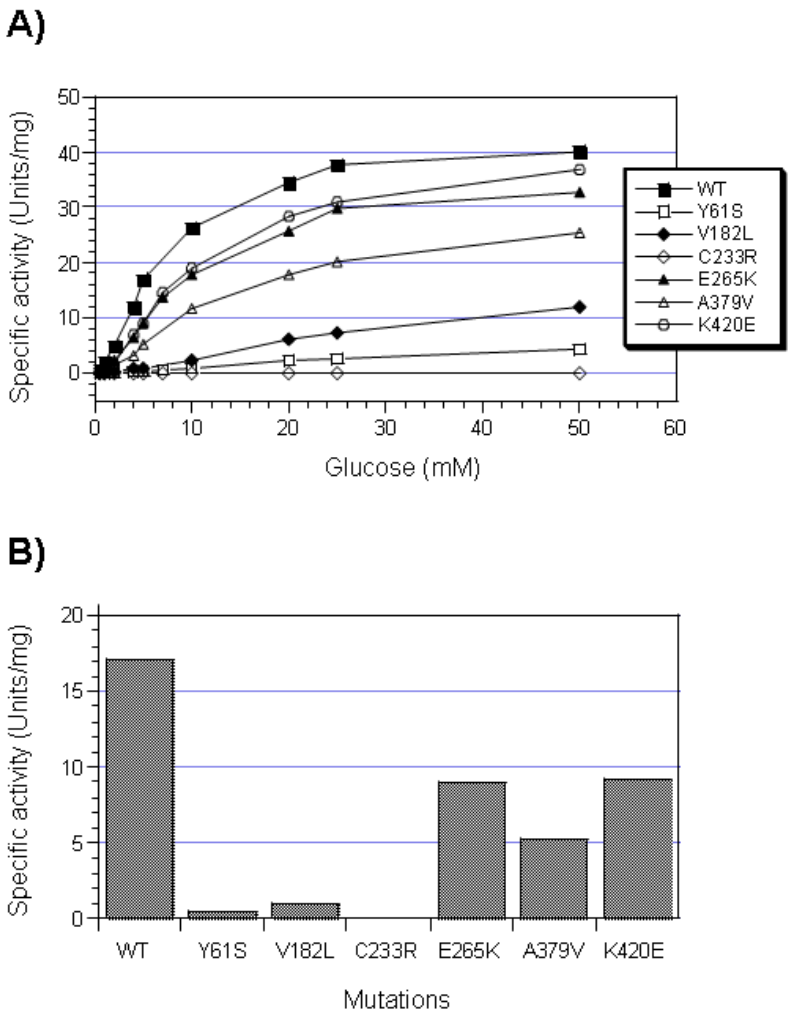

Fig. 1: Estalella et al. 2008

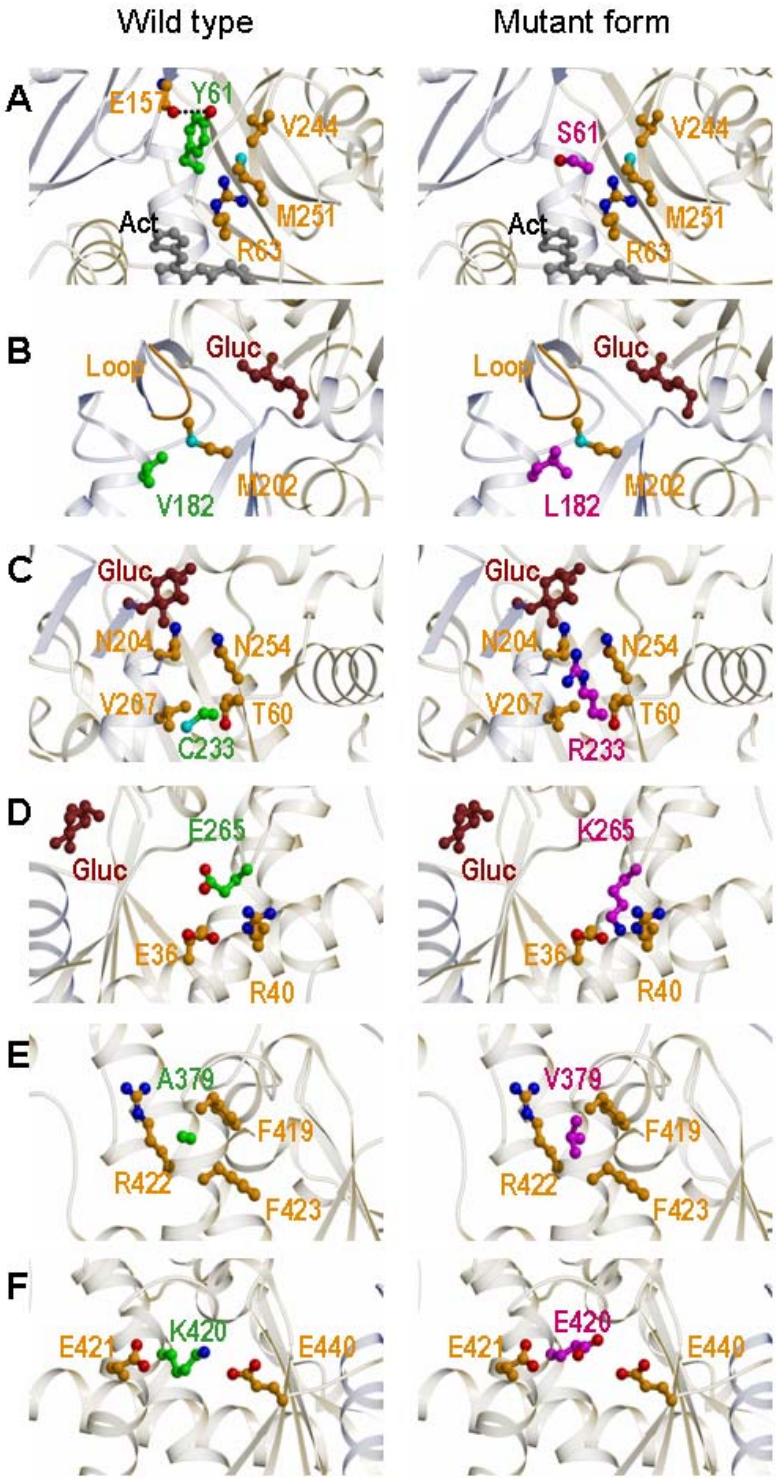

Fig. 2: Estalella et al., 2008