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ORIGINAL ARTICLE

A single-residue mutation, G203E, causes 3-hydroxy-3-methylglutaric aciduria by occluding the substrate channel in the 3D structural model of HMG-CoA lyase

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Summary 3-Hydroxy-3-methylglutaric aciduria is a rare autosomal recessive genetic disorder that affects ketogenesis and leucine metabolism. The disease is caused by mutations in the gene coding for 3-hydroxy-3-methylglutaryl-coenzyme A lyase (HL). To date 26 different mutations have been described. A $(\beta\alpha)_8$ TIM barrel structure has been proposed for the protein, and almost all missense mutations identified so far localize in the beta sheets that define the inside cavity. We report an Italian patient who bears homozygously a novel HL mutation, c.608G>A (p. G203E) in beta sheet six. A structural model of the mutated protein suggests that glutamic acid 203 impedes catalysis by blocking the entrance to the inner cavity of the enzyme. Loss of functionality has been confirmed in expression studies in *E. coli*, which demonstrate that the G203E mutation completely abolishes enzyme activity. Beta sheet six and beta sheet two are the two protein regions that accumulate most missense mutations, indicating their importance in enzyme functionality. A model for the mechanism of enzyme function is proposed.

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Introduction

3-Hydroxy-3-methylglutaric aciduria (McKusick 246450) is a rare autosomal recessive metabolic disorder that affects ketogenesis and leucine catabolism. The origin of the disease is a mutation in the gene *HMGCL* that codes for 3-hydroxy-3-methylglutaryl-coenzyme A lyase (HL) (EC 4.1.3.4), which catalyses the cleavage of 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) to form acetyl-CoA and acetoacetate. This disease appears in the first year of life, normally after a fasting period, and it is fatal in about 20% of cases. The most prominent findings are vomiting, lethargy, coma, convulsions, metabolic acidosis and hypoketotic hypoglycaemia (Gibson et al 1988). Biochemical diagnosis is based on the excretory pattern of organic acids in urine (3-hydroxy-3-methylglutaric, 3-hydroxyisovaleric, 3-methylglutaconic and 3-methylglutaric (Faull et al 1976)) and the measurement of enzyme activity in leukocytes (Wanders et al 1998). Genetic studies confirm the presence of the disease. To date, 42 patients have been described at molecular level and 26 different mutations have been identified so far, which include 3 large deletions, 4 small frameshift deletions, 1 small frameshift insertion, 4 nonsense mutations, 12 missense mutations, and 2 intron mutations (Mitchell et al 1993, 1995, 1998; Roberts et al 1996; Wang et al 1996; Buesa et al 1996; Pié et al 1997; Casals et al 1997, 2003; Casale et al 1998;

Zapater et al 1998; Muroi et al 2000; Funghini et al 2001; Pospisilova et al 2003; Cardoso et al 2004).

In 2003 a $(\beta\alpha)_8$ TIM barrel structural model was proposed for the enzyme (Casals et al 2003) using *Thermotoga maritima his A* protein as a template. All the missense mutations reported, except one, localized in the beta sheets of the barrel, which contain the catalytic domain, define the substrate cavity and stabilize the TIM barrel structure (Gromiha et al 2004). Recently, a new TIM barrel model for HL has been proposed using the aldolate dehydrogenase protein as a template (Tuinstra et al 2004). No significant variations of overall protein were detected when compared with the first model proposed. The root-mean-square deviation (RMSD) value between the corresponding backbone atoms was 1.3 Å, which indicates satisfactory superimposition and reinforces both 3D models (Puisac et al 2005).

Here we describe a new missense mutation identified in an Italian patient, which affects beta sheet six of the HL TIM barrel, and propose a mechanism by which this mutation abolishes enzyme activity.

Materials and methods

Case report

Male patient B.A. was the second child born to healthy, unrelated Italian parents. He was delivered at term in the 41st week of gestation after an uneventful pregnancy. Birth weight was 3.2 kg (10th centile), length was 49 cm (10th centile) and head circumference was 49 cm (50th centile). Apgar score was 10 and 10 at 1 and 5 min, respectively. At 3 days of life he was admitted to the local hospital because of refusal to feed and lethargy. Routine laboratory analyses revealed hypoglycaemia (1.4 mmol/L) and metabolic acidosis (BE −18.5 mEq/L, pH 7.19). Following glucose and bicarbonate infusion, clinical and biochemical conditions normalized. EEG, cerebral ultrasound and cerebrospinal fluid analysis were normal. He was then discharged after two weeks. At the age of 1 year he presented with lethargy and tonic-clonic seizures after an overnight fast. On admission his weight was 10.760 kg (50th centile), length 75 cm (>50th centile), head circumference 49.2 cm (>97th centile). Biochemical analysis revealed hypoglycaemia (0.84 mmol/L), metabolic acidosis (BE −11.4 mEq/L) and absence of urine ketones. Intravenous glucose and bicarbonate infusion normalized both clinical and biochemical parameters. Urine GC-MS analysis showed a characteristic pattern with increased excretion of 3-hydroxyisovaleric, 3-methylglutaconic, glutaric, 3-methylglutaric and 3-hydroxy-3-methylglutaric acids. Protein restriction (1 g/kg/day) and oral carnitine (100 mg/kg/day) supplementation were then started. At follow-up, present age 7 years, he shows normal auxological, cognitive and neurological development. No other episodes of hypoglycaemia, metabolic acidosis or convulsions were reported.

Mutational analysis

Mutational analysis was performed in DNA isolated from patient and control lymphocytes using 'DNAzol Reagent Kit' from Invitrogen. All exons were amplified as described in Muroi and colleagues (2000). PCR products were purified with 'Quiaquick PCR Purification Kit' or 'Quiaquick Gel Extraction Kit' of Quiagen, and sequenced with an Applied Biosystems 373 automated DNA sequencer.

Missense mutation restriction analysis

Restriction analysis was performed to confirm the new missense mutation G203E. Specific primer (Mut203F) was designed to introduce an individual nucleotide mutation, which would generate a new *Xho*I restriction site in the patient DNA. The primer used in the amplification is shown-below. The modified nucleotide is underlined. Mut203F: 5′ACTCAATGGGCTGCTACGAGATCTCCCTCG3′. Genomic fragments amplified with primers Mut203F and HLE7R (Mitchell et al 1998) in control and patient were used as substrates for analytical digestion with *Xho*I. Five units of restriction enzyme were added to 20 µl of reaction medium. Digestion was incubated for 3 h at 37°C; the resulting restriction fragments were fractionated on a 4.5% MetaPhor-agarose gel.

Construction of expression plasmids and expression of HL in *E. coli*

Mutant G203E of HL was constructed using the 'Quick Change' PCR-based mutagenesis procedure (Stratagene) with the pTr-HL-wt plasmid as a template, whose construction is described in detail elsewhere (Casals et al 2003). Primer 5′-CGAGATCTCCCTGGAGGACACCATTGGT-GTG-3′ was used to construct pTr-HL-203E. The appropriate substitution and the absence of unwanted mutations were confirmed by sequencing the insert. *E. coli* JM105 cells were transformed with the expression plasmids pTr-HL-wt, pTr-203E, or empty vector (pTr-0). Four different cell clones for G203E mutated protein and wild-type were expressed in *E. coli* as described elsewhere (Roberts et al 1994). Protein quantification was performed using the Bio-Rad protein assay with bovine albumin as standard.

HMG-CoA lyase activity

HMG-CoA lyase activity was measured by a simple spectrophotometric method that determines the amount of

acetoacetate produced (Wanders et al 1988). To measure HL-specific activity, 2 μg of soluble protein extracts and 800 nmol of substrate (HMG-CoA) were used. In the case of mutation extract, determinations were also carried out with 20 or 75 μg of soluble protein extract. The reaction assay was performed at 37°C for 15 min in a final volume of 250 μl. One enzyme unit represents the formation of 1 μmol of acetoacetate in 1 min. Results are given as mean values of at least four independent experiments.

Western blot analyses

A polyclonal rabbit antibody against residues 307–321 of human HL (which recognizes the COOH terminus of the enzyme) was raised by Sigma-Genosys. 50 μg of *E. coli* protein extract was subjected to SDS-PAGE. Electroblotting to nitrocellulose sheets was carried out for 2 h at 120 mA. Immunodetection of HL was performed using the antibody anti-HL (dilution 1:10 000), and the blots were developed with the ECL Western blotting system from Amersham Biosciences.

Structural model for HMGL-HUMAN

The 3D model of *wt* HMGL-HUMAN (amino acids 36–325) was built essentially as that described for amino acids 36–287 (Casals et al 2003) but using as modelling template the structural alignment of the available structures of the $(\beta\alpha)_8$ barrels 1QO2 and 1NVM, listed in the Protein Data Bank. The last structure corresponds to the *Pseudomonas mevalonii* 4-hydroxy-2-oxovalerate aldolase protein, which has recently been used to build an alternative model for HL (Tuinstra et al 2004). The G203E mutant was modelled using the *wt* enzyme as template. The structural quality of both models was checked using the WHAT-CHECK routines from the WHAT IF program and the PROCHECK validation program from the SWISSMODEL server facilities (Laskowski et al 1993). Protein surface topology and electrostatic potentials were calculated using GRASP (Nicholls et al 1991).

Results and discussion

Identification of mutations responsible for 3-hydroxy-3-methylglutaric aciduria in an Italian patient

DNA extracted from patient lymphocytes was used as template for amplification by PCR of the nine exons of HMGCL using primers described elsewhere (Mitchell et al 1998). The size of the amplified fragments was identical to control, which suggested that point mutations were responsible for the disease. Sequencing all of the amplified exons showed that the patient presented a new homozygous missense mutation in exon 7 (see Fig. 1a): a transversion G>A in nucleotide 608 of the cDNA (c.608G>A), which leads to the replacement of glycine 203 by glutamic acid (p.G203E). No other mutation was found in codifying sequences or in splicing consensus sequences. Restriction analysis confirmed the new missense mutation G203E. Patient DNA was completely digested by *Xho*I (Fig. 1b), which indicates that the patient bears G203E in homozygosis.

More than sixty cases of 3-hydroxy-3-methylglutaric aciduria have been described to date, being predominantly from Arabian and Mediterranean countries. In Italy, only four patients have been described at the molecular level (Mitchell et al 1995; Casals et al 1997; Funghini et al 2001; present study) and each patient carries a different mutation, indicating that there is no predominant mutation in this country, in contrast to other Mediterranean countries (Spain and Portugal) where mutation E37X predominates (Pié et al 1997; Casale et al 1998; Puisac et al 2005; Cardoso et al 2004).

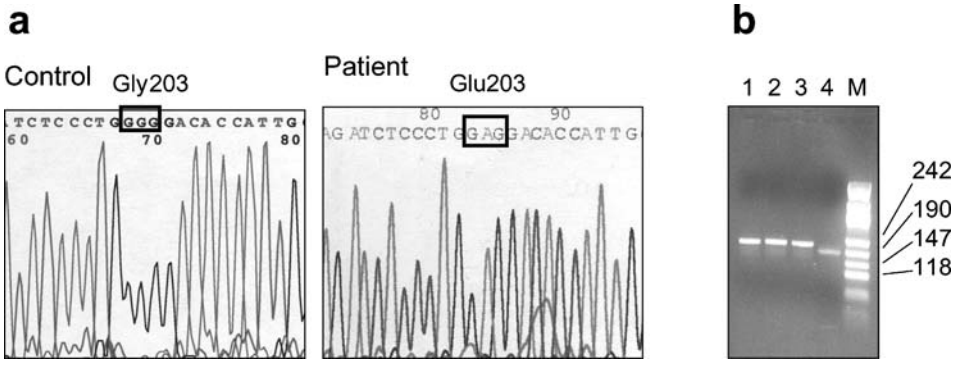

Fig. 1 Identification of mutation G203E (c.608G>A) by sequence and restriction analysis. (a) Comparison of sequence of exon 7 between patient and control, showing the replacement of Gly by Glu at position 203 of cDNA. (b) Restriction analysis of exon 7 from patient and a control DNA. Mutation c.608G>A creates a target for *Xho*I restriction enzyme. DNA fragments amplified with primers Mut203F and HLE7R were digested by *Xho*I. Only amplified DNA from the patient was digested by *Xho*I, generating two fragments of 181 and 27 bp. Lanes. 1, undigested control DNA; 2, digested control DNA; 3, undigested patient DNA; and 4, digested patient DNA

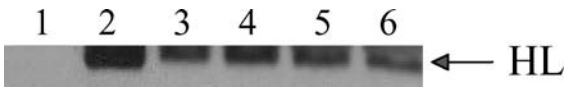

Fig. 2 Expression levels of mutated and wild-type HL proteins. Protein levels of G203E and wild-type HL expressed in *E. coli* cells were determined by Western blot in the soluble fraction of cell lysates. Antibodies used to immunodetect HL were the same as in Casals et al (2003). The band that corresponds to HL is the one marked with an arrow. The upper band is an unspecific band. Four different clones expressing G203E HL (lanes 3 to 6) and one expressing the wild-type (lane 2) were analysed. Lane 1 shows that no HL band was present in *E. coli* cells expressing the empty vector

G203E mutation impairs catalytic activity

To study the effect of the mutation on enzyme activity, G203E mutated protein and wild-type enzyme were expressed in *E. coli* JM105 cells. Four independent clones of each construct were analysed. Western blot analysis (Fig. 2) and specific HMG-CoA lyase activity were determined in the soluble fraction of the crude extracts. Mutation G203E completely abolished enzyme activity, even when 75 μg of total protein extract was assayed. It was imposible to perform saturation kinetics and determine the K_m value for this mutant. As the limit of sensitivity of the current assay was 0.002 U/mg and the specific activity of the wild-type enzyme was 9.5 U/mg, we can say that G203E mutated protein has less than 0.02% of the wild-type enzyme activity and is the cause of 3-hydroxy-3-methylglutaric aciduria in the Italian patient. All patients described so far presented an HL activity lower than 7% of healthy individuals (Pié et al 2003).

Glycine 203 is a small residue that has not been implicated in the catalytic reaction before and is far away from the catalytic residues Cys266, His233 and His235. Figure 3 shows that it is a well-conserved residue in all organisms reported from bacteria to humans and is placed in beta sheet 6 of the structural model, a region also highly conserved in all species, which suggests that glycine 203 has an important role in HL structure. Two other missense mutations that impair enzyme activity have been identified in this region, affecting serine 201 and aspartate 204, which are in close proximity to glycine 203, indicating that beta sheet six is a hot spot for HL mutations.

HMG-CoA lyase structural model explains the loss of functionality of G203E mutated enzyme

To date, two TIM-barrel models have been proposed for HL protein, one based on the structure of *T. maritima His A* protein (Casals et al 2003; Lang et al 2000), and the other based on 4-hydroxy-2-ketovalerate aldolase (Tuinstra et al 2004). RMSD value (Puisac et al 2005) indicates that both models present close structural similarity and can be used to study the effects of mutations in HL structure. Here, we have used both models to understand the effect that produces G203E mutation in HL structure, and the results are exactly the same. Figure 4 shows that mutation G203E, localized

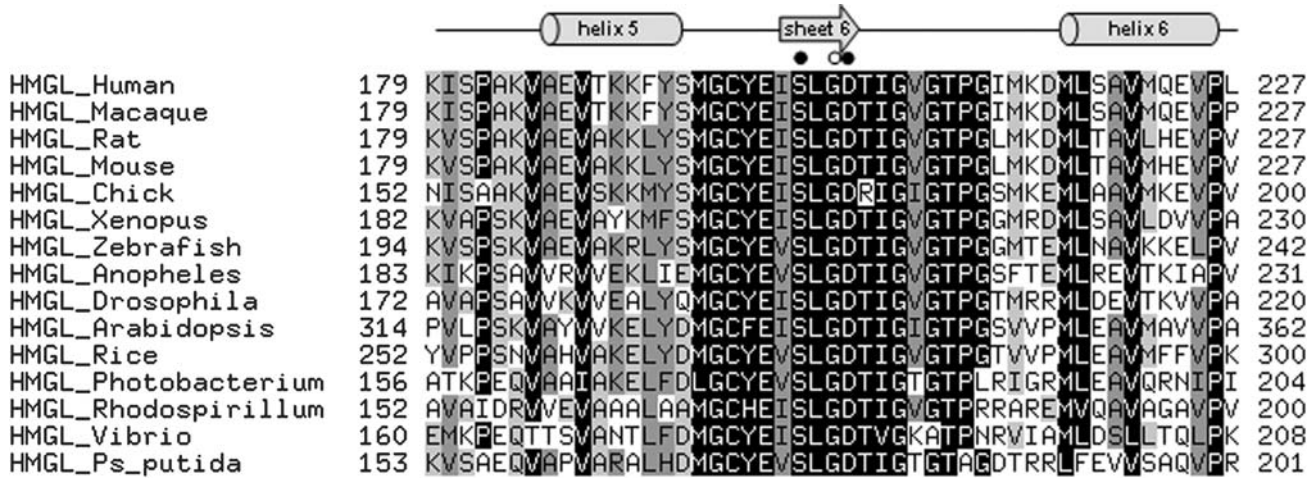

Fig. 3 Residue conservation of HL beta sheet six sequence through evolution. Structural alignment of a representative set of amino acid sequences of different HL genes, surrounding beta sheet six. Accession numbers: HMGL_Human P35914; HMGL_Macaque—*Macaca fascicularis*—Q8HXZ6; HMGL_Rat—*Rattus norvegicus*—P97519; HMGL_Mouse—*Mus musculus*—P38060; HMGL_Chick—*Gallus gallus*—P35915; HMGL_Zebrafish—*Danio rerio*—Q7ZV32; HMGL_Xenopus—*Xenopus laevis*—Q6INN1; HMGL_Rice—*Oryza sativa*—Q9LJ19; HMGL_Anopheles—*Anopheles gambiae*—Q7QLX1; HMGL_Arabidopsis—*Arabidopsis thaliana*—O81027; HMGL_Drosophila—*Drosophila melanogaster*—Q9VM58; HMGL_Photobacterium—*Photobacterium profundum*—Q6LI89; HMGL_Rhodospirillum—*Rhodospirillum rubrum*—P95639; HMGL_Vibrio—*Vibrio parahaemolyticus*—Q87H36; HMGL_Ps_putida—*Pseudomonas putida*—Q88H25. Residues are coloured by conservation according to BLOSUM62 matrix, showing higher values in the closing proximity of beta sheet six, when compared to the flanking alpha helices five and six. Positions of previously reported missense mutation locus S201 and D204 are marked with a black circle. G203 position in the alignment is indicated with an open circle

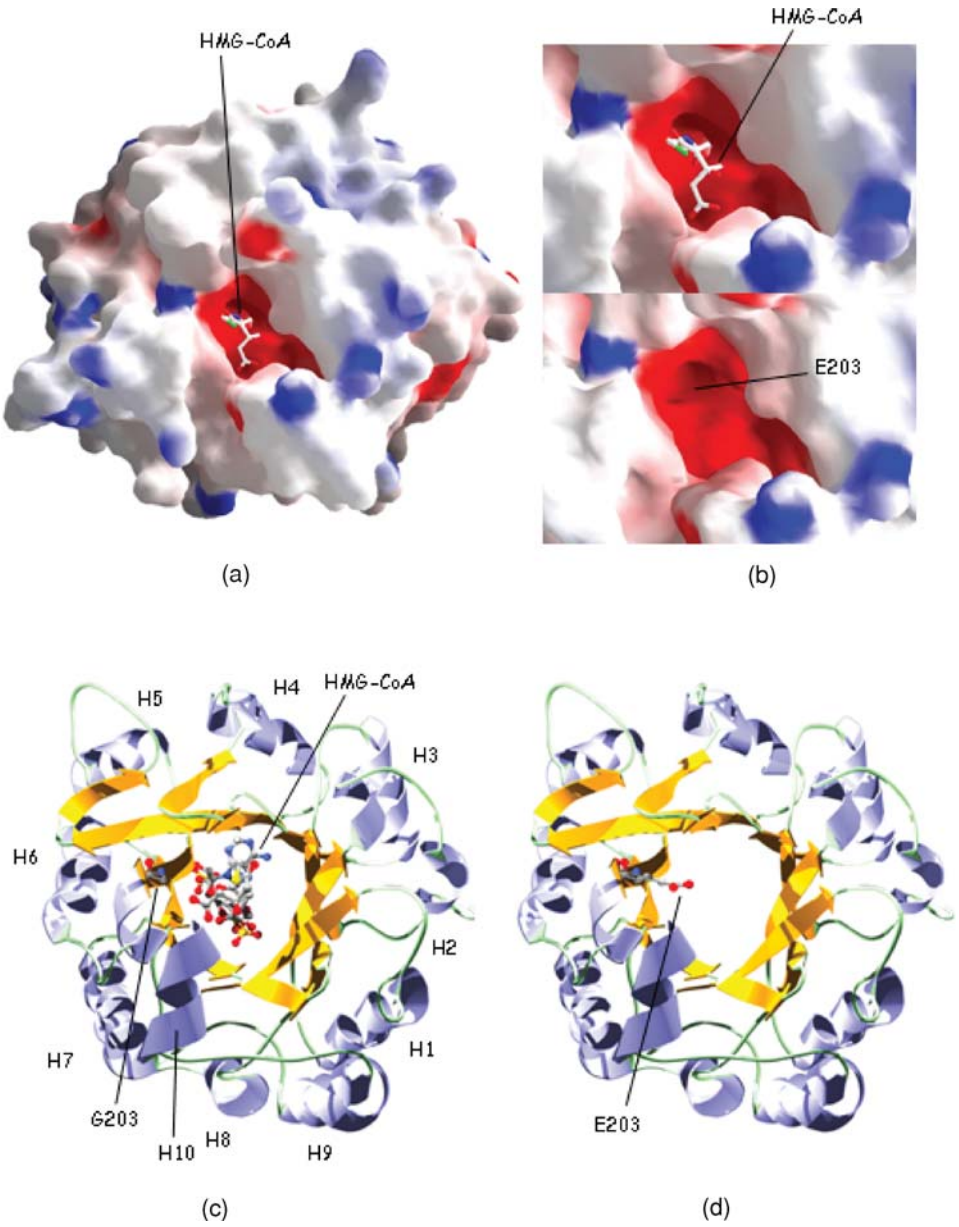

Fig. 4 Effect of mutant G203E in the structural model of the protein. The figures presented are obtained using the model based on PDB structures 1QO2 and 1NVM (Puisac et al 2005; Tuinstra et al 2004). *Upper left*, COOH-terminal view of the wild-type HL solvent-accessible surface and a ball-and-stick representation of a 3-hydroxy-3-methylglutaryl-CoA molecule. Regions of the protein surface with negative charge are shown in red and those with positive charge in blue. *Upper right*, details of the COOH-terminal face of the wild-type protein surface model (above) and G203E mutant (below). *Lower left*, ribbon plot of COOH-terminal face of wild-type HL with glycine 203 and substrate molecule shown in ball and stick representation. *Lower right*, ribbon plot of COOH-terminal face of mutated protein with glutamic acid 203 shown in ball-and-stick representation. The side chain of glutamic acid interferes with the substrate position

in beta sheet six, introduces a bulky charged amino acid, glutamic acid, at a site previously occupied by the smaller glycine. By occupying the central cavity of the TIM barrel, the glutamic acid side chain prevents the accommodation of the substrate, which indicates the importance of glycine 203 in defining the substrate cavity. Steric hindrance and changes in the charge environment may be responsible for the null activity observed in the mutant enzyme.

The 3D structure for HL and the putative locus for the HMG-CoA substrate molecule, calculated using computational docking algorithms (Casals et al 2003), generate a model for the mechanism of enzyme function. According to this model, the reaction would be induced by the insertion of a substrate molecule into the central cavity of the enzyme, as has been demonstrated for other structurally related enzymes (Mancia et al 1999). First the HMG head would traverse the entire length of the tunnel and then the adenosine tail would lie at the NH-terminal face of the TIM barrel. This position places the sulphur atom and the HMG head of the substrate in close proximity to the catalytic His233 residue. After hydrolysis, it is assumed that two enzymatic reaction products, the acetoacetyl group and acetyl-CoA, leave the reaction centre in opposite directions. This spatial arrangement of both substrate and enzyme is further supported by the finding that several previously reported missense mutations can be easily explained by simply substituting the side chains of the wild-type residues by those corresponding to the mutated amino acids. This is the case for the V70L, S75R and S201Y mutants previously described (Casals et al 2003), as well as G203E, which we report here. All these

amino acids are located on the inside of beta sheets two or six and, when substituted by a more voluminous residue, they block the central tunnel, preventing the initial positioning of the substrate and almost completely abolishing enzyme activity, as demonstrated by expression studies of G203E (this paper) or S201Y (Casals et al 2003) mutant enzymes in *E. coli*. Although, in absence of crystallographic data, some other possible locations of HMG substrate in the active centre cannot be excluded, that described above has the advantage of providing an explanation for point mutations located close to the NH-terminal end of the central beta barrel, as V70L, or in the middle, as S201Y, that cannot be easily explained by models that locate the entire substrate in the active centre cavity, as has been determined for other TIM barrel structures as *Escherichia coli* malate synthase (Anstrom et al 2003) far from the NH-face of the enzyme.

Beta sheets two and six are the regions that accumulate more missense mutations. The analysis of evolutionary conservation presented in Fig. 3 is also consistent with the observed effects of mutations in HL beta sheet six. The higher conservation values of the residues in beta sheet six and surrounding positions contrast with the lower values when comparing the sequences of alpha helices five and six, indicating stronger evolutionary restrictions on the beta-forming residues.

All these results suggest that most amino acids constituting beta sheets, and particularly those whose lateral chains are oriented to the central cavity of the molecule, are important in defining the substrate cavity. Single point mutations affecting these amino acids will probably cause 3-hydroxy-3-methylglutaric aciduria.

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