C-Terminal end and aminoacid Lys\(^{48}\) in HMG-CoA lyase are involved in substrate binding and enzyme activity

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Abstract

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase adopts a \((\beta\alpha)\)\(^8\) TIM barrel structure with an additional \(\beta 9, \alpha 11\) and \(\alpha 12\) helices. Location of HMG part of the substrate has been suggested but the binding mode for the CoA moiety remains to be resolved. As mutation F305 fs\(^{21}\), which involves the last 21 residues of the protein, and mutation K48N caused 3-hydroxy-3-methylglutaric aciduria in two patients, we examined the role of the C-terminal end and Lys\(^{48}\) in enzyme activity. Expression studies of various C-terminal-end-deleted and K48N-mutated proteins revealed that residues 311–313 (localized in the loop between \(\alpha 11\) and \(\alpha 12\) helices) and Lys\(^{48}\) are essential for enzyme activity. An \textit{in silico} docking model locating HMG-CoA on the surface of the enzyme implicates Asn\(^{311}\) and Lys\(^{313}\) in substrate binding by establishing multiple polar contacts with phosphate and ribose groups of adenosine, and Lys\(^{48}\) by contacting the carboxyl group of the panthotenic acid moiety.

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Keywords: HMG-CoA lyase; 3-Hydroxy-3-methylglutaric aciduria; TIM barrel structure; Enzyme activity; Protein mutations

Introduction

3-Hydroxy-3-methylglutaryl-coenzyme A lyase (HL, EC 4.1.3.4) is a mitochondrial enzyme that catalyzes the cleavage of HMG-CoA to acetoacetate and acetyl-CoA, the last step of ketone body synthesis and leucine metabolism. Human HL precursor contains 325 amino acids, including a 27-residue N-terminal mitochondrial leader that is removed when the enzyme reaches the mitochondrial matrix. The crystal structure of the mature form of human HMG-CoA lyase has recently been published [1]. The enzyme adopts a \((\beta\alpha)\)\(^8\) barrel fold with 9\(\beta\) strands and 12\(\alpha\) helices. The N terminal barrel end is occluded, so substrate access to the active site involves binding across the cavity located at the C-terminal end of the barrel. Cys-266, an important catalytic residue that is conserved in all HMG-CoA lyase proteins, is believed to be located in the flexible G-loop (between \(\beta 8\) and \(\alpha 10\)) that moves upon substrate binding to influence catalytic efficiency, as described by Forouhar and colleague [2] by comparing mean backbone atomic B-factors in the crystal structures of Ca\(^{2+}\)-bound HL (Brucella melitensis, PDB structure 1YDN) metal-free HL (Bacillus subtilis, 1YDO) and Mg\(^{2+}\)-bound human HL (PDB structure 2CW6). The other residues implicated in enzyme activity, Arg\(^{41}\) and His\(^{233}\)
tein quantification was performed using the Bio-Rad protein assay with and soluble crude extracts were obtained, as described elsewhere [16]. Pro-

3-Hydroxy-3-methylglutaric aciduria (MIM246450) is a rare autosomal recessive metabolic disorder that appears in the first year of life, after a fasting period, with clinical symptoms characterized by vomiting, convulsions, metabolic acidosis, hypoketotic hypoglycaemia, and lethargy. These symptoms sometimes progress to coma [7] and the disease is fatal in about 20% of cases. To date, 26 different HL mutations have been described [8–23].

Here we propose a model for enzyme substrate location that involves the C-terminal end of the protein. The model is based on the effects on enzyme structure and activity of K48N mutated protein and a series of C-terminal end deleted proteins.

Materials and methods

Materials

Qiagen Plasmid Kit (Qiagen Inc.) was used to isolate plasmid DNA from bacterial cultures. Isopropyl-1-thio-β-D-galactoside (IPTG) was pur-

3-Hydroxy-3-methylglutaryl Coenzyme A and reduced β-NAD were obtained from Sigma, and β-hydroxybutyrate dehydrogenase was obtained from Roche.

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 [16]. PCR products were purified with “Quiaquick PCR Purification Kit” or “Quiaquick Gel Extraction Kit” from Qiagen, and sequenced with an Applied Biosystems 373 automated DNA sequencer.

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

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HL mutants Mut3 (HL residues 1–322), Mut6 (residues 1–319), Mut9 (residues 1–316), Mut12 (residues 1–313) and Mut15 (residues 1–311) were constructed by introduction of premature stop codons using the “Quick Change” PCR-based mutagenesis procedure (Stratagene). DNA sequencing of the new constructs was performed to confirm the desired mutations. Enzymes were expressed in competent BL21 cells and purified from 100 ml LB-Ampicilne cultures. Expression was induced with 0.1 mM IPTG and gentle shaking at 18 °C overnight. Cells were harvested at 3000 rpm, for 10 min at 4 °C, and resuspended in 1.2 ml of STET buffer. They were then sonicated (2 cycles of 15 pulses at input 4 and 50% amplitude, with a Dr.Hielscher GmbH sonicator).

After 7400 rpm centrifugation for 10 min at 4 °C, the soluble protein fraction was obtained. Despite the optimization of the expression conditions, 90% of the recombinant protein produced remained included in the post-sonicated pellet. The lysate was isolated by adding 150 µl of glutathione sepharose-equilibrated beads, which bind to the GST domain (30 min incubation at 4 °C). Beads were harvested at 3000 rpm for 1 min, washed three times and resuspended in 240 µl of PreScission buffer. Ten micro liters of PreScission protease was added to release the enzymes from their GST domains. After an overnight incubation at 4 °C, samples were centrifuged for 3 min at 2000 rpm and the supernatant, was stored at 4 °C. Enzyme purity was verified by SDS-PAGE and Coomassie blue staining.

HMG-CoA lyase activity

HMG-CoA lyase activity was measured by a simple spectrophotometric method that determines the amount of acetocacetate produced [24]. To measure HL-specific activity, 2 µg of soluble crude extracts or 300 ng of purified HL protein was used. The reaction assay was performed with 500 nmol of substrate (HMG-CoA) at 37 °C for 15 min in a final volume of 250 µl. One enzyme unit represents the formation of one µmol of ace-

Molecular docking

A structural model of the molecular interaction between the substrate HMG-CoA and the 3-D structure of human HMG-CoA lyase (Protein Data Bank entry 2CWE) [1] was built using the programs in the Autodock package [25,26]. Protein and ligand molecules were prepared using standard procedures, as specified in the package documentation. For further steps, we only considered docking models in which the acetocetyl residue was located in the vicinity of the enzyme site determined in the crystal structure for 3-hydroxyglutaric acid molecule positioning [1]. Finally, the model with the lowest docking energy was selected.

Results

Molecular analysis of two HMG-CoA lyase mutations that cause HMG-CoA lyase deficiency

DNA extracted from lymphocytes or fibroblast cultures obtained from two HL patients were used as templates for amplification by PCR of the nine exons of hmg/l gene Examination of the amplified exons and splicing sequences revealed that one patient was homozygous for mutation g.913-15delTT and the other was heterozy-

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mutated protein expressed in E. coli cells showed less than 2% of wild type HMG-CoA lyase activity, indicating that the mutation compromises enzyme function and causes HL deficiency. No $K_m$ and $V_{max}$ determination studies could be performed because of the low mutant activity. As it can be seen in multiple sequence alignment of HL orthologues (Fig. 1c), Lys$^{48}$ is conserved in all vertebrates, plants and some bacteria species, being substituted by alanine or proline (not asparagine) in insects and in some other bacteria.

Fig. 1. Expression of C-terminal truncated proteins. (a) Diagram representing the C-terminal deletions performed in HL enzyme to study the function of the C-terminal end of the protein. HL mutants Mut3 (HL residues 1–322), Mut6 (residues 1–319), Mut9 (residues 1–316), Mut12 (residues 1–313) and Mut15 (residues 1–311) were constructed. (b) SDS-PAGE electrophoresis of 20 µg of E. coli crude extracts expressing different C-terminal truncated HL proteins. 12% acrylamide gel was stained with Coomassie blue. A band of 31kd corresponding to HL protein is present in wild type HL-expressing cells, which is absent in empty vector. Mut12 and Mut15 HL proteins are smaller than Mut3, Mut6 and Mut9 proteins. HL bands are marked with black asterisks. (c) Multiple alignment of some representative sequences from vertebrates, insects, plants or bacteria showing the conservation of residues surrounding Lys48 (left) or the C-terminal HL end (right). Position of residues K48, N311 and K313 of human HL is indicated. Residues are coloured according to conservation. Represented species are: HL_HUMAN, Homo sapiens; HL_MACFA, Macaca fascicularis; HL_RAT, Rattus norvegicus; HL_MOUSE, Mus musculus; HL_CHICK, Gallus gallus; HL_DARE, Danio rerio; HL_XENLA, Xenopus laevis; HL_FUGRU, Takifugu rubripes; HL_ANOGA, Anopheles gambia; HL_DROME, Drosophila melanogaster; HL_ORYSA, Orzya sativa; HL_ARATH, Arabidopsis thaliana; HL_PSEAE, Pseudomonas aeruginosa; HL_PSEPK, Pseudomonas putida; HL_PSESM, Pseudomonas syringae; HL_RHORU, Rhodospirillum rubrum; HL_RALSO,Ralstonia solanacearum; HL_CHRVO, Chromobacterium violaceum; HL_BRUME, Brucella melitensis; HL_BACSU, Bacillus subtilis.
C-Terminal end truncated proteins expression and activity measurement

Mutation F305 fs(–2) was previously described for two Arabian patients [10] but no physiological explanation was given for the loss of function. Recent publication of the crystal structure has revealed the exact position of amino acids 305–325 (C-terminal end of the protein), outside the Tim barrel structure, forming alpha helices 11 and 12 [1]. As mutation F305 fs(–2) does not affect the Tim barrel structure, we were interested in understanding why this mutation causes loss of enzyme function.

To examine the role of the C-terminal end of the protein in enzyme activity, we expressed a series of truncated proteins in E. coli (see Fig. 1a). We assessed the effect of the length of the regions removed on enzyme functionality. The proteins expressed were Mut3 (last 3 residues deleted), Mut6 (last 6 residues deleted), Mut9 (last 9 residues deleted), Mut12 (last 12 residues deleted) and Mut15 (last 15 residues deleted). All constructions were made from pTr-HL (wild-type HL cDNA cloned in pTr99a expression vector), as used in [16]. Fig. 1c shows a comparison of the aminoacid composition for deleted fragment in some representative HL orthologues from bacteria, plant, insect and vertebrate species.

All constructions were expressed in E. coli and specific activity was measured. Expression efficiency was determined by SDS–PAGE of crude lysates (Fig. 1b). No Western blot experiments were performed because the HMG-CoA lyase antibodies available were raised against the last fifteen amino acids of the protein. Protein expression levels were similar in all constructs, except Mut12 and Mut15 where the HL band was weaker. Enzymatic activity of Mut3, Mut6 and Mut9 proteins was similar to wild type values, but Mut12 and Mut15 presented a specific activity of 17% and less than 2% of wild-type, respectively (see Table 1).

To improve protein stability, we expressed Mut12 and Mut15 fused to the C-terminal end of GST (GST-Mut12 and GST-Mut15). The fused proteins isolated from the sepharose columns were incubated with PreScission protease to release the lyase enzymes from the GST domains.

Table 1
Catalytic activity of E.coli crude extracts expressing wild type and C-terminal-end-truncated proteins

<table>
<thead>
<tr>
<th>Clone name (residues)</th>
<th>Specific activity (U/mg)</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt (1–325)</td>
<td>1.29 ± 0.09</td>
<td>100 ± 7</td>
</tr>
<tr>
<td>Mut3 (1–322)</td>
<td>1.13 ± 0.11</td>
<td>88 ± 8</td>
</tr>
<tr>
<td>Mut6 (1–319)</td>
<td>1.20 ± 0.07</td>
<td>93 ± 5</td>
</tr>
<tr>
<td>Mut9 (1–316)</td>
<td>1.27 ± 0.08</td>
<td>99 ± 6</td>
</tr>
<tr>
<td>Mut12 (1–313)</td>
<td>0.22 ± 0.09</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>Mut15 (1–310)</td>
<td>0.03 ± 0.06</td>
<td>≤ 2 ± 5</td>
</tr>
</tbody>
</table>

Two micrograms of soluble crude extract was assayed for HMG-CoA lyase activity by the spectrophotometric assay. One enzyme unit represents the formation of one μmol of acetoacetate in one minute. Results are given as mean values of at least four independent experiments.

Enzyme purity for wild-type and mutant enzymes was verified by SDS–PAGE and Coomassie blue staining (see Fig. 2). Cleavage with PreScission allowed two residues of the site-specific cleavage domain to remain bound to the HL N-terminal end. The extension of two residues (Gly and Pro) was considered to be a slight structural perturbation which was unlikely to affect HL structural or functional integrity. On average, 9.6 μg was obtained from wild-type enzyme, 5.3 μg from Mut12 and 4.8 μg from Mut15 from LB 100 ml cultures. Specific activities and kinetic parameters were determined (Table 2). Mut12 (residues 1–313) had similar specific activity and Km to wild-type. In contrast, Mut15 (residues 1–310) lost almost all HL activity, and its kinetic parameters could not be determined, indicating that residues 311–313 are critical for enzyme activity.

As it can be shown in Fig. 1c, Arg112 is conserved in all analyzed species except for Bacillus subtilis. Instead, Asn311 is only conserved in most of vertebrate species and replaced by glycine or glutamine in plants, insects and bacteria. Lys313 is conserved or replaced by other positive charged residue, Arg, in vertebrate species, but substituted by a neutral residue or a proline in the rest of species.

Positioning of HMG-CoA in HL surface

A model is proposed for the location of HMG-CoA substrate on the surface of HL enzyme. The 3D coordinates of human HL were obtained from the Protein Data Bank (entry 2CW6, [1]). Positioning of the whole HMG-CoA substrate was modeled by the simulation docking algorithms Autodock as indicated in Materials and methods.

The only structural restraint imposed was that the acetoacetyl group of the HMG-CoA molecule should be located in the neighbourhood of the site determined in the crystal structure for 3-hydroxyglutaric acid [1]. This was the main difference to the previous published model by Forouhar...
Table 2
Kinetic parameters of purified wild type, Mut12 and Mut15 proteins

<table>
<thead>
<tr>
<th>Clone name (residues)</th>
<th>Specific activity (U/mg)</th>
<th>Km HMG-CoA (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt (1–325)</td>
<td>168</td>
<td>70</td>
</tr>
<tr>
<td>Mut12 (1–313)</td>
<td>125</td>
<td>58</td>
</tr>
<tr>
<td>Mut15 (1–310)</td>
<td>&lt;3</td>
<td>—</td>
</tr>
</tbody>
</table>

and colleague [2]. Fig. 3 shows the location of HMG-CoA on the surface of HL with the lowest docking energy. The hydroxymethylglutaric group is positioned in the entrance of the active site, close to residues Arg\textsuperscript{41}, Asp\textsuperscript{42}, Gln\textsuperscript{45}, Ser\textsuperscript{75}, Val\textsuperscript{77}, Trp\textsuperscript{81}, Val\textsuperscript{82}, Leu\textsuperscript{106}, Pro\textsuperscript{108}, Phe\textsuperscript{127} and Asn\textsuperscript{138}, all of which are located at the carboxy-terminal end of TIM barrel-beta sheets or in their neighbourhood. This position is equivalent to that of 3HG in the 2CW6 structure.

Analysis of the electrostatic properties of the HL surface, (Fig. 3a, left), indicates that the phosphate groups in the HMG-CoA substrate are close to positively-charged residues (blue surfaces). Detailed inspection of the selected location of the substrate on the surface of HL shows that

![Substrate docking in HL enzyme. Left: Proposed location of HMG-CoA molecule on the surface of HL enzyme. Electronegative and electropositive patches on the surface of HL are coloured in red and blue, respectively. Position of substrate acetoacetyl group in the active centre of the enzyme is indicated. Right: Detailed representation of HMG-CoA adenosine head in the vicinity of \(\alpha\) helix-12. Residues N311, R312, K313 (blue), T314, S315 and S316 (green), as well as \(\alpha\) helix-12 (orange) are highlighted. Predicted polar contacts between phosphate, ribose and adenine groups of the substrate and the indicated enzyme amino acids are depicted as yellow dashes. The absence of residues 311–325 in mutant Mut15 will impair the correct binding of HMG-CoA substrate. Protein and substrate representations, and vacuum electrostatics calculations for the protein surface were performed using PyMOL (DeLano Scientific, San Carlos, CA). (b) Implication of Lys\textsuperscript{48} in enzyme activity and substrate binding. Up: Interactions of NH group of Lys\textsuperscript{48} with CO groups of HMG-CoA molecule and with backbones of Asn\textsuperscript{311} and Gly\textsuperscript{264} are indicated as yellow dashes. Note the positioning of Asn\textsuperscript{311} side chain towards the active centre. Down: Detail of modelled location of HMG-CoA substrate on the enzyme surface. Lys\textsuperscript{48} is coloured in light blue. Position of residues 311–313 (blue), 314–316 (green) and \(\alpha\) helix-12 (orange) are indicated. Protein and substrate representations were performed as in Fig. 3a.]

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the adenosine head of CoA is in the vicinity (less than 5 Angstroms away) of residues 313–316, situated in the loop preceding α12 helix (Fig. 3a, right). Phosphate and ribose groups of adenosine establish multiple polar contacts with charged atoms of Lys313, in addition to the carboxyl backbone of adenosine, whereas the adenine head contacts side chain and backbone atoms of Thr314, Ser315 and Ser316.

Another notable interaction, (Fig. 3b), is that of the positively-charged NH group of Lys48. It is less than 4 Angstroms away from the catalytic residue Asp52 and the catalytic residue Asp52 (data not shown). The lack of enzymatic activity of HL mutant Lys48 → Asn can thus be explained in terms of mis-positioning of the side chains of surrounding residues and weakening of substrate binding.

**Discussion**

Although HMG-CoA lyase has been crystallized and its TIM barrel structure has been determined, the substrate location and the role of the C-terminal end in HL activity remained unclear. Our results now clarify these two aspects.

Expression studies with C-terminal-end-truncated proteins revealed that proteins with up to nine deletions (affecting the α12 helix) maintained almost all wild-type activity. In contrast, when residues located between α11 and α12 were removed (Mut12 and Mut15), protein expression and specific activity of cell extracts were strongly reduced. These results indicate that alpha helix 12 does not participate in catalysis. It appears, rather, to be involved in the maintenance of protein stability, since protein expression was strongly reduced in its absence.

On the other hand, kinetic studies with Mut12 (residues 1–313) and Mut15 (residues 1–310) pure enzymes (Table 2) clearly showed that residues 311–313, in the loop between α11 and α12, play an important role in enzyme activity, since their elimination produces an inactive protein. Nevertheless, an inappropriate folding of Mut15 protein could also explain the lack of enzymatic activity. To elucidate the role of these three amino acids in enzyme activity, we built a structural model of the molecular interaction between substrate HMG-CoA and human HMH-CoA lyase enzyme. The CoA moiety that binds to enzyme is unknown, because the lyase crystal structure was assessed with 3-hydroxyglutarate, a hydrolysis product of the competitive inhibitor 3-hydroxyglutarate-CoA. The docking model gives several positions for substrate binding, but one of the most probable (the one with lowest docking energy) locates the CoA head of the substrate close to residues 311–316, just before α12 helix (Fig. 3a).

The proposed location of HMG-CoA on the surface of HL was obtained by imposing the restraint that the acetoacetyl head should be located in approximately the same place as the co-crystallised inhibitor 3-hydroxyglutaric acid (3HG) molecule in the human HL crystal [1]. This initial restriction was forced in order to find an alternate position for the substrate, different to the previously proposed by Forouhar and colleague [2]. They constructed an elegant and feasible model for substrate location, with the acetoacetyl group located in a deeper position in enzyme active centre, based on similarity to other TIM barrel metalloenzymes. Although both models locates adenosine head of substrate contacting different loci in HL surface, both of them could be compatible if co-existence of different locations of HMG-CoA in the surface of HL reflects a complex approximation route of substrate to active site, previous to the catalytic event. Our model could illustrate the initial substrate location (the one detected in the human enzyme crystal [1]) previous to the definite position in the deeper active centre (as proposed by Forouhar and colleague [2]). Both should be considered theoretical approximations, and experiments should be performed to test the predictions.

Our model gives some clues on the measured activities of Mut3, Mut6, Mut9, Mut12 and Mut15 mutants. Former four ones lack α helix 12, but maintain residues Asn311 and Lys313, which are responsible for most contacts of ribose and phosphate groups of adenosine according to the proposed model. Interestingly, multiple alignment of some HL orthologues (Fig. 1c) shows that the only invariant feature in this sequence region is the existence of a basic aminoacid between positions 312–313 suggesting that a basic residue in position 312–313 must be required for HL activity. In the case of human HL, the docking model presented in Fig. 1b indicates that basic residue Lys313 contacts phosphate group of substrate while basic residue Arg312 is situated in the surface of the substrate channel, contributing to generate a local basic environment necessary to allocate the three phosphate groups of the substrate.

In agreement with structural constraints, all Mut3, Mut6, Mut9 and Mut12 activities are similar to that measured for the wt enzyme (Tables 1 and 2). Although Thr314, Ser315 and Ser316 maintain contacts with the substrate adenine head, and Ser316 is conserved in all HL orthologues, they do not appear to be essential for catalysis, at least in the in vitro assay conditions used, as Mut12, which lacks all three residues, retains most of its activity.

The proposed substrate location is also supported by the other clinical mutation found in the second HMG-CoA lyase patient, K48N, which completely abolishes enzyme activity. As residues Lys and Asn have similar size and hydrophilic characteristics, this mutation would have little effect on folding or stability. Lys48 is located in the loop between β strand 1 and α helix 1 on the COOH face of the TIM barrel structure, not far from the active centre. In the proposed docking model, the NH group of Lys48...
contacts CO groups in both the substrate molecule and the backbone of residues Asn46 and Gly264 (present in the flexible G-loop [2]) contributing to substrate binding and enzyme conformation. Notice that the side chain of Asn46 is oriented towards the active centre cavity, at less than 4 Angstroms from Asn46 and the catalytic residue Asp47. This orientation indicates a key role for Lys48 not only in substrate positioning but also in catalysis, since it maintains the conformation of the active site. Mutation of Lys48 to Asn48 probably alters both substrate binding and active site conformation causing dysfunction of enzyme activity. An alternative explanation for the lack of activity of K48N mutant would be a protein misfolding issue, but the structural positioning of Lys48 in a non-structured loop apparently discards this possibility.

All these results indicate that Lys48, Asn311 and Lys313 play an important role in enzyme activity, probably ensuring the correct position of substrate, especially the CoA moiety. In addition, we propose a putative model for HMG-CoA binding to the enzyme. Analysis of the presence of these residues in HLs of different species (Fig. 1c) indicates that none of them is fully conserved across all studied sequences. On the contrary, vertebrate-specific conservation is shown. It is difficult to demonstrate whether the observed differences in the distribution of Lys48, Asn311 and Lys313 between vertebrates and the rest of analyzed species is given to simple lack of conservation or, in contrast, to a particular residue distribution due to an specialized behaviour of vertebrate HLs. New experiments of point mutation of selected residues and the analysis of their kinetic parameters must be done in the future to elucidate this point.

The study of new clinical mutations found in HMG-CoA lyase deficient patients, using the rationale provided by the crystal structure and experimental results from expressed mutated proteins, will contribute to our understanding of the functional mechanism of HL enzyme.

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