Modeling of a Mutation Responsible for Human 3-Hydroxy-3-methylglutaryl-CoA Lyase Deficiency Implicates Histidine 233 as an Active Site Residue*

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3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase is inactivated by diethyl pyrocarbonate (DEPC); activity can be fully restored by incubation with hydroxylamine. Protection against DEPC inactivation is afforded by a substrate analogue, suggesting an active site location for a DEPC target. Included in the inherited defects that map within the HMG-CoA lyase gene is a point mutation that results in an arginine substitution for histidine 233, one of only two invariant histidines. These observations prompted a functional test of the importance of His-233. The mutant lyases H233R, H233A, and H233D were overexpressed in Escherichia coli, isolated, and kinetically characterized. In H233D, DEPC targets one less histidine than was measured using wild-type lyase, supporting the assignment of wild-type lyase His-233 as one of the DEPC targets. Substitution of His-233 results in diminution of activity by ∼4 orders of magnitude. K values of the mutant lyases for both substrate HMG-CoA and activator divalent cation (Mg2⁺ or Mn2⁺) are comparable to the values measured for wild-type enzyme, indicating that these enzymes retain substantial structural integrity. This conclusion is reinforced by the observation that the affinity label, 2-butylnonyl-CoA, stoichiometrically modifies the mutant lyases, indicating that they contain a full complement of active sites. In view of these data suggesting that the structures of these mutant lyases closely approximate that of the wild-type enzyme, their observed 103–104-fold diminution in catalytic efficiency supports assignment to His-233 of a role in the chemistry of HMG-CoA cleavage.

3-Hydroxy-3-methylglutaryl-CoA lyase (EC 4.1.3.4) catalyzes the cleavage of HMG-CoA into acetoacetate and acetyl-CoA. This reaction is an important step in both the ketogenic HMG-CoA cycle (1) and the leucine catabolic pathway (2). The chemistry of HMG-CoA cleavage (Equation 1) is believed to involve a retro-Claisen condensation in which both a general acid and base are required for catalysis.

E + HMG-CoA → EAH + acetyl-CoA

As depicted in Equation 1, a general base abstracts a proton from the C3 hydroxyl of HMG-CoA, which leads to ketone formation and cleavage of the C2–C3 bond. This generates a transient carbanionic form of acetyl-CoA, which is quenched by a proton provided by a general acid. Neither the general acid nor the base has been unequivocally assigned.

Using affinity labeling techniques, a cysteine residue (Cys-237) was mapped to the active site of prokaryotic HMG-CoA lyase (3). Human HMG-CoA lyase has recently been expressed in Escherichia coli cells and purified to homogeneity (4). Using this expression system, the active site cysteine (Cys-266) of human HMG-CoA lyase was altered by site-directed mutagenesis to determine whether this amino acid was a key component of the catalytic apparatus (5). Upon conservative substitution with serine or alanine, a 103–104-fold decrease in the rate of catalysis and an altered pH/rate profile was observed (5). The mutagenesis data were in accord with the protein chemistry data suggesting that Cys-266 maps within the active site of HMG-CoA lyase and indicating an important function in catalysis for this residue.

The metabolic importance of HMG-CoA lyase is underscored by clinical studies which demonstrate that inherited defects in the HMG-CoA lyase gene can lead to the metabolic disease hydroxymethylglutaric aciduria (6). Recent efforts have led to the isolation and sequencing of the cDNA encoding HMG-CoA lyase from human (7), mouse (8), and chicken (7). Anderson and Rod kell (9) have sequenced the gene encoding Pseudomonas mevalonii HMG-CoA lyase. Based on the information from the human cDNA, a number of inherited mutations have been mapped to introns within the HMG-CoA lyase gene (10). In the course of our ongoing studies of mutations in HMG-CoA lyase-deficient patients (7, 10, 11), we discovered a missense mutation in the codon of the highly conserved His-233 residue. This information prompted our investigation of the sensitivity of HMG-CoA lyase to histidine modification, which, in turn, suggested that a test of the function of His-233 by directed mutagenesis would be informative. As documented in this report, the results of these studies implicates His-233 in catalysis of HMG-CoA cleavage.

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Experimental Procedures

Materials

DEPC was purchased from Aldrich. Phenyl agarose was purchased from Sigma. Q-Sepharose anion-exchange resin and Superose-12 (pregrade) resin are products of Pharmacia Biotech, Inc. Bacto-tryptone and yeast extract are products of Difco. Isopropylthiogalactoside was bought from Aldrich. Phenyl agarose was purchased from Phenomenex (Torrance, CA). Q-Sepharose anion-exchange resin and Superose-12 (Hi-Prep grade) resin are products of Pharmacia Biotech, Inc. Bacto-tryptone and yeast extract are products of Difco. Isopropylthiogalactoside was bought from Aldrich. Phenyl agarose was purchased from Phenomenex (Torrance, CA). Q-Sepharose anion-exchange resin and Superose-12 (Hi-Prep grade) resin are products of Pharmacia Biotech, Inc. Bacto-tryptone and yeast extract are products of Difco. Isopropylthiogalactoside was bought from Aldrich. Phenyl agarose was purchased from Phenomenex (Torrance, CA).

Methods

Construction of Mutants—The region of the HMG-CoA lyase gene (900 bp) that encodes His-233 was flanked by two unique restriction enzymes, PstI and SacI. Using primers listed in Table I, mutants H233A and H233D were constructed by overlap extension PCR (14) using Pfu DNA polymerase. Internal complementary oligonucleotides, primers B and C, were altered to encode the particular mutant of choice. Primers A and D, which flank the region for mutagenesis, were used in both rounds of PCR to generate the mutagenic fragment. After overlap extension PCR, the 200-bp fragment was purified and digested with PstI and SacI. The resulting 100 bp PstI-SacI fragment was gel purified using Qiaex and following the protocol described by the manufacturer (Qiagen).

To obtain the other fragments, the original expression plasmid, pTrcHL-1 (4), was digested with AvoI (located 40 bp into the HMG-CoA lyase coding sequence) and BamHI (located 20 bases downstream of the stop codon). The resulting 875-bp fragment (containing 95% of the coding region) and the 4.1 kilobase fragment (containing the vector and the initial 5% of the HMG-CoA lyase coding region) were gel purified as described above. The 875-bp piece was then digested with the restriction enzymes PstI and SacI to produce three fragments; two of these fragments (AvoI-BamHI, 630 bp; PstI-BamHI, 240 bp) were gel purified. The 100-bp SacI-PstI fragment generated from overlap extension PCR, along with the three purified fragments isolated from the original pTrcHL-1 construct (the 4.1-kb BamHI-AvoI, 630 bp AvoI-BamHI, and 240-bp PstI-BamHI) were ligated and transformed into competent E. coli (JM105) cells. The mutated regions of the resulting expression vectors, pTrcHL-H233A and pTrcHL-H233D, were sequenced in both directions by the method of Sanger et al. (15) using a Pharmacia ALF automated sequencer.

A mutant human HMG-CoA lyase cDNA encoding H233R2 was obtained by amplification of cDNA prepared from a H233R homozygote and insertion of a mutant SacI-HindIII restriction cassette containing the H233R mutation into the pTrc-HL-C323S vector.

Purification of Mutants—E. coli (JM105) was used as a host for expression of the human lyase His-233 variants. Growth of bacterial cells was performed as described by Roberts et al. (4); cells were cultured at 37 °C in LB broth supplemented with 50 μg/ml ampicillin. After the culture reached an A600 nm ~ 0.6, expression was induced by the addition of isopropylthioligosaccharide (1 mM). After induction, the bacteria were grown overnight in a thermoregulated shaker at 22 °C. The recombinant human HMG-CoA lyase His-233 variants were purified as described by Roberts et al. (4). Upon lysis of each pellet, the crude extract was subjected to high speed centrifugation. The high speed supernatant was passed over an anion exchange (Q-Sepharose) column, to which the lyase does not bind. The recovered enzyme was fractionated by (NH4)2SO4 precipitation (40–65% saturation). The recombinant protein was further purified on a hydrophobic (Phenyl-Agarose) column. The final step of purification involved elution from a gel filtration (Superose-12) column. Each His-233 variant purified identically to the wild-type enzyme.

Protein and Enzyme Assays—Protein concentrations were determined following the method of Bradford (16) using bovine serum albumin as a standard. SDS-polyacrylamide gel electrophoresis was performed under denaturing conditions as described by Laemmli (17) using an 11% acrylamide running gel and a 4.5% acrylamide stacking gel. Coomassie Blue dye (0.2%) was used to stain the gel.

HMG-CoA lyase activity of the engineered variants was determined using the citrate synthase-coupled spectrophotometric assay of Stegink and Coon (18) as modified by Kramer and Miziorko (19) except that 100 μM HMG-CoA was used to initiate the reaction. Also, HMG-CoA lyase was incubated for 10–15 min at room temperature in the presence of 20 μM dithiothreitol prior to addition to the assay mixture. After addition of the reduced lyase variants, the reaction was immediately initiated with HMG-CoA. Determination of the kcat for HMG-CoA was performed using 0.20 μg of wild type, 16 μg of H233R, 16 μg of H233A, or 32 μg of H233D enzymes. The HMG-CoA concentration was varied between 10 and 200 μM.

To determine the kcat for Mn2+ or Mg2+ with the H233 variants, the radioactive assay under standard pH conditions (Tris-HCl, pH 8.2) was used (5). HMG-CoA lyase (8 μg of H233R, 8 μg of H233A, 16 μg of H233D) was preincubated with 20 μM dithiothreitol and 100 μM [14C]HMG-CoA in a final volume of 200 μl. The reaction was initiated by addition of the enzymatically synthesized [14C]HMG-CoA (6000 dpm/μl). The protein concentration for all samples was 1.25 μM. K values for the acid-stable radioactive ([14C]HMG-CoA) to form the acid-volatile product (acetoacetyl-CoA) was measured as a function of time to determine enzymatic rate. All components of the assay mixture were passed over a Chelex-100 column to remove any trace metal contaminants. The concentration of Mn2+ and Mg2+ ranged from 125 mM to 20 μM and 10 μM to 20 μM, respectively.

CD Spectroscopy—Circular dichroism spectra of human HMG-CoA lyase and the His-233 variants were measured using a Jasco J-700 spectropolarimeter. The protein concentration for all samples was 1.25 μM in 10 mM phosphate, pH 7.8, 10 mM NaCl, 0.1 mM dithiothreitol, and 20% glycerol. Measurements were made with a cylindrical 0.1-cm pathlength quartz cuvette at 22 °C.

DEPC Inactivation—Diethyl pyrocarbonate solutions were freshly prepared by dilution of the reagent into cold absolute ethanol. The concentration of a stock DEPC solution was determined by the addition of an aliquot to 10 mM imidazole buffer, pH 7.0. The reaction to form N-carbethoxyimidazole is accompanied by a change in absorbance at 230 nm with an extinction coefficient of 3000 m -1 cm -1 (20). A typical DEPC modification used a sample of human HMG-CoA lyase (5.0–8.0 μM substrate concentration) in 0.02 M potassium phosphate, pH 6.8, at 25 °C. To initiate the reaction, DEPC (0.25–2.5 mM in ethanol) was added (final reaction mixture volume was 500 μl). At appropriate time intervals, aliquots were removed and residual DEPC was quenched in 5 mM imidazole buffer, pH 7.0. A control experiment using equivalent volumes of ethanol (without DEPC) was performed under comparable conditions. Each aliquot was assayed for HMG-CoA lyase activity as described above except that potassium phosphate buffer, pH 7.8, was substituted for Tris-HCl, pH 8.2. The concentration of ethanol in the assay mixture never exceeded 5%.

After an aliquot was removed for assay, the remaining reaction mixture was scanned from 235–340 nm using a Perkin-Elmer Lambda 19 spectrophotometer with a thermoregulated temperature-controlled water bath set at 22 °C. The stoichiometry of N-carbethoxyhistidine

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**Table I**

| Oligonucleotides used for site-directed mutagenesis |
|-----------------------------------------------|
| **Flanking primers (for both mutations)**       |
| **Upstream**                                   |
| Primer A                                       |
| **Downstream**                                 |
| Primer B                                       |
| **Mutagenic primers**                         |
| H233A                                         |
| Primer C                                       |
| H233D                                         |
| Primer D                                       |
was determined by the difference spectroscopy using the extinction coefficient of 3200 M⁻¹cm⁻¹ at 240 nm (21).

Hydroxylamine Treatment—DEPC was incubated with HMG-CoA lyase (6.7 μM subunit concentration) as described above except that hydroxylamine, pH 7.0 (100 mM) was added after 30 min. Prior to the addition of hydroxylamine, 5 mM imidazole was added to the mixture to quench unreacted DEPC. A control was run in the absence of DEPC to test the effect of NH₂OH on the reaction. After addition of hydroxylamine, activity was immediately measured by addition of an aliquot to an HMG-CoA lyase assay mixture.

Protection against DEPC Inactivation—Human HMG-CoA lyase in 0.02 M potassium phosphate buffer, pH 6.5, was preincubated with HG-CoA (1–2 mM) for 10 min at 25 °C prior to the addition of DEPC (1 mM). Control samples were run without the addition of DEPC after incubation with HG-CoA and also in the absence of HG-CoA. In all cases, aliquots were removed at various time points and quenched with imidazole to deplete unreacted DEPC. At each time point, an aliquot was assayed for HMG-CoA-lyase activity as described above.

Butynoyl-CoA Modification—HMG-CoA lyases were fully reduced with 60 mM dithiothreitol prior to removal of excess reductant from protein by centrifugal gel filtration in an anaerobic chamber. Modification of enzyme with [³⁵Cl]-butynoyl-CoA (16,000 dpm/nmol) was performed under anaerobic conditions, as described previously (3).

RESULTS

DEPC Inactivation of HMG-CoA Lyase—Upon incubation of HMG-CoA lyase with DEPC (0.25–2.5 mM) in potassium phosphate buffer, pH 6.8, a time-dependent loss of activity was observed (Fig. 1). The rate at which inactivation occurred increased with added amounts of DEPC. Fig. 2 depicts the time-dependent difference spectra obtained upon incubation of HMG-CoA lyase with 1 mM DEPC. The spectra display an absorption maximum at 240 nm characteristic of N-carboxylation of a histidine residue (20). No modification of tyrosine, which would have been suggested by large progressive decreases (ε = 13,000 M⁻¹cm⁻¹) in 278 nm absorbance of the difference spectra, is apparent (22). When modification was performed at [DEPC] = 1 mM, the maximum absorbance at 240 nm was obtained after 30 min. Based on the extinction coefficient of 3200 M⁻¹cm⁻¹ (21), the number of histidine residues modified per monomer was estimated to be 2.4 ± 0.3.

Restoration of Activity with Hydroxylamine—To further test whether modification of a histidine residue was responsible for inactivation of HMG-CoA lyase, hydroxylamine was added 30 min after incubation of enzyme with 1 mM DEPC. After addition of NH₂OH (Fig. 3), the activity was rapidly restored to >95% of the original activity. Control experiments confirmed that hydroxylamine alone did not have a significant effect on HMG-CoA lyase. The data shown in Fig. 3 suggest that inactivation did not involve modification of a tyrosine residue, since breakdown of O-(ethoxyformyl)-tyrosine with hydroxylamine occurs at a very slow rate (23). Furthermore, modification of either cysteine residues or primary amines with DEPC is not reversible by NH₂OH treatment (20). This test also eliminates the possibility that inactivation is attributable to enzyme denaturation or to biscardethoxylation of histidine by two DEPC molecules, since activity loss due to either of these events would not be reversed by NH₂OH (20). The collected results (Figs. 2 and 3) argue that the loss of HMG-CoA-lyase activity is the result of monocarbethoxylation of histidine by diethyl pyrocarbonate.

Protection against Inactivation by Hydroxyglutaryl-CoA—To determine whether loss of activity upon DEPC treatment correlates with modification of an active site histidine, protection experiments were performed. HMG-CoA-lyase uses a single substrate and turnover occurs at an appreciable rate. Thus, since only transient protection would be afforded by substrate, a strategy that involved reversible formation of a dead-end complex was pursued. Kramer and Miziorko (13) established that an analogue of HMG-CoA, HG-CoA, is a competitive inhibitor of avian lyase (Ki = 50 μM). HG-CoA has also been used for protection of bacterial HMG-CoA lyase; a Ki of 130 μM was estimated in those experiments (3). When HG-CoA was preincubated with human HMG-CoA-lyase, the rate of inactivation by DEPC was greatly reduced (Fig. 4), indicating that the substrate analogue, HG-CoA, was able to afford significant protection. These results strongly suggest that a histidine targeted by DEPC is situated within the active site pocket of HMG-CoA lyase.

Rational for Construction of His-233 Mutations—In addition to the protein modification work presented above, genetic evidence suggested the possibility that a histidine residue influences HMG-CoA lyase activity. Hydroxymethylglutaric acid-
HMG-CoA lyase activities; as a similar conclusion is drawn on the basis of studies performed on the His-233 variants. Binding constants determined in Table II, the activity of the naturally occurring point mutant, H233R, is approximately 4 orders of magnitude lower than that measured for wild-type enzyme. When a more conservative change to an alanine was engineered, the specific activity was slightly lower than observed with either the arginine or alanine substitutions.

The large observed diminution in catalytic activity would appear to be significant provided that the mutant enzymes retain structural integrity. Three lines of evidence argue that this is indeed the case. Previous work with human (4), avian (3), bovine (18), and bacterial (25) HMG-CoA lyases has demonstrated that a dissociable divalent cation (Mg$^{2+}$ or Mn$^{2+}$) stimulates catalysis. Both protein (5) and substrate (26) donate ligands to the activator cation. Magnesium and manganese are the only divalent cations observed to stimulate human HMG-CoA lyase activity; a similar conclusion is drawn on the basis of studies performed on the His-233 variants. Binding constants for Mg$^{2+}$ (233 μM) were determined to be 1000-fold weaker than Mn$^{2+}$ (0.34 μM) with the wild-type human enzyme (4). However, identical $V_{max}$ values were obtained using either
detailed study of the function of this residue. Our studies with recombinant H233R lyase indicate that this clinically detected deficiency (30) is not attributable to a destabilized protein but rather to a catalytically impaired but otherwise stable enzyme.

In the absence of any indication of substantial structural differences between wild-type enzyme and H233R, H233A, or H233D lyases, these mutants’ diminution in catalytic efficiency by 4 orders of magnitude suggests that a consideration of possible catalytic roles for His-233 would be appropriate. Cleavage of HMG-CoA involves general acid/base catalysis of the removal of a proton from the substrate’s C3 hydroxy group, as well as protonation to quench the carbamion that, following C2–C3 bond cleavage, develops at C2 of product acetyl-CoA (Equation 1). Given the distinctly alkaline pH optimum for HMG-CoA lyase (5), His-233’s imidazole might receive consideration as a catalytic base. However, a similar catalytic functionality could be envisioned for the thiol group of the active site residue Cys-266 (5). Substitution of this residue results in a diminution of catalytic efficiency comparable in magnitude to that observed with the His-233 lyase variants (5). While other active site residues could conceivably function in such a capacity, the only amino acids that have, to date, been mapped to the HMG-CoA lyase active site and passed mutagenesis tests of function are His-233 and Cys-266.

Literature precedent qualifies both His-233 and Cys-266 for consideration as a candidate for the general base. In the case of cysteine, the most obvious example is provided by the work of Walsh and co-workers (31) on Zoogloea ramigera β-ketothiolase, which supports the involvement of active site cysteines not only in forming the acetyl-S-enzyme but also in deprotonation of the second acetyl-CoA prior to its condensation with the acetyl-S-enzyme. Recently, the structure of a yeast peroxisomal thiolase has become available (32). Sequence comparison between the yeast and Z. ramigera thiolases indicates only two conserved cysteines; these are the residues implicated in acyl enzyme formation and general base catalysis. While the peroxisomal thiolase structure contains no substrate in the active site, the sulfur atoms of these conserved cysteines are, nevertheless, separated by only 6.1 Å, qualifying them for the mechanistic roles that have been proposed. In the case of histidine, work with chloramphenicol acetyltransferase (33) has clearly implicated imidazole in deprotonation of the substrate’s alcohol functionality. More relevant to our study of HMG-CoA lyase is the aldol cleavage reaction catalyzed by isocitrate lyase. Mutagenesis of the E. coli enzyme has indicated the importance of His-197 (34). These data extend and confirm the predictions concerning histidine involvement that are based on DEPC modification of the Cephalosporium acremonium isocitrate lyase (28). Available data for isocitrate lyase do not yet permit a structural test of the type discussed above for β-ketothiolase.

In conclusion, our results with the HMG-CoA lyase variants containing substitutions for His-233 confirm that replacement of this single residue is sufficient to account for a clinically observed metabolic disease. Moreover, the stability and structural integrity of the His-233-substituted variants argue that their marked diminution in activity qualifies His-233 for a catalytic role. At present, it is unclear whether the residue functions directly as a general base catalyst or more indirectly, e.g. by polarizing an adjacent functional group that deprotonates the substrate’s C3 hydroxyl.

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