Aminoethylcysteine Can Replace the Function of the Essential Active Site Lysine of *Pseudomonas mevalonii* 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase†

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Received February 4, 1999; Revised Manuscript Received April 26, 1999

ABSTRACT: The biodegradative 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase of *Pseudomonas mevalonii* catalyzes the NAD^+-dependent conversion of (*S*)-HMG-CoA to (*R*)-mevalonate. Crystallographic analysis of abortive ternary complexes of this enzyme revealed lysine 267 located at a position in the active site, suggesting that it might serve as the general acid/base for catalysis. Site-directed mutagenesis and subsequent chemical derivatization were therefore employed to investigate this active site lysine. Replacement of lysine 267 by alanine, histidine, or arginine resulted in mutant enzymes that lacked detectable activity. Lysine 267 was next replaced by the lysine analogues aminoethylcysteine and carboxyamidomethylcysteine. Using instead of the wild-type enzyme the fully active, cysteine-free mutant enzyme C156A/C296A, lysine 267 was first replaced by cysteine. Cysteine 267 of mutant enzyme C156A/C296A/K267C was then treated with bromoethylamine or iodoacetamide to insert aminoethylcysteine (AEC) or carboxyamidomethylcysteine at position 267. The carboxyamidomethylcysteine derivative was inactive, whereas mutant enzyme C156A/C296A/K267AEC exhibited high catalytic activity. That aminoethylcysteine, but not other basic amino acids, can replace the function of lysine 267 documents both the importance of this residue and the requirement for a precisely positioned positive charge at the active site of the enzyme.

HMG-CoA reductase,[1] the catalyst for the rate-limiting reaction of isoprenoid biosynthesis in higher eukaryotes, represents the target for control of cholesterol biosynthesis in human subjects by "statin" drugs such as Lovastatin (*1*). The reductive deacylation of (*S*)-HMG-CoA to (*R*)-mevalonate is believed to involve the successive formation of enzyme-bound mevaldyl-CoA and mevaldehyde. The reaction proceeds in three stages, the first and third of which are reductive (Scheme 1).

The enzyme can also catalyze two reactions of free mevaldehyde, reduction to mevalonate and oxidative acylation to HMG-CoA, reactions that appear to model the third stage and the reverse of the first and second stages of the overall reaction, respectively.

Recent advances in genome sequencing that permitted us to compare the sequences of 50 eukaryotic, prokaryotic, and archaeal HMG-CoA reductases revealed the previously unsuspected existence of two distinct classes of the enzyme (*2*). Sequence conservation is highest within each class, and the previously identified histidine (*3*), glutamate (*4*), and aspartate (*5*) are conserved in both classes of the enzyme. Class II forms of the enzyme include the HMG-CoA reductases of several bacterial pathogens and the biodegradative, NAD(H)-dependent HMG-CoA reductase of *Pseudomonas mevalonii* that catalyzes the conversion of mevalonate to HMG-CoA (Scheme 2).

Inspection of the crystal structure of the *P. mevalonii* enzyme, the only form of the enzyme whose crystal strcuture has been solved (*6*), revealed the presence of a previously unsuspected active site residue, lysine 267. First apparent in the crystal structure of a binary complex with HMG-CoA (*6*), subsequent solution of the HMG-CoA/NAD^+ ternary complex (*7*) revealed that the ϵ-nitrogen of lysine 267 is located within 2.6 Å of the thioester oxygen of HMG-CoA. These observations formed the basis for a revised catalytic mechanism in which lysine 267 is proposed to serve as the general acid/base during hydride transfer (*7*). We therefore have employed site-directed mutagenesis to replace lysine 267 with different amino acids to inquire whether lysine 267 is essential, whether a different amino acid might serve the same function, and to obtain biochemical evidence to bear on the structure-based inference of the function of lysine 267.

EXPERIMENTAL PROCEDURES

Materials. Purchased reagents included mevalonolactone, NADH, NAD^+, coenzyme A, phenylmethylsulfonyl fluoride, anti-chicken IgG coupled to horseradish peroxidase, 4-chloronaphthol, DEAE Sepharose FF, 2-bromoethylamine, and iodoacetamide (Sigma); restriction enzymes (New England

† Funded in part by National Institutes of Health Grants HL 47113 (V.W.R.) and HL 52115 (C.V.S.) and by a Predoctoral Fellowship awarded to D.A.B. by the American Heart Association, Indiana Affiliate. The data are from the Ph.D. thesis of D.A.B. This is journal paper 15928 from the Purdue University Agricultural Experiment Station.

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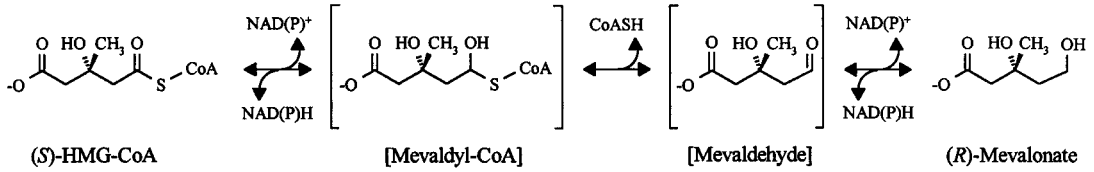
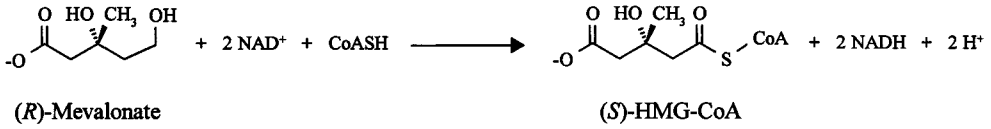
[1] Abbreviations: HMG, 3-hydroxy-3-methylglutaryl; CoASH, coenzyme A.

10.1021/bi9902687 CCC: $18.00
Published on Web 06/19/1999

Scheme 1



Scheme 2



Biolabs, Promega, or Gibco); QIAquick Gel Extraction Kit, QIAprep Spin Plasmid Miniprep Kit, QIAquick PCR Purification Kit (Qiagen); T4 DNA ligase, Vent DNA polymerase (New England Biolabs); prestained low-range protein standards (Bio-Rad); HMG-CoA agarose (PL Biochemicals). Additional purchased materials were from previously listed sources (*5*, *8*). Determination of protein concentration employed the method of Bradford (*9*) using bovine serum albumin (Bio-Rad) as a protein standard. PEG buffer contained 1 mM EDTA and 10% glycerol in 10 mM K_xPO_4, pH 7.3.

Vectors and Bacterial Strains. The expression vector pHMGR (pKK177-3-RED) that encodes *P. mevalonii* HMG-CoA reductase has been previously described (*10*). *Escherichia coli* strain TG1 served as a host during mutagenesis, cloning, and plasmid preparation, and *E. coli* strain BL21 as the host for protein expression.

DNA Manipulations. Oligonucleotides were synthesized in the Purdue University Laboratory for Macromolecular Structure and were purified prior to use by thin-layer chromatography on silica gel in 15 N NH_4OH/2-propanol/1-propanol/H_2O, 35:28:28:9 (v/v). Mutant genes were constructed using the polymerase chain reaction-based overlap extension method of oligonucleotide-directed mutagenesis (*11*) using pHMGR as template. All mutant constructs were subcloned into pUC19 for verification by DNA sequencing (*12*) at the Purdue Department of Biochemistry DNA Sequencing Facility.

Expression and Purification of Enzymes. Genes encoding wild-type and mutant HMG-CoA reductases were expressed in *E. coli* BL21. Expressed proteins were purified to apparent homogeneity (Figure 1) by the protocol employed for purification of the wild-type enzyme (*13*) but substituting 1 mM EDTA and 10% glycerol in 20 mM Tris, pH 8.5, for PEG buffer.

Assay of HMG-CoA Reductase Activity. Assays employed a Hewlett-Packard Model 8452 diode array spectrophotometer to monitor the reduction of NAD^+ at 340 nm. All assays were conducted at 37 °C in a final volume of 200 μL. Standard conditions for assay of the oxidative acylation of mevalonate employed 6 mM (*R,S*)-mevalonate, 2 mM CoASH, 4 mM NAD^+, 100 mM KCl, 100 mM Tris, pH 8.1. Reactions were initiated by the addition of mevalonate. One microunit represents the turnover, in 1 min, of 1 μmol of NAD^+.

Derivatization of Cysteine. Exposure to bromoethylamine or iodoacetamide was conducted both under native and under

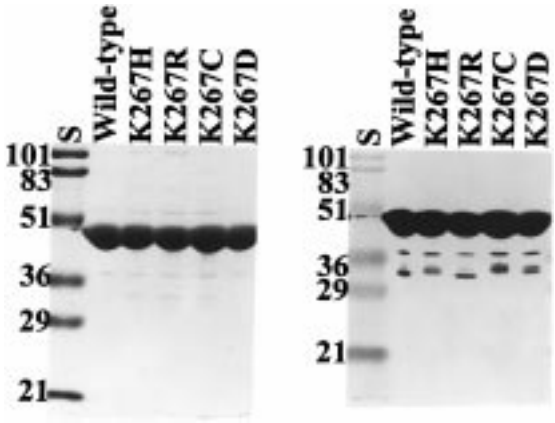


FIGURE 1: SDS−PAGE and Western blotting of purified wild-type and mutant *P. mevalonii* HMG-CoA reductases. Left: Approximately 10 μg portions of purified wild-type enzyme (WT) or the indicated purified mutant enzyme were subjected to SDS−PAGE. Lane S contained protein standards of the indicated molecular mass in kDa. The gel was stained with Coomassie blue. Right: Western blot of an unstained duplicate of the preceding gel employed chicken polyclonal antibodies raised against *P. mevalonii* HMG-CoA reductase as the primary antibody and anti-chicken IgG horseradish peroxidase conjugate as the secondary antibody. The blot was developed using 4-chloronaphthol. Low molecular weight reacting bands represent minor proteolytic fragments of HMG-CoA reductase.

denaturing conditions. Enzyme solutions, 2 mg/mL, were diluted 1:10 either into 1 mM EDTA−500 mM Tris, pH 8.0 (native conditions), or into 8 M urea−1 mM EDTA−500 mM Tris, pH 8.0 (denaturing conditions). Following addition of iodoacetamide (final concentration 10 mM) or bromoethylamine (final concentration 100 mM), samples were maintained at 16 °C in the dark for 1 h (iodoacetamide) or for 12 h (bromoethylamine). Reactions were terminated by addition of β-mercaptoethanol (100 mM for bromoethylamine; 10 mM for iodoacetamide), dialyzed for 12−24 h against three changes of PEG buffer containing 400 mM KCl, and concentrated to approximately 2 mg/mL by ultrafiltration prior to analysis.

Chromatography on HMG-CoA Agarose. Portions, 10−100 μg, of enzyme were applied to a 0.5 mL bed volume column of HMG-CoA hexane agarose in PEG buffer. Economic considerations dictated use of coenzyme A or KCl as eluant, rather than HMG-CoA. Following application of 10 column volumes of PEG, elution was initiated with 2 mM coenzyme A or 1 M KCl in PEG. Similar results were obtained using CoASH or KCl.

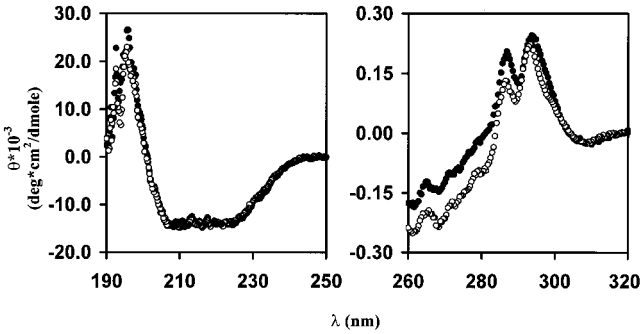


FIGURE 2: Circular dichroism spectra of wild-type enzyme and mutant enzyme K267A. The spectra shown are for the wild-type enzyme (●) and for mutant enzyme K267A (○). Mean residue elipticity (θ) data at the indicated wavelengths are average values for 6 scans. Spectra in 25 mM K_xPO_4, pH 7.6, were measured at room temperature using a Jasco 600 spectropolarimeter. Left: Far-UV CD spectra, 1 mm path length cell, 0.05 mg of protein/mL. Right: Near-UV CD spectra, 10 mm path length cell, 1.0 mg of protein/mL.

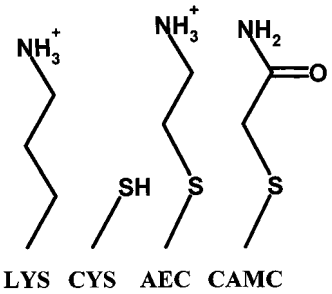


FIGURE 3: Structures of the side chains of naturally occurring and modified residues present at position 267. Planar representation of the extended structures of the R-groups of lysine (LYS), cysteine (CYS), aminoethylcysteine (AEC), and carboxamidomethylcysteine (CAMC). C−C and C−S bond distances are 1.54 and 1.82 Å, respectively. C−C−C and C−S−C bond angles are 109° and 105°, respectively. The precise location of functional groups relative to the ϵ-amino group of lysine will depend on how the flexible alkyl chain folds in modified enzymes.

RESULTS

Characterization of Mutant Enzyme K267A. Lysine 267 was first mutated to alanine to determine whether it is essential for catalysis. Following expression and purification to apparent homogeneity, mutant enzyme K267A was found to catalyze the oxidative acylation of mevalonate at less than 0.05% the rate of the wild-type enzyme. While inactivity is consistent with the proposed role for lysine 267 in catalysis (*7*), replacement of lysine by alanine could have been accompanied by adverse conformational changes. We therefore employed size-exclusion chromatography, UV circular dichroism, and affinity chromatography to compare enzyme K267A to the wild-type enzyme. Size-exclusion HPLC profiles and the far-UV circular dichroism spectrum of enzyme K267A matched those for the wild-type enzyme. However, the near-UV circular dichroism spectrum differed from that of the wild-type enzyme (Figure 2). In addition, and unlike the wild-type enzyme, mutant enzyme K267A failed to bind to an HMG-CoA affinity support.

Replacement of Lysine 267 by Other Naturally Occurring Amino Acids. We next asked whether a different amino acid could replace lysine 267. Following their expression and purification, mutant enzymes K267H, K267R, K267C, or K267D had undetectable (less than 0.05% wild-type) activity, and like enzyme K267A, also failed to bind to HMG-CoA agarose.

Introduction of Nonnaturally Occurring Amino Acids. The inability of arginine, aspartate, cysteine, or histidine to substitute for lysine 267 prompted its replacement by two nonnaturally occurring amino acids. A limitation of site-directed mutagenesis is that only protein amino acids can be substituted. Substitution of a nonprotein amino acid can be achieved by mutagenesis of the residue of interest to cysteine and subsequent modification by halogenated reagents. Examples include modification of cysteine with iodoacetic acid to replace glutamate (*14*), with 2,2′-dithiobis-(acetamidine) to replace arginine (*15*), or with bromoethylamine to replace lysine (*16*−*19*). Wild-type *P. mevalonii* HMG-CoA reductase contains only two cysteinyl residues, neither of which is essential for catalytic activity (*20*). We therefore mutated lysine 267 of the fully active, cysteine-free mutant enzyme C156A/C296A so as to avoid subsequent modification of cysteines other than that present at position 267. Lysine 267 of enzyme C156A/C296A was therefore replaced by cysteine, and as a control by alanine. The resulting mutant enzymes C156A/C296A/K267C and C156A/C296A/K267A were then subjected to chemical modification (Figure 3).

Carboxyamidomethylcysteine Cannot Substitute for Lysine 267. The carboxyamido group of carboxyamidomethylcysteine can form multiple hydrogen bonds and is of similar size to lysine. To convert cysteine 267 to carboxyamidomethylcysteine, mutant enzyme C156A/C296A/K267C was treated with iodoacetamide. Chemical modification was conducted both under native conditions and under denaturing conditions followed by renaturation. While titration with 5,5′-dithiobis(2-nitrobenzoate) indicated complete reaction, modification failed to restore detectable activity or the ability to bind to HMG-CoA agarose. Enzyme C156A/C296A treated in the same way retained 95% of its initial activity and the ability to bind to HMG-CoA agarose. As anticipated, similar treatment of enzyme C156A/C296A/K267A failed to restore activity or binding to HMG-CoA agarose.

Replacement of Lysine 267 by Aminoethylcysteine Yields Active Enzyme. To introduce the lysine structural analogue aminoethylcysteine (AEC) at position 267, enzyme C156A/C296A/K267C was treated with bromoethylamine. As controls, enzymes C156A/C296A and C156A/C296A/K267A were also treated with bromoethylamine. Treatment of enzyme C156A/C296A/K267C under native conditions yielded an enzyme with low but significant (1.3% wild-type) activity and K_m values for mevalonate, coenzyme A, and NAD^+ that differed little from those for the wild-type enzyme or for identically treated enzyme C156A/C296A. However, titration of free −SH groups with 5,5′-dithiobis(2-nitrobenzoate) revealed that almost 97% of Cys267 had remained unmodified. As expected, treatment of enzyme C156A/C296A/K267A with bromoethylamine failed to restore detectable activity.

To attempt to enhance the reactivity of Cys267, modification of enzyme C156A/C296A/K267C was next conducted under denaturing conditions. Subsequent titration with 5,5′-dithiobis(2-nitrobenzoate) indicated that Cys267 had been completely modified. Following renaturation, mutant enzyme C156A/C296A/K267AEC had a specific activity of 6.0 microunits/mg, a value equal to that of similarly treated

Table 1: Kinetic Parameters for Enzymes C156A/C296A and C156A/C296A/K267AEC

enzyme[a]	V_{max} (microunits/mg)	K_m (μM) for mevalonate	K_m (μM) for NAD^+	K_m (μM) for CoASH
C156A/C296A	6.0 ± 1.6	390 ± 13	210 ± 30	60 ± 16
C156A/C296A/K267AEC	6.0 ± 1.1	930 ± 84	660 ± 75	89 ± 26

[a] Enzyme C156A/C296A/K267AEC is enzyme C156A/C296A/K267C that had been denatured, treated with bromoethylamine, and renatured. Enzyme C156A/C296A was subjected to parallel treatment. Data are ±SEM for triplicate determinations. For comparison, the K_m values for unmodified wild-type enzyme are 260 μM (mevalonate), 300 μM (NAD^+), and 60 μM (CoASH).

enzyme C156A/C296A, and about one-fifth that of unmodified wild-type enzyme. As anticipated, parallel treatment of enzyme C156A/C296A/K267A did not restore detectable activity. The completely modified enzyme C156A/C296A/K267AEC had K_m values for mevalonate, coenzyme A, and NAD^+ similar to those for identically treated enzyme C156A/C296A (Table 1) and for the wild-type enzyme, and behaved similarly during chromatography on HMG-CoA agarose.

DISCUSSION

Several lines of evidence bear on the identity of the residue that functions as the active site acid/base during catalysis by HMG-CoA reductase. While no structure is available for any Class I HMG-CoA reductase, prior mutagenic and kinetic analyses of the catalytic domain of the Syrian hamster enzyme inferred that aspartate 766 may serve as the general acid/base during catalysis (*5*). Sequence comparisons implicated Asp283 of the *P. mevalonii* enzyme as the cognate of Asp766. However, subsequent solution of the crystal structure of the Class II HMG-CoA reductase from *P. mevalonii* revealed that the δ-oxygens of Asp283 are too far from the oxygen of the thioester moiety of HMG-CoA for this residue to function as the active site acid/base (*7*).

Inspection of the crystal structure of the HMG-CoA/NAD^+ ternary complex of *P. mevalonii* HMG-CoA reductase indicated that a more likely candidate for this function is lysine 267. This residue was therefore subjected to mutational analysis. Following mutation of lysine 267 to alanine, enzyme K267A lacked detectable activity and failed to bind to HMG-CoA agarose. While this is consistent with lysine 267 playing a critical role in catalysis and/or substrate binding, other reasons could account for the observed inactivity. Lysine 267 is located at the center of a hydrogen bond network that orients several other active site residues. Its replacement could disrupt this network, repositioning residues critical for ligand recognition and/or catalysis. A role for lysine 267 in maintaining the structure of the active site is consistent with the near-UV CD spectrum of enzyme K267A, which suggested an altered tertiary structure.

While both histidine and arginine provide positive charge at the active site, neither could replace lysine 267 for catalysis or binding to HMG-CoA agarose. We therefore wished to introduce more appropriate analogues of lysine at position 267. Lysine 267 of mutant enzyme C156A/C296A, which contains no cysteinyl residues and retains full catalytic activity, was replaced by cysteine. Subsequent reaction of cysteine 267 with bromoethylamine or iodoacetamide introduced the nonnatural lysine analogues aminoethylcysteine and carboxyamidomethylcysteine at this position. Carboxyamidomethylcysteine, while of similar size to lysine and capable of forming multiple hydrogen bonds, failed to restore detectable activity or the ability to bind to HMG-CoA agarose. By contrast, replacement of lysine 267 by aminoethylcysteine restored binding to HMG-CoA agarose and yielded a mutant enzyme with essentially full catalytic activity.

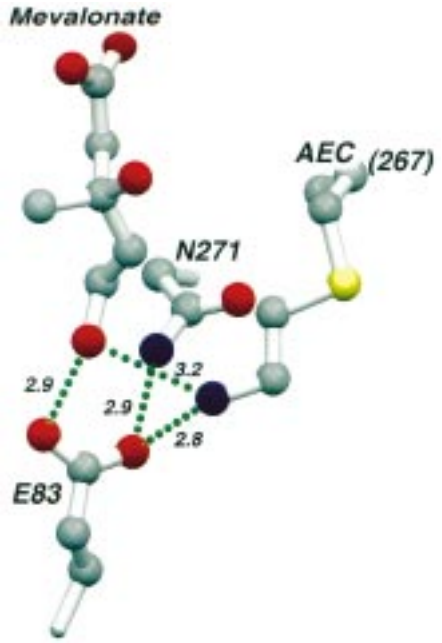


FIGURE 4: Shown is a portion of the active site of HMG-CoA reductase in which lysine 267 has been replaced by aminoethylcysteine (AEC). AEC was modeled into the X-ray structure of the mevalonate−NADH ternary complex of *P. mevalonii* HMG-CoA reductase (*7*). The model was then subjected to energy minimization using X-PLOR (*21*). Atoms are color-coded by atom type. Hydrogen bond interactions are indicated by dotted green lines. The figure was prepared using SETOR (*22*).

We have shown that an aminoacyl residue critical for maintaining structure and catalytic activity can be replaced by a nonnaturally occurring amino acid. Of the substitutions examined, only aminoethylcysteine could successfully replace lysine 267. Substitution of aminoethylcysteine generated a mutant enzyme with both high catalytic activity and essentially wild-type kinetic parameters. A precisely positioned positive charge at position 267 of *P. mevalonii* HMG-CoA reductase thus is essential for catalytic activity. We conclude, in addition, that mutant enzyme C156A/C296A/K267C provides a promising point of departure for future investigation of the functional role of lysine 267.

To provide insight into why aminoethylcysteine can successfully replace lysine 267, we modeled aminoethylcysteine into the X-ray structure of the mevalonate/NADH abortive ternary complex of the native enzyme (*7*). As shown in Figure 4, the wild-type interactions with glutamate 83 and binding and appropriate positioning of mevalonate are conserved in the mutant enzyme.

ACKNOWLEDGMENT

We thank our colleagues John Burgner, Jon Friesen, Matija Hedl, C. Martin Lawrence, Dong-Yul Kim, and Kenneth Li for helpful suggestions.

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BI9902687