Investigation of the Conserved Lysines of Syrian Hamster 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase†

Daniel A. Bochar,‡,§ Cynthia V. Stauffacher,‖ and Victor W. Rodwell*,‡

Departments of Biochemistry and Biological Sciences, Purdue University, West Lafayette, Indiana 47907

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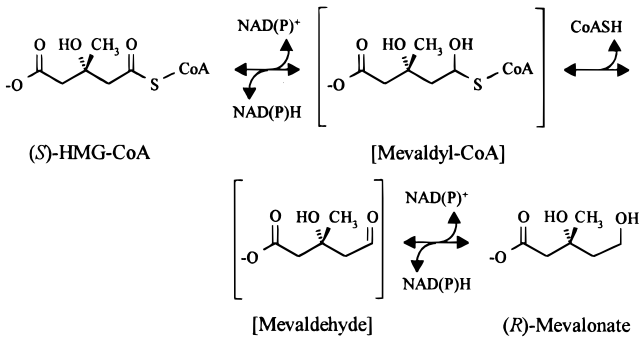
ABSTRACT: Sequence analysis has revealed two classes of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Crystal structures of ternary complexes of the Class II enzyme from *Pseudomonas mevalonii* revealed lysine 267 critically positioned at the active site. This observation suggested a revised catalytic mechanism in which lysine 267 facilitates hydride transfer from reduced coenzyme by polarizing the carbonyl group of HMG-CoA and subsequently of bound mevaldehyde, an inference supported by mutagenesis of lysine 267 to aminoethylcysteine. For this mechanism to be general, Class I HMG-CoA reductases ought also to possess an active site lysine. Three lysines are conserved among all Class I HMG-CoA reductases. The three conserved lysines of Syrian hamster HMG-CoA reductase were mutated to alanine. All three mutant enzymes had reduced but detectable activity. Of the three conserved lysines, sequence alignments implicate lysine 734 of the hamster enzyme as the most likely cognate of *P. mevalonii* lysine 267. Low activity of enzyme K734A did not reflect an altered structure. Substrate recognition was essentially normal, and both circular dichroism spectroscopy and analytical ultracentrifugation implied a native structure. Enzyme K734A also formed an active heterodimer when coexpressed with inactive mutant enzyme D766N. We infer that a lysine is indeed essential for catalysis by the Class I HMG-CoA reductases and that the revised mechanism for catalysis is general for all HMG-CoA reductases.

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA)[1] reductase (E. C. 1.1.1.34) catalyzes the reductive deacylation of (*S*)-HMG-CoA to (*R*)-mevalonate, a reaction central to biosynthesis of isoprenoids in eukaryotes, archaea, and certain true bacteria (*1*).



A reversible four-electron oxidoreduction, the reaction involves three stages, the first and third of which are reductive, and successive formation of enzyme-bound mevaldyl-CoA and mevaldehyde. HMG-CoA reductase also catalyzes two reactions that resemble the third stage and the reverse of the first stage of the overall reaction, respectively. These reactions are the reduction of free mevaldehyde to mevalonate and its oxidative acylation to HMG-CoA.

As the catalyst for the rate-limiting reaction of isoprenoid biosynthesis, HMG-CoA reductase represents the site of action of the "statin" drugs that ameliorate hypercholesterolemia in human subjects by inhibiting HMG-CoA reductase activity competitive with HMG-CoA (*1*). The enzyme also performs key functions in other life forms. Plants elaborate multiple HMG-CoA reductase isozymes that are regulated differentially during development and in response to environmental stress. While absent from *Escherichia coli* and many other eubacteria, genes that may be inferred to encode HMG-CoA reductase are present in several pathogenic bacteria (*2*). HMG-CoA reductase thus presents a potential target for the control of isoprenoid metabolism in forms of life as diverse as human subjects, plants, and selected bacterial pathogens.

We previously employed sequence comparisons to identify genes that encode two distinct classes of HMG-CoA reductase (*2*). Class I includes the HMG-CoA reductases of all eukaryotes and of several archaea. Class II presently includes several eubacterial and one archaeal form of the enzyme. Both classes of HMG-CoA reductase share an active site aspartate, glutamate and histidine, and several conserved motifs such as the DAMG loop involved in nucleotide

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* To whom correspondence and request for reprints should be addressed: Telephone: 765-494-1608. Fax: 765-494-7897. E-mail: vrodwell@purdue.edu.

‡ Department of Biochemistry, Purdue University.

‖ Department of Biological Sciences, Purdue University.

§ Present Address: The Wistar Institute, 3601 Spruce St., Philadelphia, Pennsylvania 19104-4268.

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

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coenzyme binding (*1*, *3*). Crystallographic analysis of two abortive ternary complexes of the Class II enzyme from *Pseudomonas mevalonii* revealed a lysyl residue, $Lys267_P$,[2] critically positioned in the active site and hydrogen bonded to catalytic residues $Glu83_P$ and $Asp283_P$ (*4*). On the basis of the structure of these ternary complexes, a revised mechanism of catalysis was proposed. In this mechanism, $Lys267_P$ serves as the general acid−base, facilitating hydride transfer from NADH during catalysis of both reductive stages of the reaction by polarizing the carbonyl oxygen of the HMG-CoA, and subsequently of bound mevaldehyde. As anticipated for a residue involved in catalysis, mutation of $Lys267_P$ to alanine was accompanied by a decrease in activity to less than 0.06% that of the wild-type enzyme (*4*). Mutation to cysteine of $Lys267_P$ of an otherwise cysteine-free mutant form of the enzyme and subsequent chemical modification with bromoethylamine replaced $Lys267_P$ by the nonnaturally occurring lysine analogue aminoethylcysteine (AEC). Consistent with the proposed role of $Lys267_P$ in catalysis by the *P. mevalonii* enzyme (*4*), mutant enzyme K267AEC had high catalytic activity (*5*). On the basis of these observations, we anticipated the presence of an active site lysine also in Class I forms of the enzyme. Although sequence alignments implicate $Lys734_H$ as the most likely cognate of $Lys267_P$, three lysines ($Lys690_H$, $Lys691_H$, and $Lys734_H$) are conserved among all Class I HMG-CoA reductases (*2*). We, therefore, employed site-directed mutagenesis to investigate whether these conserved lysines are essential for catalysis.

EXPERIMENTAL PROCEDURES

Materials. Purchased reagents included (*R*,*S*)-HMG-CoA, NADPH, phenylmethylsulfonyl fluoride, dithiothreitol, anti-rat immunoglobulin G-alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium chloride, and Pharmalyte pH 5−8 (Sigma); Blue Sepharose CL-6B (Pharmacia); restriction enzymes (New England 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); rat monoclonal antibody raised against yeast α-tubulin (EEF antibodies) (Serotec); HMG-CoA agarose (PL Biochemicals); and silica gel thin-layer chromatography sheets (Kodak). Additional purchased materials were from previously listed sources (*6*, *7*). Determination of protein concentration employed the method of Bradford (*8*) and protein standards from Bio-Rad. Buffer A contained 50 mM NaCl, 10% (v/v) glycerol, 100 mM sucrose, and 10 mM dithiothreitol in 20 mM Na_xPO_4, pH 7.3.

Vectors and Bacterial Strains. Expression vectors p*K*FT7−21 (*9*) and pACT7-D766N (*10*) have been previously described. *E. coli* strain TG1 served as host during mutagenesis, cloning, and plasmid preparation. *E. coli* strain BL21-(DE3) or strain SP1515 served as host during protein expression. *E. coli* strain SP1515, a *recA*$^-$ strain of BL21-(DE3), was a generous gift from Ihor Skrypka and Ronald Somerville of Purdue University. Coexpression of genes encoding two inactive mutant enzymes involved simultaneous transformation of SP1515 cells with DNA that encoded mutant enzymes K734A and D766N.

Growth of Bacterial Strains. Media for growth of *E. coli* strains included LB (*11*), LB_{amp} (LB + 75 μg/mL ampicillin), LB_{kan} (LB + 40 μg/mL kanamycin), and $LB_{amp,kan}$ (LB + 75 μg/mL ampicillin and 40 μg/mL kanamycin). *E. coli* TG1 or BL21(DE3) cells were grown in LB or LB_{amp} medium, *E. coli* strain SP1515 cells on LB, LB_{amp}, or LB_{kan} medium, and SP1515 cells cotransformed with both PKFT7−21-(K734A) and pACT7(D766N) on $LB_{amp,kan}$ medium.

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/*n*-propanol/H_2O 35:28:28:9 (v/v). Mutant genes constructed using the polymerase chain reaction-based overlap extension method of oligonucleotide-directed mutagenesis (*12*) were subcloned into pUC19 for verification by DNA sequencing (*13*) at the Purdue Department of Biochemistry DNA Sequencing Facility.

Assay of HMG-CoA Reductase Activity. Standard conditions for assay of the reductive deacylation of HMG-CoA contained, in 200 μL, 32 μM (*R*,*S*)-HMG-CoA, 270 μM NADPH, 100 mM NaCl, 10 mM dithiothreitol, and 20 mM K_xPO_4, pH 6.75. The decrease in absorbance at 340 nm that accompanies the oxidation of NADPH was monitored in a Hewlett-Packard model 8452 diode array spectrophotometer maintained at 37 °C. One eu represents the turnover in one min of one μmole of NADPH, and hence of 0.5 μmole of HMG-CoA.

RESULTS

Expression and Purification of Wild-type and Mutant Enzymes. Site-directed mutagenesis was employed to independently alter the codons for lysines 690_H, 691_H, and 734_H to alanine. Genes encoding wild-type enzyme and mutant enzymes K690A, K691A, and K734A were expressed in *E. coli* BL21(DE3) cells. The encoded enzymes were then purified to apparent homogeneity as judged by SDS-PAGE and Western blotting (Figure 1) by the protocol developed for purification of the wild-type enzyme (*9*).

Catalysis of the Overall Reaction. All mutant enzymes were assayed both under standard conditions and at several concentrations of HMG-CoA and NADPH. Following replacement of lysine by alanine, mutant enzymes K690A, K691A, and K734A all were severely impaired for catalysis. Despite low activity, it was nevertheless possible to determine K_M values for HMG-CoA and NADPH for all three mutant enzymes (Table 1). Mutation to alanine thus failed to unequivocally implicate a single lysine as the cognate of lysine 267_P. Since sequence alignments (*2*) implicated $Lys734_H$ as the most likely candidate for the active site lysine of the hamster enzyme, we asked whether the low activity of enzyme K734A might have resulted from structural changes rather than from the loss of a residue critical for catalysis. We, therefore, employed physical and biochemical techniques to investigate the integrity of the structure of mutant enzyme K734A.

Mutant Enzyme K734A Has Native Overall Secondary, Tertiary, and Quaternary Structure. Circular dichroism and analytical ultracentrifugation were employed to examine the

[2] The suffixes H and P refer to residues from Syrian hamster and *P. mevalonii* HMG-CoA reductase, respectively.

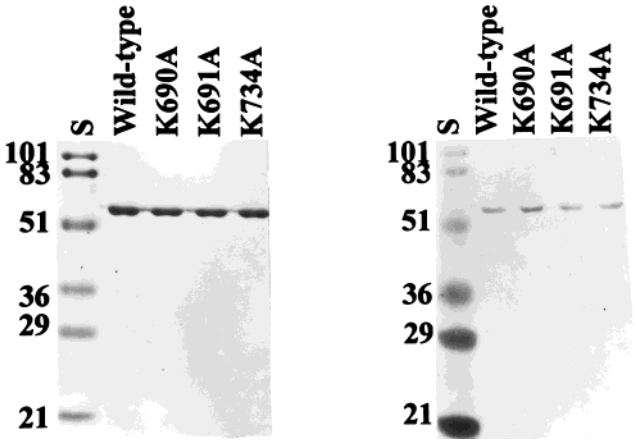

FIGURE 1: SDS-PAGE and Western blot analysis. Approximately 5 μg portions of purified wild-type enzyme (WT) or the indicated purified mutant enzyme were subjected to SDS-PAGE. Lane S contained prestained protein standards of the indicated molecular mass in kDa. Left: The gel was stained with Coomassie blue. Right: Western blot of an unstained duplicate of the preceding gel employed monoclonal antibodies against yeast α-tubulin, which recognizes the C-terminal Glu-Glu-Phe epitope of the hamster enzyme (*9*), and anti-mouse IgG alkaline phosphatase conjugate as the secondary antibody. The blot was developed using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride (*11*).

Table 1: Kinetic Parameters for Wild-Type Hamster HMG-CoA Reductase and Mutant Enzymes K734A, K690A, and K691A

enzyme	V (μU/mg)[a]	V/V_{wt} (%)[b]	K_M (μM) HMG-CoA	K_M (μM) NADPH
wild-type	10.4	100	40	75
K734A	0.017	0.16	8.0	120
K691A	0.17	1.6	82	180
K690A	0.021	0.20	5.4	100

[a] $V = V_{max}$. [b] V/V_{wt} = activity relative to that of the wild-type enzyme.

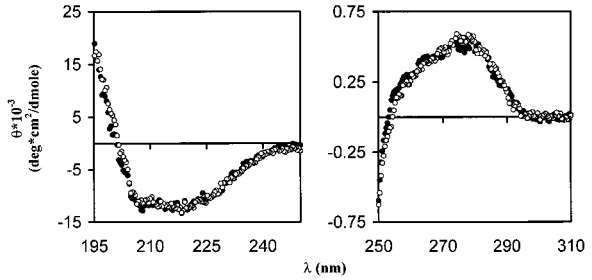

FIGURE 2: Circular dichroism. Shown are the spectra of the wild-type enzyme (●) and mutant enzyme K734A (○). Mean residue elipticity (θ) data at the indicated wavelengths are average values for six scans. Spectra in 25 mM K_xPO_4, pH 7.6, were measured at room temperature in a Jasco 600 spectropolarimeter. Left: Far UV CD spectra, 0.05 mg protein/ml, 1 mm path length cell. Right: Near UV CD spectra, 10 mm path length cell, 1.0 mg protein/ml.

structural integrity of mutant enzyme K734A. Both the near and far UV circular dichroism spectra of enzyme K734A matched those of the wild-type enzyme (Figure 2). The overall secondary and tertiary structure of enzyme K734A thus appear similar to that of the wild-type enzyme. The quaternary structure of mutant enzyme K734A also matched that of the wild-type enzyme. Sedimentation velocity experiments revealed that enzyme K734A and the wild-type enzyme had similar sedimentation coefficients (Figure 3). On the basis of these physical criteria, mutation of lysine

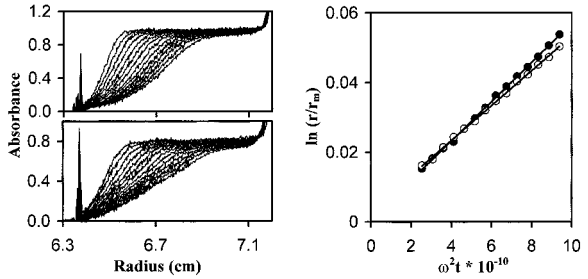

FIGURE 3: Analytical ultracentrifugation. Sedimentation velocity experiments employed a Beckman XL-A analytical ultracentrifuge. Samples, approximately 1 mg/mL in 100 mM K_xPO_4, pH 6.9, 200 mM KCl, 10 mM 2-mercaptoethanol, were centrifuged at 40,000 rpm. After 20 min, scans were measured at 280 nm every 5 min for 85 min. Left: Representative profiles for wild-type enzyme (top) and mutant enzyme K734A (bottom). Right: The data on the left for wild-type enzyme (●) and mutant enzyme K734A (○) are shown as the log of the radius (r) at the midpoint of the curves divided by the radius at the meniscus (r_m) versus $\omega^2t \times 10^{-10}$.

734_H to alanine thus did not appear to have been accompanied by major changes in secondary, tertiary, or quaternary structure.

Coexpression of Mutant Enzymes K734A and D766N Yields a Functional Enzyme. The crystal structure of the Class II HMG-CoA reductase of *P. mevalonii* revealed that the minimal functional unit is a homodimer. The active site resides at the subunit interface with residues critical for catalysis contributed by each subunit (*3*). The minimal functional unit of the hamster enzyme is also known to be a dimer that recruits catalytic residues from both subunits. This was shown by coexpression of genes encoding two mutant enzymes, each of which had been mutated in a different residue known to be essential for catalysis (*7*). By analogy to the *P. mevalonii* enzyme, each active site of the hamster enzyme should contain an aspartate from one subunit and a lysine from the other. If lysine 734_H is indeed the active site lysine, coexpression of genes encoding inactive mutant enzymes D766N (*7*) and K734A should yield a mixture of inactive and active enzymes. Providing that both mutant enzymes are expressed coequally, this should result in equal quantities of the nonfunctional homodimeric enzymes K734A/K734A and D766N/D766N and of the heterodimeric enzyme K734A/D766N. The K734A/D766N heterodimer should contain one functional and one impaired active site. The overall specific activity thus should be 25% that of the wild-type enzyme. We, therefore, exploited coexpression of two nonfunctional mutant enzymes to show that mutant enzyme K734A can form a functional dimer. Figure 4 illustrates the presence of approximately equal quantities of the two mutant subunits. Formation of an active heterodimer following coexpression of mutant enzymes K734A and D766N was shown by the recovery of activity that was 17% that of the wild-type enzyme (Table 2).

DISCUSSION

Attention was initially drawn to an active site lysine by determination of the unliganded structure of *P. mevalonii* HMG-CoA reductase (*3*). The structure revealed $Lys267_P$ in the vicinity of the previously identified active site aspartate and glutamate, $Asp283_P$ and $Glu83_P$ (*7*, *14*). The subsequently solved structures of the HMG-CoA/NAD^+ and

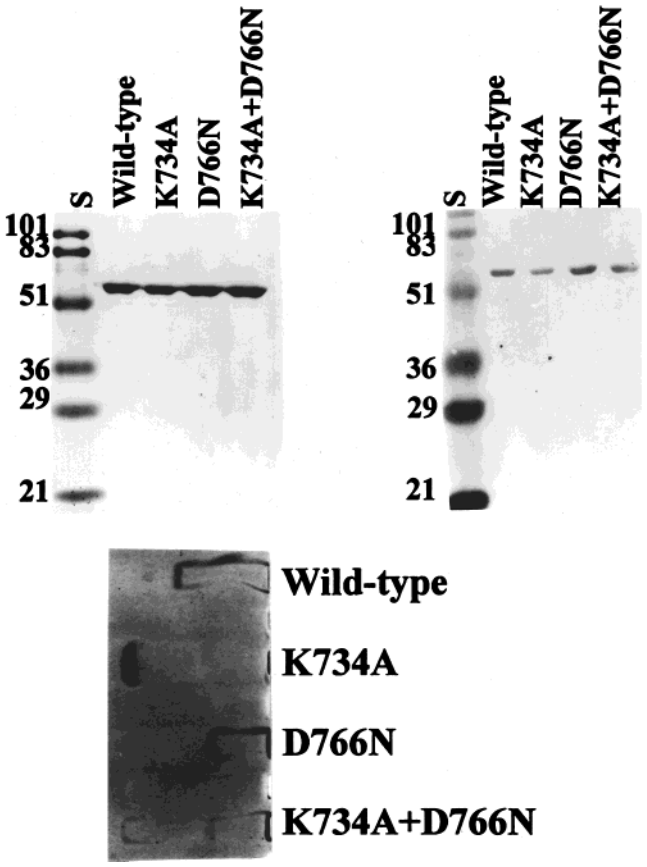

FIGURE 4: SDS-PAGE, Western blot analysis, and isoelectric focusing of purified wild-type, mutant, and coexpressed mutant hamster HMG-CoA reductases. Top: Approximately 5 μg portions of purified wild-type enzyme (WT) or the indicated purified mutant enzyme were subjected to SDS-PAGE. Lane S contained prestained protein standards of the indicated molecular mass in kDa. Top left: The gel was stained with Coomassie blue. Top Right: Western blotting was conducted as in Figure 1. Bottom: Approximately 2 μg portions of purified wild-type enzyme (WT), the indicated purified mutant enzyme, and coexpressed mutant enzymes were subjected to isoelectric focusing as described by O'Farrell (*15*). The gel contained 8 M urea, 2% Nonident P-40, and 2.5% Pharmalyte pH 5−8. Focusing was conducted at 400 V for 8 h followed by 500 V for 1 h. The gel was stained with Coomassie blue.

Table 2: Specific Activity of Wild-Type Hamster HMG-CoA Reductase, Enzymes K734A, D766N, and Co-expressed Enzymes K734A+D766N

enzyme	specific activity (μU/mg)	percent wild-type (%)
wild-type	11.0	100
K734A	0.017	0.16
D766N	0.034	0.31
K734A+D766N	1.9	17

mevalonate/NADH ternary complexes of the *P. mevalonii* enzyme revealed that $Lys267_P$ also participates in binding the substrate HMG-CoA. The ϵ-amino group of $Lys267_P$ is positioned within hydrogen bonding distance of $Glu83_P$ and $Asp283_P$ and is about 2.7 Å from the carbonyl oxygen of HMG-CoA (*4*). $Lys267_P$ was, therefore, proposed as a key player in catalysis that polarizes the carbonyl double bond of HMG-CoA, and subsequently of bound mevaldehyde, facilitating both stages of hydride transfer from NADH (*4*). Mutagenesis of the enzyme to remove the lysyl side chain and reconstruction of the active site by substitution of aminoethylcysteine (*5*) provided additional confirmation of the role of lysine 267 in the mechanism of catalysis by the *P. mevalonii* enzyme.

Class I HMG-CoA reductases possess only three conserved lysines, $Lys690_H$, $Lys691_H$, and $Lys734_H$. An analysis of the sequence alignments of Class I and Class II HMG-CoA reductases gives no unequivocal choice for the cognate of $Lys267_P$, since all three conserved lysines of the Class I HMG-CoA reductases reside in regions of low identity. The alignment predicts, however, that lysines 690_H and 691_H would be found in the junction between two domains of the structure (*3*) and that lysine 734_H is the most likely candidate for the active site lysine.

Site-directed mutagenesis was, therefore, employed in an attempt to identify the active site lysine of the Class I enzymes. Following the independent mutation of lysines 734_H, 691_H, and 690_H to alanine, the mutant enzymes retained the ability to catalyze the overall reaction at significant but severely attenuated, rates. All mutant enzymes also possessed K_M values for substrates that did not differ greatly from those of the wild-type enzyme, implying that all three are somehow critical for catalytic activity. While these mutations failed to unambiguously identify the active site lysine, lysine 734_H remained the most probable cognate of lysine 267_P (*2*, *4*). Enzyme K734A was then tested for structural integrity to address whether its low activity was a consequence of altered structure. The overall secondary, tertiary, and quaternary structure of enzyme K734A as judged by near and far UV circular dichroism and by analytical ultracentrifugation, respectively, was indistinguishable from that of the wild-type enzyme. The ability of mutant enzyme K734A to form a catalytically active heterodimer with the catalytically inactive mutant enzyme D766N also documented the integrity of the dimer interface in the neighborhood of the active site. From these combined observations, we infer that $Lys734_H$ is the probable active site lysine of the Class I HMG-CoA reductases and that the mechanism proposed for catalysis by the *P. mevalonii* enzyme appears to be valid for the hamster enzyme, and by extension for all Class I and Class II HMG-CoA reductases.

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