Evaluation of 3-Hydroxy-3-methylglutaryl-Coenzyme A Lyase Arginine-41 as a Catalytic Residue: Use of Acetyldithio-Coenzyme A To Monitor Product Enolization†

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ABSTRACT: 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) lyase catalyzes the divalent cation-dependent cleavage of HMG-CoA to produce acetyl-CoA and acetoacetate. Arginine-41 is an invariant residue in HMG-CoA lyases. Mutation of this residue (R41Q) correlates with human HMG-CoA lyase deficiency. To evaluate the functional importance of arginine-41, R41Q and R41M recombinant mutant human HMG-CoA lyase proteins have been constructed, expressed, and purified. These mutant proteins retain structural integrity based on Mn^{2+} binding and affinity labeling stoichiometry. R41Q exhibits a 10^5-fold decrease in V_{max}; R41M activity is ≥10-fold lower than the activity of R41Q. Acetyldithio-CoA, an analogue of the reaction product, acetyl-CoA, has been employed to test the function of arginine-41, as well as other residues (e.g., aspartate-42 and histidine-233) implicated in catalysis. Acetyldithio-CoA supports enzyme-catalyzed exchange of the methyl protons of the acetyl group with solvent; exchange is dependent on the presence of Mg^{2+} and acetoacetate. In comparison with wild-type human enzyme, D42A and H233A mutant enzymes exhibit 4-fold and 10-fold decreases, respectively, in the proton exchange rate. In contrast, R41Q and R41M mutants do not catalyze any substantial enzyme-dependent proton exchange. These results suggest a role for arginine-41 in deprotonation or enolization of acetyldithio-CoA and implicate this residue in the HMG-CoA cleavage reaction chemistry that leads to acetyl-CoA product formation. Assignment of arginine-41 as an active site residue is also supported by a homology model for HMG-CoA lyase based on the structure of 4-hydroxy-2-ketovalerate aldolase. This model suggests the proximity of arginine-41 to other amino acids (aspartate-42, glutamate-72, histidine-235) implicated as active site residues based on their function as ligands to the activator cation.

3-Hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA)[1] lyase catalyzes the cleavage of HMG-CoA into acetyl-CoA and acetoacetate (*1*). This reaction represents a key step in both ketogenesis (*2*) and leucine catabolism (*3*). Homologous C−C bond cleavage/condensation reactions are catalyzed by various members of the HMG-CoA lyase family of enzymes. Included in this family are homocitrate synthase, isopropylmalate synthase, and 4-hydroxy-2-ketovalerate aldolase (*4*).

The cleavage of HMG-CoA has been proposed to involve general acid/base catalysis and requires the presence of a divalent cation such as Mg^{2+} or Mn^{2+}. The divalent cation is believed to ligate to the thioester carbonyl and 3-hydroxyl groups of HMG-CoA (*5*), bridging the enzyme and substrate. A plausible reaction mechanism (Scheme 1) involves the deprotonation of the C3 hydroxyl group by a general base, followed by C−C bond cleavage and divalent cation-assisted enolization of acetyl-CoA to stabilize the negative charge that would develop on C2 after C−C bond cleavage. The reaction is completed by the protonation of C2 by a general acid (*4*).

Several active site residues, including two divalent cation ligands, have been identified on the basis of site-directed mutagenesis, kinetic, and electron spin resonance (ESR) studies. Histidine-235 (*6*) and glutamate-72 (*7*) are proposed to ligate to the divalent cation, while aspartate-42 (*7*), histidine-233 (*8*), and cysteine-266 (*9*−*11*) are important to catalytic efficiency. However, the exact roles of aspartate-42, histidine-233, and cysteine-266 have not yet been determined. Site-directed mutagenesis and kinetic studies using recombinant human HMG-CoA lyase have shown that D42A exhibits a V_{max} that is diminished by 130 000-fold in comparison with the estimate for wild-type enzyme (*7*); V_{max} estimates for H233A and C266A indicate diminutions of 7600-fold and 13 000-fold, respectively (*8*, *11*). These residues are conserved in all HMG-CoA lyase sequences reported to date (Figure 1).

Sequence alignments also show the conservation of arginine at residue 41. Mutation of this residue to glutamine has been correlated with human HMG-CoA lyase deficiency (*13*).

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[1] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; DTT, dithiothreitol; IPTG, isopropyl β-D-1-thiogalactopyranoside; 3D-PSSM, 3D-protein specific scoring matrix.

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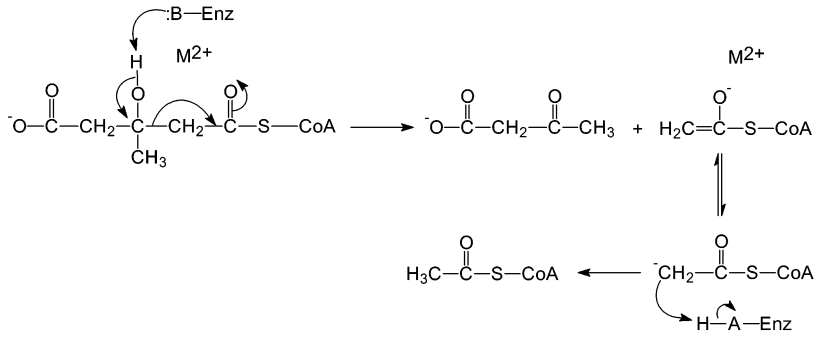
Scheme 1: Reaction Catalyzed by HMG-CoA Lyase



A.

	41 / 42	72	233 / 235	266
HMGL *H.sapiens*	GP**RD**GLQ	VI**E**T	V**H**C**H**DTY	GG**C**PY
HMGL *P.mevalonii*	GP**RD**GLQ	HI**E**A	G**H**F**H**DTW	GG**C**PY
HMGL *C.elegans*	GA**RD**GLQ	TV**E**T	V**H**F**H**DTY	GG**C**PY
HMGL *S.coelicolor*	GA**RD**GLQ	TI**E**A	V**H**F**H**DTY	GG**C**PF
HMGL *A. thaliana*	GP**RD**GLQ	VV**E**A	V**H**F**H**DTY	GG**C**PY
HMGL *B. subtilis*	GP**RD**GLQ	YI**E**I	L**H**F**H**DTR	GG**C**PY
HMGL *P. alcaligenes*	SP**RD**GFQ	RV**E**I	F**H**G**H**DTY	GG**C**PF

B.

	41 / 42	72	233 / 235
IMS *H.influenzae*	TL**RD**GEQ	VM**E**V	V**H**C**H**NDL
IMS *L.lactis*	SL**RD**GEQ	VI**E**A	P**H**C**H**DDL
IMS *E. coli*	TL**RD**GEQ	VM**E**V	V**H**T**H**DDL
IMS *B. aphidicola*	TL**RD**GEQ	II**E**V	V**H**C**H**NDL
HCS *A.chroococcum*	TL**RD**GEQ	EL**E**I	V**H**A**H**DDF
HCS *R.sphaeroides*	TL**RD**GEQ	EV**E**V	F**H**G**H**NDL
HOA *B. cepacia*	TL**RD**GMH	LI**E**V	F**H**G**H**HNL
HOA *P.putida*	SL**RD**GMH	LI**E**I	F**H**G**H**HNM
HOA *P. sp (strain CF600)*	TL**RD**GSH	YI**E**A	M**H**A**H**HNL

FIGURE 1: Primary amino acid sequence alignment of various HMG-CoA lyases with HMG-CoA lyase family members. A primary amino acid alignment of several HMG-CoA lyase (HMGL) enzymes (A) is compared with the alignment of members of the HMG-CoA lyase family (B). Included in the HMG-CoA lyase protein family are homocitrate synthase (HCS), isopropylmalate synthase (IMS), and 4-hydroxy-2-oxovalerate aldolase (HOA). Residues that are invariant among HMG-CoA lyases and homologous in the HMG-CoA lyase protein family are in bold and underlined. Numbers appearing above sequence alignments correspond to the amino acid numbering of human HMG-CoA lyase. Only a representative number of sequences have been shown. All sequences were obtained from public databases. Alignment was generated using the Pfam protein family database (*12*). Sequences from the following organisms and accession numbers are included: *Homo sapiens*, P35914 (residues 39−45, 70−73, 232−238, 264−268); *Pseudomonas mevalonii*, P13703 (residues 10−16, 41−44, 203−209, 235−239); *Caenorhabitis elegans*, AAK73908 (residues 25−31, 56−59, 218−224, 250−254); *Streptomyces coelicolor*, CAB87215 (residues 27−33, 58−61, 220−226, 252−256); *Arabidopsis thaliana*, AAK15568 (residues 59−65, 90−93, 252−258, 284−288); *Bacillus subtilis*, D69893 (residues 13−19, 44−47, 206−212, 238−242); *Pseudomonas alcaligenes*, AAG39454 (residues 13−19, 44−47, 206−212, 238−242); *Haemophilus influenzae*, P43861 (residues 11−17, 42−45, 201−207); *Lactococcus lactis*, Q02141 (residues 10−16, 41−44, 200−206); *Escherichia coli*, P09151 (residues 11−17, 42−45, 201−207); *Buchnera aphidicola*, P48571 (residues 11−17, 42−45, 201−207); *Azotobacter chroococcoccum*, P23122 (residues 10−16, 41−44, 191−197); *Rhodobacter sphaeroides*, Q01181 (residues 25−31, 56−59, 207−213); *Burkholderia cepacia*, P51015 (residues 14−20, 45−48, 198−204); *Pseudomonas putida*, P51017 (residues 14−20, 45−48, 198−204); *Pseudomonas* sp. (strain CF600), P51016 (residues 15−21, 72−75, 199−204).

To examine the influence of arginine-41 mutations on the efficiency of the enzyme reaction and the corresponding human disease, human HMG-CoA lyase proteins containing side chain substitutions for arginine-41 were purified and characterized. The product analogue, acetyldithio-CoA, was employed for functional tests of arginine-41. Results of these studies, as well as a homology model for this enzyme, argue for the assignment of arginine-41 as an active site residue in HMG-CoA lyase and suggest its participation in the terminal steps of the reaction that account for formation of the product, acetyl-CoA.

EXPERIMENTAL PROCEDURES

Materials. Escherichia coli (JM109) cells were obtained from Promega. HPLC-purified deoxyoligonucleotides were purchased from Operon. Qiagen plasmid kits (Qiagen Inc.) were used to isolate plasmid DNA from bacterial cultures. Bacto-tryptone and yeast extract are products of Difco Laboratories. Isopropyl-1-thio-β-D-galactoside (IPTG) was bought from U. S. Biochemical Corp. Phenyl agarose was purchased from Sigma and Q-Sepharose anion-exchange resin from Pharmacia Biotech. Malate dehydrogenase and citrate synthase were obtained from Sigma. HMG-CoA was

synthesized from the anhydride, prepared from the free acid (Fluka) according to Goldfarb and Pitot (*14*); concentration of (*S*)-isomer of HMG-CoA was determined by enzymatic end-point analysis. 3-Hydroxyglutaryl-CoA was synthesized as described by Kramer and Miziorko (*15*). [^{14}C]HMG-CoA (8700 dpm/nmol) was enzymatically synthesized from acetoacetyl-CoA and 1-[^{14}C]acetyl-CoA as described by Vinarov and Miziorko (*16*). Wild-type β-ketothiolase from *Zoogloea ramigera* (*17*) was a generous gift from Dr. Vernon Anderson; the C378G-encoding plasmid was a generous gift from Dr. Anthony Sinskey (MIT). C378G thiolase was expressed and purified as described by Vinarov et al. (*18*). All other chemicals were reagent grade.

Site-Directed Mutagenesis. The expression construct for human HMG-CoA lyase (pTRC-HL1) encodes the mature mitochondrial isoform (lacking the transit peptide) and is derived from the pTRC-99a expression vector, as previously described (*19*). Generation of plasmids encoding the D42A (*7*), H233A (*8*), and R41Q (*13*) point mutations have been described previously. Generation of the R41M point mutation in HMG-CoA lyase was performed by full-circle PCR using the QuikChange Site-Directed Mutageneis Kit from Stratagene. The 5′ to 3′ directional primer used to construct R41M was 5′- G GAA GTT GGT CCC ATG GAT GGA CTA CAA AAT G-3′. The underlined characters indicate the base changes that were required to generate the mutagenic codon. Mutagenic plasmid DNA was isolated from selected transformants and analyzed by restriction mapping and DNA sequencing to confirm the desired mutation and the absence of any artifactual changes in the coding sequence. DNA sequencing was performed by the Protein and Nucleic Acid Facility at the Medical College of Wisconsin. Plasmids were transformed into competent JM109 cells for subsequent expression of mutant lyase enzymes.

Expression and Purification of Wild-Type and Mutant Enzymes. Enzymes were expressed and purified from 3 L cultures as described by Roberts et al. (*19*). Enzyme purity for wild-type and each mutant enzyme was assessed by SDS−PAGE and Coomassie blue staining. SDS−PAGE was performed according to Laemmli (*20*) using an 11.5% acrylamide running gel and a 4.5% acrylamide stacking gel.

Protein and Enzyme Assays for HMG-CoA Lyase. Protein concentration was determined following the method of Bradford (*21*) using the Bio Rad protein assay reagent (BioRad Laboratories) and bovine serum albumin as the standard. A subunit molecular mass of 33 000 Da was used to calculate the molar concentration of enzyme. Modification of enzyme with [^{14}C]2-butynoyl-CoA (16 000 dpm/nmol) was performed as previously described by Hruz and Miziorko (*22*).

HMG-CoA lyase enzymatic activity was measured using the citrate synthase-coupled assay of Stegink and Coon (*1*) as modified by Kramer and Miziorko (*23*) or using a radioactive assay (*24*) to improve sensitivity. All reaction components, including buffer, water, and substrate, were passed over a Chelex-100 column (Bio-Rad) to remove trace metals. V_{max} and K_m values for HMG-CoA lyase were determined from initial velocity data fit by nonlinear regression analysis using the Grafit program (Erithacus software).

ESR Spectroscopy. The binding of Mn^{2+} to human HMG-CoA lyase was measured on a Varian Century-Line 9 GHz spectrometer with a TE_{102} cavity as described by Roberts et al. (*11*). Each spectrum was recorded at 22 °C with a modulation amplitude of 10 G, a modulation frequency of 100 kHz, a microwave power of 60 mW, a field sweep of 1000 G, and a time constant of 0.25 s. Prior to ESR, protein samples were concentrated to 200 μM, and glycerol was removed using a Sephadex G-25 centrifugal column equilibrated with 25 mM Tris-Cl, pH 8.2, containing 150 mM NaCl and 1 mM DTT. A quartz capillary tube was used for all measurements. The ESR samples contained variable concentrations of Mn^{2+} (10−150 μM) with constant HMG-CoA lyase sites (50 μM). The amount of bound Mn^{2+} was determined by directly comparing the spectral amplitudes of samples containing HMG-CoA lyase to the corresponding amplitudes observed with a buffered solution containing an equal concentration of Mn^{2+} in the absence of enzyme. Scatchard plots of the data were subjected to linear regression analysis using Grafit to determine both the Mn^{2+} binding stoichiometry and the binding affinity of the HMG-CoA lyase mutants.

HMG-CoA Lyase Catalyzed Exchange of Acetyldithio-CoA C2 Methyl Protons. Synthesis of acetyldithio-CoA from phenyl thioacetate (Aldrich) was performed as described by Wlassics et al. (*25*) with the following modifications. The exchange reaction between *S*-phenylthioacetate and CoASH was performed in 300 mM Li_2CO_3, pH = 8.5. The reaction was terminated by acidification to pH = 4 with 1 N HCl followed by evaporation to reduce the volume to 3 mL. After extraction with 3 mL of ethyl acetate, the aqueous phase was concentrated to 200 μL; 1.5 mL of cold methanol was added, and acetyldithio-CoA was precipitated as the lithium salt by addition of 10 mL of cold acetone. After a total of four precipitations from cold methanol/acetone, the product was dried under a stream of nitrogen. The acetyldithio-CoA eluted as a single peak on RP-HPLC using an isocratic elution, (70% 50 mM sodium phosphate, pH 4.5/30% methanol) indicating the absence of additional CoA species. Purity was also verified by ^{1}H NMR and UV/vis spectroscopy (ratio of A_{260}/A_{306} = 1.3).

The ability of HMG-CoA lyase to catalyze the enolization of acetyldithio-CoA was monitored by ^{1}H NMR. HMG-CoA lyase proteins were twice concentrated in an Amicon ultrafiltration device to 250 μL and resuspended in 3 mL of a D_2O solution of 20 mM potassium phosphate buffer, pD 7.8, containing 100 mM NaCl, 1 mM DTT, and 20% glycerol. Exchange reactions for HMG-CoA lyase proteins were measured using samples that included 7 mM acetyldithio-CoA, 100 mM acetoacetate, 10 mM $MgCl_2$, and 0.5 mM DTT in 50 mM potassium phosphate buffer, pD = 7.9, and various concentrations of enzyme. The enzyme dilution is such that glycerol carryover does not interfere with NMR measurements. Thiolase proteins and reactions were prepared identically to HMG-CoA lyase samples but with the omission of acetoacetate, $MgCl_2$, and DTT. The nonenzymatic exchange rates were measured by omitting HMG-CoA lyase or thiolase from the reaction mixture. ^{1}H NMR experiments were performed on a Bruker AC-300 instrument operating at 300 MHz for ^{1}H. After addition of enzyme to the reaction mixture, samples were placed in a 5 mm NMR tube, and spectra were recorded at 22 °C. The ^{1}H NMR spectra of the reaction mixture were recorded every 3 min over a period of 3 h. Each spectrum is comprised of a total of 64 acquisitions.

Proton exchange was estimated (*26*, *27*) from the time-dependent change of the ratio of the 2.8 ppm α-hydrogen signal to the 0.75 ppm panthetheine methyl resonance (which is unaffected by the exchange process). k_{obs} was calculated from eq 1:

$$k_{obs} = k_{enzyme} - k_{control} \quad (1)$$

where k_{enzyme} is the slope of the least-squares fit of the data plotted as the log of the peak ratios versus time (SigmaPlot) for exchange reactions containing enzyme while $k_{control}$ is the slope measured in background controls performed without enzyme. The exchange rate, k_{exch}, was calculated (*28*) using eq 2:

$$k_{exch} = 3[\text{acetyldithio-CoA}]k_{obs}/[\text{enzyme sites}] \quad (2)$$

Development of a Structural Model for HMG-CoA Lyase. The sequence of the precursor form of human HMG-CoA lyase (including the transit peptide) and the sequence of *Pseudomonas mevalonii* HMG-CoA lyase were analyzed to produce secondary structure and three-dimensional folding predictions based on the available structure of 4-hydroxy-2-ketovalerate aldolase. Analyses were performed using the 3D-protein specific scoring matrix algorithm (*29*) available through the 3D-PSSM web server of the Structural Bioinformatics Group at Imperial College of Science, Technology, and Medicine; side chain positions were modeled using the SCWRL method of Bower et al. (*30*). While alignment of human HMG-CoA lyase residues 25−316 (of 325 total residues) with 4-hydroxy-2-ketovalerate aldolase indicates an overall sequence identity of only 17%, the homology model produced by threading HMG-CoA lyase onto the fold exhibited by this template corresponds to an *E*-value of 1.77×10^{-14} suggesting an extremely high level of confidence in the predicted fold. For the slightly smaller bacterial enzyme (301 residues), alignment of residues 1−301 with 4-hydroxy-2-ketovalerate aldolase also exhibited 17% identity; the homology model produced in this analysis is characterized by an *E*-value of 3.13×10^{-13}. While this result is slightly lower that the value for the human protein, the model is more useful in that extended gaps were minimal and all eight β strands of the β/α barrel are apparent. No other fit from the fold library used for the analysis resulted in *E*-values better than 6.5×10^{-1}. For these reasons, the model depicted was generated by fitting bacterial HMG-CoA lyase to the 4-hydroxy-2-ketovalerate aldolase fold; residue numbering corresponds to the human HMG-CoA lyase protein.

RESULTS

Rationale for Construction, Expression, and Isolation of Arginine-41 Substituted HMG-CoA Lyase Proteins. The R41Q HMG-CoA lyase mutant protein was generated to model the clinically detected mutation that has been correlated with human disease (*13*). While preliminary studies indicated a substantial diminution in specific activity of this mutant enzyme, no substantial enzyme characterization had been previously reported. Thus, it was not possible to predict whether functional or structural defects account for the low activity measured for enzyme containing this human mutation. Additional significance for arginine-41 derives from the observation that not only is this residue invariant in all deduced sequences for HMG-CoA lyase proteins (Figure 1) but it is also highly conserved in the various members of the HMG-CoA lyase family of proteins (Pfam 00682). Members of this family include isopropylmalate synthase and homocitrate synthase, both of which catalyze reactions requiring acetyl-CoA, the product of the HMG-CoA lyase reaction. Additionally, this family includes 4-hydroxy-2-ketovalerate aldolase, which catalyzes a homologous cation-dependent aldol cleavage and represents a useful template for homology modeling and prediction of a folded structure for HMG-CoA lyase. In anticipation of the need for more detailed tests of the functional significance of arginine-41, the R41M mutant was also generated to remove any hydrogen bonding capability while minimizing steric differences between side chains. Both R41Q and R41M mutant proteins were expressed in soluble form and were purified to a high degree of purity using the isolation procedure developed for wild-type HMG-CoA lyase (Figure 2).

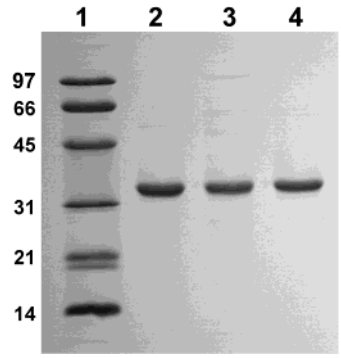


FIGURE 2: SDS−PAGE of purified R41 mutant HMG-CoA lyases. Three micrograms of purified proteins were loaded onto 11.5% SDS−polyacrylamide gel as follows: lane 1, molecular weight markers; lane 2, wild-type; lane 3, R41Q; lane 4, R41M. Bands were visualized by Coomassie blue staining.

Evaluation of Structural Integrity of Arginine-41 Mutant Proteins. Two approaches that have previously proven useful in evaluating whether mutant HMG-CoA lyases retain active site integrity include evaluation of the stoichiometry of modification by the affinity label 2-butynoyl-CoA and measurement of divalent cation binding affinity and stoichiometry upon formation of a binary enzyme−cation complex. The active site integrity of the arginine-41 mutants is supported by the observation of stoichiometric modification exhibited by R41Q (0.9/site) and R41M (0.8/site) upon incubation with the affinity label 2-butynoyl-CoA (Table 1) under conditions that result in comparable modification (0.9/site) of wild-type enzyme. Mn^{2+} binding properties of the mutant enzymes can be evaluated by using ESR to detect cation binding and Scatchard analysis to determine binding affinity and stoichiometry. Both R41M and R41Q exhibit a K_d for the binary enzyme−metal complex that is comparable to the estimate for wild-type enzyme (Table 1). Binding stoichiometry for R41M (1.3/site) is similar to the value measured for wild-type enzyme (1.2/site). While the stoichiometry of cation binding to the R41Q mutant (1.9/site) is somewhat elevated, a stoichiometry (1.2/site) comparable to the estimate for wild-type enzyme is measured upon inclusion of the competitive inhibitor 3-hydroxyglutaryl-CoA, suggesting that specificity in cation binding to this mutant would occur in the presence of substrate HMG-CoA. The

Table 1: Kinetic and Cation Binding Parameters of Wild-Type and R41 Mutant HMG-CoA Lyases

enzyme	V_{max} (U/mg)	$K_{m\ HMG-CoA}$ (μM)[b]	$K_{d\ Mn^{2+}}$ (μM)[c]	$K_{d\ Mn^{2+}}$ (+HG-CoA) (μM)[d]	2-butynoyl-CoA stoichiometry[e]
wt	191 ± 10	48 ± 6	1.9 ± 0.3 (1.2 ± 0.1)		0.88 ± 0.01
R41Q	0.0007 ± 0.0001	62 ± 12	3.8 ± 0.6 (1.9 ± 0.1)	1.3 ± 0.4 (1.2 ± 0.1)	0.89 ± 0.10
R41M	<0.0001[a]		2.1 ± 0.3 (1.3 ± 0.1)		0.78 ± 0.01

[a] Activity for R41M was too low to be accurately determined. The value given is the estimated limit of detection using the radioactive assay. The low level of enzymatic activity for R41M precluded analysis at subsaturating concentrations of substrate; therefore, K_m for HMG-CoA could not be determined. [b] $K_{m\ HMG-CoA}$ was determined under standard assay conditions using 10 mM Mg^{2+}. Therefore, these are apparent K_m values. V_{max} and K_m values and errors were determined by nonlinear regression fits of the data (>8 data points). [c] Dissociation constants and Mn^{2+} binding stoichiometry were calculated by Scatchard analysis using ESR spectroscopy to determine free Mn^{2+}. Stoichiometry is given in parentheses and is calculated on the basis of a 33 kDa subunit. $K_{d\ Mn^{2+}}$ values and errors were determined by linear regression fits to Scatchard plots (≥6 data points). [d] Dissociation constant and binding stoichiometry for Mn^{2+} were determined as described above, except 1 mM of the competitive inhibitor 3-hydroxyglutaryl-CoA was included. Stoichiometry is given in parentheses. [e] For butynoyl-CoA modification stoichiometries, error is reported as a standard error of the mean of duplicate measurements.

results of these affinity labeling and cation binding studies suggest that the substrate binding site is not substantially impaired in the R41M and R41Q mutants and argue that the results of kinetic characterization experiments can be interpreted without the concern that gross structural changes in the mutant proteins influence those data.

Kinetic Characterization of Arginine-41 Mutants. Measurement of kinetic parameters for the R41Q and R41M mutants (Table 1) demonstrates a significant diminution in enzymatic activity. R41Q exhibits a V_{max} that is 270 000-fold lower than the estimate for wild-type enzyme. However, no significant perturbation in K_m for the substrate is measured. Similarly, comparison of the $K_{m\ Mg}$ values for wild-type ($K_{m\ Mg}$ = 200 μM) and R41Q ($K_{m\ Mg}$ = 88 μM) proteins does not suggest substantial differences. The higher activity observed with R41Q versus the R41M mutant enzyme may be influenced by the ability of glutamine side chain to provide a partial electropositively charged nitrogen, which would be eliminated upon mutation to methionine. Removing not only this amide nitrogen but also any hydrogen bonding capability in the R41M mutant results in at least an additional 10-fold decrease in specific activity. This low level of activity approaches the limit of sensitivity for the radioactive assay, and therefore, precise V_{max} and $K_{m\ HMG-CoA}$ values for the R41M mutant could not be determined. The k_{cat} value (0.0004 s^{-1}) for R41Q is the most perturbed (270 000-fold diminution) of any catalytically deficient HMG-CoA lyase mutant examined to date. In comparison, D42A, H233A, and C266A mutants exhibit 130 000-fold, 7700-fold, and 13 500-fold decreases in k_{cat}, respectively, as compared with wild-type enzyme.

Evaluation of Arginine-41 as an Active Site Residue in the Context of a Structural Model for HMG-CoA Lyase. Initial attempts at homology modeling produced disappointing results. While β/α barrel folds were predicted, poor probabilities characterized the models, and the predicted juxtaposition of residues previously established to strongly influence enzyme catalysis was incompatible with such function. Recently, addition of the structure of 4-hydroxy-2-ketovalerate aldolase (*31*; PDB coordinates 1nvm) to the fold library used by the 3D-PSSM method resulted in identification of this template and production of a homology model for HMG-CoA lyase that is self-consistent with much of the existing functional data. This model is, as anticipated, a $(\beta/\alpha)_8$ barrel (Figure 3A). For predicted folds that exhibit a high level of confidence (e.g., *E*-value of 3.13 × 10^{-13}), side chain positions are also modeled using the method of

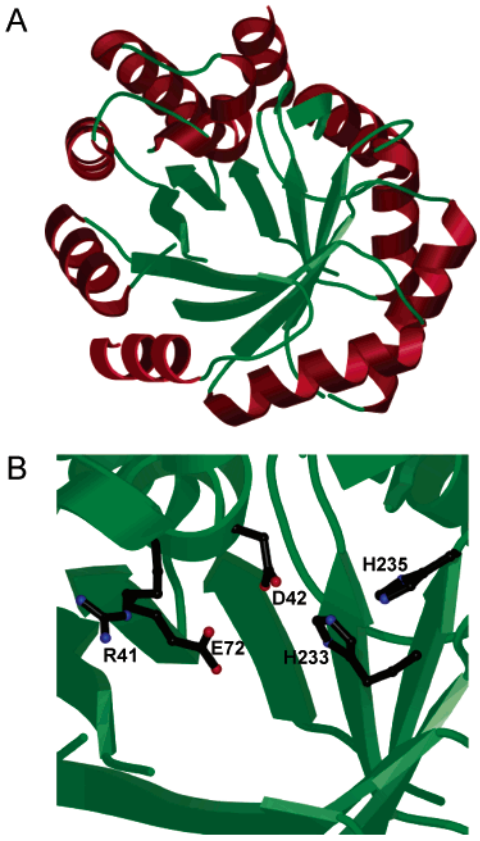


FIGURE 3: A homology model for HMG-CoA lyase, based on the prediction of the 3D-PSSM algorithm for folding of bacterial HMG-CoA lyase on a template corresponding to 4-hydroxy-2-ketovalerate aldolase. Panel A depicts a C-terminal view of the predicted $(\beta/\alpha)_8$ barrel structure for HMG-CoA lyase. Panel B depicts a view of the C-terminal edge of the (β/α) barrel and indicates the side chain positions of the active site residues arginine-41, aspartate-42, glutamate-72, histidine-233, and histidine-235. Side chain positions were modeled using the SCWRL method of Bowers et al. (*30*). Numbering of active site residues corresponds to the human HMG-CoA lyase protein. Molscript (*32*) and Raster3D (*33*) were used to produce the structural figures.

Bower et al. (*30*). Figure 3B depicts a view of the C-terminal end of the barrel and shows the positions predicted for Arg-41, Asp-42, Glu-72, His-233, and His-235; all of these residues have been implicated in reaction chemistry or activator cation binding or both. The proposed orientation of the modeled side chains may be subject to some error, but clearly all of these critical residues are situated at the consensus region for catalytic sites of β/α barrel enzymes. The proximity of Arg-41 to established cation ligands such as Glu-72, Asp-42, and His-235 not only strengthens the argument for assignment of Arg-41 as an active site residue but also suggests that several sources of electropositive charge are situated in the active site; these could represent an "anion hole" that would stabilize any enolized form of acetyl-CoA that is produced upon cleavage of the C3−C4 bond of HMG-CoA (Scheme 1). Additionally, positioning of residues with well demonstrated function at the C-terminal end of the barrel makes plausible the docking of substrate to the C-terminal face of the barrel, as observed for acetyl-CoA binding to malate synthase (*34*), which catalyzes a homologous Claisen reaction. This mode of substrate binding is common for enzymes that exhibit β/α barrel folds but contrasts with the observation for methylmalonyl-CoA mutase (*35*), where the acyl pantetheine portion of the substrate passes through the barrel cavity.

Utilization of the Product Analogue, Acetyldithio-CoA, To Evaluate the Function of Arginine-41. The cleavage reaction catalyzed by HMG-CoA lyase (Scheme 1) is considered to be effectively irreversible. However, previous studies (*15*) have demonstrated that enzyme, in the presence of divalent cation and acetoacetate, catalyzes a slow exchange of protons from the methyl group of acetyl-CoA. This reaction reflects enolization of a carbanionic form of acetyl-CoA that is generated as a result of cleavage of HMG-CoA. Reformation of a C2 carbanion from the enolized species is followed by protonation by an active site general acid or by a solvent molecule and release of the product, acetyl-CoA. Active site residues could participate in this process either by stabilizing the negatively charged oxygen of the enolate, by functioning as a general acid in donation of a proton to quench the carbanionic form of acetyl-CoA, or by polarizing a solvent molecule to facilitate protonation of the carbanion. Identification of a mutant enzyme that is defective in the enolization could implicate a function for the substituted residue. However, proton exchange from acetyl-CoA is slow, complicating comparisons between wild-type and mutant enzymes. These observations prompted evaluation of acetyldithio-CoA, which exhibits a decreased pK_a (*25*) for the C2 protons (pK_a = 12.5 versus 18−20 for acetyl-CoA) and potentially could support a more facile HMG-CoA lyase catalyzed exchange reaction. The ability of acetyldithio-CoA to support enzyme catalyzed proton exchange has been documented for citrate synthase (*27*) and β-ketothiolase (*36*).

Wild-type HMG-CoA lyase enzyme alone does not account for a substantial increase in proton exchange from the C2 (methyl) group of acetyldithio-CoA over the rate measured in buffer (Figure 4). However, inclusion of 10 mM Mg^{2+} or 100 mM acetoacetate (the other product of the cleavage reaction) stimulated exchange, which is optimally enhanced when both Mg^{2+} and acetoacetate are present (Figure 4; Table 2). These concentrations of Mg^{2+} and acetoacetate are comparable to those used in earlier work on enolization of acetyl-CoA (*15*) and are adequate to optimize the exchange from acetyldithio-CoA. This conclusion is supported by control experiments performed at higher levels of Mg^{2+} and acetoacetate as well as by the determination of K_i values for acetoacetate (K_i = 21 mM) and acetyldithio-CoA (K_i = 170 μM) as inhibitors of the normal HMG-CoA cleavage reaction. Under these conditions, it is also possible to demonstrate a linear dependence of the exchange reaction upon enzyme concentration (Figure 5).

As a prelude to investigating whether HMG-CoA lyase mutants might exhibit contrasts in the rates of proton exchange from acetyldithio-CoA, attempts were made to measure and compare the exchange rates supported by wild-type thiolase and the C378G mutant (which lacks the thiol group needed for deprotonation of acetyl-CoA). While it was possible to confirm the earlier report that wild-type thiolase catalyzes proton exchange from this acetyl-CoA analogue,

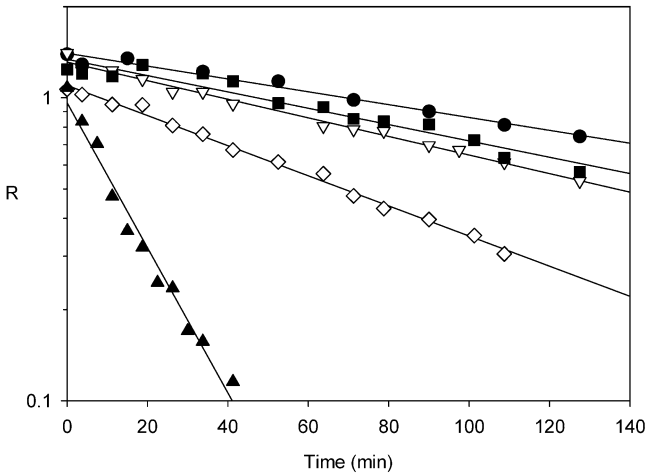


FIGURE 4: Dependence of the HMG-CoA lyase catalyzed exchange of α-hydrogens from acetyldithio-CoA with acetoacetate and Mg^{2+}. Enzyme-catalyzed exchange of acetyldithio-CoA was conducted using 7 mM acetyldithio-CoA with 75 μM wild-type HMG-CoA lyase in 50 mM potassium phosphate buffer, pD 7.9, containing 0.5 mM DTT in the absence of acetoacetate and Mg^{2+} (■), in the presence of 10 mM Mg^{2+} only (▽), in the presence of 100 mM acetoacetate only (◇), or in the presence of both 100 mM acetoacetate and 10 mM Mg^{2+} (▲). The control reaction (●) contained 100 mM acetoacetate and 10 mM Mg^{2+} in the absence of enzyme. Data were plotted as the ratio (R) of the 2.8 and 0.75 ppm proton signals versus time.

Table 2: Dependence on Acetoacetate and Mg^{2+} of the Enzyme-Catalyzed Proton Exchange from Acetyldithio-CoA[a]

sample	k_{obs} (min^{-1})[b]	k_{exch} (min^{-1})[c]
wt alone	1.1×10^{-3}	0.33
wt + Mg^{2+}	0.7×10^{-3}	0.24
wt + acetoacetate	3.0×10^{-3}	0.90
wt + acetoacetate + Mg^{2+}	22×10^{-3}	6.60

[a] Enzyme-catalyzed proton exchange of acetyldithio-CoA was conducted using 75 μM enzyme and 7 mM acetyldithio-CoA in 50 mM potassium phosphate buffer, pD 7.9, containing 0.5 mM DTT in combination with either 100 mM acetoacetate or 10 mM $MgCl_2$ or both. [b] k_{obs} was calculated using the following equation: $k_{enzyme} - k_{control}$, where k is the first-order rate constant determined from the slope of the least-squares fit of the log of the ratio of the α-hydrogen signal at 2.8 ppm to the pantetheine methyl signal at 0.75 ppm versus time. The control sample was identical to enzyme samples, except enzyme was excluded from the reaction mix; value of $k_{control} = 2 \times 10^{-3}$ min^{-1}. [c] k_{exch} was calculated from the following equation: 3[acetyldithio-CoA]k_{obs}/[enzyme sites].

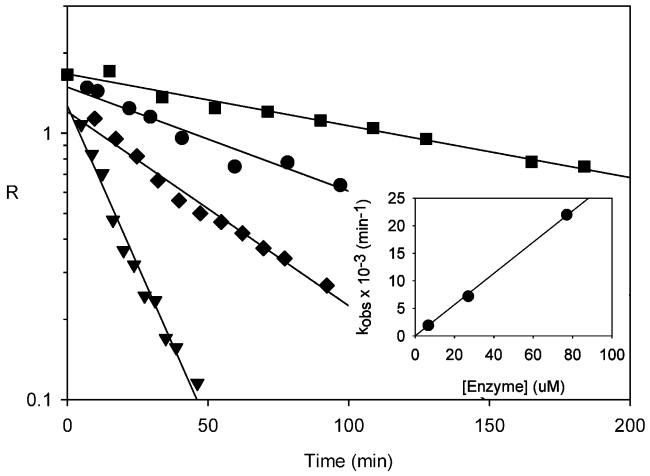


FIGURE 5: Protein concentration dependence of the HMG-CoA lyase catalyzed exchange of the α-hydrogens of acetyldithio-CoA. Enzyme-catalyzed exchange of acetyldithio-CoA was conducted by using 7 mM acetyldithio-CoA, 100 mM acetoacetate, and 10 mM Mg^{2+} in 50 mM potassium phosphate buffer, pD 7.9, containing 0.5 mM DTT. Data were plotted as the ratio (*R*) of the 2.8 and 0.75 ppm proton signals versus time. Control reaction (■) was identical to conditions above, except enzyme was excluded. Concentrations of HMG-CoA lyase used were (●) 6.7, (◆) 27, and (▼) 75 μM.

Table 3: Comparison of Enzyme-Catalyzed Proton Exchange From Acetyldithio-CoA by Wild-Type and Mutant HMG-CoA Lyase and β-Ketothiolase Proteins

enzyme	k_{exch} (min^{-1})[c]	$k_{exch\ wt}/k_{exch\ mutant}$
wt HMG-CoA lyase[a]	6.40 ± 0.20	
R41Q	*d*	
D42A	1.88 ± 0.26	3.5
H233A	0.65 ± 0.05	10
wt β-ketothiolase[b]	2.59 ± 0.54	
C378G β-ketothiolase	*d*	

[a] Enzyme-catalyzed proton exchange of acetyldithio-CoA with HMG-CoA lyase proteins was conducted using 7 mM acetyldithio-CoA, 100 mM acetoacetate, and 10 mM $MgCl_2$ in 50 mM potassium phosphate buffer, pD 7.9, containing 0.5 mM DTT. [b] Enzyme-catalyzed proton exchange with β-ketothiolase proteins was conducted as described above except acetoacetate, $MgCl_2$, and DTT were excluded from the reaction mix. [c] k_{exch} was calculated from the following equation: 3[acetyldithio-CoA]k_{obs}/[enzyme]. k_{obs} was calculated using the following equation: $k_{enzyme} - k_{control}$, where *k* is the first-order rate constant determined from the slope of the least-squares fit of the log of the ratio of the α-hydrogen signal at 2.8 ppm to the pantetheine methyl signal at 0.75 ppm versus time. The control sample was identical to enzyme samples, except enzyme was excluded from the reaction mix; value of $k_{control} = 2.0 \times 10^{-3}$ min^{-1}. [d] Not detectable.

no substantial enzyme concentration dependent exchange could be detected for the C378G mutant (Table 3). This observation supports the assigned role (*37*) of cysteine-378 in acetyl-CoA deprotonation.

The thiolase results suggested that extension of these measurements to mutant HMG-CoA lyase proteins could be useful in detecting active site residues that are involved in product formation either by stabilizing an enolized form of acetyl-CoA or by facilitating protonation of C2 of the carbanionic species. R41Q, R41M, D42A, and H233A mutant enzymes are characterized by large (7000 to >270 000-fold) decreases in catalytic rate in the overall HMG-CoA cleavage reaction. For each mutant, the efficiency in catalyzing proton exchange from acetyldithio-CoA was determined and can be compared with wild-type enzyme (Table 3). D42A and H233A mutants exhibited measurable exchange rates that were lower than the value for wild-type enzyme by approximately 3-fold and 10-fold, respectively. For D42A, the exchange rate is only 1.5-fold lower than the rate for wild-type enzyme when elevated concentrations of acetyldithio-CoA (10 mM) or acetoacetate (250 mM) are employed. For H233A, an increase in acetyldithio-CoA concentration (from 7 to 10 mM) does not affect exchange rate. An increase in acetoacetate (from 100 to 250 mM) increases the exchange rate to a level which is <4-fold lower than the rate for wild-type enzyme. This observation is compatible with an increase in the K_i for acetoacetate (measured for the HMG-CoA cleavage reaction) from 21 mM for wild-type enzyme to 168 mM for H233A protein. For R41Q and R41M, no substantial enzyme concentration dependent proton exchange was detectable. Increases in the concentrations of acetyldithio-CoA (from 7 to 10 mM) or acetoacetate (from 100 to 250 mM) did not stimulate R41Q-dependent exchange above background levels. Thus, while aspartate-42, histidine-233, and arginine-41 all influence HMG-CoA cleavage efficiency, involvement in the specific steps that result in product acetyl-CoA formation and stabilization is most clearly attributable to arginine-41.

DISCUSSION

The high frequency of the human R41Q mutation in HMG-CoA lyase deficiency has been documented, and preliminary experiments with bacterial expression of this mutant enzyme suggested that the protein is stable but exhibits low activity. It was unclear at this level of characterization whether this mutant exhibits structural changes that do not affect stability but change tertiary or active site structure. The results of more extensive characterization are now reported, and they indicate that the active site is reasonably intact. For R41Q, K_m values for HMG-CoA or Mg^{2+} do not exhibit substantial changes in comparison with wild-type enzyme. Stoichiometry of affinity labeling and of cation binding in the presence of substrate analogue also suggest that major changes in active site structure do not account for low catalytic efficiency. These observations suggest that arginine-41 is an active site residue that influences reaction chemistry.

Any assignment of potential functions to arginine-41 is influenced by the positively charged side chain guanidinium group. In principle, based on an expected high pK_a value, arginine might function as a general acid to quench a C2 carbanion prior to release of product acetyl-CoA. There is some precedent with fumarate reductase (*38*, *39*) to support such a role for arginine. Perhaps a stronger possibility involves interaction of the positively charged guanidinium group with developing negative charge on the C1 oxygen of the enolate form of acetyl-CoA. Such a function is supported by substantial precedent for homologous Claisen and aldol condensation/cleavage reactions. In the homologous malate synthase reaction, recent structural information (*34*) indicates the importance of hydrogen bonding of an active site arginine to the thioester carbonyl oxygen of bound acetyl-CoA. Structural information on a complex of malate synthase with malic acid and coenzyme A (*40*) has also prompted speculation that the active site arginine may support the condensation reaction between glyoxylate and acetyl-CoA by interacting with the thioester carbonyl oxygen to stabilize the enolate form of acetyl-CoA. Related cation-dependent

aldol cleavages include reactions catalyzed by 2-dehydro-3-deoxygalactarate aldolase and 4-hydroxy-2-ketovalerate aldolase. The structure of enzymes that catalyze each of these reactions has recently been reported, and the data reveal active site arginines. In both cases, interaction of an active site arginine with oxygen of a bound ligand has been proposed. In the case of 2-dehydro-3-deoxygalactarate aldolase (*41*), modeling of dehydrodeoxygalactarate into the active site leads to the prediction that an arginine hydrogen bonds to oxygen on C4 of the substrate. For 4-hydroxy-2-ketovalerate aldolase (*31*), modeling of substrate suggests hydrogen bonding of active site arginine-17 to the oxygen of the 4-hydroxyl group of the substrate. Interestingly, alignment of 4-hydroxy-2-ketovalerate aldolase with HMG-CoA lyase indicates that arginine-17 corresponds to arginine-41 of HMG-CoA lyase (Figure 1). Thus, while precedent does not prove function, the proposition that arginine-41 of HMG-CoA lyase is an active site residue that stabilizes an enolized form of product acetyl-CoA is supported by the acetyldithio-CoA exchange experiments as well as by homologous functions for active site arginines in these related enzyme reactions.

Recently, there has been a report of a homology model for HMG-CoA lyase (*42*) that relies on a template corresponding to the β/α barrel fold exhibited by phosphoribosylformamino-5-aminoimidazole carboxamide ribonucleotide (PFAR) isomerase. While this model is useful in providing a rationale for human mutations that correlate with HMG-CoA lyase deficiency, the unrelated reaction catalyzed by the enzyme used as a modeling template limits the ability of this model to provide insight into active site function. Additionally, to explain how several human mutations depress enzyme activity, the model requires the assumption that the acyl-pantetheine portion of the substrate passes through the barrel cavity.

In contrast, not only do malate synthase, 4-hydroxy-2-ketovalerate aldolase, and 2-dehydro-3-deoxygalactarate aldolase exhibit the $(\beta/\alpha)_8$ barrel fold but each enzyme also catalyzes a reaction similar to the HMG-CoA lyase catalyzed reaction. Additionally, like HMG-CoA lyase, each requires a divalent cation to support catalysis of the corresponding reaction. In the case of 2-dehydro-3-deoxygalactarate aldolase, Mg^{2+} is the preferred cation and two acidic residues contribute ligands to cation. For 4-hydroxy-2-ketovalerate aldolase, Mn^{2+} activates the enzyme and ligands are provided by aspartate-18, histidine-200, and histidine-202. Alignment of HMG-CoA lyase family proteins indicates that these residues correspond to aspartate-42, histidine-233, and histidine-235 of HMG-CoA lyase.

In a homology model of HMG-CoA lyase (Figure 3A,B) that is based on a 4-hydroxy-2-ketovalerate aldolase template, an active site assignment to arginine-41 is well supported by its proximity to aspartate-42, glutamate-72, histidine-233, and histidine-235, which have large demonstrated effects on either catalytic efficiency (aspartate-42, histidine-233) or cation binding (aspartate-42, glutamate-72, histidine-235). Active site residues may be expected to deprotonate the substrate C3 hydroxyl group and to protonate the carbanion form of acetyl-CoA prior to product release (Scheme 1). Histidine-233 and aspartate-42 remain good candidates for such functions, but precise assignments must await more direct tests of the type reported here for arginine-41. Functional assignment of arginine-41 as a interacting partner with a substrate oxygen atom is reasonable on the basis of not only proximity to other residues that could contribute to an "anion hole" either by contributing positively charged side chains or by creating a binding site for divalent cation but also independent proton exchange evidence for arginine-41 that supports such a functional hypothesis.

It may be possible to extrapolate from the proposed function of arginine-41 in HMG-CoA lyase to roles for corresponding arginines (Figure 1) in the HMG-CoA lyase family of enzymes. Both isopropylmalate synthase and homocitrate synthase catalyze acetyl-CoA-dependent Claisen condensations. It certainly seems plausible that the homologous arginines interact with substrate acetyl-CoA to form an enolized species. Rearrangement of the enolate to form a C2 carbanion that subsequently reacts with the ketone carbon of the respective cosubstrates (2-ketoisobutyrate or 2-ketoglutarate, respectively) would account for the C−C bond formation that results in reaction products (isopropylmalate or homocitrate).

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