Pseudomonas mevalonii 3-Hydroxy-3-methylglutaryl-CoA Lyase: Testing the Function of the Active Site Cysteine by Site-Directed Mutagenesis†

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ABSTRACT: *Pseudomonas mevalonii* 3-hydroxy-3-methylglutaryl-CoA lyase is affinity labeled by 2-butynoyl-CoA; peptide sequence analysis demonstrates C237 to be the site of modification [Hruz *et al.* (1992) *Biochemistry 31*, 6842−6847]. In order to evaluate whether C237 functions in the chemistry of hydroxymethylglutaryl-CoA cleavage, cassette mutagenesis has been employed to alter wild-type DNA to encode serine or alanine at residue 237. ESR measurements indicate that the purified mutant enzymes bind stoichiometric amounts of the spin-labeled substrate analog, R•CoA, which has been established as a competitive inhibitor. Binding affinities measured with C237S (K_d = 92 μM) and C237A (K_d = 97 μM) lyases are comparable to that observed with wild-type lyase. The rotational dynamics of R•CoA bound to mutant enzymes are also very similar to those for R•CoA bound to wild-type lyase. These observations suggest that the mutant enzymes are structurally intact. In view of this demonstrated structural integrity, it is significant that the V_{max}s of C237A and C237S are ≈4 × 10^4- and ≈725-fold lower, respectively, than the value measured for wild-type hydroxymethylglutaryl-CoA lyase. The C237S enzyme exhibits a K_m = 53 μM for substrate; this value is only 2-fold higher than theK_m of the wild-type enzyme. Additionally, we report that the residual activity in C237S hydroxymethylglutaryl-CoA lyase is unaffected by 2-butynoyl-CoA under conditions which support inactivation of wild-type enzyme. These results are consistent with an active site assignment to C237, confirming the prediction based on the affinity labeling/peptide mapping data. Moreover, they suggest that C237 is a crucial residue in the catalytic apparatus and qualify it for consideration as the active site residue that deprotonates the 3-hydroxyl group of hydroxymethylglutaryl-CoA.

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA)[1] lyase (EC 4.1.3.4) plays a vital role in hepatic ketogenesis by catalyzing the cleavage of HMG-CoA into acetoacetate and acetyl-CoA in the final step of the HMG-CoA cycle (Lynen et al., 1958). In addition to its role in the formation of ketone bodies (Robinson & Williamson, 1980), the enzyme also is crucial to the catabolism of leucine (Coon et al., 1955). The enzyme has been partially purified from various sources such as pig heart (Bachhawat et al., 1955) and beef liver (Stegink & Coon, 1968) and has been isolated in homogenous form from avian liver (Kramer & Miziorko, 1980). After partial purification (Scher & Rodwell, 1989) of the enzyme from *Pseudomonas mevalonii*, Anderson and Rodwell (1989) cloned and sequenced DNA encoding this protein. Subsequently, the recombinant form of this enzyme was purified to homogeneity and partially characterized (Narasimhan & Miziorko, 1992; Narasimhan et al., 1994).

The overall reaction catalyzed by HMG-CoA lyase,

$$\text{HMG-CoA} \xrightarrow{Mn^{2+}} \text{acetoacetate} + \text{acetyl-CoA}$$

has been well established, and the mechanism is usually postulated to involve a retro-Claisen condensation. Abstraction of a proton from the 3-hydroxyl group of HMG-CoA by an amino acid residue acting as a general base triggers formation of a ketone at C3 concomitant with cleavage of the C2−C3 bond. A carbanionic form of acetyl-CoA is transiently formed and quenched upon protonation of C2 of this product by a general acid. Neither the base nor the acid involved in the HMG-CoA lyase reaction mechanism has been identified.

On the basis of the observation that lyase activity in beef liver preparations was influenced by the presence of mercaptans, Stegink and Coon (1968) postulated that an active site sulfhydryl was important in HMG-CoA cleavage. By affinity labeling of *P. mevalonii* HMG-CoA lyase, Hruz et al. (1992) demonstrated the presence of a reactive C237 residue within the active site. While overall identity between the amino acid sequences of *P. mevalonii* and eukaryotic HMG-CoA lyases is only approximately 50%, C237 is located within a 10 amino acid section that is invariant. Such an observation supports an active site assignment to this residue. In order to more directly evaluate the significance of C237 in the catalytic mechanism of HMG-CoA lyase,

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[1] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; R•, 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl; EPR, electron paramagnetic resonance; IPTG, isopropyl thiogalactoside; PMSF, phenylmethanesulfonyl fluoride.

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cassette mutagenesis has been used to replace this residue in the *P. mevalonii* lyase with serine or alanine. In this report, we demonstrate that stable and structurally intact mutants are generated. Characterization of the isolated enzyme variants provides considerable insight into the involvement of C237 in catalysis.

EXPERIMENTAL PROCEDURES

Materials. Sephadex G-50, NAD^+, NADH, Q-Sepharose, and ampicillin were purchased from Sigma (St. Louis, MO). Isopropyl thiogalactoside (IPTG) was bought from U.S. Biochemicals (Cleveland, OH). Bactotryptone and yeast extract were purchased from Difco Labs (Detroit, MI). *Escherichia coli* BL21(DE3) was supplied by Novagen (Madison, WI). R• (3-carboxy-2,2,5,5-tetramethyl-1-pyrrolydinyloxyl; 3-carboxy-PROXYL) was obtained from Aldrich (Milwaukee, WI). R•CoA thioester (Weidman et al., 1973) was synthesized using the mixed anhydride (Misra et al., 1993) prepared by activation of the free acid using the method of Bernert and Sprecher (1977). HMG-CoA was synthesized from the anhydride prepared from the free acid (Fluka, Switzerland) according to the procedure of Goldfarb and Pitot (1971). 2-Butynoyl-CoA was synthesized as described by a modification (Hruz & Miziorko, 1992) of the general method of Freund et al. (1985). Deoxyoligonucleotides were synthesized by the Protein/Nucleic Acid Facility at the Medical College of Wisconsin. All other chemicals were reagent grade. Determination of protein concentration was done following the method of Bradford (1976) using bovine serum albumin as the standard.

Construction of the Mutant HMG-CoA Lyases (C237S & C237A). The strategy for the construction of the lyase mutations C237A and C237S is presented in Figure 1, and the sequences of the deoxyoligonucleotide duplex cassettes used for constructing these lyase mutants are given in Table 1. The pT7-2600 plasmid (a generous gift of Dr. Victor Rodwell, Purdue University) that encodes bacterial HMG-CoA lyase was digested with *Xba*I and *Bst*EII. The two corresponding fragments (1400 and 3700 bp) were isolated and purified using a Qiaex kit (Qiagen, Chatsworth, CA). The bases encoding C237 (TGC) lie in a region between unique *Sph*I and *Bgl*II restriction sites within the 1400-bp coding fragment. The isolated *Xba*I/*Bst*EII piece containing the 1400-bp coding fragment was, therefore, further digested with *Sph*I/*Bgl*II. Two of the three fragments (*Xba*I−*Sph*I, 700 bp; *Bgl*II−*Bst*EII, 600 bp) generated from this digest were isolated and purified as described above. The synthetic cassettes that encode the appropriate mutations (GCC for C237A and TCC for C237S) span the 100-bp region between *Sph*I and *Bgl*II. Each cassette was used in a four-way ligation with the 3700-bp *Xba*I−*Bst*EII, 700-bp *Xba*I−*Sph*I, and 600-bp *Bgl*II−*Bst*EII fragments to generate the plasmids required for production of the HMG-CoA lyase variants. Both strands of the resulting expression vectors, pT7-C237A and pT7-C237S, were sequenced (Sanger et al., 1977) to confirm that the Cys to Ala or Cys to Ser mutations were encoded. These plasmids were subsequently used to transform *E. coli* BL21 (DE3).

Bacterial Growth and Purification of the Wild-Type and Mutant HMG-CoA Lyases. Recombinant bacterial HMG-CoA lyase was expressed in *E. coli* BL21 (DE3) transformed with either a wild-type (pT7-2600) or a mutant (pT7-C237A;

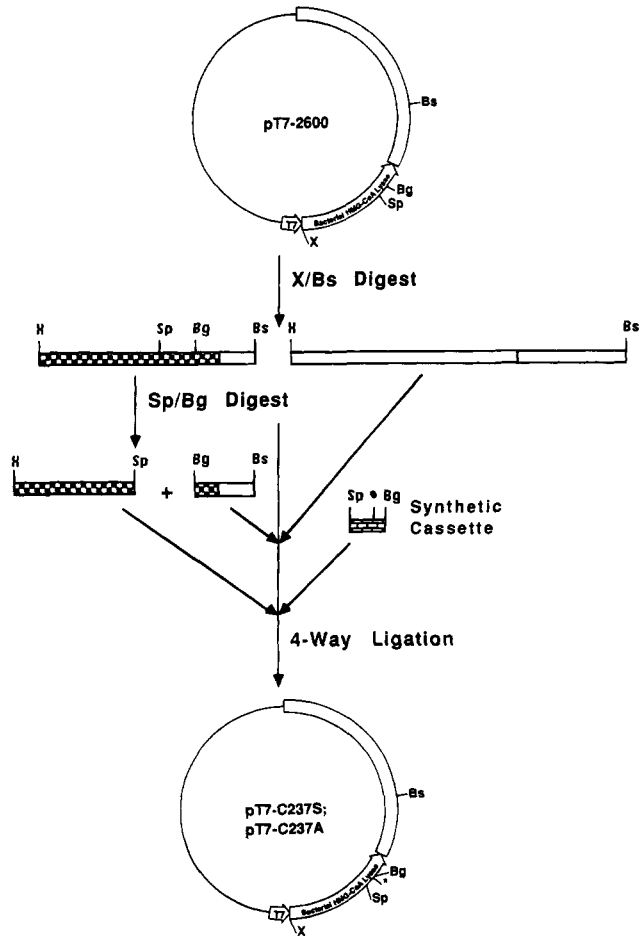

FIGURE 1: Construction of plasmids encoding mutant *P. mevalonii* HMG-CoA lyase. Restriction enzymes utilized: *Xba*I, X; *Bst*EII, Bs; *Sph*I, Sp; *Bgl*II, Bg.

pT7-C237S) expression plasmid. The procedure for expression and purification was essentially the same as described by Narasimhan and Miziorko (1992). In brief, IPTG-induced bacteria harboring an expression plasmid were harvested at late log phase by centrifugation at 3000*g* (4 °C), and the pellets were stored at −20 °C prior to cell lysis. The bacterial pellet was resuspended in the lysis buffer (10 mM phosphate, pH 7.2, 1 mM EDTA, 100 μM PMSF, 10 μg/mL DNAse, and 10 μg/mL RNAse) and lysed using a French pressure cell operating at 16 000 psi. The lysed cells were centrifuged at 100 000*g* for 1 h at 4 °C. The high-speed supernatant was then brought to 40% saturation with ammonium sulfate and stirred slowly for 2 h. The precipitated protein was separated by centrifugation, and the resulting pellet was resuspended in 10 mM phosphate buffer, pH 7.2, containing 20% glycerol; ammonium sulfate was removed using a centrifugal desalting column (Penefsky, 1977). This sample was then chromatographed on a Q-Sepharose anion-exchange column with a phosphate gradient of 10−100 mM. The fractions containing lyase activity were pooled and concentrated with an Amicon stirred cell and stored at −80 °C. The purity of the enzyme preparations was evaluated by SDS−PAGE (Laemmli, 1970) using an 11% acrylamide running gel and a 4.5% acrylamide stacking gel.

Characterization of HMG-CoA Lyase. Kinetic characterization of the purified wild-type as well as the mutant HMG-CoA lyases was performed using either the spectrophotometric assay (Stegink & Coon, 1968; Kramer & Miziorko,

Table 1: Deoxyoligonucleotide Duplex Sequences Used for Cassette Mutagenesis[a]

Wild-Type

```
SphI                                                                    BglII
    CCGCACTCGCGCAGGGTGTACGCACCTTCGACAGCTCGGTCGCGGGCCTCGGCGGCTGCCCCTACTCGCCGGGTGCCAGCGGTAACGTGGCCACGGAA
GTACGGCGTGAGCGCGTCCCACATGCGTGGAAGCTGTCGAGCCAGCGCCCGGAGCCGCCGACGGGGATGAGCGGCCCACGGTCGCCATTGCACCGGTGCCTTCTAG
H  A  A  L  A  Q  G  V  R  T  F  D  S  S  V  A  G  L  G  G  C  P  Y  S  P  G  A  S  G  N  V  A  T  E  D
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C237A

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SphI                                                                    BglII
    CCGCACTCGCGCAGGGTGTACGCACCTTCGACAGCTCGGTCGCGGGCCTCGGCGGCGCCCCCTACTCGCCGGGTGCCAGCGGTAACGTGGCCACGGAA
GTACGGCGTGAGCGCGTCCCACATGCGTGGAAGCTGTCGAGCCAGCGCCCGGAGCCGCCGCGGGGGATGAGCGGCCCACGGTCGCCATTGCACCGGTGCCTTCTAG
H  A  A  L  A  Q  G  V  R  T  F  D  S  S  V  A  G  L  G  G  A  P  Y  S  P  G  A  S  G  N  V  A  T  E  D
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C237S

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SphI                                                                    BglII
    CCGCACTCGCGCAGGGTGTACGCACCTTCGACAGCTCGGTCGCGGGCCTCGGCGGCTCCCCCTACTCGCCGGGTGCCAGCGGTAACGTGGCCACGGAA
GTACGGCGTGAGCGCGTCCCACATGCGTGGAAGCTGTCGAGCCAGCGCCCGGAGCCGCCGAGGGGGATGAGCGGCCCACGGTCGCCATTGCACCGGTGCCTTCTAG
H  A  A  L  A  Q  G  V  R  T  F  D  S  S  V  A  G  L  G  G  S  P  Y  S  P  G  A  S  G  N  V  A  T  E  D
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[a] The synthetic cassette was generated by annealing the six oligonucleotides (differentiated by underlining) listed above. The shaded areas represent the codons altered for mutagenesis.

1980) or the radioactive assay (Clinkenbeard et al., 1975). Data were analyzed by nonlinear regression analysis (Marquardt, 1963).

EPR Experiments. Conventional X-band EPR spectra were recorded using a Varian Century-Line 9-GHz spectrometer with a TE_{102} cavity. The spectra were recorded at 6 °C using a modulation amplitude of 1 G, a modulation frequency of 100 KHz, a time constant of 0.5 s, and a microwave power of 5 mW. The samples contained variable concentrations of R•CoA (20−120 μM) and either 100 μM (C237A) or 70 μM (C237S) HMG-CoA lyase sites in 10 mM phosphate buffer, pH 7.2, containing 20% glycerol. R•CoA bound to HMG-CoA lyase mutants was calculated from the fractional differences between the amplitudes of the high-, center-, and low-field lines for the unbound R•CoA in the enzyme-containing sample spectra and the amplitudes of the corresponding lines observed using solutions containing the same concentration of R•CoA in buffer. Scatchard plots of these data were fit using linear regression analysis to obtain K_d and binding stoichiometry. Direct observation of bound R•CoA was possible under conditions of elevated gain and increased modulation amplitude (5 G). Rotational correlation time (τ_c) for the enzyme-bound spin label (at 6 °C) was estimated by modeling the signal attributable to bound species using the spectral simulation program of Freed and co-workers (Freed, 1976; Schneider & Freed 1989).

RESULTS

Mutagenesis Strategy and Production of C237A and C237S HMG-CoA Lyases. On the basis of the affinity labeling studies of bacterial HMG-CoA lyase (Hruz et al., 1992), which mapped cysteine 237 at the substrate binding site, this residue was targeted for site-directed mutagenesis. The presence of unique *Xba*I and *Bst*EII sites in the expression vector pT7-2600, as well as *Sph*I and *Bgl*II sites that were unique within the coding region of the *Xba*I/*Bst*EII fragment, allowed the efficient design of a mutagenesis experiment directed at C237. Using a synthetic cassette to replace the wild-type *Sph*I/*Bgl*II fragment, plasmids were reconstructed (Figure 1) to encode the proteins in which C237 was replaced by either an alanine or a serine. Competent *E. coli* BL21 (DE3) cells were then transformed for subsequent expression of these vectors. After growth in LB broth at 22 °C to $A_{600\ nm}$ = 0.6, T7 RNA polymerase-dependent protein synthesis was induced by addition of IPTG (1 mM). Cells were harvested at late log phase. *E. coli* harboring the mutant lyase expression plasmids produced the protein variants at about the same levels as previously reported for the wild-type enzyme (Narasimhan & Miziorko, 1992). The mutant enzymes purified similarly to the wild-type lyase. Figure 2 shows the SDS−PAGE analysis of the purified HMG-CoA lyase mutants.

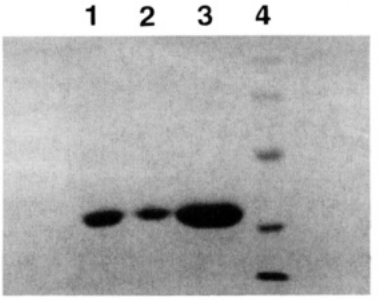

FIGURE 2: SDS−PAGE of purified *P. mevalonii* HMG-CoA lyase mutants. The samples were run under reducing conditions: lane 1, C237S lyase; lane 2, C237A lyase; lane 3, wild-type *P. mevalonii* lyase; lane 4, molecular mass standards [phosphorylase b (top band), 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21 kDa].

Evaluation of the Structural Integrity of Lyase Mutants. It has been shown previously that the spin-labeled substrate analog R•CoA is a competitive inhibitor of *P. mevalonii* HMG-CoA lyase (Narasimhan et al., 1994) as well as HMG-CoA synthase (Miziorko et al., 1979; Misra et al., 1993). R•CoA binds *P. mevalonii* HMG-CoA lyase with a K_d of 103 μM. In order to quantitatively evaluate the substrate binding properties of the mutant lyases C237A and C237S, binding of R•CoA to these mutants was studied using EPR spectroscopy. Figure 3 shows the spectrum of a sample of R•CoA (50 μM) in the absence or presence of the mutant C237A (100 μM sites). The diminution in the amplitude of the signal attributable to the free R•CoA is easily seen (Figure 3B), and the concentration of unbound R•CoA is accurately and straightforwardly determined from the amplitude of the residual signal. A series of similar binding measurements was performed for both C237A and C237S lyases. Samples contained different amounts of R•CoA in the presence of a fixed concentration of mutant enzyme. Binding data calculated from the amplitude of the residual free R•CoA signal

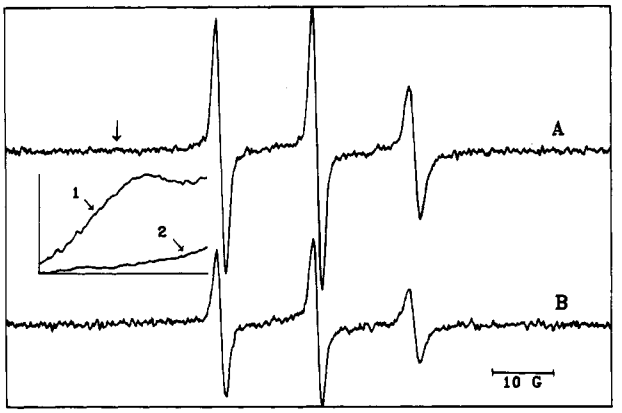

FIGURE 3: Binding of R•CoA to bacterial HMG-CoA lyase mutant C237A. Conventional X-band EPR spectra are shown for R•CoA (50 μM) in buffer (A) or R•CoA (50 μM) in the presence of 100 μM C237A HMG-CoA lyase (B). The inset shows 20-G regions centered at the marker arrow shown in the low-field side of the 100 G spectrum (A). Inset trace 1 shows the spectrum of R•CoA (50 μM) bound to C237A lyase (100 μM), and inset trace 2 shows the identical concentration of R•CoA (50 μM) in buffer. Spectral conditions: scan range, 100 G for the full spectra (center field, 3250 G) and 20 G for the spectra shown in the inset (center field, 3218 G); modulation amplitude, 1 G for the full spectra and 5 G for the spectra in the inset. Inset spectra represent the average of 25 scans, recorded at 4-fold higher gain than used for the full spectra. Measurements were performed at 6 °C.

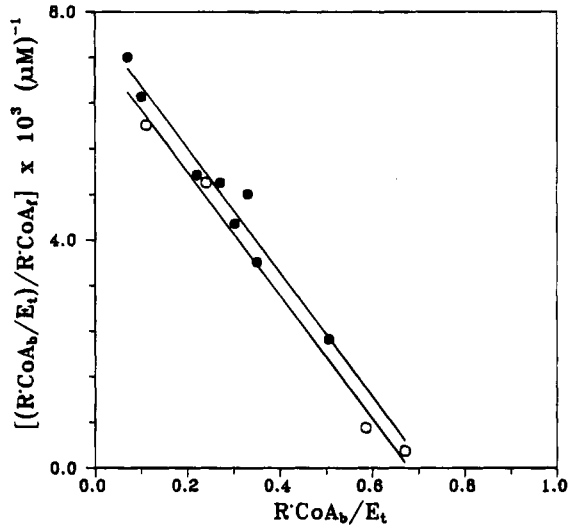

FIGURE 4: Scatchard plot of the paramagnetic resonance data for R•CoA binding to HMG-CoA lyase mutants. Closed circles (●) represent C237S, and open circles (○) represent C237A lyase. Binding stoichiometry and K_d were determined by linear regression analysis of the data points. The binding parameters are summarized in Table 2.

are displayed as a Scatchard plot (Figure 4). Analysis of the data indicates (Table 2) that, under equilibrium conditions, not only C237A ($n = 0.7$, $K_d = 97$ μM) but also C237S ($n = 0.7$; $K_d = 92$ μM) exhibits binding properties very similar to that reported for wild-type enzyme ($n = 0.65$; $K_d = 103$ μM; Narasimhan et al., 1994). These observations indicate that conservative mutations at residue 237 do not significantly affect the structural integrity of the acyl-CoA binding site and imply that the enzyme's tertiary structure is intact. Under appropriate gain and modulation conditions, features of the EPR spectrum of R•CoA bound to each mutant enzyme could be detected. The inset in Figure 3 shows the low-field signal attributable to R•CoA bound to the C237A mutant (trace 1) and R•CoA in buffer (trace 2). The rotational dynamics of this immobilized species were simulated using an algorithm developed by Freed and co-workers (Freed, 1976; Schneider & Freed, 1989). Spin probe bound to C237A and C237S mutants tumbles with rotational correlation times (τ_c) of 22 and 20 ns, respectively (Table 2). These values are in very good agreement with the rotational correlation time observed for R•CoA bound to the wild-type enzyme (20 ns; Narasimhan et al., 1994). Thus, not only are binding affinities and site occupancies for wild-type and mutant lyases comparable, but so is the degree to which the acyl group is immobilized. This observation represents a stringent validation of our earlier assertion concerning the integrity of the acyl-CoA binding site in the C237 mutants.

Table 2: R•CoA Binding to HMG-CoA Lyase Mutants

	enzyme sample		
parameter	wild type	C237A	C237S
K_d (μM)[a]	103	97	92
n[a]	0.65	0.7	0.7
τ_c (ns)[b]	20	22	20

[a] Value obtained by linear regression analysis of the Scatchard data (error: ±8%). [b] The rotational correlation time of bound R•CoA was estimated using the spectral simulation (error, ±15%) program of Freed and co-workers (Freed, 1976; Schneider & Freed, 1989).

Table 3: Kinetic Characterization of *P. mevalonii* HMG-CoA Lyase Mutants

	enzyme sample		
parameter	wild-type	C237A	C237S
V_{max} (units/mg)[a]	80	0.0018[b]	0.11[b]
K_m (μM)[a]	20	N/D[c]	53
k_{cat}/K_m (M^{-1} s^{-1})	2.1×10^6		1.1×10^3

[a] The values for V_{max} and K_m were obtained by nonlinear regression analyses of the experimentally measured kinetic data (Marquardt, 1963). [b] Value obtained from radioactive assay. [c] Not determined.

Kinetic Characterization of P. mevalonii HMG-CoA Lyase Mutants. The kinetic parameters of the mutant lyases were measured using either the spectrophotometric assay or the equivalent radioactive assay, which affords improved sensitivity; the results are summarized in Table 3. The V_{max} for the C237S mutant is about 725-fold less than that measured for wild-type enzyme. C237A lyase exhibits $\approx 4 \times 10^4$-fold diminution in V_{max}. Due to the magnitude of these effects and to the signal to noise limitations inherent in the radioactive assay when levels of [^{14}C]HMG-CoA are reduced below those adequate for rate saturation, an accurate estimate of the K_m value for C237A lyase could not be obtained. Thus, in the case of this mutant, an independent demonstration (cf. above) that the enzyme has a full complement of binding sites with affinity comparable to that measured with wild-type protein is crucial to interpretation of the kinetic data. For C237S, residual activity is somewhat higher, allowing an estimate of $K_m = 53$ μM (Table 3), a value which only exceeds that estimated for wild-type enzyme by about 2-fold. This observation, together with the agreement between binding affinities measured in the Scatchard analyses, argues that the observed diminution in catalytic rate is significant and that the magnitude of the effect is interpretable in the context of a catalytic role for the C237 sulfhydryl.

Sensitivity of Wild-Type and C237S HMG-CoA Lyases to Affinity Labeling. Upon incubation of wild-type HMG-CoA

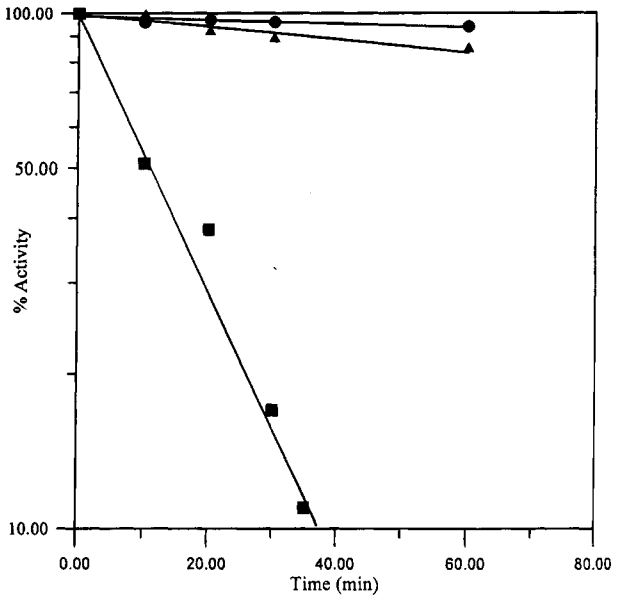

FIGURE 5: Sensitivity of *P. mevalonii* HMG-CoA lyases to 2-butynoyl-CoA. Wild-type (■) or C237S (▲) enzymes were incubated at 23 °C in 10 mM potassium phosphate buffer, pH 7.2, containing 20% glycerol. After addition of 500 μM 2-butynoyl-CoA, enzyme activities were measured at the times indicated. In a control experiment (●), C237S was incubated in buffer without 2-butynoyl-CoA.

lyase with 2-butynoyl-CoA at concentrations established as adequate to inactivate the enzyme, loss of activity was indeed observed (Figure 5) with kinetics ($t_{1/2}$ = 10 min) matching those reported earlier (Hruz et al., 1992). In contrast, when C237S lyase was subjected to identical treatment, activity was retained (extrapolated $t_{1/2}$ > 180 min) at levels comparable to those measured in a control sample of C237S enzyme that was incubated in buffer only (Figure 5). These observations indicate the absence in C237S lyase of a highly reactive nucleophile within the active site. Failure of the serine hydroxyl group to react with the affinity label (as suggested by the retention of activity) is not unexpected given the ~5 unit elevation in pK_a for the amino acid side chain. As discussed below, the data impact on any speculation concerning the function of the C237 sulfhydryl. Moreover, they indicate that the residual activity measured in purified C237S HMG-CoA lyase is *not* attributable to a low level of wild-type revertant.

DISCUSSION

The development of methodology for overexpression of an active form of HMG-CoA lyase (Narasimhan & Miziorko, 1992) has accounted for the rapid improvement in our understanding of this enzymic reaction. Availability of the homogeneous *P. mevalonii* enzyme at milligram levels made possible the identification of cysteine-237 as the target of the affinity label, 2-butynoyl-CoA (Hruz et al., 1992). In tests of the function of cysteine-237, preliminary screening indicated that reasonably large amounts (milligram levels) of recombinant HMG-CoA lyase would be required for any accurate estimate of the low residual activity. The level of overexpression achieved for the *P. mevalonii* lyase made such analyses practical. Demonstration of the significance of the diminution in catalytic activity observed for C237 mutants has relied on a physical approach (i.e., EPR spectroscopy). A different strategy would have been required if limitations on the available levels of homogeneous enzyme had been more stringent.

In the context of documenting the structural integrity of engineered enzymes, the utility of spin-labeled probes warrants wider consideration. Two different categories of information become potentially available through use of a dissociable spin-labeled metabolite analog. In all cases, it should be possible to extract both K_d and binding stoichiometry information by use of Scatchard analysis or similar approaches. Since such information may also be generated by other methodology, the advantage of using a spin-labeled analog becomes more apparent when rotational dynamics information can be generated. In some cases, the analog binds tightly, facilitating straightforward measurement of the EPR spectrum of bound species and estimation of a rotational correlation time for the probe. When analog binding is weaker, rotational dynamics of the bound species may still be estimated. Depending on the subunit size and oligomeric nature of the protein, the low- or high-field signals attributable to the bound probe may be detectable due to their appearance outside the spectral region dominated by unbound analog. The rotational correlation time can be estimated using a simulation approach (Schneider & Freed, 1989) to model those spectral features. Extraction of rotational dynamics information requires a high degree of immobilization of the nitroxyl portion of the bound analog. Our ability to detect the bound component of R•CoA on dimeric (64 kDa) HMG-CoA lyase represents an example of the situation outlined above. Comparison of binding stoichiometry, binding affinity, and rotational correlation time measured for wild-type protein with corresponding values for engineered mutants represents a stringent test not only of the integrity of the tertiary and quaternary structure but of the local active site structure as well.

Since, in the case of *P. mevalonii* HMG-CoA lyase, the active sites of the C237A and C237S mutants appear to be unperturbed, the task of interpreting the diminution in catalytic activity upon elimination of the thiol remains. In many cases where cysteine has been shown to be crucial to catalysis, its involvement has been limited to supporting formation of covalent EnzS−substrate intermediates (Thompson et al., 1989; Davis et al., 1994). There is no evidence to suggest that HMG-CoA cleavage involves covalent catalysis, and thus, the question of whether cysteine functions in general acid/base catalysis arises. HMG-CoA cleavage has been demonstrated (Messner et al., 1975) to proceed with inversion of stereochemistry at C2. Such a consequence is the general observation for enzymes catalyzing Claisen-type reactions, prompting the proposal that they utilize as general acid and general base catalysts two amino acids situated on opposite sides of the bound substrate (Hanson and Rose, 1975). In principle, an active site cysteine could function in either capacity. However, the pH/rate profiles for both prokaryotic (Scher & Rodwell, 1989) and eukaryotic (Kramer & Miziorko, 1980) HMG-CoA lyases exhibit optima at pH > 9, conditions under which a thiolate anion would exist at C237. While precise pK_a values have not been reported, those data support an estimate of 8.0−8.5. Thus, on chemical grounds, it does not seem unreasonable to propose a general base function for a cysteinyl thiolate. Specific precedent for participation of a thiolate in deprotonation of an alcohol is not abundant, but considerable precedent for thiolate as a general base has been

documented. Such a role for cysteine is clear in both glutamate racemase (Tanner et al., 1993) and proline racemase (Belasco et al., 1986). Additionally, a role for cysteine as an active site base has been proposed for β-keto thiolase (Palmer et al., 1990) which, like HMG-CoA lyase, catalyzes a Claisen reaction. In contrast, HMG-CoA synthesis involves a cysteine in the formation of reaction intermediates (Misra et al., 1993) but not in acid/base chemistry (Misra et al., 1995). Moreover, the citrate synthase and chloramphenicol acetyltransferase reactions, which are catalyzed by enzymes for which high-resolution structures have been established (Remington, 1992; Leslie, 1990), do not offer any precedent for participation of a cysteine. Thus, returning to the issue of catalysis of HMG-CoA cleavage, an assignment of function to C237 cannot yet be made with certainty. However, the magnitude of the effect resulting from mutation of this cysteine is compatible with its function either as an active site base or in polarizing a neighboring group that is more directly involved in deprotonation of substrate. The insensitivity of C237S lyase to inactivation by 2-butynoyl-CoA (Figure 5) does not suggest the presence in the active site of any reactive base other than the sulfhydryl normally contributed by C237. On these grounds, the provisional assignment of C237 as the active site base becomes an attractive and unifying explanation for all currently available affinity labeling and mutagenesis data on HMG-CoA lyase.

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