Evidence Supporting a Role for Histidine-235 in Cation Binding to Human 3-Hydroxy-3-methylglutaryl-CoA Lyase†

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ABSTRACT: Histidine-235 of human 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase is the second basic residue in a conserved HXH motif. This residue is solvent accessible, readily reacting with the group specific reagent diethyl pyrocarbonate. Site-directed mutagenesis has been employed to substitute alanine or aspartate for H235. Characterization of the isolated H235A and H235D lyase mutants indicates that their tertiary structure is substantially intact. The mutant proteins, like the wild-type enzyme, are stoichiometrically modified by the affinity label, 2-butynoyl-CoA. Catalytic activity of the mutants is diminished by 15-fold and K_m for HMG-CoA elevated ≈4-fold in comparison with the values for wild-type enzyme. The function of H235 is suggested by investigation of the interaction of these enzymes with the dissociable divalent cation (e.g. Mg^{2+} or Mn^{2+}) that is required for activity. ESR experiments show that wild-type enzyme forms a stable binary E*M complex. In contrast, H235A and H235D proteins do not efficiently form a binary complex. Significant interaction with cation (Mn^{2+}) only occurs in the presence of the substrate analog, 3-hydroxyglutaryl-CoA. Similarly, when cation interaction is estimated in the presence of substrate using steady-state kinetic approaches, activator constants (K_a) and divalent cation K_m values are measurable but are elevated by 15−90-fold over comparable estimates for the wild-type enzyme. The data confirm our earlier suggestion that both protein and substrate contribute ligands to HMG-CoA lyase's divalent cation activator. More specifically, the current observations suggest that H235 has an important function in cation binding.

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA)[1] lyase catalyzes the Claisen cleavage of HMG-CoA which forms acetoacetate and acetyl-CoA, a reaction which represents one of the final steps in both ketone body formation (Robinson & Williamson, 1980) and leucine catabolism (Coon et al., 1955). Deficiency in human HMG-CoA lyase can lead to a buildup of branched chain organic acids, which has been the cause of death in a number of cases [as reviewed by Gibson et al. (1988)]. The cDNA sequence corresponding to human HMG-CoA lyase has been elucidated (Mitchell et al., 1993), facilitating the identification of point mutations in the coding region (Robert et al., 1994). In addition to the human sequence, three other HMG-CoA lyase sequences have been deduced to date; these encode the mouse (Wang et al., 1993), chicken (Mitchell et al., 1993), and bacterial [*Pseudomonas mevalonii* (Anderson & Rodwell, 1989)] enzymes.

Past studies of mammalian HMG-CoA lyase have been performed using partially purified enzyme from pig heart (Bachhawat et al., 1955) and cow liver (Stegink & Coon, 1968) or using homogeneous avian liver enzyme (Kramer & Miziorko, 1980). A prokaryotic HMG-CoA lyase has been expressed and purified from *P. mevalonii* (Scher & Rodwell, 1989) and as a recombinant protein from *Escherichia coli* (Narasimhan & Miziorko, 1992). Recently, recombinant human HMG-CoA lyase has been expressed in *E. coli* and purified to homogeneity (Roberts et al., 1994). Utilizing the expression system developed for human HMG-CoA lyase, two active site residues have been identified by site-directed mutagenesis (Roberts et al., 1995, 1996). One of these residues is H233 (Roberts et al., 1996), the first residue of a HXH motif that contains the only two histidine residues conserved among all HMG-CoA lyase sequences.

In a variety of studies with HMG-CoA lyases prepared from avian liver (Kramer & Miziorko, 1980), beef liver (Stegink & Coon, 1968), and with either recombinant *P. mevalonii* (Narasimhan & Miziorko, 1992) or recombinant human (Roberts et al., 1994) enzymes, a marked stimulation of activity was observed upon addition of a dissociable cation (Mg^{2+} or Mn^{2+}). In addition, Kramer and Miziorko (1983), utilizing the avian enzyme, found that Mg^{2+} stimulated enzyme-catalyzed tritium exchange from acetyl-CoA. Hruz et al. (1993) determined that a dithioester analog of HMG-CoA, 3-hydroxy-3-methyl-1-thionoglutaryl-CoA (HMG[=S]-CoA), acted as an alternative substrate. Divalent cations stimulated the reaction with HMG[=S]CoA, but a marked preference for Mn^{2+} was observed with the dithioester analog, in contrast to the observation that Mg^{2+} optimally supports cleavage of the physiological substrate, HMG-CoA. On the basis of the above observations, the suggestion that the metal ligates not only to the protein but also to the carbonyl of the substrate (Hruz et al., 1993) was made.

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[1] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HG-CoA, 3-hydroxyglutaryl-CoA; DEPC, diethyl pyrocarbonate; ESR, electron spin resonance; CD, circular dichroism; OD, optical density; IPTG, isopropyl thiogalactoside; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; oePCR, overlap extension PCR.

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Table 1: Primers Utilized for Overlap Extension PCR of the H235 Variants

Flanking Primers		
Upstream	5′ GCT ACG AGA TCT CCC T 3′	Primer A
Downstream	5′ TGC GTA GGG AGA GCC TCC 3′	Primer D
Mutagenic Primers		
H235A		
	5′ CTG GCT GTC CAC TGC GCT GAC ACC TAT G 3′	Primer C
3′ GAC CGA CGG GAC CGA CAG GTG ACG CGA CT 5′		Primer B
H235D		
	5′ CTG GCT GTC CAC TGC GAT GAC ACC TAT G 3′	Primer C
3′ GAC CGA CGG GAC CGA CAG GTG ACG CTA CT 5′		Primer B

To date, very little is known about which residues in HMG-CoA lyase are involved in binding the dissociable cation. On the basis of group specific modification of enzyme with diethyl pyrocarbonate (DEPC), Roberts et al. (1996) determined that ~2.4 histidine residues are modified in the human enzyme. H233 has been assigned as one of these reactive residues and has been implicated as a participant in catalysis. The role of the second histidine residue (H235) in the HXH motif remained unclear. We now report evidence supporting the assignment of H235 as a ligand to the dissociable cation needed for catalysis of HMG-CoA cleavage.

EXPERIMENTAL PROCEDURES

Materials

Phenylagarose was purchased from Sigma (St. Louis, MO). Q-Sepharose anion exchange resin and Superose-12 (prep grade) resin are products of Pharmacia Biotech, Inc. (Piscataway, NJ). DEPC was purchased from Aldrich Chemical Co. (Milwaukee, WI). Bactotryptone and yeast extract were obtained from Difco Laboratories (Detroit, MI). Isopropyl thiogalactoside (IPTG) was purchased from U.S. Biochemicals (Cleveland, OH). Qiaex and Qiagen were purchased from Qiagen (Chatsworth, CA). Restriction enzymes are products of New England Biolabs, with the exception of *Rca*I which was purchased from Boehringer Mannheim. DNA polymerase, *pfu*, was obtained from Stratagene. Oligonucleotides were synthesized at Operon Laboratories (Alameda, CA). HMG-CoA was synthesized from the anhydride, prepared from the free acid (Fluka) according to Goldfarb and Pitot (1971). 3-Hydroxyglutaryl-CoA (HG-CoA) was synthesized as described by Kramer and Miziorko (1983). All other chemicals were reagent grade.

Methods

Generation of Mutations at Histidine-235. Utilizing the primers in Table 1, mutants H235A and H235D were generated by overlap extension PCR (Ho et al., 1989) with *pfu* DNA polymerase and pTrcHL-1 (Roberts et al., 1994) as the template. The internal complementary oligonucleotides, primers B and C, were engineered to encode H235A and H235D (Table 1). Primers A and D, which anneal upstream and downstream, respectively, of the region for mutagenesis, were utilized in both rounds of PCR, to generate a 200 bp fragment. The restriction enzymes, *Pst*I and *Rca*I, which cut pTrcHL-1 at sites flanking the H235 codon, were used to produce a 100 bp *Pst*I−*Rca*I fragment that was gel purified utilizing QiaexII as described by the manufacturer (Qiagen).

The other fragments were obtained by digesting the original expression plasmid, pTrcHL-1 (Roberts et al., 1994), with *Ava*I (located 40 bp downstream of the start codon for HMG-CoA lyase) and *Bam*HI (located 20 bases downstream of the stop codon). The resulting two fragments, an 875 bp piece (95% of the HMG-CoA lyase gene) and a 4.1 kilobase fragment (the vector and the rest of the HMG-CoA lyase coding region), were gel purified as described above. Unique restriction cuts were made using the enzymes *Pst*I and *Rca*I to further digest the 875 bp piece. The resulting digest generated three fragments, of which two (630 bp *Ava*I−*Rca*I, 240 bp *Pst*I−*Bam*HI) were gel purified. A four-way ligation was performed with the 100 bp *Rca*I−*Pst*I mutagenic fragment generated from oePCR and the 4.1 kb *Bam*HI−*Ava*I, the 630 bp *Ava*I−*Rca*I, and the 240 bp *Pst*I−*Bam*HI fragments. The resulting expression plasmids (pTrcHL-H235D and pTrcHL-H235A) were transformed into competent *E. coli* (JM105) cells. To validate the mutations, the region obtained by oePCR was sequenced (Sanger et al., 1977) in both directions utilizing a Pharmacia ALF automated sequencer.

Purification of H235 Variants. The growth of the H235 variants was performed as described by Roberts et al. (1994). Each transformant was cultured at 37 °C in LB broth, supplemented with 50 μg/mL ampicillin until an $OD_{600\ nm}$ of ≈0.6 was reached. At this point, expression was induced by the addition of IPTG (1 mM) and the bacteria were grown overnight at 22 °C. The H235 HMG-CoA lyase variants were purified as described by Roberts et al. (1994). After each suspended cell pellet was lysed with a French press, the crude extract was subjected to high-speed centrifugation. The supernatant was passed through an anion exchange (Q-Sepharose) column, to which the lyase does not bind at a pH of 8.2. The eluted enzyme was fractionated by $(NH_4)_2SO_4$ precipitation (40−65% saturation). The ammonium sulfate pellet was redissolved and the protein loaded onto a hydrophobic (phenylagarose) column. After reverse gradient elution, the recovered enzyme was isolated in a highly homogeneous form using a gel filtration (Superose-12) column. Each H235 variant was purified in a fashion indistinguishable from that described for the wild-type enzyme (Roberts et al., 1994).

Analytical Procedures. Using bovine serum albumin as a standard, the method of Bradford (1976) was utilized to determine the concentration of both wild-type and the H235 variants of HMG-CoA lyase. All SDS−PAGE (Laemmli, 1970) was run under reducing conditions (5% β-mercaptoethanol) using a 11% acrylamide running gel and a 4.5% stacking gel. Modification of enzyme with [^{14}C]butynoyl-CoA (16 000 dpm/nmol) was performed under anaerobic conditions, as previously described by Hruz et al. (1992).

Enzymatic Assays for HMG-CoA Lyase. The activity of the engineered H235 variants of HMG-CoA lyase was determined utilizing the citrate synthase-coupled spectrophotometric assay of Stegink and Coon (1968) as modified by Kramer and Miziorko (1980), with the exception that 200 μM HMG-CoA was used to initiate the reaction. All assays were performed in 0.2 M Tris-HCl at pH 7.8. Prior to addition to the assay mixture, HMG-CoA lyase was incubated with 20 mM DTT for 10−15 min at room temperature.

Upon addition of the reduced variants, the reaction was immediately initiated with 200 μM HMG-CoA. For both H235A and H235D, 2 μg was utilized for the determination of the K_m value for HMG-CoA, which was varied between 40 and 300 μM.

The pH/rate profiles for the H235 variants were determined as described above, except that the buffers employed in the assay mixture were either 100 mM Tris-HCl/0.10 mM EDTA for pH values between 6.6 and 8.9 or 100 mM glycine/0.10 mM EDTA for pH values between 8.7 and 10.0. The concentration of HMG-CoA used to initiate the reactions was appropriately varied at each pH value. The V_{max} was determined at the specified pH values by linear regression analysis of the Lineweaver−Burk plots. The overall pH/rate profile was fit to a nonlinear regression analysis algorithm as described earlier (Roberts et al., 1995).

When either the K_m or the K_a for the divalent cations was determined, all components of the assay mix, including buffers, were passed over a Chelex-100 column to remove all other trace metals. The assays were conducted as described earlier. The concentration of either Mn^{2+} or Mg^{2+} ranged from 1 to 1000 μM and 0.5 to 40 mM, respectively. To determine the K_a of Mn^{2+}, HMG-CoA was varied between 25 and 125 μM, while the Mn^{2+} was varied from 5 to 200 μM.

DEPC Modification. The concentrations of stock solutions of DEPC, which were freshly prepared in cold absolute ethanol, were determined with 10 mM imidazole buffer at 22 °C. This reaction generates an absorbance change at 230 nm (ϵ = 3000 M^{-1} cm^{-1}) characteristic of *N*-carbethoxyimidazole (Miles, 1977). HMG-CoA lyase (5.9 μM wild type and 7.9 μM H235A subunit concentrations) in 0.02 M potassium phosphate at pH 6.8 and 25 °C was reacted with 2.5 mM DEPC to a final volume of 0.50 mL. At various time intervals, the reaction mixture was scanned from 235 to 340 nm. The stoichiometry of *N*-carbethoxyhistidine formation was determined by difference spectroscopy utilizing the extinction coefficient of 3200 M^{-1} cm^{-1} at 240 nm (Ovádi et al., 1967). A control experiment using equivalent volumes of ethanol (without DEPC) was performed under comparable conditions.

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. 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. The protein samples were prepared as described in Roberts et al. (1995). Each sample was concentrated to ≈100 μM (subunit concentration) using an Amicon Centriflo membrane cone. During concentration, the samples were buffered in 10 mM potassium phosphate (pH 7.8) containing 100 mM NaCl, 20% glycerol, and 1.0 mM DTT. Prior to ESR measurements, the glycerol was removed using Sephadex G-50 centrifugal columns. A quartz flat cell was used for all measurements. The ESR samples contained variable concentrations of Mn^{2+} (25−150 μM) with 90 μM HMG-CoA lyase sites (H235A or H235D). HG-CoA was added to each sample (final concentration of 1 mM). 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 to determine both the Mn^{2+} binding stoichiometry and binding affinity of the human HMG-CoA lyase H235 variants.

RESULTS

Mutagenesis Strategy, Expression, and Isolation of HMG-CoA Lyase H235 Variants. Recent work (Roberts et al., 1996) suggested that HMG-CoA lyase contains at least two histidines that are accessible and reactive with DEPC. The observations that modified enzyme is catalytically inactive and that a substrate analog protects against modification and loss of activity suggested that one or both of the histidine targets may be situated within the enzyme's active site. H233, a residue clinically implicated in HMG-CoA lyase deficiency and the first of two invariant histidines found in a HXH motif, has been assigned as one target of DEPC (Roberts et al., 1996). In an attempt to identify the second DEPC target, H235 represented an attractive candidate, since its proximity to H233 suggested accessibility to the reagent and its invariant status argued that it could potentially influence enzyme activity.

To determine the role of H235, site-directed mutagenesis, by overlap extension PCR (Table 1), was employed. The original pTrcHL-1 expression plasmid encoding the gene for wild-type HMG-CoA lyase was utilized as the template for oePCR. The first mutation involved a conservative substitution of alanine for the invariant histidine-235. A second variant was produced that had an aspartic acid at residue 235. The acidic residue was chosen as a potential metal ligand, since Narasimhan et al. (1994) found that a nitrogen ligand may play an important role in cation binding to the bacterial enzyme.

The plasmids encoding the H235 variants were transformed into the JM105 strain of *E. coli*, and protein expression was induced by addition of IPTG. Both variants were subjected to the purification procedure described by Roberts et al. (1994) for wild-type HMG-CoA lyase. The salt precipitation properties and chromatographic elution profiles of H235A and H235D were identical to that determined for wild-type enzyme (Roberts et al., 1994). On the basis of SDS−polyacrylamide gel electrophoresis, subunit molecular weights of 34 000 were estimated for H235A and H235D, identical to the value determined for wild-type lyase (Roberts et al., 1994). The purity of each variant was greater than 95%, which matches the results obtained for wild-type enzyme (Roberts et al., 1994).

DEPC Modification of H235A. Both wild-type and H235A enzymes were subjected to treatment with 2.5 mM DEPC as described by Roberts et al. (1996). The resulting absorbance profile for the modified H235A variant was similar to that measured using wild-type enzyme, in that a distinctive maximum was observed at approximately 240 nm. This is characteristic of carbethoxylation of histidine residues. No spectral features indicative of tyrosine modification (i.e. a valley at 278 nm) were observed with the modified H235A enzyme. On the basis of the extinction coefficient at 240 nm (ϵ = 3200 M^{-1} cm^{-1}), the number of modified histidines was calculated. Figure 1 depicts the time-dependent modification of both wild-type and H235A enzymes by DEPC. It is apparent that the extent of modification is lower with

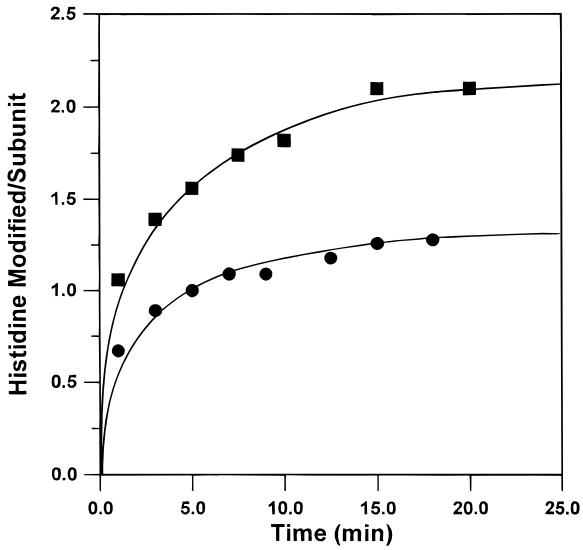

FIGURE 1: Stoichiometry of diethyl pyrocarbonate modification of H235A (●) and wild-type (■) HMG-CoA lyase. Either H235A (7.9 μM subunit) or wild-type (5.9 μM subunit) enzymes were incubated in the presence of 2.5 mM DEPC. An extinction coefficient ϵ_{240} of 3200 M^{-1} cm^{-1} was used to determine the extent of histidine modification. A subunit molecular weight of 32 000 was utilized to calculate the number of carbethoxyhistidine residues per subunit.

Table 2: Kinetic Parameters of the H235 Variants of Human HMG-CoA Lyase

property	wild type	H235A	H235D
specific activity (units/mg)	159	10	10
K_M (μM) for HMG-CoA	24	97	95
K_M (μM) for Mg^{2+}	233	4240	3600
K_M (μM) for Mn^{2+}	0.34	25	15
stoichiometry of Butynoyl-CoA modification	1.1	1.1	1.4

the mutant. Upon completion of the reaction (20 min), one less histidine was modified in the H235A enzyme versus the wild-type lyase, suggesting that H235 is one of the histidine residues sensitive to DEPC modification.

Enzymological Characterization of the H235 Variants. The specific activity of each of the purified mutants was determined using the enzymatically coupled spectrophotometric assay. As shown in Table 2, the activities of both H235A and H235D were diminished by only 15-fold, an effect much smaller than the decrease of 4 orders of magnitude observed for the catalytic residue H233 (Roberts et al., 1996). The Michaelis constant for the substrate, HMG-CoA, was determined for H235A and H235D. In both cases, the measured values are slightly higher than those obtained for wild-type enzyme (Table 2). pH/rate profiles measured for the mutant enzymes are similar in shape to that measured for wild-type lyase but shift to reflect p*K* values about 0.5 unit higher than that measured for wild-type enzyme.

A dissociable cation stimulates the activity of human (Roberts et al., 1994), bacterial (Narasimhan & Miziorko, 1992), avian (Kramer & Miziorko, 1980), and bovine (Stegink & Coon, 1968) HMG-CoA lyases. The only two cations that function in this capacity are Mg^{2+} and Mn^{2+}, which exhibit K_m values of 233 and 0.34 μM, respectively, with the human enzyme (Roberts et al., 1994). However, when the K_m values for these two metals were determined for each of the H235 variants, significant differences were observed. The effect was most pronounced in the case of manganese, where a 40−70-fold increase in K_m values was observed (Table 2). The observation of such effects for both the alanine and the aspartic acid mutant is somewhat surprising, since carboxylates are relatively good ligands for hard metals. This is the first case where a change in the amino acid sequence of HMG-CoA lyase has led to an alteration in the binding of the divalent cation.

Structural tests were performed to confirm that the decrease in metal affinity was a direct effect of the change in amino acid, and not merely the consequence of a gross conformation alteration. Each of the H235 variants, along with wild-type enzyme, was subjected to CD spectroscopy. In all cases, the spectra were indistinguishable from one another (data not shown). The estimated α-helical and β-sheet contents were within experimental error of the values obtained with wild-type enzyme. This analysis illustrates that the overall secondary structures of the histidine-235 variants are very similar to wild-type protein. As a more stringent test, the protein conformation around the active site was tested by utilizing the affinity label 2-butynoyl-CoA. Hruz et al. (1992) found that 2-butynoyl-CoA labels a conserved cysteine residue (C237) found in the active site of bacterial lyase. Later studies demonstrated that 2-butynoyl-CoA also stoichiometrically labels the human enzyme (Roberts et al., 1994). When H235A and H235D lyases were incubated with the affinity label, modification stoichiometries similar to that measured using wild-type enzyme were observed (Table 2). This demonstrates that each of the variants at residue 235 contains an intact binding site for coenzyme A and, even more importantly, that the catalytic cysteine is in the proper orientation for modification. On the basis of these observations, and the fact that catalytic activity is diminished by only 15-fold, it is reasonable to conclude that the tertiary structure of these mutants is not grossly altered.

Kinetic and Spectroscopic Determinations of the Manganese Binding Properties of H235A and H235D. To further functionally characterize these mutant enzymes, additional cation binding parameters were determined for both H235A and H235D. In particular, manganese binding was characterized, since past studies have demonstrated that Mn^{2+} can be a useful structural probe for HMG-CoA lyase (Roberts et al., 1995). Since Mn^{2+}, unlike the spectroscopically inactive Mg^{2+}, contains five unpaired d electrons, an ESR spectroscopic method could be employed in addition to kinetic approaches.

The K_m value for Mn^{2+} (Table 2) is measured in the presence of saturating substrate. As a more stringent test of enzyme−cation interaction, the activator constant (K_a) for manganese, which is measured at varying concentrations of HMG-CoA, was determined for H235A and H235D. The activator constant can be calculated from the intersection point of the double-reciprocal plot (Mildvan & Cohn, 1965); this affinity estimate is independent of HMG-CoA concentration. Figure 2 depicts the results obtained for purified H235D; a value of 45 μM was determined (Table 3), which is much larger than the K_a estimate of 0.5 μM for wild-type enzyme. Thus, replacement of the imidazole ring with an carboxylate does not efficiently support metal binding. When a similar experiment was performed with H235A, a K_a of 30 μM was observed (Table 3). This observation confirms that replacement of the histidine at position 235 significantly weakens (60−90-fold) metal binding.

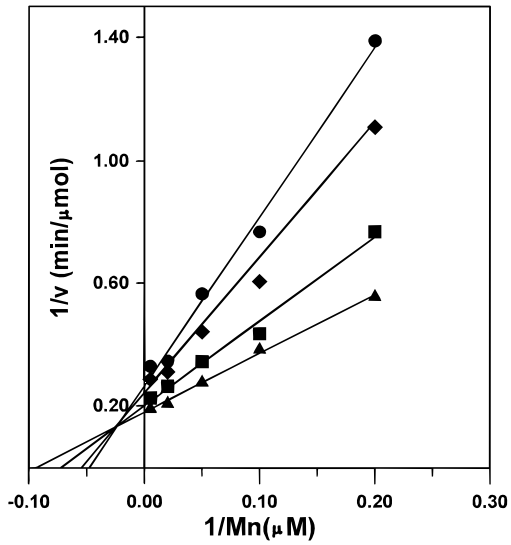

FIGURE 2: Double-reciprocal plot of the rate of H235D HMG-CoA lyase as a function of Mn^{2+} concentration. The substrate, HMG-CoA, was varied as follows: (●) 25, (◆) 50, (■) 75, and (▲) 125 μM. The activator constant (K_a) was determined from the intersection point of the lines fit to the experimental data.

Table 3: Mn^{2+} Binding Properties of the H235 Variants of Human HMG-CoA Lyase

	kinetic assays		ESR spectroscopy	
source	K_M (μM)	K_a (μM)	K_D (μM)	n
wild type	0.34	0.5	1.5	0.7
H235A	25	30	25[a]	0.8
H235D	15	45	34[a]	0.8

[a] Measured in the presence of 1 mM 3-hydroxyglutaryl-CoA.

To determine both the stoichiometry and the K_d for binding of the activator cation to HMG-CoA lyase, ESR spectroscopy was employed. In order to cleanly perform Mn^{2+} ESR experiments, glycerol must be removed from the samples or signal broadening due to viscosity effects complicates the analyses. Fortunately, human HMG-CoA lyase, unlike the prokaryotic enzyme, is stable in the absence of glycerol for more than 12 h (Roberts et al., 1995). Immediately prior to the start of the ESR experiments, glycerol was removed from the protein samples by centrifugal gel filtration. In the case of wild-type enzyme, previous experiments demonstrated that manganese binding occurred in the absence of substrate, with a K_d value of 1.5 μM and a stoichiometry of 0.7 metal bound per subunit (Roberts et al., 1995; Table 3). Utilizing conditions similar to those previously employed for wild-type enzyme, the H235 mutants were examined. When the ESR spectrum of the free cation in buffer was compared with the spectrum from samples containing cation and one of the H235 mutants, only a small change in amplitude was observed. Therefore, neither H235A nor H235D demonstrated a significant amount of binary E−M complex formation, an observation that is in sharp contrast with the results measured using wild-type enzyme (Roberts et al., 1995).

The above results were surprising since, kinetically, an activator constant for manganese was easily determined for both mutants (Table 3). However, the ESR experiment was different in one major respect; there was no substrate present. Due to the fast turnover rates, the physiological substrate, HMG-CoA, could not be utilized in these ESR experiments.

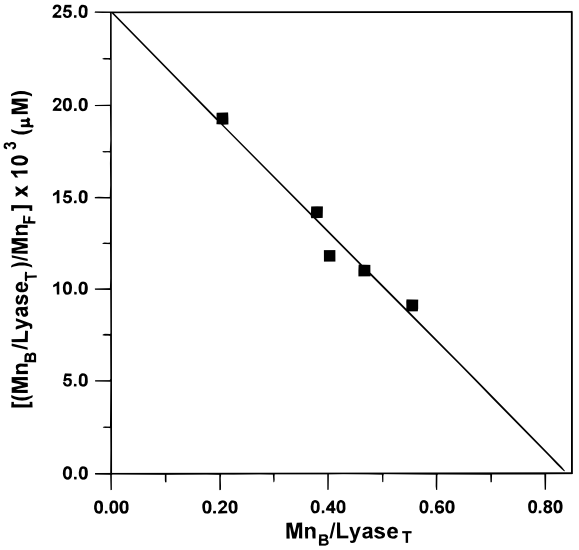

FIGURE 3: Scatchard plot of the ESR data for manganese binding to H235D HMG-CoA lyase. 3-Hydroxyglutaryl-CoA (1 mM) was included in the each of the samples. The amount of bound metal was determined by comparing the amplitudes of the Mn^{2+} ESR spectra measured in the absence and presence of the H235 variants. The data were fit by linear regression analysis, and the binding constant (K_d) and stoichiometry (n) were determined from the slope and x-intercept, respectively.

Instead, HG-CoA, a substrate analog which is a competitive inhibitor with respect to HMG-CoA (Kramer & Miziorko, 1983) and does not tightly bind Mn^{2+}, was added to the all of the samples. When the spectra of Mn^{2+} in the absence of enzyme or in the presence of one of the H235 variants were compared, significant decreases in the signal amplitude were observed for the enzyme-containing samples. By comparing the spectral peak amplitudes measured for samples without and with enzyme, the amount of bound manganese could be calculated and the binding data subjected to Scatchard analysis. Figure 3 depicts the data obtained for the H235D mutant in the presence of 1 mM HG-CoA. A similar Scatchard plot was obtained for H235A. Upon linear regression analysis of the data, the binding stoichiometry was determined from the x-axis intercept and the dissociation constant was calculated from the slope of the resulting theoretical fit. Like wild-type enzyme, both H235A and H235D have a full complement of metal sites; a stoichiometry of 0.8 is measured for both mutants (Table 3). The dissociation constants for the mutants are similar to the kinetically determined K_a values. These K_d values are much higher for the mutant lyases than the K_d of wild-type enzyme in forming the binary E*M complex (Table 3), but perhaps the most interesting difference is the observation that each of the mutants requires the presence of an analog of HMG-CoA for metal binding to become substantial.

DISCUSSION

There are numerous examples of metalloenzymes in which histidine has proven to be essential for metal coordination. In particular, the HXH pattern has been implicated as an important metal binding motif in a number of metalloenzymes (Vallee & Auld, 1993). A *dissociable* cation (Mg^{2+} or Mn^{2+}) is required for optimal activity in all species of HMG-CoA lyases characterized to date. Since mutation of H235 leads to decreases of up to 70-fold in the kinetically determined K_m for the activator cation, a role for the second

histidine in the conserved motif that involves coordinating the dissociable divalent cation seemed worthy of consideration. However, both manganese and magnesium, which are hard metals, generally prefer oxygen for coordination. Nonetheless, there are a number of enzymes in which histidine acts as a ligand for either Mn^{2+} or Mg^{2+}. The refined atomic structure of glutamine synthetase has demonstrated that a histidine ligand is important in coordinating Mn^{2+} (Yamashita et al., 1989). The crystal structure of cytochrome c oxidase (Tsukihara et al., 1995) supports the mutagenesis work suggesting that a histidine is a Mn^{2+} ligand (Hosler et al., 1995). X-ray crystallography and site-directed mutagenesis were also utilized to implicate histidine in coordination to the second metal (Mn^{2+}, Mg^{2+}, or Co^{2+}) in D-xylose isomerase (Cha et al., 1994).

The perspective provided by the aforementioned metal binding precedents and hypotheses should not be formulated in an isolated context but rather in concert with a consideration of the data on the *function* of the divalent cation in the HMG-CoA lyase reaction. The first extensive investigation of the functional requirement for a divalent cation in HMG-CoA cleavage was reported by Stegink and Coon (1968), who surveyed various cations for stimulation of activity and determined the K_m of Mg for enzyme purified from bovine liver. Kramer and Miziorko (1983), utilizing purified avian HMG-CoA lyase, demonstrated that the divalent cation stimulated not only the cleavage reaction but also the rate of enzyme-catalyzed tritium exchange from acetyl-CoA, possibly by stabilizing an enolate intermediate through binding to the carbonyl oxygen. Later, Hruz et al. (1993) proposed that the dissociable cation was coordinated not only to the protein but also to the substrate. On the basis of K_m differences for Mg and Mn, measured kinetically using the physiological substrate or the dithioester substrate analog, HMG[=S]CoA, it was suggested that cation coordination involved the thioester carbonyl and possibly the C3 hydroxyl oxygen of the substrate. Recent work using biophysical methods with human HMG-CoA lyase has demonstrated that enzyme will form a binary E*M complex in the absence of substrate (Roberts et al., 1995). The binding constant obtained for the binary complex was similar in magnitude to the K_m for the cation, a kinetically determined parameter that is more complex than the physically determined binding constant. Thus, a variety of approaches suggest that HMG-CoA lyase employs both substrate and enzyme ligands in binding the activator divalent cation.

In a direct test of the function of H235, when formation of a binary E*M complex for either H235A or H235D was studied by ESR spectroscopy, very little E*M complex was observed. This result was unexpected since it was not difficult to kinetically determine K_a constants which, although measured in an activity assay, have values that are independent of substrate concentration. Significant divalent cation binding to these mutant proteins was only observed when HG-CoA, an analog which contains both the thioester carbonyl and the C3 hydroxyl oxygen, was added (Figure 3). Thus, in contrast to the wild-type enzyme, the H235 variants required the addition of a substrate or an analog to afford functional binding of the divalent cation. In the case of H235 variants, it is possible that additional ligands from the substrate generated binding energy needed to replace the contribution from the missing nitrogen ligand. While the 64-fold effect on k_{cat}/K_m upon elimination of H235's imidazole is a significant effect, this corresponds to a change in binding energy of less than 3 kcal. Such an effect may be due to loss of a hydrogen bond or due to a perturbation in tertiary structure too subtle to be detected by our physical approaches. The binding energy contribution due to the substrate analog, 3-hydroxyglutaryl-CoA, or to substrate, HMG-CoA, is clearly sufficient to support reasonably efficient catalysis by the H235 mutants.

The true magnitude of the effect attributable to the histidine-235 ligand is probably best estimated from comparison of K_d values for the binary E*M complex. Since the E*M complex is virtually undetectable when H235 is absent, only a lower limit to the effect of eliminating H235 on binary complex formation may be estimated; >100-fold weaker Mn^{2+} binding characterizes these mutants. Stability constants for Mn^{2+}−imidazole complexes (Smith & Martell, 1989) suggest that an effect of this magnitude is somewhat larger than expected for elimination of one imidazole ligand from enzyme-bound Mn^{2+}. It remains possible that other ligands, which in wild-type enzyme contribute to binary E*M complex formation, may not be optimally oriented in the mutant lyases. Less direct kinetic analyses support the physical data, although the difference estimates based on measurements in the presence of CoA derivatives are smaller than the intrinsic differences that become apparent upon comparison of the binary E*M complexes. Based on the collected evidence, a functional assignment of histidine-235 as a ligand for HMG-CoA lyase's dissociable divalent cation seems well justified.

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