Published in final edited form as:

Arch Biochem Biophys. 2011 July ; 511(1-2): 48–55. doi:10.1016/j.abb.2011.04.004.

Influence of multiple cysteines on human 3-hydroxy-3-methylglutaryl-CoA lyase activity and formation of intersubunit adducts

Christa Montgomery and Henry M. Miziorko

Division of Molecular Biology and Biochemistry, University of Missouri – Kansas City, Kansas City, MO 64110

Abstract

Human 3-Hydroxy-3-methylglutaryl-CoA lyase catalyzes formation of acetyl-CoA and acetoacetate in a reaction that requires divalent cation and is stimulated by sulfhydryl protective reagents. The enzyme is a homodimer and intersubunit adducts form in the absence of reducing agents or upon treatment with cysteine selective crosslinking agents. To address the influence of cysteines on enzyme activity and formation of intersubunit and intrasubunit adducts, single serine substitutions have been engineered for each enzyme cysteine. Enzyme activity varies for each cysteine→serine mutant protein and different mutations have widely different effects on recovery of activity upon DTT treatment of nonreduced enzyme. These levels of enzyme activity do not strongly correlate with formation of intersubunit adducts by these HMGCL mutants. C170S, C266S, and C323S proteins do not form intersubunit disulfide adducts but such an adduct is restored in the C170S/C174S double mutant. Coexpression of HMGCL proteins encoded by C266S and C323S expression plasmids supports formation of a C266S/C323S heterodimer which does form a covalent intersubunit adduct. These observations are interpreted in the context of competition between cysteines in formation of intrasubunit and intersubunit heterodisulfide adducts.

Keywords

HMG-CoA lyase; Hydroxymethylglutaryl-CoA; cysteine; disulfide; enzyme activity; intersubunit adduct

3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase (HMGCL; EC 4.1.3.4) catalyzes the cleavage of HMG-CoA into acetyl-CoA and acetoacetate [1, 2] (Scheme 1).

Cleavage of HMG-CoA is involved in the generation of ketone bodies to support the energy requirements of non-hepatic animal tissues [3]. This accounts for the hypoketotic conditions [4] that develop in humans as a result of the many reported inherited HMGCL mutations [5].

© 2011 Elsevier Inc. All rights reserved.

Corresponding author: Henry M. Miziorko, Univ. of Missouri-Kansas City, 5007 Rockhill Road, Kansas City, MO 64110, phone: 816-235-2246; fax: 816-235-5595; miziorkoh@umkc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

¹Abbreviations used are: HMGCL, 3-hydroxy-3-methylglutaryl-CoA lyase; DTT, dithiothreitol; kan, kanamycin; amp, ampicillin; IPTG, isopropylthiogalactoside; ECL, enhanced chemiluminescence; NEM, N-ethyl maleimide; TIM, triosephosphate isomerase; PMSF, phenylmethylsulfonyl fluoride.

Identification of several inherited point mutations [6] and characterization of recombinant human enzymes mutated to incorporate the corresponding amino acid substitutions [7-9] led to the identification of functionally important active site residues (e.g. R41, D42, H233). The enzyme also functions in catalyzing the terminal step of leucine catabolism [10], which accounts for broad distribution of the protein in prokaryotes and eukaryotes. HMGCL activity is absolutely dependent on the presence of a divalent cation (e.g. Mg^{2+} , Mn^{2+}) and activity is stimulated by reducing agents (e.g. DTT). The influence of reducing agents may be explained by the observation that animal HMGCL proteins contain eight cysteine residues [11]. Affinity labeling results [12] identified one of these residues (C266) as an active site residue in the avian enzyme. This residue is located in a flexible loop positioned over the active site [13, 14] and catalysis would be blocked if C266 mobility was constrained by participation in any disulfide adduct, providing a clear rationale for the enzyme's reducing agent requirement. Additionally, the sensitivity of one or more HMGCL cysteines to diminution in cellular reductant levels was suggested by observations that multiple physiological perturbations occur in a superoxide dismutase knock out mouse [15]. These include a 36% decrease in HMGCL activity and a consequent organic aciduria due to degradation of the pool of accumulating HMG-CoA metabolite.

Endogenously expressed enzyme, purified from avian liver tissue [16] was employed in protein chemistry experiments which demonstrated that the dimeric enzyme (32 kDa subunits) forms a covalent intersubunit adduct upon treatment with the sulfhydryl selective reagent, *N,N'*-ortho-phenylenedimaleimide [17]. Difference peptide maps of tryptic digests prepared from crosslinked and non-crosslinked proteins implicated two peptides as harboring the modification targets. Edman degradation and amino acid composition analyses demonstrated that these are related, C-terminal region peptides which correspond either to residues 318-324 (VSQAACR) or residues 318-325 (VSQAACRL). The simplest explanation for all data available at that time involved the hypothesis that an intersubunit crosslink formed between a C-terminal C323 residue from each subunit of the dimeric enzyme. When a recombinant form of human HMGCL became available, wild-type and C323S proteins were used to extend studies on the effects of reducing agents and cysteine selective protein crosslinkers [18]. Results of these studies, which focused only on the function of C323, were consistent with the proposed role for a C-terminal cysteine in formation of a covalent intersubunit adduct.

Subsequently, the 2.1 Å resolution structure of human HMGCL became available [13]. The structure did not clearly identify cysteines (including C323) that would participate in formation of the interchain adduct observed upon disulfide formation or chemical crosslinking [17, 18]. These observations suggested that it would be informative to expand the investigation to include the possible contribution of other cysteine residues in formation of either *intrasubunit* or *intersubunit* covalent adducts. This report presents the results of these studies, which suggest that multiple cysteine residues influence covalent adduct formation in HMG-CoA lyase as well as the dependence of enzyme activity on reducing agent.

Experimental Procedures

Materials

E. coli JM109 and BL21 competent cells, miniprep, midiprep, and gel purification kits were purchased from Promega. dNTPs and Pfu DNA polymerase used for mutagenesis were purchased from Stratagene. Primers used for mutagenesis were synthesized by Integrated DNA Technologies. BamHI, NcoI, and DpnI endonucleases were obtained from New England Biolabs. DNA sequencing was performed at the DNA Core Facility, University of Missouri-Columbia. Ni-Sepharose was purchased from GE Healthcare, Bradford reagent

and unstained protein standards from Bio-Rad, NEM from Eastman-Kodak, ECL reagents and PMSF from Pierce, and autoradiography film from MIDSCI (St. Louis, MO). Secondary antibodies, Tween 20, NAD, NADH, malic acid, and coupling enzymes were purchased from Sigma-Aldrich. DNA ligase, DNase I, media components, buffers, DTT, and all other reagents were obtained from Fisher Scientific.

Plasmid Construction

The open reading frame encoding the mature mitochondrial form of human HMGCL was sub-cloned into the expression vector pET30b (Novagen) using standard molecular biology techniques. Briefly, the HMGCL coding sequence was excised from pTrc99 HL [18] using the restriction endonucleases BamHI and NcoI and gel purified. The purified restriction fragment was ligated with a similarly digested and purified pET30b vector. The ligation produced an expression construct, pET30HL, which encodes mature mitochondrial HMGCL containing N-terminal His₆ and S tags. DNA sequence analysis was used to verify the integrity of the final product.

Protein Expression

Chemically competent *E. coli* BL21 (DE3) cells (Promega) were transformed with pET30HL, plated onto LB agar containing 50 µg/ml kanamycin (Kan), and incubated overnight at 37°C. A single colony was used to inoculate 6 ml of LB/Kan for overnight growth. Glycerol stocks were made from the overnight culture by combining 1 ml of culture with 0.5 ml of sterile 50% glycerol and storing at -80°C. A 50 ml starter culture of LB/Kan was inoculated from glycerol stock, incubated overnight at 37°C, and 2-3 ml used to inoculate a 1 L culture of LB/Kan. After incubation at 37°C until the OD₆₀₀ was 0.5 – 0.6, protein expression was induced by the addition of sterile IPTG (RPI; final concentration, 1 mM). After overnight incubation at 22°C, the induced cells were harvested by centrifugation and pellets were stored at -80°C until protein purification. Similar conditions were used for the expression of mutant proteins.

Mutagenesis

Mutants were generated using full circle PCR according to Stratagene's QuickChange site-directed mutagenesis protocol. The WT pET30HL construct was used as a template for single mutants and the pET30HL C170S mutant construct was used as a template for double mutants. Mutations were verified by DNA sequence analysis. Forward and reverse mutagenic primer sequences (mutagenic bases underlined) are as follows:

C141S for: 5'- CCAAGAAGAACATCAATAGTTCCATAGAGGAGAG -3'

C141S rev: 5'- CTCTCCTCTATGGAACTATTGATGTTCTTCTTGG -3'

C170S for: 5'- GGTACGTCTCCTCTGTCTTTGGCTGC -3'

C170S rev: 5'- GCAGCCAAGAGCAGAGGAGACGTACC -3'

C174S for: 5'- CCTGTGCTCTTGGCAGCCCTTATGAAGGG -3'

C174S rev: 5'- CCCTTCATAAGGGCTTGCCAAGAGCACAGG -3'

C197S for: 5'- CTACTCAATGGGCTCTACGAGATCTCCCTGG -3'

C197S rev: 5'- CCAGGGAGATCTCGTAGGAGCCCATTGAGTAG -3'

C234S for: 5'- CCTGGCTGTCCACTCCCATGACACCTATGG -3'

C234S rev: 5'- CCATAGGTGTCATGGGAGTGGACAGCCAGG -3'

C266S for: 5'- GGACTTGGAGGCTCTCCCTACGCACAGG -3'

C266S rev: 5'- CCTGTGCGTAGGGAGAGCCTCCAAGTCC -3'

C307S for: 5'- GCTGGAACTTTATCTCTCAAGCCCTGAACAG -3'

C307S rev: 5'- CTGTTTCAGGGCTTGAGAGATAAAGTTTCCAGC -3'

C323S for: 5'- GGCTCAGGCTACCTCTAAACTCTAGGATCCG -3'

C323S rev: 5'- CGGATCCTAGAGTTTAGAGGTAGCCTGAGCC -3'

Enzyme Purification

All steps were carried out at 4°C. Bacterial pellets from 1 L of expression culture were resuspended in 100 ml of ice cold lysis buffer containing 50 mM NaPi pH 7.8, 300 mM NaCl, 5% glycerol, and 5 mM imidazole. Protease inhibitors (1 mM PMSF, 1 uM pepstatinA, and 10 uM leupeptin), 1 U/ml DNaseI, and 5mM mercaptoethanol were added immediately before cell disruption. Cells were mechanically disrupted by passing twice through a microfluidizer at ~17 kpsi. The lysate was clarified by centrifugation at 10,000 ×g for 10 min. and the supernatant was loaded onto Ni-Sepharose Fast Flow resin (~1 ml). The column was washed with 50 mM NaPi pH 7.8, 300 mM NaCl, 10% glycerol, 40 mM imidazole, and 5 mM mercaptoethanol until the $A_{280} < 0.010$. The protein was eluted slowly overnight with 50 mM NaPi pH 7.8, 300 mM NaCl, 20% glycerol, 300 mM imidazole and 5 mM mercaptoethanol. Fractions containing HMGCL were pooled and the concentration was determined by the method of Bradford [19]. The homogeneous wild type enzyme was found to have a V_{max} of 123 U/mg and a K_m for HMG-CoA of 26 μ M, values which are comparable with those for pTrc99 HL-expressed enzyme. These conditions were also utilized for the purification of mutant proteins.

Enzyme Activity Measurement

Enzyme activity was determined using the method of Stegink and Coon [1] as modified by Kramer and Miziorko [16]. HMG-CoA was synthesized using the method of Goldfarb and Pitot [20] Briefly, this spectrophotometric assay couples the acetyl-CoA produced upon the cleavage of HMG-CoA to the reactions of malate dehydrogenase and citrate synthase. For each acetyl-CoA that condenses with oxaloacetate to form citrate, one malate is oxidized to oxaloacetate, producing one NADH. The rate of NADH production is determined by measuring the increase in A_{340} and is proportional to the amount of HMG-CoA lyase added. DTT was omitted from assays of nonreduced enzyme but included at 5 mM levels in measuring activity under reducing conditions.

Preparation of nonreduced enzyme

Rapid buffer exchange via gel filtration, centrifugal ultrafiltration, or dilution resulted in variability in measured activity and levels of covalently linked dimer in nonreduced enzyme, perhaps due to slow exchange of trace levels of reductant with bulk solvent or slow decay of a mixed DTT-enzyme adduct. Therefore, extensive dialysis was employed to fully deplete reducing agents, permitting spontaneous formation of disulfide linkages. Purified protein was diluted in cold, air equilibrated, non-reducing dialysis buffer consisting of 50 mM NaPi pH 6.8, 100 mM NaCl, and 20% glycerol to a concentration of 1 mg/ ml. A volume < 1 ml of protein was dialyzed against 4 L of buffer for at least 16 hrs at 4°C. Longer dialysis and/or multiple exchanges of buffer did not increase the enzyme's dependence on thiol for activity or intensity of the dimer band upon SDS PAGE analysis.

NEM Modification

Free enzyme thiols were alkylated with NEM to prevent thiol-disulfide exchange upon denaturation. Small aliquots of reduced or nonreduced dialyzed protein were diluted into

reaction mixtures containing final concentrations of 100 mM NaPi pH 6.8 and 100 mM NEM. After 10 minutes, SDS was added to a concentration of 0.05% to unfold protein and maximize NEM modification. Reactions were allowed to proceed at 23°C for an additional 60-90 minutes. Longer incubation times resulted in some formation of non-reducible protein dimer.

SDS-PAGE and Western Blotting

SDS-PAGE was performed using a 12% resolving gel and a 4% stacking gel as described by Laemmli [21]. For reducing gels, samples were heated (95 °C) for 5 min in loading buffer containing a minimum of 50 mM mercaptoethanol. For non-reducing gels, samples were prepared as above except mercaptoethanol was omitted from the loading buffer. Protein bands were visualized using Coomassie blue staining. In western blot experiments, proteins were transblotted from SDS PAGE gels to nitrocellulose overnight at 23V in the cold box. Blots were blocked in 5% non-fat milk (NFM) dissolved in tris-buffered saline with 0.1% Tween-20 (TTBS) for 30 min. at room temperature (RT) with agitation. Blots were incubated in a 1:5000 dilution of rabbit anti-HMGCL serum in 3% NFM/TTBS for 1 hr. at RT. Rinsed blots were then incubated in a 1:10,000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG in 3% NFM/TTBS for 1 hr. at RT. Finally, rinsed blots were incubated for 5 min. at RT in enhanced chemi-luminescence (ECL) Western Blotting Substrate (single mutants) or West Pico ECL Substrate (double mutants), and exposed to autoradiology film for 5 min. in a darkroom. A Bio-Rad or Fermentas PageRuler Plus prestained protein ladder was used to estimate molecular weights.

Results

Location of cysteine residues in native human HMGCL

Human HMGCL (RCSB coordinates 2CW62) crystallizes with six monomers in the asymmetric unit. The protein exhibits a (β/α)₈ barrel fold, with a highly conserved signature sequence (harboring C266) located in a very dynamic loop situated over the C-terminal end of the barrel. For two of the six monomers in the asymmetric unit, this loop is disordered, suggesting that it is highly flexible in solution. Nevertheless, the published data [13] make possible mapping the position of each of the eight cysteines in each subunit of the physiologically relevant dimer (Figure 1A). Using this structural model, it is possible to determine the distances between the different cysteine sulfhydryls that would have to be spanned if any intrachain (Figure 1B) or interchain (Figure 1C) covalent (e.g. disulfide) adduct was to be formed. For intrachain interactions, there are three sulfur-sulfur pair distances that are in the 4-10 Å range (C170/C174; C266/C170; C266/C174). For interchain interactions, two sulfur-sulfur pair distances (C234/C234; C266/C323) are in the 13-20 Å range. Formation of a C170/C174 intrachain disulfide adduct seems likely on the basis of close proximity but a multiplicity of such adducts could result if dynamics of the C266-containing loop or other mobile cysteine-containing structural regions close the distances between pairing partners.

Reduction of microheterogeneity in SDS PAGE mobility upon N-ethylmaleimide (NEM) modification of nonreduced HMGCL

Sequence alignment of a diverse selection of eukaryotic and prokaryotic HMGCL proteins (Figure 2) suggests that, among the eight residues of animal HMGCL proteins, only C266 (human sequence numbering) is invariant and C174 is very highly conserved. Calculated

²The structure of enzyme without substrate or inhibitor acyl-CoA ligands (only bound Mg²⁺ and 3-hydroxyglutarate) is the appropriate model for the protein samples used in these studies.

molecular mass for a monomer of the recombinant human protein produced using the pET30b expression construct is 36.6 kDa (in reasonable agreement with a MALDI estimate of 36.7 kDa); apparent mass calculated from SDS PAGE mobility (figure 3, lane 1) is 39 kDa. Some indication of the possible multiplicity of disulfide bonds in nonreduced HMGCL is apparent upon comparison of such protein samples that are either unmodified or treated with N-ethylmaleimide (NEM) to modify any non-disulfide linked cysteines prior to sample analysis by SDS PAGE. NEM blocking of accessible cysteines prior to and during sample denaturation reduces the possibility of additional new extraneous inter- or intrachain disulfide formation. Also reduced is the exchange of available free cysteines with any existing disulfides in samples of nonreduced protein to form different disulfide linkages as the remaining cysteines become exposed. NEM sharpens the covalently linked dimer SDS PAGE band (Figure 3, lane 5) that is otherwise quite diffuse and difficult to observe (Figure 3, lane 4). Similarly, the monomer band from the untreated nonreduced sample (Figure 3, lane 4) is more diffuse than observed for the sample which is NEM treated and subsequently reduced to eliminate any disulfide bonds prior to SDS PAGE (Figure 3, lane 2). Such sharpening of the monomer band is compatible with the microheterogeneity that would be expected if intrasubunit disulfide linkages also form within the monomer of the nonreduced protein. Therefore, to optimize detection of covalent interchain adducts, as well as to sharpen monomer bands, NEM modification of free cysteines was routinely performed prior to and during denaturation of samples for analysis by SDS PAGE.

Strategy for evaluation of cysteine residue contributions to disulfide bond formation and to enzyme activity in the presence or absence of reductant

A mass spectrometry/proteomics (LC-ESI-MS/MS) approach was evaluated by preliminary experiments that identified and compared peptides from either tryptic or chymotryptic HMGCL digests. Tryptic and chymotryptic digests were prepared from samples of NEM modified HMGCL that were either reduced with DTT or untreated after extensive dialysis in air equilibrated buffer. Due to the high sensitivity of the MS approach and the presence of eight cysteine residues in HMGCL, widely ranging peak intensities attributable to various disulfide linked candidate peptides were detectable. Such observations characterized not only digests of nonreduced samples but even digests of NEM modified reduced control protein, which should only contain trace levels of disulfide linkages. The difficulty in accurately estimating relative abundance of candidate disulfide linked peptides for comparisons between samples complicated identification of functionally important disulfides. These developments prompted us to initiate another approach for a less ambiguous evaluation of the formation of disulfide adducts in nonreduced HMGCL as well as the influence of individual cysteines on the dependence of activity on exogenous reducing agents.

Site directed mutagenesis was employed to generate eight Cys→Ser mutant HMGCL proteins with a single substitution for each individual cysteine residue. The mutant proteins were expressed (pET 30b constructs) at levels comparable to wild type enzyme. The soluble fractions of lysates containing these N-his-tagged proteins were purified by nickel affinity chromatography. Detection of monomeric and covalently linked dimeric enzyme bands after SDS-PAGE was performed on western blots using polyclonal rabbit antiserum prepared against avian liver enzyme [16]. As described below, the mutant enzymes were suitable for both activity analyses as well as for detection of covalently linked dimers, validating the decision to pursue an expanded mutagenesis approach. Additionally, the availability of the pET 30b expression constructs (in addition to selected pTrc 99 expression plasmids) for HMGCL mutant proteins enabled production of a C266/C323S heterodimer (*vide infra*). This protein supported an experimental test of the formation of a C266-C323 intersubunit

adduct, which seemed plausible (*vide ante*) on the basis of structural proximity considerations (Fig. 1C).

Influence of cysteine substitutions on intersubunit adduct formation in nonreduced protein

When wild-type and each of the eight Cys→Ser mutant HMGCL proteins are reduced in the presence of 2% mercaptoethanol prior to denaturing SDS PAGE and western blotting, each protein exhibits mobility expected for a monomer (Fig. 4A). When samples of extensively dialyzed protein are not reduced prior to SDS PAGE, interesting contrasts are apparent (Fig. 4B). A substantial band corresponding to a covalently linked dimer of subunits is apparent for wild type protein, as well as for C141S, C174S, and 307S mutant proteins; a dimer band of diminished intensity is also observed for C197S and C234S proteins. As previously reported for the pTrc99 expressed C323S protein [18], no dimer is observed for C323S protein. However, in extending this study to other Cys→Ser mutants, it now becomes clear that a dimer adduct is also undetectable in nonreduced samples of either C266S or C170S mutants. Thus, multiple cysteines (e.g. C170, C266, and C323) can strongly influence intersubunit adduct formation in nonreduced HMGCL. Such a possibility had not previously been considered.

Coexpression of HMGCL C266S and C323S proteins supports formation of a heterodimer that forms a covalent intersubunit adduct

E. coli BL21 (DE3) cells were transformed with both purified pET 30 HL C266S and pTrc HL C323S plasmids. Double transformants were selected on LB agar plates containing both kanamycin (pET 30 marker) and ampicillin (pTrc 99 marker). After IPTG expression of HMGCL in a culture of a double transformant, a soluble lysate fraction which could include C266S homodimer, C323S homodimer, and/or C266S/C323S heterodimer was prepared. This sample was subjected to nickel sepharose chromatography. Any pTrc expressed C323S homodimer is untagged and elutes in unbound fractions. The affinity (imidazole) eluted protein includes dimeric enzyme containing at least one pET 30 expressed his tagged C266S subunit. Any C266S homodimer in the affinity eluate will not exhibit significant catalytic activity (Table 1). The specific activity of the affinity eluted protein is >20-fold higher than attributable to C266S homodimer. This suggests elution of a heterodimeric species comprised of not only a his tagged, low activity C266S subunit but also an untagged C323S subunit, which retains high catalytic activity [18]. Such a heterodimer should contain on one subunit a C266 sulfhydryl juxtaposed toward the adjacent subunit's C323 sulfhydryl (a potential disulfide forming residue pair; Fig 1A). Additionally, the heterodimer contains on adjacent subunits one pair of S266 and S323 residues. After extensive dialysis of the purified protein, nonreduced and reduced samples were subjected to SDS PAGE and western transfer. Immunodetection indicated the presence of a covalent HMGCL dimer attributable to C266S/C323S protein (figure 5, lane 4) and, as expected, no covalent dimer in any reduced HMGCL sample (figure 5, lanes 1, 2). As already demonstrated in Figure 4, nonreduced C266S and C323S homodimers fail to form any covalent dimer. Thus, the detection of a covalent dimer, combined with the observation of catalytic activity in the nickel resin purified protein are most straightforwardly explained by formation of a heterodimer formed from one subunit containing a C266 sulfhydryl and another containing a C323 sulfhydryl. This empirical observation validates the prediction of such an adduct that was based on the proximity of these residues (Fig. 1C) indicated by the X-ray structure of the enzyme.

Influence of individual cysteines on HMGCL catalytic activity

Previous studies on HMGCL cysteines were focused by the results of affinity labeling experiments [12] which implicated C266 as a residue important to catalytic function, an

observation that was supported by results of mutagenesis experiments [22]. A pET-30b based expression plasmid offered improvements in yield and simplification of purification of N-his tagged HMGCL. Wild type enzyme prepared using this methodology exhibits (Table 1) specific activity (123 U/mg), K_m HMG-CoA (26 μ M) and DTT stimulation of activity ($\sim$ 10-fold) comparable to pTrc expressed enzyme used in previous work [18]. A series of eight individual Cys $\rightarrow$ Ser mutant proteins was prepared; dialyzed samples of the purified mutants supported activity measurements on reduced and nonreduced forms of each protein (Table 1). The C266S mutant, which contains at the catalytic site a side chain alcohol of higher pK than the normal cysteine thiol of wild type enzyme, is diminished in specific activity by $\sim$ 550 fold, as expected. None of the other reduced mutants exhibits more than a 6-fold change of specific activity, which indicates some influence on, but no crucial role in, catalytic function. Their specific activity values range from 17-107% of wild type activity.

If dialyzed mutant enzymes are not pre-incubated with reductant prior to or during assay, activity is reduced for all of the enzymes to different extents, confirming the requirement for a reducing agent in order to optimize activity but also indicating that multiple cysteines contribute to the requirement. For the non-catalytic mutant proteins (i.e. excluding C266S which, regardless of the presence/absence of reductant, exhibits minimal activity due to disruption of reaction chemistry [14, 22]) the relative ability of dialyzed enzyme to recover activity under reducing conditions is most noteworthy for C170S. The effect (Table 1) is much larger for C170S (636-fold) than for C323S (9.6-fold), even though nonreduced samples of both mutants fail to form a covalent dimeric adduct (Fig. 4B). In comparison with C323S, C141S does form a covalent dimer; it exhibits a modest ($\sim$ 5 fold) stimulation of activity by DTT. From these contrasts, it seems apparent that HMGCL *activity* is not strictly correlated with the level of covalently linked dimer detected in denatured, nonreduced protein. Rebound in enzyme activity upon reduction is also substantial for C197S and C234S mutants. Nonreduced samples of both of these proteins exhibit covalent dimer formation that is somewhat diminished (Fig. 4B) in comparison with observations for wild type, C141S, C174S, or C307S proteins. However, the collected assay results most clearly implicate C170, a previously uninvestigated residue, as a key residue in affecting HMGCL activity as the level of reduction of the protein changes.

Investigation of the possible interaction between cysteines C170 and C174 as an influence on formation of intersubunit dimer adducts

In animal and plant HMGCL proteins, C170 and C174 are conserved but, while homologous residues are found in some bacterial forms of the protein, strict conservation across a broad spectrum of HMGCL proteins is not observed. Three residues separate these cysteines in human HMGCL so they do not conform to the CXXC motif [23] implicated in many enzymes sensitive to oxidation/reduction. Nonetheless, these sulfhydryls are separated by only $\sim$ 4 Angstroms (Figure 1B; [13]) and the serine substitution in C170S strongly influenced both the HMGCL activity levels in reduced versus nonreduced protein and also the ability of nonreduced protein to form a covalent dimer. Therefore, the influence of C170 on interactions with C174 and formation of covalent adducts between subunits was examined by expression, purification, and partial characterization of the C170S/C174S double mutant. Double mutants involving C170 as well as other residues (C266, C233) that affect detection of intersubunit adducts were also prepared and studied in parallel experiments. Activity measurements on dialyzed purified double mutants indicate that the large dependence on reductant observed for C170S (636-fold stimulation) is minimized in C170S/C174S; the more modest 11.4 fold stimulation (Table 2) that is observed is closer to the effects observed for double mutants C170S/C266S (2.6-fold), C170S/C323S (27.9-fold), and for other single Cys $\rightarrow$ Ser mutants (Table 1). The possibility of C170-C174 interactions that was suggested by these results was also investigated by SDS PAGE analysis of the

double mutants under reducing/non-reducing conditions. The results (fig. 6) indicate that the serine substitution of both C170 and C174 side chains restores some formation of the intersubunit dimer in nonreduced protein. In contrast, comparable samples of C170S/C266S and C170S/C323S lack any detectable covalent dimer adduct, as is the case for single mutants C170S, C266S, and C323S (Fig. 4). Thus, the absence of a thiol at residues 170 and 174 not only diminishes the large stimulation of activity by reductant that is observed for C170S but also alters the remaining cysteine-cysteine interactions that influence the formation of a covalently linked dimer.

Discussion

Magnitude of dependence on exogenous reductants does not correlate with formation of intersubunit adducts

HMGCL's requirement for a reducing agent in order to optimize activity had been previously attributed to the participation of C266 in catalysis and to C323's involvement in formation of the disulfide linked dimer in nonreduced C323S, which exhibits a diminution (~10-fold for recombinant protein) in activity. The absence of any interchain adduct after treatment of *Pseudomonas mevalonii* enzyme (which lacks a homologous C-terminal region cysteine; [24]) with a bifunctional chemical crosslinker [17] appeared to be consistent with the role proposed for avian HMGCL's C323. More recently, structural observations indicated that intersubunit disulfide linkages involving residues other than C323 (notably C266) required consideration due to their higher proximity to C323 on an adjacent subunit of the native enzyme dimer. The results of the more detailed experiments described above indicate that there is not any direct correlation between the formation of an intersubunit disulfide linked dimer in nonreduced protein and the magnitude of changes in HMGCL activity for reduced/nonreduced forms of wild type and mutant enzymes. For example, the magnitude of activity stimulation upon reduction of C323S, which forms no covalent intersubunit dimer, is comparable to activity stimulation observed for wild type enzyme, which does form such a dimer. A nonreduced sample of C170S, like C323S, shows no formation of covalent dimer (Fig. 4B) but exhibits a very large activity stimulation upon DTT treatment (Table 1) in comparison with the effect observed for either wild-type or C323S enzymes. Comparisons of these data with earlier observations are not complicated by the use of pTrc-99 versus pET-30b expressed protein. For samples subjected to dialysis in air equilibrated buffer, the magnitude of activity stimulation upon DTT treatment is comparable for HMGCL proteins produced by either expression method. Extended dialysis (>16 hours) with air equilibrated buffer produces pET-30b expressed wild type enzyme that completely rebounds to its original activity level upon incubation with DTT. This observation agrees with earlier work using pTrc-99 expressed enzyme, which employed a more rapid centrifugal gel filtration method to deplete residual reductant. The more extensive survey of the magnitude of sensitivity of each of eight cysteine substituted mutant enzymes to stimulation of activity upon reduction indicates that cysteines such as C197, C234 have intermediate effects on activity stimulation as well as the level of formation of covalently linked dimers. Such effects may indicate some possible involvement of these residues in disulfide exchange or in direct formation of intrasubunit disulfides but their influence is secondary to the larger effects discussed above in the context of C323S and C170S proteins.

Competition for C266 may reflect participation of several HMGCL cysteines in formation of disulfide bonds

Single Cys→Ser substitutions eliminate formation of a covalent intersubunit adduct for nonreduced C266S, C323S, and C170 proteins, suggesting that each of these three residues influences formation of the disulfide linked dimer. Involvement of C323 does not seem

surprising, given its reasonable proximity (~ 14 Å) to C266 located in a flexible and highly conserved “signature” loop of the adjacent HMGCL subunit. Proximity between this C323-C266 pair of residues is unmatched by any other intersubunit cysteine pair (Figure 1C). Given the involvement of C266 in reaction catalysis, tethering the C323-C266 residues together accounts for a diminution in catalytic rate. Coexpression of C266S and C323S proteins to produce a C266S/C323S heterodimeric protein has been essential to generating *experimental* evidence (Figure 5) that the proposed C266-C323 adduct can, in fact, explain the formation of a covalent dimer in nonreduced HMGCL.

The role of C170 in influencing covalent adduct formation is less obvious. Given the proximity between C170 and C174 (~ 4 Å; Figure 1B), it is certainly plausible that they could form an intrachain disulfide in nonreduced protein. C266 is more distant (8-10 Å) from these residues. In C170S, given the dynamic nature of the flexible loop that harbors C266, the absence of C170's sulfhydryl may increase the possibility of formation of an intrachain disulfide between C174 and C266 in competition with any interchain C266-C323 dimer adduct. In contrast, for C174S good formation of a covalent dimer is observed for nonreduced protein. This suggests that C170 is not as effective as C174 in competing for the intrachain C266, even though both are in reasonable proximity. A structure based HMGCL alignment [13] indicates that C170 is positioned at the end of a beta strand ($\beta 5$) while C174 is positioned in the middle of the loop between beta strand 5 and alpha helix 7, which may slightly improve its access to C266.

The double mutant C170S/C174S exhibits strong formation of a covalently linked dimer (Fig. 6B, lane 3), reversing the elimination of such an adduct that is observed for the single mutant C170S. The hypothesis presented above straightforwardly accommodates this observation. The absence of thiol containing side chains at both residues 170 and 174 eliminates the possibility that *either* residue forms an intrachain disulfide with C266. Thus in nonreduced C170S/C174S, without competition by these residues for C266, an increase in formation of the interchain adduct between C266 and C323 of the adjacent subunit is favored, accounting for the SDS PAGE detection of a substantial formation of this adduct.

Conclusion

Results of this study (Table 1) indicate that, in nonreduced human HMGCL, multiple cysteine residues significantly influence catalytic activity. Thus, interpretation of *in vivo* activity estimates (e.g. HMGCL assays performed in the context of a gene knockout or characterization of an inherited HMGCL mutation) should consider effects not only due to the reduced status of C266 and/or C323 but also of other cysteine residues. The ability of enzyme to form, *in vitro*, a covalent adduct between subunits of the physiological dimer does not directly correlate with the level of catalytic activity. Coexpression of C266S and C323S plasmids supports formation of a C266S/C323S heterodimer which can form a covalent intersubunit adduct that is not observed for either C266S or C323S protein homodimers. C170 and C174 compete with other cysteines, influencing formation of intrasubunit or intersubunit heterodisulfide adducts. These observations indicate the complexity of animal HMGCL stimulation by reducing agents.

Acknowledgments

The authors appreciate the assistance of Ms. Catherine You, who expressed and purified many of the HMGCL mutant proteins used for the experiments reported in this paper; these include C266S, C323S, C170S/C174S, and others. Dr. Leslie Hicks (Danforth Plant Science Center Proteomics and Mass Spectrometry Facility, St. Louis, MO) and Dr. Andrew Keightley (UMKC) conducted the evaluation of the feasibility of a mass spectrometric/proteomics approach to identify intra- and intersubunit disulfide adducts in HMGCL. Dr. Ted White (Cell Biology & Biophysics, Univ. of Missouri – Kansas City) suggested the attempt to engineer a C266S/C323S heterodimer.

References

1. Stegink LD, Coon MJ. *J Biol Chem*. 1968; 243:5272–5279. [PubMed: 5702047]
2. Bachhawat B, Robinson WG, Coon MJ. *J Biol Chem*. 1955; 216:727–736. [PubMed: 13271348]
3. Robinson AM, Williamson DH. *Physiol Rev*. 1980; 60:143–187. [PubMed: 6986618]
4. Gibson KM, Breuer J, Nyhan WL. *European Journal of Pediatrics*. 1988; 148:180–186. [PubMed: 3063529]
5. L-V E, Pié J, Puisac B, Menao S, Pié A, Casale C, Ramos FJ, Hegardt FG, C N, Gómez-Puertas P. *Mol Genet Metab*. 2007; 92:198–209. [PubMed: 17692550]
6. Mitchell GA, Ozand PT, Robert MF, Ashmarina L, Roberts J, Gibson KM, Wanders RJ, Wang S, Chevalier I, Plochl E, Miziorko H. *Am J Hum Genet*. 1998; 62:295–300. [PubMed: 9463337]
7. Roberts JR, Mitchell GA, Miziorko HM. *J Biol Chem*. 1996; 271:24604–24609. [PubMed: 8798725]
8. Tuinstra RL, Miziorko HM. *J Biol Chem*. 2003; 278:37092–37098. [PubMed: 12874287]
9. Tuinstra RL, Wang CZ, Mitchell GA, Miziorko HM. *Biochemistry*. 2004; 43:5287–5295. [PubMed: 15122894]
10. C R, Massey LK, Sokatch JR. *J Bacteriol*. 1974; 118:112–120. [PubMed: 4150714]
11. Mitchell GA, Robert MF, Hruz PW, Fontaine G, Behnke CE, Mende-Mueller LM, Wang S, Schappert K, Lee CS, Gibson KM, Miziorko HM. *J Biol Chem*. 1993; 268:4376–4381. [PubMed: 8440722]
12. Hruz PW, Narasimhan C, Miziorko HM. *Biochemistry*. 1992; 31:6842–6847. [PubMed: 1637819]
13. Fu Z, Runquist JA, Forouhar F, Hussain M, Hunt JF, Miziorko HM, Kim JJ. *J Biol Chem*. 2006; 281:7526–7532. [PubMed: 16330550]
14. Fu Z, Runquist JA, Montgomery C, Miziorko HM, Kim JJ. *J Biol Chem*. 2010; 285:26341–26349. [PubMed: 20558737]
15. Melov S, Coskun P, Patel M, Tuinstra R, Cottrell B, Jun AS, Zastawny TH, Dizdaroglu M, Goodman SI, Huang TT, Miziorko H, Epstein CJ, Wallace DC. *Proc Natl Acad Sci U S A*. 1999; 96:846–851. [PubMed: 9927656]
16. Kramer PR, Miziorko HM. *J Biol Chem*. 1980; 255:11023–11028. [PubMed: 6159358]
17. Hruz PW, Miziorko HM. *Protein Sci*. 1992; 1:1144–1153. [PubMed: 1304393]
18. Roberts JR, Narasimhan C, Hruz PW, Mitchell GA, Miziorko HM. *J Biol Chem*. 1994; 269:17841–17846. [PubMed: 8027038]
19. Bradford MM. *Anal Biochem*. 1976; 72:248–254. [PubMed: 942051]
20. Goldfarb S, Pitot HC. *J Lipid Res*. 1971; 12:512–515. [PubMed: 5164097]
21. Laemmli UK. *Nature*. 1970; 227:680–685. [PubMed: 5432063]
22. Roberts JR, Narasimhan C, Miziorko HM. *J Biol Chem*. 1995; 270:17311–17316. [PubMed: 7615532]
23. Chivers P, Laboissiere MC, Raines RT. *EMBO J*. 1996; 15:2659–1667. [PubMed: 8654363]
24. Anderson DH, Rodwell VW. *Journal of Bacteriology*. 1989; 171:6468–6472. [PubMed: 2687236]
25. DeLano, W. The PyMOL Molecular Graphics System. 2002. <http://www.pymol.org>
26. Guex N, Peitsch MC. *Electrophoresis*. 1997; 18:2714–1723. [PubMed: 9504803]

Highlights

- Stimulation of HMG-CoA lyase activity by reducing agents
- Intersubunit adduct formation in HMG-CoA lyase
- Specific Cys→Ser mutations eliminate intersubunit adducts in HMG-CoA lyase
- HMGCL C266S/C323S heterodimer forms an intersubunit adduct
- Restoration of intersubunit adducts in a double Cys→Ser HMG-CoA lyase mutant

Figure 1.

Crystal structure of HMGCL and distances between cysteine sulfurs. Panel A displays a Pymol [25] ribbon drawing representation of the crystal structure of human HMGCL (2CW6) which was solved at a resolution of 2.1 Å [13]. The physiological dimer consists of two (β/α)₈ TIM barrels, oriented with the axes of the two barrels perpendicular to each other. This structure contains no bound acyl-CoA substrate or inhibitor; one chain (A) is occupied by bound Mg²⁺ and 3-hydroxyglutarate, which indicate the active site at the C-terminal end of the barrel. The view of the monomer on the left is from the C-terminal end of the barrel cavity. The barrel of the monomer on the right is rotated by about 90° relative to the left monomer. Intrachain and interchain distances between HMGCL cysteines are displayed in

panels B and C, respectively. The Swiss PDB viewer Deep View (4.01) [26] was used to determine the distance (in Angstroms) between each cysteine sulfur of the physiological HMGCL dimer. The shortest intrachain distances are highlighted in the upper table. Highlighted in the lower table are the shortest interchain and the shortest homo-disulfide distances.

	141	170/174	197	234	266	307	323
<i>H. sapiens</i>	INCSI	CALGCPYE	MGCYE	VHCHD	VAGLGCCPYA	FICQA	ATCKL
<i>G. gallus</i>	INCSI	CVLGCPYE	MGCYE	VHCHD	VAGLGCCPYA	FICNA	AACRL
<i>D. melanogaster</i>	VNCTA	TVVGCPEY	MGCYE	VHCHD	VSGLGCCPYA	YICTE	ARVK-
<i>P. mevalonii</i>	INCSI	CVLGCPFS	LGCYE	GHFHD	VAGLGCCPYS	ALCQT	RAGLA
<i>R. rubrum</i>	INCSI	CVLGCPYE	MGCHE	AHFHD	VAGLGCCPYA	ASRAA	-----
<i>B. subtilis</i>	INKST	TVFGCPYE	FGISE	LHFHD	AGGLGCCPYA	WIEEK	-----

Figure 2.

Sequence alignment of diverse eukaryotic and prokaryotic HMG-CoA lyase proteins. Residues flanking cysteine residues in the animal proteins are depicted. Alignment was generated using ClustalW. Residue numbering corresponds to human HMGCL (uncleaved mitochondrial isoform). Sequences from the following organisms are included: *Homo sapiens*, *Gallus gallus*, *Drosophila melanogaster*, *Pseudomonas mevalonii*, *Rhodospirillum rubrum*, and *Bacillus subtilis*. Amino acid sequences were obtained from the UniProt database using the following accession numbers: P35914, P35915, Q9VM58, P13703, Q2RT05, and O34873 respectively. A more extensive alignment would indicate infrequent substitutions for C174 (e.g. aspartate in *Streptomyces coelicolor* HMGCL).

Figure 3.

SDS-PAGE of human HMGCL under reducing or nonreducing conditions. Purified HMGCL was subjected to dialysis (>16 hr) in air equilibrated buffer. An aliquot was incubated with the sulfhydryl-specific alkylating reagent NEM (lanes 2, 5) while another aliquot was mock treated (lanes 1, 4). Reactions were split in half and denatured in an equal volume of SDS loading buffer and treated with (lanes 1-3) or without (lanes 4-5) mercaptoethanol (2%) prior to heating (95 °C) for 5 min. Five ug of protein was loaded in each lane of a 12% SDS-PAGE gel. Molecular weight markers (lane 3) include: phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa.

Figure 4.

Western blots of human HMGCL Cys→Ser mutants under reducing [A] or nonreducing [B] conditions. Purified HMGCL and Cys→Ser mutants were dialyzed (>16 hr) in air equilibrated buffer. Free cysteines were alkylated with the sulfhydryl-specific reagent NEM prior to and during denaturation with SDS loading buffer. Equal volumes of each reaction were denatured in 2× SDS loading buffer and then incubated in the presence [A] or absence [B] of mercaptoethanol (2%) prior to heating (95 °C) for 5 min. Proteins (0.25 ug each except C197S, 0.75 ug) were run in separate lanes of two 12% SDS-PAGE gels and transblotted to nitrocellulose. Protein bands were detected as described in experimental procedures. Prestained molecular weight markers include: phosphorylase b, 104.4 kDa;

bovine serum albumin, 97.3 kDa; ovalbumin, 50.4 kDa; carbonic anhydrase, 37.2 kDa; trypsin inhibitor, 29.2 kDa; lysozyme, 20.2 kDa. Positions indicated for pre-stained molecular weight standards are determined when exposed autoradiography film is overlaid on the western blot.

Figure 5.

Western blot of human HMGCL and pET30HL C266S / pTrcHL C323S heterodimer under reducing and nonreducing conditions. Wild type HMGCL and pET30HL C266S / pTrcHL C323S heterodimer were extensively dialyzed (>16 hr) in air equilibrated buffer. Free cysteines were alkylated with the sulfhydryl-specific reagent NEM prior to and during denaturation with SDS loading buffer. Aliquots of each reaction were denatured in 2× SDS loading buffer and then incubated in the presence (lanes 1 and 2) or absence (lanes 4 and 5) of mercaptoethanol (2%) prior to heating (95 °C) for 5 min. Proteins (0.4 ug WT and 1 ug pET30HL C266S / pTrcHL C323S heterodimer) were run in separate lanes of a 12% SDS-PAGE gel and transblotted to nitrocellulose. Protein bands were detected as described in experimental procedures. The PageRuler Plus prestained protein ladder (Fermentas) was used to estimate molecular weights, as indicated in lane 3. The high molecular weight band in lane 4 is attributable to a heterodimeric form of HMGCL and is not observed (fig. 4) for either C266S or C323S homodimers.

Figure 6.

Western blots of human HMGCL C170S double mutants under reducing [A] or nonreducing [B] conditions. Purified HMGCL, C170S, and C170S double mutants were extensively dialyzed (16 hr) in air equilibrated buffer. Free cysteines were alkylated with the sulfhydryl-specific reagent NEM prior to and during denaturation with SDS loading buffer. Equal volumes of each reaction were denatured in 2× SDS loading buffer and then incubated in the presence [A] or absence [B] of mercaptoethanol (2%) prior to heating (95 °C) for 5 min. Proteins (0.13 ug WT; 0.13 ug C170S; 0.17 ug [A] or 0.33 ug [B] C170S/C174S; 0.17 ug C170S/C266S, and 0.26 ug C170S/C323S) were run in separate lanes of two 12% SDS-PAGE gels and transblotted to nitrocellulose. Protein bands were detected as described in

experimental procedures. Positions indicated for pre-stained molecular weight standards are determined when exposed autoradiography film is overlaid on the western blot.

Scheme 1.
Reaction catalyzed by HMG-CoA lyase

Table 1
Dependence of Activity of Human HMGCL Single Cys→Ser Mutants on Exogenous Thiol

	Activity Recovered Following Reduction of Dialyzed Enzyme (U/mg)	Activity Following Dialysis (U/mg)	Stimulation of Activity by Exogenous Thiol	Intersubunit Dimer Formation ^a
Enzyme	Reducing Assay Conditions	Non-Reducing Assay Conditions		Non-Reducing Assay Conditions
WT HMGCL	123 (± 7)	11.5 (± 1.4)	10.7 fold	Moderate
C141S HL	42.0 (± 3.7)	8.83 (± 0.23)	4.76 fold	Strong
C170S HL	53.1 (± 8.3)	0.084 (± 0.012)	636 fold	None
C174S HL	42.1 (± 5.9)	2.66 (± 0.30)	15.8 fold	Moderate
C197S HL	21.2 (± 0.6)	0.141 (± 0.012)	150 fold	Weak
C234S HL	128 (± 6)	0.742 (± 0.062)	172 fold	Weak
C266S HL	0.224 (± 0.010)	0.137 (± 0.005)	1.63 fold	None
C307S HL	132 (± 5)	13.8 (± 0.1)	9.56 fold	Moderate
C323S HL	132 (± 2)	13.8 (± 1.6)	9.57 fold	None

^aStrong indicates a SDS PAGE dimer band (Fig. 4B) which is >65% of the total intensity in that lane; moderate, 34-65%; weak, <34%.

Table 2
Dependence of Activity of HMGCL Double Cys→Ser Mutants on Exogenous Thiol

Activity Recovered Following Reduction of Dialyzed Enzyme (U/mg)		Activity Following Dialysis (U/mg)	Stimulation of Activity by Exogenous Thiol	Intersubunit Dimer Formation ^a
Enzyme	Reducing Assay Conditions	Non-Reducing Assay Conditions	Non-Reducing Assay Conditions	
WT HMGCL	123 (± 7)	11.5 (± 1.4)	10.7 fold	Strong
C170S HL	53.1 (± 8.3)	0.084 (± 0.012)	636 fold	None
C170S/C174S HL	1.06 (± 0.01)	0.093 (± 0.022)	11.4 fold	Moderate
C170S/C266S HL	0.123 (± 0.021)	0.047 (± 0.008)	2.62 fold	None
C170S/C323S HL	18.5 (± 1.3)	0.662 (± 0.024)	27.9 fold	None

^aStrong indicates a SDS PAGE dimer band (Fig. 6B) which is >65% of the total intensity in that lane; moderate, 34-65%; weak, <34%.