Protein Science (1992), *1*, 1144–1153. Cambridge University Press. Printed in the USA.

Avian 3-hydroxy-3-methylglutaryl-CoA lyase: Sensitivity of enzyme activity to thiol/disulfide exchange and identification of proximal reactive cysteines

PAUL W. HRUZ AND HENRY M. MIZIORKO
Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

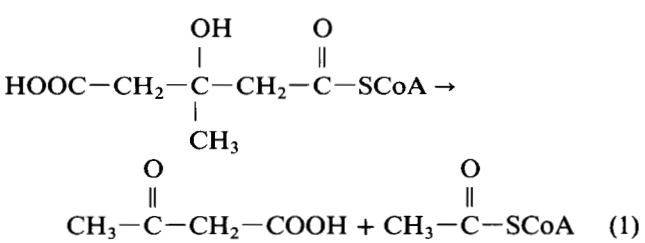
(RECEIVED January 21, 1992; REVISED MANUSCRIPT RECEIVED April 14, 1992)

Abstract

Catalysis by purified avian 3-hydroxy-3-methylglutaryl-CoA lyase is critically dependent on the reduction state of the enzyme, with less than 1% of optimal activity being observed with the air-oxidized enzyme. The enzyme is irreversibly inactivated by sulfhydryl-directed reagents with the rate of this inactivation being highly dependent upon the redox state of a critical cysteine. Methylation of reduced avian lyase with 1 mM 4-methylnitrobenzene sulfonate results in rapid inactivation of the enzyme with a k_{inact} of 0.178 min^{-1}. The oxidized enzyme is inactivated at a sixfold slower rate ($k_{inact} = 0.028$ min^{-1}). Inactivation of the enzyme with the reactive substrate analog 2-butynoyl-CoA shows a similar dependence upon the enzyme's redox state, with a sevenfold difference in k_{inact} observed with oxidized vs. reduced forms of the enzyme. Chemical cross-linking of the reduced enzyme with stoichiometric amounts of the bifunctional reagents 1,3-dibromo-2-propanone (DBP) or *N,N'*-ortho-phenylenedimaleimide (PDM) coincides with rapid inactivation. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of enzyme treated with bifunctional reagent reveals a band of twice the molecular weight of the lyase monomer, indicating that an intersubunit cross-link has been formed. Differential labeling of native and cross-linked protein with [1-^{14}C]iodoacetate has identified as the primary cross-linking target a cysteine within the sequence VSQAACR, which maps at the carboxy-terminus of the cDNA-deduced sequence of the avian enzyme (Mitchell, G.A., et al., 1991, *Am. J. Hum. Genet. 49*, 101). In contrast, bacterial HMG-CoA lyase, which contains no corresponding cysteine, is not cross-linked by comparable treatment with bifunctional reagent. These results provide evidence for a potential regulatory mechanism for the eukaryotic enzyme via thiol/disulfide exchange and identify a cysteinyl residue with the reactivity and juxtaposition required for participation in disulfide formation.

Keywords: affinity labeling; avian HMG-CoA lyase; cross-linking; ketogenesis; reactive cysteines; sulfhydryls/cysteine; thiol/disulfide exchange

HMG-CoA lyase (EC 4.1.3.4) catalyzes the cleavage of HMG-CoA into acetoacetate and acetyl-CoA in the final step of the ketogenic HMG-CoA cycle.



(1)

In addition to its vital role in the generation of ketone bodies, which are used as alternative sources of metabolic fuel during periods of diminished glucose availability

Reprint requests to: Henry M. Miziorko, Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226.

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-Coenzyme A; DBP, 1,3-dibromo-2-propanone; PDM, *N,N'*-ortho-phenylenedimaleimide; IAA, iodoacetate; MNBS, 4-methylnitrobenzene sulfonate; HPLC, high pressure liquid chromatography; DTT, dithiothreitol; TPCK, tosyl-L-phenylalanine chloromethyl ketone; Tris-Cl, tris(hydroxymethyl)aminomethane hydrochloride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

(Robinson & Williamson, 1980), the enzyme is also essential in the catabolism of leucine (Faull et al., 1976). The serious consequences of an inability to produce ketone bodies have been described in a number of recent reports of inherited HMG-CoA lyase deficiency (Gibson et al., 1988). Despite this recent appreciation of the requirement for proper ketogenic control, little is known about the potential for regulation within the HMG-CoA cycle.

In the case of HMG-CoA lyase, the purification of sufficient quantities of homogenous enzyme to carry out chemical studies has been difficult in part because of the low amounts of the enzyme protein normally present in tissues. As a result, little is known about important structural and potential regulatory features of this enzyme. The avian enzyme has been purified to homogeneity by Kramer and Miziorko (1980); SDS-PAGE and gel filtration chromatography suggest that the enzyme exists as a homodimer of 27-kDa subunits. Characterization of this enzyme has revealed that activity is highly stimulated by the addition of exogenous thiol. Reports on HMG-CoA lyase from a number of other sources including pig heart (Bachawat et al., 1955), beef liver (Stegink & Coon, 1968), *Pseudomonas mevalonii* (Scher & Rodwell, 1989), and *Tetrahymena piriformis* (Prasana & Holmlund, 1987) have shown varying levels of stimulation by exogenous thiol. This stimulation of enzymatic activity by reduction with added thiols as well as our initial observation of reversible inactivation by oxidation following thiol removal suggested that a thiol/disulfide exchange reaction may have an effect upon substrate binding and/or catalysis. The work described in this report was initiated to determine if such a mechanism is operative in vitro and to identify cysteines that are reasonable candidates for participation in such an exchange process.

Results

Reversible inactivation by air oxidation

Reduction of avian HMG-CoA lyase by incubation of enzyme with reduced thiols has been shown to be essential for maximal activity (Kramer & Miziorko, 1983). In the absence of added thiol, less than 1% of optimal enzyme activity is observed. Upon addition of 5 mM DTT to the enzyme in Tris-Cl, pH 8.2 at 30 °C, maximal activity is gradually obtained within 10 min. Following complete reduction, if the added thiol is rapidly removed using a centrifugal desalting column (Penefsky, 1977), enzyme is readily converted back to the oxidized and inactive form. This oxidation is spontaneous and does not require the addition of oxidizing reagents. The lack of detectable avian lyase activity in the absence of added thiol contrasts with that observed with purified recombinant *P. mevalonii* HMG-CoA lyase. Although the bacterial enzyme is stimulated by addition of reduced DTT, it retains nearly 50% of its maximal activity in the absence of added thiol.

Irreversible inactivation with group-specific reagents

Avian HMG-CoA lyase is highly sensitive to irreversible inactivation by group-specific reagents such as IAA and MNBS, which are capable of selectively modifying sulfhydryl residues. As shown in Table 1, the rate of this inactivation is highly dependent upon the redox state of the enzyme. In the reduced state, inactivation with 1.0 mM MNBS proceeds at a rate that is at least six times faster than with the oxidized counterpart. A similar although less pronounced effect is also seen with 200 μM IAA (Table 1).

Modification with 2-butynoyl-CoA

The correlation of rates of inactivation of HMG-CoA lyase by sulfhydryl-directed reagents with the oxidized or reduced status of the enzyme suggests that a thiol/disulfide exchange reaction may have an effect upon the enzyme's active site. Examination of inactivation rate differences with an active site-directed inhibitor would represent a more direct test of this hypothesis. A number of halogenated and unsaturated acyl-CoA compounds have been used to affinity label CoA-utilizing enzymes. 3-Alkynoyl derivatives in particular have been shown to inactivate pig heart thiolase (Holland et al., 1973), butyryl-CoA dehydrogenase (Fendrich & Abeles, 1982), and pig liver general acyl-CoA dehydrogenase (Frerman et al., 1980). 2-Octynoyl-CoA has also been demonstrated to act as a suicide substrate for general acyl-CoA dehydrogenase (Freund et al., 1985).

2-Butynoyl-CoA was synthesized and examined as a substrate analog and potential active site-directed inhibitor of HMG-CoA lyase. Selective labeling of the active

Table 1. *Effect of the redox state of HMG-CoA lyase on the rates of inactivation by chemical modification*

Reagent	k_{inact} (min^{-1})[a]	
	Reduced[b]	Oxidized[c]
MNBS (1.0 mM)	0.178	0.028
IAA (200 μM)	0.030	0.011
DBP (8 μM)	0.175	0.024
PDM (5 μM)	0.198	0.011
2-Butynoyl-CoA	0.220	0.030

[a] Rates of inactivation for all reagents except PDM were measured at 30 °C, pH 7.8, with the indicated concentration of inhibitor. PDM inactivation was measured at 0 °C, pH 7.0. For 2-butynoyl-CoA, a limiting k_{inact} was calculated from a plot of $1/k_{inact}$ vs. $1/[I]$.

[b] Enzyme was fully reduced by preincubation with 40 mM DTT at pH 8.2. DTT was removed under anaerobic conditions prior to the addition of inhibitor, and incubation mixes were kept anaerobic to prevent reoxidation of enzyme sulfhydryls.

[c] Enzyme was treated as with the reduced samples with the exception that DTT was omitted from the preincubation mix.

site of HMG-CoA lyase by this reagent is supported by a number of observations. First, inactivation of reduced enzyme by 2-butynoyl-CoA follows pseudo-first-order kinetics (Fig. 1). Such kinetics are typical for active site-directed reagents. Presumably due to the presence of the CoA moiety, a reversible enzyme-inhibitor complex (E * I) is formed initially, followed by irreversible covalent modification of an active site residue as shown in Equation 2.

$$\text{Enz} + \text{I} \rightleftharpoons \text{Enz} * \text{I} \rightarrow \text{Enz-I} \qquad (2)$$

The failure of 2-butynoic acid to inhibit the enzyme at concentrations comparable to those effective for 2-butynoyl-CoA indicates that the CoA moiety is essential for effective labeling. The ability of 3-hydroxyglutaryl-CoA, a competitive inhibitor of HMG-CoA lyase (Kramer & Miziorko, 1983), to protect against inactivation is highly indicative of active site-directed inhibition by 2-butynoyl-CoA. The saturability of the process (shown in Fig. 2) is further evidence for affinity labeling. Because $k_{inact} = k_3[I]/(K_I + [I])$, k_3 and K_I can be determined from the intercepts of the plot of $1/k_{inact}$ vs. $1/[I]$. Although enzyme oxidation results in only a small change in K_I (K_I = 320 μM and 355 μM for the reduced and oxidized enzyme, respectively), a nearly sevenfold difference in the limiting k_{inact} (k_3) is seen between oxidized and reduced forms of the enzyme (Table 1). These results support the hypothesis that thiol/disulfide exchange affects access to or reactivity of a key amino acid within the enzyme's active site.

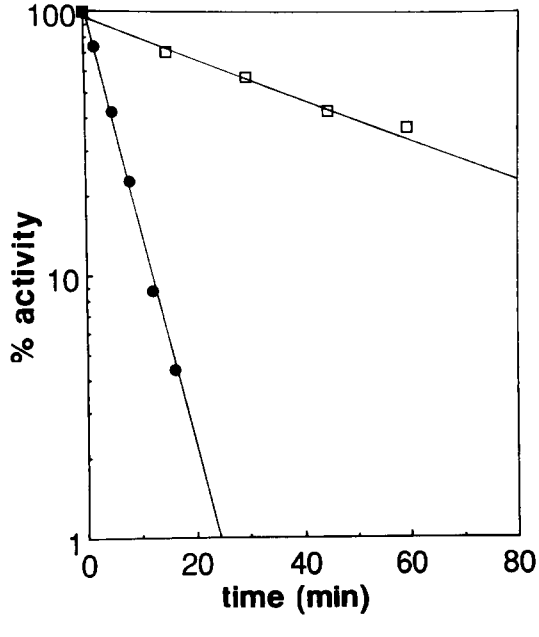


Fig. 1. Inactivation of reduced (•) and oxidized (□) avian HMG-CoA lyase by 2-butynoyl-CoA. Reduced enzyme was preincubated with DTT and kept under anaerobic conditions following thiol removal. Oxidized enzyme was preincubated in the absence of DTT. Inhibition mixtures (80 μL) were prepared containing potassium phosphate buffer, pH 7.8, HMG-CoA lyase (0.1 unit; 350 U/mg), and 2-butynoyl-CoA (320 μM). Reactions were initiated by the addition of 2-butynoyl-CoA and were performed at 30 °C. Aliquots were removed at the indicated times and assayed using the citrate synthase-coupled assay as described by Kramer and Miziorko (1983).

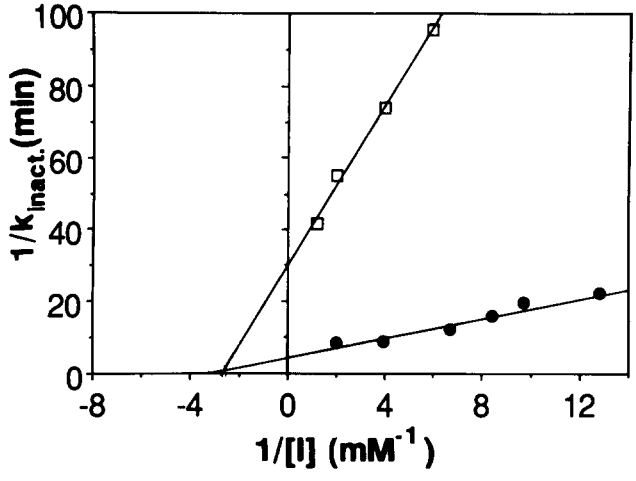


Fig. 2. Double reciprocal plot of k_{inact} of oxidized (□) and reduced (•) avian HMG-CoA lyase as a function of 2-butynoyl-CoA concentration. All incubations were carried out at 30 °C in potassium phosphate buffer, pH 7.8. Values of k_{inact} were obtained from plots of % activity vs. time. Intercepts indicate a K_i = 320 μM and a limiting k_{inact} = 0.22 min^{-1} for the reduced enzyme. K_i and limiting k_{inact} for the oxidized enzyme are 355 μM and 0.033 min^{-1}, respectively.

Sensitivity of HMG-CoA lyase to bifunctional reagents

The observations that the enzyme's catalytic efficiency as well as its reactivity with a variety of inactivators depends on redox status are compatible with the hypothesis that thiol/disulfide exchange occurs in this protein. Any cysteine residues that can be considered as candidates for participation in such exchange must be proximal and appropriately reactive. Because air oxidation is sufficient to largely eliminate avian HMG-CoA lyase activity, a differential comparison of cysteines accessible in reduced vs. air-oxidized enzyme might be envisioned as a productive approach to the identification of residues involved in any exchange reaction. However, given the reaction conditions normally used for identifying free cysteinyl sulfhydryl, it is possible for cysteines involved in an original disulfide linkage to exchange with other reactive sulfhydryls that become accessible as protein denatures. Such a development complicates straightforward assignment of the target residues. Preliminary evidence suggested that such complications would preclude productive analysis of avian HMG-CoA lyase. Therefore, modification of reactive, proximal sulfhydryls with bifunctional reagents that produce stable adducts was explored.

DBP modification

DBP, which has a demonstrated potential to chemically cross-link vicinal thiols (Stoops & Wakil, 1981), was tested as an inhibitor of HMG-CoA lyase. DBP rapidly and irreversibly inactivates the enzyme at concentrations significantly lower than those employed with the monofunctional reagents MNBS and IAA (Table 1). Furthermore, the rate of inactivation is dependent upon the redox state of the enzyme, with rapid labeling dependent upon enzyme being in the reduced state. These results suggested that it would be valuable to identify the putative sulfhydryl targets of this bifunctional reagent by isolation and sequencing of the peptides that harbor any modified cysteines.

A differential modification approach was taken in which the sulfhydryls of the denatured protein were carboxymethylated with ^{14}C-IAA either using sample without prior modification or following prior modification with DBP at reagent concentrations approaching those of the enzyme protomer. Figure 3A shows that following tryptic digestion, seven radioactive peaks corresponding to cysteine-containing peptides are seen on the HPLC map of the protein carboxymethylated without prior DBP treatment. Following DBP labeling, the first two peaks are no longer observed (Fig. 3B). This indicates that the cysteines in these two peptides are selectively modified by DBP. In addition to the reactivity of the cysteine in peptide 1 to modification by DBP, this residue is also preferentially labeled by IAA as shown in Figure 3C. Isolation and sequencing of carboxymethylated peptides 1 and 2 reveal that the two peaks represent peptides that both contain the sequence VSQAACR (Table 2). As a result of the relatively low repetitive yield encountered upon Edman degradation of these small peptides, no residue assignment was obvious for sequence cycle 8, nor was one anticipated since observation of arginine at cycle 7 accounts for the C-terminal residue of a tryptic peptide. Thus, the reason for the difference in HPLC mobilities for peaks 1 and 2 is not readily apparent. However, amino acid composition analysis (Table 3) indicates that peptide 2 differs from peptide 1 by having an additional leucine residue. This suggests that the sequence of peptide 2 is actually VSQAACRL. It appears that this peptide results from variable and incomplete cleavage of the C-terminal leucine. In the Edman sequence analysis, washout of the sample from the reaction cartridge explains why PTH-leu is not observed in the eighth sequencing cycle.

Because a bifunctional reagent was used for modification and inactivation, one would expect to find two cysteine residues modified if intra-subunit cross-linking had occurred between two unique sulfhydryls. The failure to identify a second cysteine residue upon modification by DBP could be explained by the formation of a cross-link between identical residues in the two subunits. An SDS-PAGE gel (Fig. 4A) of purified avian HMG-CoA lyase before and after treatment with stoichiometric amounts of DBP demonstrates that following DBP modification the enzyme runs at a molecular weight of exactly twice that of the lyase monomer indicating that an inter-subunit cross-link has indeed been formed. In contrast, iden-

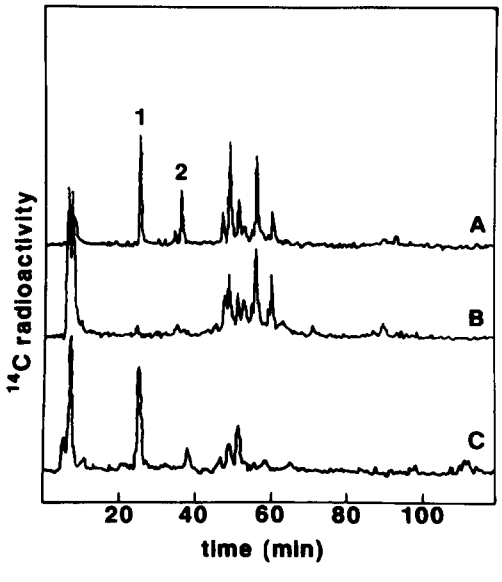


Fig. 3. Radioactivity profiles of ^{14}C-carboxymethylated tryptic peptides resolved by reverse-phase HPLC. A Synchropak RP-18 column (5 × 250 mm) equilibrated with 0.1% trifluoroacetic acid was loaded and eluted at a flow rate of 0.7 mL/min over 110 min using a linear gradient (0–70% acetonitrile in 0.1% trifluoroacetic acid) with a 5-min delay at 0% acetonitrile. A: Reduced HMG-CoA lyase was ^{14}C-carboxymethylated with ^{14}C-IAA (27,000 dpm/nmol) without prior treatment with 1,3-dibromo-2-propanone. B: Enzyme was incubated in the presence of DBP at 1.2 mol/mol enzyme protomer at 30 °C until enzyme activity was <5% initial activity. The reaction was quenched with DTT, and unmodified sulfhydryls were carboxymethylated with ^{14}C-IAA (27,000 dpm/nmol). C: Reduced enzyme was selectively titrated with ^{14}C-IAA (22,000 dpm/nmol) until <10% of initial enzyme activity remained. Peaks 1 and 2 correspond to peptides whose sequence and composition are presented in Tables 2 and 3, respectively.

Table 2. *Amino acid sequence of DBP-modified cysteine-containing peptides*

	Peptide 1		Peptide 2	
Cycle no.	Residue	pmol[a]	Residue	pmol[b]
1	Val	192.8	Val	179.2
2	Ser	100.5	Ser	69.8
3	Gln	52.9	Gln	33.0
4	Ala	46.7	Ala	24.0
5	Ala	42.1	Ala	16.0
6	CM-Cys[c]	23.5	CM-Cys[c]	9.3
7	Arg	7.4	Arg	4.6

[a] Initial yield was ≥87%; average repetitive yield was 63%.
[b] Initial yield was ≥81%; average repetitive yield was 51%.
[c] Assignment was made on the observation of a peak corresponding to PTH carboxymethyl-Cys.

Table 3. *Amino acid composition of DBP-modified peptides*

Amino acid	mol amino acid/mol peptide[a]		Sequence[b]
	Peptide 1	Peptide 2	
CM-Cys	0.8	1.0	1
Asx	0.3	0.3	0
Thr	0.2	0.2	0
Ser	1.6	1.2	1
Glx	1.4	1.2	1
Pro	0.1	0.2	0
Gly	0.7	0.8	0
Ala	1.7	2.1	2
Val	1.0	1.2	1
Met	n.d.[c]	n.d.	0
Ile	0.1	0.1	0
Leu[d]	0.3	1.0	0
Tyr	0.4	0.3	0
Phe	0.2	0.1	0
His	0.2	0.0	0
Lys	0.3	0.1	0
Arg	1.0	1.0	1

[a] Normalized to pmol Arg.
[b] Amino acids determined from Edman degradation (Table 2).
[c] n.d., not determined.
[d] Note discrepancy between peptides 1 and 2.

tical treatment of *P. mevalonii* HMG-CoA lyase does not produce a protein that runs as a dimer on an SDS-PAGE gel (Fig. 4B); similar results are obtained even when the bacterial enzyme is exposed to higher concentrations (20-fold excess with respect to enzyme protomer) of the modification reagent. Absence in the prokaryotic enzyme (Anderson & Rodwell, 1989) of a cysteine-containing peptide homologous to that targeted in the avian enzyme (Table 2) would appear compatible with these observations.

PDM modification

Because DBP is a highly reactive compound that is not necessarily sulfhydryl specific (Husain & Lowe, 1968) and the differential labeling approach only detects modified cysteines, the failure to identify two unique cysteine residues upon modification by DBP could be the result of modification of a functional group other than a sulfhydryl. Therefore, speculation that cross-linking of the enzyme occurs between identical cysteines on adjacent subunits required further testing. At neutral and acidic pH, phenylenedimaleimides represent bifunctional reagents documented to be selective for sulfhydryl modification (Reisler et al., 1974; Wells et al., 1980). When tested with avian HMG-CoA lyase, PDM was shown to be a potent inactivator (Table 1). As with DBP and the monofunctional sulfhydryl-directed reagents, the rate of inactivation is highly dependent upon the enzyme's redox state. Complete inactivation of the reduced enzyme could be achieved using stoichiometric amounts of inhibitor with respect to the enzyme dimer. Differential labeling of the enzyme with ^{14}C-IAA either prior to or following modification with PDM (Fig. 5) reveals an HPLC profile similar to that observed with DBP. Furthermore, SDS-PAGE of reduced avian enzyme treated with stoichiometric levels of PDM indicates that an inter-subunit cross-link is formed using the sulfhydryl-directed cross-linking reagent (Fig. 6, lane 2). Treatment of oxidized enzyme with identical concentrations of PDM fails to produce a species that runs as a dimer on an SDS-PAGE gel (Fig. 6, lane 3). These observations indicate that inter-subunit cross-linking is indeed occurring between two cysteine residues. Based upon the cross-linking span of PDM, the reactive sulfhydryls must be separated by less than 9 Å. Both the reactivity and close spatial proximity of these residues make this pair of cysteines likely candidates to undergo thiol/disulfide exchange.

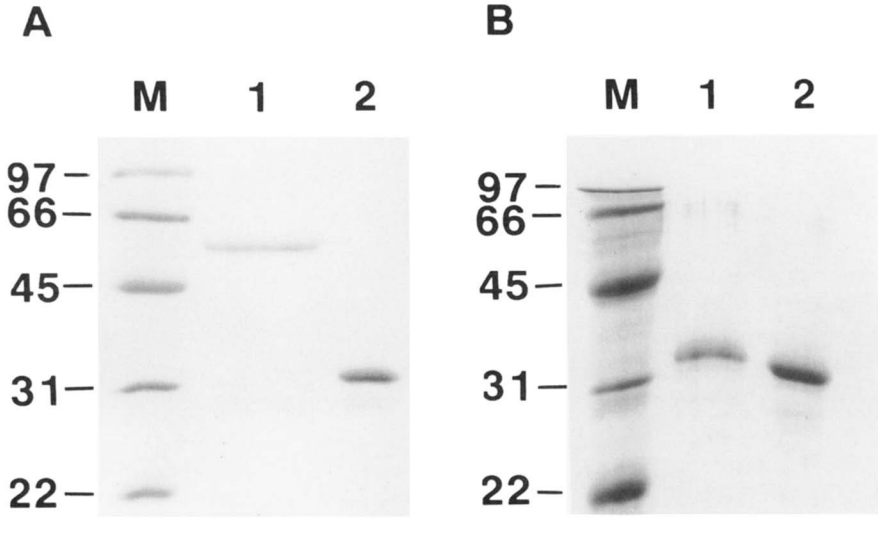


Fig. 4. SDS polyacrylamide gel of DBP-modified HMG-CoA lyase. **A**: Lane 1: Reduced avian HMG-CoA lyase that was incubated with DBP (1.1 mol/mol enzyme dimer) in 100 mM potassium phosphate buffer, pH 7.8, at 30 °C until <5% initial activity was observed. Lane 2: Unmodified affinity-purified avian HMG-CoA lyase. **B**: Lane 1: *Pseudomonas mevalonii* HMG-CoA lyase treated with DBP as described in A above. Lane 2: Unmodified *P. mevalonii* HMG-CoA lyase. Molecular weight markers (M) are rabbit muscle phosphorylase b, bovine serum albumin, hen egg white ovalbumin, bovine carbonic anhydrase, and soybean trypsin inhibitor.

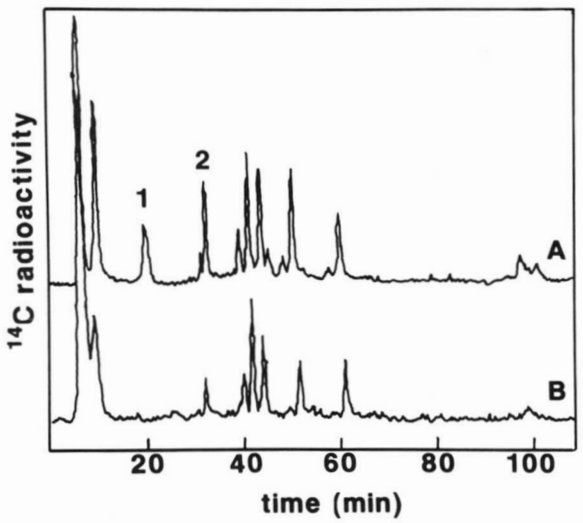


Fig. 5. Radioactivity profiles of ^{14}C-carboxymethylated tryptic peptides resolved by HPLC: Differential labeling with PDM. A: Reduced lyase was ^{14}C-carboxymethylated with ^{14}C-IAA (27,000 dpm/nmol) without prior treatment with PDM. B: Reduced lyase was incubated in the presence of PDM at 2 mol/mol enzyme dimer at 4 °C until enzyme activity was reduced by >95%. Unbound reagent was removed by centrifugal gel filtration and remaining sulfhydryls were carboxymethylated with ^{14}C-IAA (27,000 dpm/nmol). Chromatography conditions were as described in Materials and methods.

Discussion

The schematic model of HMG-CoA lyase shown in Figure 7 is consistent with the differential sensitivity of oxidized and reduced avian HMG-CoA lyase to inactivation by both sulfhydryl-directed reagents and the substrate analog 2-butynoyl-CoA as well as with the protein chemistry data identifying reactive proximal cysteines. In this model, disulfide formation occurs between identical cysteine residues in each of the two subunits. Upon disulfide formation, the juxtaposition of subunits changes such that HMG-CoA is rendered inaccessible to the catalytic apparatus. Consistent with the observation that oxidation does not markedly alter the K_I of 2-butynoyl-CoA, no perturbation of the CoA binding domain is depicted. Alternatively, a more subtle effect may account for the experimental data; oxidation could result in a change in subunit interaction such that the catalytic base (B:) is no longer properly aligned to abstract a proton from the 3-hydroxyl group of HMG.

The observation that ^{14}C-IAA will preferentially label the same cysteine that is modified by DBP and PDM suggests that the monofunctional reagents may be inactivating the enzyme by modifying the cysteine involved in thiol/disulfide exchange. As shown in Figure 7, such inactivation may be due to impaired access to the catalytic domain as a result of steric perturbations that coincide with modification. Such an interpretation would imply that the cysteine is in an extremely sensitive region of the enzyme since introduction of a relatively small methyl group by MNBS correlates with complete inactivation of the enzyme. In a number of cases, such as with phosphoribulokinase (Porter et al., 1990), the methylation of a nonessential cysteine results in an enzyme with reduced but still measurable activity. Although the modification of this residue may be sufficient to inactivate the protein, another sulfhydryl not involved in disulfide formation must also be accessible to modification since the oxidized enzyme shows limited susceptibility to inactivation by these reagents. An active site cysteine identified in the bacterial enzyme from affinity labeling studies (Hruz et al., 1992) is a likely candidate for this additional cysteine residue.

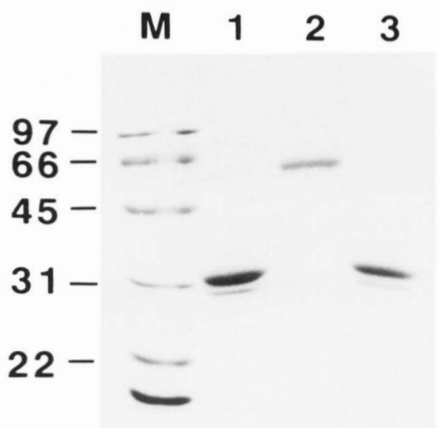


Fig. 6. SDS polyacrylamide gel of PDM-modified HMG-CoA lyase. Lane 1: Unmodified affinity-purified HMG-CoA lyase. Lane 2: Reduced avian lyase treated with PDM (1 mol/mol enzyme dimer). Lane 3: Oxidized avian lyase treated with PDM under the same conditions as the reduced enzyme. Molecular weight markers are shown in lane M.

The proximal reactive cysteines involved in subunit cross-linking can be mapped to the carboxy-terminus of the cDNA-deduced sequence of the avian enzyme (Mitchell et al., 1991). This region shows considerable homology with the carboxy-terminal sequence of the human enzyme with conservation of this critical cysteine residue. Studies of HMG-CoA lyase in human skin fibroblasts (Wanders et al., 1988) have indicated that, like the avian enzyme, this enzyme is stimulated in the presence of reduced thiols. Therefore, it is likely that any thiol/disulfide exchange reaction identified in the avian enzyme also occurs in the human enzyme. However, it is interesting to note that the protein sequence deduced from analysis of *P. mevalonii* DNA that encodes HMG-CoA lyase (Anderson & Rodwell, 1989) does not indicate a comparable cysteine in this C-terminal region. The fact that this cysteine is not conserved clearly argues against a catalytically essential role for this residue. Because the bacterial lyase, unlike the avian enzyme, is not susceptible to chemical cross-linking by DBP it is unlikely that the proposed regulatory mechanism is operative in the prokaryotic en-

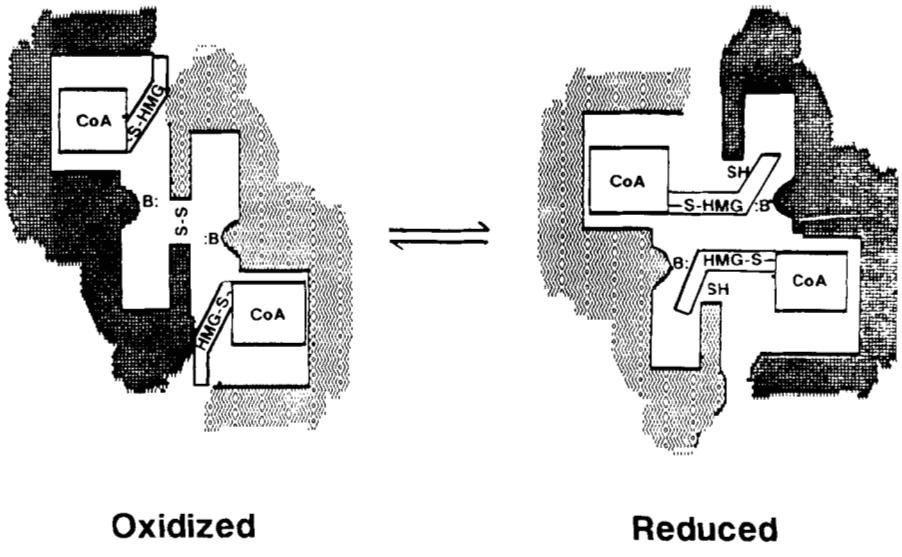


Fig. 7. Schematic model of avian HMG-CoA lyase.

zyme. Instead, *P. mevalonii* induces HMG-CoA lyase in order to grow in the presence of mevalonate as an energy source. Regulation of bacterial enzyme activity by modulation of enzyme protein level may make unnecessary the short-term regulatory mechanism proposed for the eukaryotic protein.

A number of other enzymes involved in carbohydrate and lipid metabolism have been shown to be modified by thiol/disulfide exchange in vitro (Ziegler, 1985). The modification that alters activity in many cases involves the formation of a mixed disulfide between a protein sulfhydryl and a low molecular weight thiol such as glutathione as shown in Equation 3. In other proteins, an intramolecular disulfide bond is formed between solvent-accessible cysteines (Equation 4).

$$\mathrm{EnzSH} + \mathrm{RSSR} \rightleftharpoons \mathrm{EnzSSR} + \mathrm{RSH}; \qquad (3)$$

$$\mathrm{Enz(SH)_2} + \mathrm{RSSR} \rightleftharpoons \mathrm{EnzS_2} + 2\mathrm{RSH}. \qquad (4)$$

Precedent for enzyme inactivation as a result of inter-subunit cross-linking of vicinal cysteines has been demonstrated in the dimeric enzyme II^{mtl} of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system (Roossien et al., 1986).

Although the physiologic significance of this thiol/disulfide exchange reaction remains to be evaluated for HMG-CoA lyase, the possibility for regulation via this mechanism is attractive. Several enzymes that catalyze key metabolic reactions in photosynthetic organisms (Buchanan, 1980) have been convincingly demonstrated to be regulated in vivo by such a process. The physiologic role of this type of modification in other enzymes remains speculative. Nevertheless, the evidence for a thiol/disulfide exchange reaction in avian HMG-CoA lyase, which influences enzymatic activity by controlling access to the active site, provides the first suggestion of potential regulation of this enzyme.

Materials and methods

Blue-Sepharose, NAD^+, NADH, PDM, and TPCK-trypsin were purchased from Sigma (St. Louis, Missouri). 2-Butynoic acid, 4-methylnitrobenzene sulfonate, and IAA were obtained from Aldrich (Milwaukee, Wisconsin). DBP was purchased from Lancaster. DTT was purchased from Research Organics, Inc. [1-^{14}C]IAA was purchased from New England Nuclear. Synchropak and Lichrospher RP-18 HPLC columns were purchased from Synchrom, Inc., and Merck, respectively. Coenzyme A and Sephadex G-50 were obtained from Pharmacia.

Enzyme purification and assays

Avian HMG-CoA lyase was isolated according to the procedure of Kramer and Miziorko (1980) with the following modification. Following hydroxylapatite chromatography, the enzyme was loaded onto a blue-Sepharose column equilibrated in 20 mM sodium phosphate, pH 7.0, containing 0.5 mM DTT and eluted with the same buffer containing 100 μM HMG-CoA. *Pseudomonas mevalonii* HMG-CoA lyase was isolated from *E. coli* containing the pT7-2600 plasmid reported by Anderson and Rodwell (1989) using the method of Narasimhan and Miziorko (1993). For assays with reduced HMG-CoA lyase, enzyme (350 U/mg) was preincubated in 40 mM Tris-Cl, pH 8.2, with 40 mM DTT for >30 min at 30 °C. Following complete reduction, the enzyme was placed in an anaerobic chamber and rapidly passed over a Sepha-

dex G-50 centrifugal desalting column (Penefsky, 1977) equilibrated in oxygen-free 100 mM potassium phosphate, pH 7.8. Incubation mixes of 100–200 μL were then set up in the individual 1.0-mL Eppendorf microcentrifuge tubes. The tubes were sealed with rubber septa and removed from the anaerobic chamber. Aliquots (10–20 μL) were removed using a gas-tight syringe and added directly to the citrate synthase-coupled assay mixture containing 5 mM DTT as described by Kramer and Miziorko (1980). For incubations with the oxidized enzyme, preincubations were carried out in the absence of DTT, and anaerobic procedures were not employed. In order to fully reactivate oxidized enzyme and obtain an accurate determination of maximal activity, all assays were initiated by the addition of 0.3 μmol HMG-CoA 10 min after the enzyme aliquot was added to the 1.0-mL DTT-containing assay mix.

Synthesis and purification of 2-butynoyl-CoA

2-Butynoyl-CoA was synthesized by the mixed anhydride procedure of Bernert and Sprecher (1977) as modified by Freund et al. (1985) in their synthesis of 2-octynoyl-CoA. Freshly redistilled triethylamine (150 μmol) and 150 μmol ethylchloroformate were added to 125 μmol of 2-butynoic acid in 1.0 mL benzene. After stirring for 1 h at 20 °C under nitrogen, the benzene, triethylamine, and ethylchloroformate were removed by evaporation. The residue was dissolved in 2.5 mL dry tetrahydrofuran, and the insoluble salts were pelleted by centrifugation.

Ten milligrams CoA (Li^+ salt; 12 μmol) was dissolved in 0.5 mL ice-cold nitrogen-purged H_2O and the pH adjusted to 8.0 by dropwise addition of 1.0 M $KHCO_3$. Anhydride (25–36 μmol) was added and the pH maintained at 8.0 with additional $KHCO_3$. After stirring under N_2 for 3–5 min, reaction completeness was determined by a negative nitroprusside test, which became positive following addition of methanolic NaOH. The pH was then adjusted to 2.0 by dropwise addition of 0.5 M HCl to allow extraction of the free acid and any unreacted reagent with diethyl ether. Following extraction, the solution was readjusted to pH 4.5 with $KHCO_3$.

Further purification was performed using an RP-18 Sep-pak cartridge equilibrated in 50 mM ammonium acetate, pH 5.3. After loading the sample and washing with 50 mM ammonium acetate, the acyl-CoA was eluted with 10% (v/v) acetonitrile/50 mM ammonium acetate, pH 5.3. HPLC analysis on a Lichrospher RP-18 column equilibrated in 25% methanol/100 mM sodium phosphate, pH 3.0 (flow rate = 1.0 mL/min), resulted in a single A_{260} peak eluting at 6.9 min, indicating that the product was free of CoA or CoA dimer. Concentrations of 2-butynoyl-CoA were calculated from A_{260} using $\epsilon = 20.9\ M^{-1}\ cm^{-1}$ as reported by Freund et al. (1985) for 2-octynoyl-CoA.

Protein modification and trypsin digestion

Modification of avian lyase with dibromopropanone was performed by incubating 42 μg (1.6 nmol subunit) of reduced enzyme (350 U/mg) with 2.0 nmol recrystallized 1,3-dibromo-2-propanone at 30 °C in the dark. When <5% of the initial enzyme activity remained, the reaction was quenced by the addition of 1 μmol DTT. To label the cysteines unmodified by dibromopropanone, the enzyme was precipitated from the original modification mixture by the addition of 20 volumes ice-cold acetone. After the sample was pelleted in a microcentrifuge for 15 min, the supernatant was removed and the pellet resuspended in 100 μL potassium phosphate, pH 7.8, containing 6 M urea. The solubilized enzyme was then incubated with 1.2 mM ^{14}C-IAA (27,000 dpm/nmol) for 35 min at 30 °C in the dark. The reaction was then quenced by the addition of 5 μL mercaptoethanol. Urea and unbound reagents were removed by precipitating the enzyme with 20 volumes ice-cold acetone and washing the pellet three times with acetone. In a control experiment, a sample of lyase (1.0 nmol) that had not been incubated with DBP was carboxymethylated under identical conditions. Enzyme pellets were suspended in 200 μL 100 mM ammonium bicarbonate (pH 8.2) and incubated with 1% (w/w) TPCK-treated trypsin at 37 °C. After 2 h, an additional 1% trypsin was added and the digestion allowed to proceed for an additional 2 h at 37 °C. The suspension gradually became clear with digestion. Samples were frozen on dry ice and stored at −20 °C. Prior to HPLC analysis, the samples were lyophilized to remove bicarbonate and dissolved in 5% trifluoroacetic acid. For isolation and sequencing of cysteine-containing peptides, 480 μg (18 nmol) of affinity-purified HMG-CoA lyase was ^{14}C-carboxymethylated as described above.

PDM-modified enzyme was prepared by incubating 60 μg (2.0 nmol) reduced avian lyase with 1.0 nmol PDM at 0 °C in 100 mM potassium phosphate pH 7.0. After 10 min, >95% inactivation had been achieved and the reaction was terminated by passing the sample over a Sephadex G-50 centrifugal desalting column equilibrated in 100 mM potassium phosphate, pH 7.8. Urea was added to a final concentration of 6 M and the enzyme was carboxymethylated and digested with trypsin as described for the DBP-treated sample.

Selective carboxymethylation of reduced avian lyase was performed by incubating 60 μg of enzyme (400 U/mg) with 100 mM Tris-Cl, pH 8.0, and 1 mM DTT for 10 min at 30 °C. ^{14}C-IAA (2 mM; 22,000 dpm/nmol) was added, and the incubation was continued in the dark at 23 °C until less than 10% of the original enzyme activity remained. The reaction was quickly quenched by the addition of 5 μL β-mercaptoethanol. Following acetone precipitation, the enzyme pellet was solubilized in 6 M urea and 100 mM Tris-Cl, pH 8.0. To modify remaining sulfhydryls, unlabeled IAA was added to a final concen-

tration of 2.5 mM, and the mixture was allowed to incubate at 30 °C for 30 min. The reaction was quenched by the addition of 2 μL β-mercaptoethanol. Acetone precipitation and trypsin digestion were then performed as described above.

Peptide isolation and analysis

Analysis of ^{14}C-carboxymethylated tryptic peptides (100–200 pmol) was performed on a Synchropak RP-18 column (5 × 250 mm) using a linear gradient (0–70% acetonitrile/0.1% trifluoroacetic acid) over 110 min at a flow rate of 0.7 mL/min. Absorbance at 215 nm and ^{14}C radioactivity were recorded in tandem using an Altex flow cell-equipped Hitachi spectrophotometer (Model 100-10) and Radiomatic Flo-one/β radioactivity detector.

For preparative isolation of the cysteine containing peptides, ^{14}C-carboxymethylated tryptic peptides (4 nmol in 5% trifluoroacetic acid) were chromatographed as described above. Absorbance at 215 nm was monitored and the effluent collected in 0.5-min fractions. Five-microliter aliquots were removed for radioactivity determination by liquid scintillation counting. Radioactive fractions from the peptide that eluted at 34 min were combined from two successive HPLC runs and rechromatographed using a linear gradient from 5 to 35% acetonitrile/0.1% trifluoroacetic acid over 100 min at a flow rate of 0.7 mL/min. UV absorbance profiles of the isolated peptides suggested homogeneous samples. Homogeneity was verified upon Edman degradation of approximately 140 pmol of the isolated peptides using an automated protein sequencer (Applied Biosystems Model 477A) equipped with an on-line PTH analyzer (Model 120A). Amino acid composition (100 pmol sample) was determined using a Beckman model 6700 amino acid analyzer equipped for ninhydrin detection. Cysteine was detected as its carboxymethylated derivative. Samples were hydrolyzed in 5.7 N HCl for 20 h at 110 °C under nitrogen and reduced pressure.

Acknowledgments

We thank Dr. Victor Rodwell for providing us with the pT7-2600 plasmid and Dr. Chakravarthy Narisimhan for the gift of purified recombinant *P. mevalonii* HMG-CoA lyase. We are grateful to Dr. Claire Kennedy and Dr. Helmut Beinert for the use of their anaerobic chamber. Dr. Liane Mende-Mueller (Medical College of Wisconsin Protein/Nucleic Acid Shared Facility) performed the composition and sequence analyses of the DBP-modified cysteine containing peptides. We appreciate Dr. Grant Mitchell's timely communication and discussion of the cDNA sequence data.

This work was supported, in part, by a grant from the National Institutes of Health (DK-21491). P.W.H. is a recipient of a predoctoral fellowship from the American Heart Association—Wisconsin Affiliate.

References

Anderson, D.H. & Rodwell, V.W. (1989). Nucleotide sequence and expression in *Escherichia coli* of the 3-hydroxy-3-methylglutaryl Coenzyme A lyase gene of *Pseudomonas mevalonii*. *J. Bacteriol. 171*, 6468–6472.

Bachawat, B.K., Robinson, W.G., & Coon, M.J. (1955). The enzymatic cleavage of β-hydroxy-β-methylglutaryl Coenzyme A to acetoacetate and acetyl Coenzyme A. *J. Biol. Chem. 216*, 727–736.

Bernert, J.T. & Sprecher, H. (1977). An analysis of partial reactions in the overall chain elongation of saturated and unsaturated fatty acids by rat liver microsomes. *J. Biol. Chem. 252*, 6736–6744.

Buchanan, B.B. (1980). Role of light in the regulation of chloroplast enzymes. *Annu. Rev. Plant Physiol. 31*, 341–374.

Faull, K.F., Bolton, P., Halpern, P., Hammond, J., Danks, D.M., Hahnel, R., Wilkinson, S.P., Wysocki, S.J., & Mansters, P.L. (1976). A patient with defect in leucine metabolism. *N. Engl. J. Med. 294*, 1013.

Fendrich, G. & Abeles, R.H. (1982). Mechanism of action of butyryl-CoA dehydrogenase: Reactions with acetylenic, olefinic and fluorinated substrate analogs. *Biochemistry 21*, 6685–6695.

Frerman, F.E., Miziorko, H.M., & Beckmann, J.D. (1980). Enzyme-activated inhibitors, alternate substrates, and a dead end inhibitor of the general acyl-CoA dehydrogenase. *J. Biol. Chem. 255*, 11192–11198.

Freund, K., Mizzer, J., Dick, W., & Thorpe, C. (1985). Inactivation of general acyl-CoA dehydrogenase from pig kidney by 2-alkynoyl Coenzyme A derivatives: Initial aspects. *Biochemistry 24*, 5996–6002.

Gibson, K.M., Breuer, J., & Nyhan, W.L. (1988). 3-Hydroxy-3-methylglutaryl-Coenzyme A lyase deficiency: Review of 18 reported patients. *Eur. J. Pediatr. 148*, 180–186.

Holland, P.C., Clark, M.G., & Bloxham, D.P. (1973). Inactivation of pig heart thiolase by 3-butynoyl Coenzyme A, 3-pentynoyl Coenzyme A and 4-bromocrotonyl Coenzyme A. *Biochemistry 12*, 3309–3315.

Hruz, P.W., Narasimhan, C., & Miziorko, H.M. (1992). 3-Hydroxy-3-methylglutaryl Coenzyme A lyase: Affinity labeling of the *Pseudomonas mevalonii* enzyme and assignment of cysteine-237 to the active site. *Biochemistry 31*, 6842–6847.

Husain, S.S. & Lowe, G. (1968). Evidence for histidine in the active site of ficin and stem-bromelain. *Biochem. J. 110*, 53–57.

Kramer, P. & Miziorko, H. (1980). Purification and characterization of avian liver 3-hydroxy-3-methylglutaryl-Coenzyme A lyase. *J. Biol. Chem. 255*, 11023–11028.

Kramer, P. & Miziorko, H. (1983). 3-Hydroxy-3-methylglutaryl-CoA lyase: Catalysis of acetyl Coenzyme A enolization. *Biochemistry 22*, 2353–2357.

Mitchell, G.A., Roberts, M.F., Hruz, P., Fontaine, G., Behnke, C., Mende-Muller, L., Gibson, K.M., & Miziorko, H. (1991). Hydroxymethylglutaryl CoA lyase (HL): Initial molecular characterization in man. *Am. J. Hum. Genet. 49*, 101.

Narasimhan, C. & Miziorko, H.M. (1993). 3-Hydroxy-3-methylglutaryl Coenzyme A lyase: Characterization of the isolated recombinant protein and investigation of the enzyme's cation requirements. *Biochemistry*, in press.

Penefsky, H.S. (1977). Reversible binding of P_i by beef heart mitochondrial adenosine triphosphatase. *J. Biol. Chem. 252*, 2891–2899.

Porter, M.A., Potter, M.D., & Hartman, F.C. (1990). Affinity labeling of spinach phosphoribulokinase subsequent to S-methylation at Cys-16. *J. Protein Chem. 9*, 445–451.

Prasana, P. & Holmlund, C.E. (1987). Identification in *Tetrahymena pyriformis* of 3-hydroxy-3-methylglutaryl Coenzyme A lyase: Its purification and properties. *Int. J. Biochem. 19*, 385–389.

Reisler, E., Burke, M., Himmelfarb, S., & Harrington, W. (1974). Spatial proximity of the two essential sulfhydryl groups of myosin. *Biochemistry 13*, 3837–3840.

Robinson, A.M. & Williamson, D.H. (1980). Physiological roles of ketone bodies as substates and signals in mammalian tissues. *Physiol. Rev. 60*, 143–187.

Roossien, F.F., van Es-Spiekman, W., & Robillard, G.T. (1986). Dimeric enzyme II^{mtl} of the *E. coli* phosphoenolpyruvate-dependent phosphotransferase system; cross-linking studies with bifunctional sulfhydryl reagents. *FEBS Lett. 196*, 284–290.

Scher, D.S. & Rodwell, V.W. (1989). 3-Hydroxy-3-methylglutaryl co-

enzyme A lyase from *Pseudomonas mevalonii*. *Biochim. Biophys. Acta 1003*, 321–326.

Stegink, L.D. & Coon, M.J. (1968). Stereospecificity and other properties of highly purified β-hydroxy-β-methylglutaryl Coenzyme A cleavage enzyme from bovine liver. *J. Biol. Chem. 243*, 5272–5279.

Stoops, J.K. & Wakil, S.J. (1981). Animal fatty acid synthetase; a novel arrangement of the β-ketoacyl synthetase sites comprising domains of the two subunits. *J. Biol. Chem. 256*, 5128–5133.

Wanders, R.J., Schutgens, R.B., & Zoeters, P.H. (1988). 3-Hydroxy-3-methylglutaryl-CoA lyase in human skin fibroblasts: Study of its properties and deficient activity in 3-methyl-3-glutaric aciduria patients using a simple spectrophotometric assay. *Clin. Chim. Acta 171*, 95–101.

Wells, J.A., Knoeber, C., Sheldon, M.C., Werber, M.M., & Yount, R.G. (1980). Cross-linking of myosin subfragment 1: Nucleotide-enhanced modification by a variety of bifunctional reagents. *J. Biol. Chem. 255*, 11135–11140.

Ziegler, D.M. (1985). Role of reversible oxidation-reduction of enzyme thiols–disulfides in metabolic regulation. *Annu. Rev. Biochem. 54*, 305–329.