Inactivation of 3-Hydroxy-3-methylglutaryl-CoA Synthase and Other Acyl-CoA-Utilizing Enzymes by 3-Oxobutylsulfoxyl-CoA†

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Received November 4, 1996⊗

ABSTRACT: 3-Oxobutylsulfoxyl-CoA has been produced by oxidation of *S*-3-oxobutyl-CoA, the thioether analog of acetoacetyl-CoA. Avian hydroxymethylglutaryl-CoA (HMG-CoA) synthase is inactivated by oxobutylsulfoxyl-CoA in a time-dependent fashion. Protection against inactivation is afforded by the substrate, acetyl-CoA, suggesting that inactivation involves modification of the enzyme's active site. Pretreatment of HMG-CoA synthase with the inactivator blocks the enzyme's ability to form Michaelis and acetyl-S-enzyme intermediates, supporting the hypothesis that modification is active-site directed. Incubation of enzyme with oxobutylsulfoxyl-[^{32}P]CoA followed by precipitation with trichloroacetic acid indicates that inactivation correlates with stoichiometric formation of a covalent adduct between enzyme and a portion of the inactivator that includes the CoA nucleotide. The observation of reagent partitioning suggests that HMG-CoA synthase catalyzes conversion of oxobutylsulfoxyl-CoA into a reactive species that modifies the protein. Treatment of inactivated enzyme with DTT or other mercaptans restores enzyme activity and reverses the covalent modification with release of CoASH. Oxobutylsulfoxyl-CoA inactivates β-ketothiolase and HMG-CoA lyase in a process that is also reversed by DTT. These three enzymes all contain active site cysteines, suggesting that inactivation results from disulfide formation between a cysteine and the CoA moiety of the inhibitor. The data are consistent with the hypothesis that enzymatic cleavage of oxobutylsulfoxyl-CoA results in the transient formation of a sulfenic acid derivative of CoA which subsequently reacts to form a stable disulfide linkage to protein.

The role of Coenzyme A in activating carboxylic acids is well illustrated in a variety of the pathways of intermediary metabolism. Details concerning how acyl-CoAs support such metabolism have been elucidated in numerous enzymological studies. One approach to such investigations has involved the development of acyl-CoA analogs in which the chemistry of the thioester linkage has been modified. For example, Stewart and Wieland (1978) eliminated the sulfur atom in preparing acetonyldethio-CoA, which they demonstrated to be an inhibitor of citrate synthase. Recently, Drueckhammer and co-workers have synthesized related dethio analogs that are much more potent inhibitors of citrate synthase; these analogs (Usher et al., 1994; Schwartz et al., 1995) contain free carboxy or amide groups at their terminus. More commonly, the sulfur atom has been retained and the thioester carbonyl has been modified or replaced in design of acyl-CoA analogs. Anderson and colleagues (Wlassics et al., 1988; Anderson et al., 1990) reported a series of dithioester analogs in which sulfur replaces the thioester carbonyl oxygen to produce compounds that, in several cases, function as alternative substrates. Replacement of the carbonyl moiety with a methylene group produces thioether analogs which can efficiently function as alternative substrates (Miziorko et al., 1982), allosteric effectors (Blaschkowski et al., 1979), reversible inhibitors (Rubenstein & Dryer, 1980; Bayer et al., 1981), or irreversible inactivators (Owens & Barden, 1978) of acyl-CoA utilizing enzymes. A recent and interesting extension of the thioether analogs involves oxidation of the thioether sulfur to either a sulfoxyl or sulfonyl moiety (Sixt & Eggerer, 1992; Karl et al., 1995). Several of these compounds are reported to function as reversible inhibitors that exhibit higher affinity ($\sim 10^3$-fold) than the parent thioethers.

HMG-CoA synthase catalyzes the formation of a key intermediate in cholesterogenic and ketogenic pathways in a three-step process (Miziorko & Lane, 1977).

$$\text{ESH} + \text{acetyl-CoA} \rightleftharpoons \text{acetyl-SE} + \text{CoASH} \quad (1)$$

$$\text{acetoacetyl-CoA} + \text{acetyl-SE} \rightarrow \text{ES-HMG-CoA} \quad (2)$$

$$\text{ES-HMG-CoA} + \text{H}_2\text{O} \rightarrow \text{ESH} + \text{HMG-CoA} \quad (3)$$

In pursuit of our investigations on HMG-CoA synthase, we have utilized a mechanism-based inhibitor, 3-chloropropionyl-CoA, that alkylates an active site cysteine-129 (Miziorko & Behnke, 1985a,b) but contains a thioester linkage that eventually hydrolyzes to release CoASH. Studies on the structure/function correlations that account for enzymatic synthesis of HMG-CoA confirm the function of the active site cysteine-129 in formation of the acetyl-S-enzyme reaction intermediate (eq 1; Vollmer et al., 1988; Misra et al., 1993). Additionally, histidine-264 has recently been implicated in stabilizing binding of the substrate, acetoacetyl-CoA (eq 2; Misra & Miziorko, 1996). The identity of the active site base that deprotonates the acetyl-S-enzyme intermediate to facilitate condensation with acetoacetyl-CoA (eq 2)

† This work was supported, in part, by a research grant to H.M.M. from NIH (DK21491) and a predoctoral fellowship award to H.A.C. (American Heart Association/Wisconsin Affiliate).

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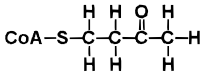
⊗ Abstract published in *Advance ACS Abstracts,* February 1, 1997.

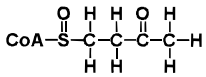
[1] Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HPLC, high-pressue liquid chromatography; DTT, dithiothreitol.

S0006-2960(96)02751-1 CCC: $14.00

Chart 1



3-Oxobutyl-CoA



3-Oxobutylsulfoxyl-CoA

remains to be determined. For a variety of mechanistic studies, including further tests of the active site histidine, availability of a tight binding analog that contains a stable linkage to CoA would be useful. Since *S*-3-oxobutyl-CoA, the thioether analog of acetoacetyl-CoA, is an alternative substrate for HMG-CoA synthase (Miziorko et al., 1982), the possibility that an oxidized form of this analog, namely, 3-oxobutylsulfoxyl-CoA, might function as a high-affinity inhibitor seemed worthy of investigation. This report describes the preparation and evaluation of this analog, which exhibits novel inhibitory properties with HMG-CoA synthase, β-ketothiolase, and HMG-CoA lyase.

EXPERIMENTAL PROCEDURES

Materials

Homogeneous recombinant avian cytosolic HMG-CoA synthase was prepared by the method of Misra et al. (1993). Homogeneous wild-type and C237A *Pseudomonas mevalonii* HMG-CoA lyases were prepared according to the method of Narasimhan and Miziorko (1992). Homogenous *Zoogloea ramigera* β-ketoacyl thiolase (Palmer et al., 1991) was a generous gift of Dr. Vernon Anderson. 3′-Nucleotidase from rye grass was purchased from Sigma (St. Louis, MO). Dephospho-CoA kinase (Worral & Tubbs, 1983) was a gift from Drs. Dale Drueckhammer and Ben Schwartz. Coenzyme A was obtained from Calbiochem (La Jolla, CA). [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from DuPont NEN Research Products (Boston, MA). All other reagents were purchased from Aldrich (Milwaukee, WI) or Bio-Rad (Richmond, CA).

Methods

Synthesis of 3-Oxobutyl-Coenzyme A. 3-Oxobutyl-CoA was typically prepared by adding a 3-fold molar excess of methyl vinyl ketone to a solution of CoASH (30 μmol; trilithium salt), adjusted with LiOH to pH 8.0−8.5 at 0 °C. After the free sulfhydryl was depleted, as judged by a negative response to nitroprusside reagent, the pH was adjusted to 4.0−5.0 with HCl and the entire reaction mixture was taken to dryness to remove any unreacted methyl vinyl ketone. The dry 3-oxobutyl-CoA was dissolved in minimal methanol and was then precipitated as the lithium salt by the addition of ice-cold acetone (5 volumes over methanol). Reverse-phase HPLC [Spherisorb S5 ODS2; 100 mM sodium phosphate (pH 3.0)/methanol (80:20)] analysis of the product revealed one nucleotide peak, suggesting a high degree of purity. Mass spectroscopy analysis revealed a parent ion peak of *m*/*z* 838.1, which is the molecular weight of 3-oxobutyl-CoA. Enzymatic assay demonstrated the product to be a substrate for HMG-CoA synthase, providing additional verification of its identity as 3-oxobutyl-CoA (Chart 1; Miziorko et al., 1982; Misra & Miziorko, 1996).

Synthesis of 3-Oxobutylsulfoxyl-CoA. 3-Oxobutylsulfoxyl-CoA was synthesized using a variation of the method of Sixt and Eggerer (1992). A 1.3-fold molar excess of chloramine-T was added to a solution of 3-oxobutyl-CoA; the mixture was stirred at room temperature for 0.5 h. The sample was taken to dryness, and the nucleotide species precipitated as the lithium salt from methanol/acetone (1:5). HPLC analysis revealed a single peak distinct from that of the 3-oxobutyl-CoA starting material, indicating that the oxidation reaction had gone to completion and suggesting a high level of purity in the isolated nucleotide. Mass spectroscopy analysis of the sample indicated a parent ion with an *m*/*z* of 854.1. The 16 amu increase over that of 3-oxobutyl-CoA precursor corresponds to the incorporation of a single oxygen atom, which suggests that over-oxidation to the sulfone did not occur. FTIR analysis of the product was performed in KCl pellets. Comparison between 3-oxobutyl-CoA and its sulfoxide analog revealed a single new peak for the sulfoxide at 1054 cm^{-1}. This is further evidence that over-oxidation to the sulfone did not occur since sulfoxides show strong bands in the 1050−1060 cm^{-1} region whereas sulfones show strong bands in the 1300−1320 and 1140−1160 cm^{-1} regions (Durst, 1978).

Synthesis of 3-Oxobutylsulfoxyl-[3′-^{32}P]CoA. Dephospho-CoA was prepared according to the method of Wang et al. (1954). 97 μmol of CoASH (lithium salt) was dissolved in 1.4 mL of 25 mM Li_2CO_3, pH 8.0, prior to addition of diamide (105 μmol) and incubation for 2 h at room temperature. The resulting CoA dimer was precipitated twice from 1:5 methanol/acetone with 54 μmol being recovered. The precipitate was dissolved in 2.5 mL of 15 mM Li_2CO_3 (final pH 8.5), and 0.8 unit of 3′-nucleotidase was added. After 15 h at 37 °C, the conversion to dephospho-CoA was 70% complete as determined by HPLC. An additional 0.4 unit of enzyme was added; the reaction was allowed to continue for 2 h, at which time complete conversion had been accomplished. 9 μmol of the dephospho-CoA dimer was incubated with 40 μmol of DTT for 2 h at 30 °C to convert the dimer to free dephospho-CoA.

[3′-^{32}P]CoASH was made from dephospho-CoA as described by Miziorko and Behnke (1985a). 18 μmol of dephospho-CoA was added to a 4 mL reaction mixture containing 47.6 μmol of [γ-^{32}P]ATP (24 000 dpm/nmol) and 50 milliunits of dephospho-CoA kinase (Worrall & Tubbs, 1983) in 100 mM Tris-HCl, pH 8.2, 100 mM $MgCl_2$, and 8 mM DTT. After a 4 h incubation at 37 °C, an additional 17 milliunits of enzyme was added and the sample was incubated for another 11 h, at which time all of the nucleotide had been converted to [3′-^{32}P]CoA, as determined by HPLC. The product was purified by column chromatography on DE52-cellulose (1 × 50 cm) using a 10−200 mM LiCl linear gradient containing 3 mM HCl. The [3′-^{32}P]CoASH-containing fractions (verified by HPLC) were pooled and evaporated to dryness. The residue was dissolved in cold H_2O and immediately converted to 3-oxobutyl-CoA and its sulfoxide as described above.

Protein and Enzymatic Assays. Determination of protein concentration was done following the method of Bradford (1976) using bovine serum albumin as the standard. Measurements of HMG-CoA synthase activity employed the standard spectrophotometric assay (Clinkenbeard et al., 1975) using mixtures containing either 20 or 50 μM acetoacetyl-CoA. HMG-CoA lyase activity was measured using the citrate synthase-coupled assay of Stegink and Coon (1968) as modified by Kramer and Miziorko (1980). β-Ketoacyl thiolase activity was determined by following the cleavage

of acetoacetyl-CoA according to the method of Thompson et al. (1989).

Efficiency of HMG-CoA Synthase Inactivation by 3-Oxobutylsulfoxyl-CoA. The partition ratio of HMG-CoA synthase was determined with 3-oxobutylsulfoxyl-CoA. Enzyme (1.22 nmol) was incubated at room temperature at various concentrations of inhibitor in 50 mM Tris-HCl, pH 8.2. Activity estimates were performed 2 min after the initiation of inactivation. The partition ratio was calculated by subtracting 1.0 from the turnover number obtained from the *x*-intercept of the plot of % activity vs the initial ratio of inhibitor to enzyme (I/E) (Silverman, 1995).

Covalent Modification of HMG-CoA Synthase with 3-Oxobutylsulfoxyl-[γ-^{32}P]CoA. The labeling reaction contained the specified amounts of enzyme and 3-oxobutylsulfoxyl-[γ-^{32}P]CoA in 100 mM Tris-HCl, pH 8.2. When the residual activity was at the desired level, 0.05−0.1 mL aliquots of the reaction mix were added to 0.9 mL of chilled 10% trichloroacetic acid. The precipitated protein samples were centrifuged, resuspended in 1.0 mL of 10% trichloroacetic acid, transferred to 2.5 cm glass fiber filters, and washed as previously described (Miziorko et al., 1975). Radioactivity was determined by liquid scintillation counting.

Isolation and Identification of the DTT-Released Adduct. HMG-CoA synthase (1.7 μmol; 99 mg) was added to a solution of 18.8 μmol of 3-oxobutylsulfoxyl-CoA in 100 mM sodium phosphate, pH 7.0, at room temperature. In a parallel reaction, HMG-CoA synthase (10 nmol) was incubated at room temperature in 100 mM sodium phosphate, pH 7.0, at room temperature to serve as the fully active enzyme control. At the specified times, aliquots of each were assayed for enzymatic activity by standard spectrophotometric procedures (Clinkenbeard et al., 1975) using mixtures containing 20 μM acetoacetyl-CoA. After 25 h, 28% of the control activity remained. At this point, an additional 5 μmol of 3-oxobutylsulfoxyl-CoA was added. At 48 h, less than 10% of the control activity persisted.

To separate the inactivated protein from the free inhibitor, the reaction mix was applied to a G-50 size exclusion column (1.5 × 100 cm) and was eluted with 25 mM potassium phosphate, pH 7.0. Two peaks with A_{280} were pooled and analyzed for protein content. Only the earliest peak fractions contained protein; these were combined into a pool containing 1.7 μmol of enzyme (and accounting for the 10% residual activity) in 39 mL of 25 mM potassium phosphate, pH 7.0. DTT (78 μmol) was added to this sample prior to incubation at 4 °C for 17 h, which restored 31% of the control activity. Subsequent addition of 700 μmol of DTT and incubation for 4 h at room temperature resulted in restoration of activity to 74% of the control value.

The protein was removed by passing the reaction mix through a centrifugal concentration cone at 1090*g*. The cone was repeatedly rinsed with 25 mM potassium phosphate, pH 7.0. The resulting filtrates were added to the original deproteinized sample and applied to a DE52 anion exchange column (1 × 20 cm) equilibrated in 20 mM LiCl/3 mM HCl. The effluent was monitored spectrophotometrically for absorbance at 232, 250, 257, and 280 nm. DTT eluted in the breakthrough fractions. The column was eluted with a 200 mL linear gradient ranging from 20 to 200 mM LiCl in 3 mM HCl followed by a 1 M LiCl/3 mM HCl wash. Only one nucleotide-containing peak (λ_{max} at 257 nm) was eluted at 60 mM LiCl. These fractions were pooled, and the sample reduced in volume to 1.5 mL had a concentration based on A_{257} (ϵ = 15.4 mM^{-1}) of 0.59 mM. Reverse-phase HPLC analysis revealed a single peak with a retention time identical to that of CoASH. To verify the released adduct's identity, a CoASH-specific end point assay utilizing phosphotransacetylase was employed (Bergmeyer, 1985). Aliquots of the sample were added to a reaction containing 5 milliunits of phosphotransacetylase, 70 mM Tris-HCl, pH 7.6, and 10 mM acetyl phosphate. The change in A_{233} (ϵ_{max} = 4.44 mM^{-1}) was used to calculate the amount of thioester formed as all of the CoASH was converted to acetyl-CoA. The concentration of CoASH in the sample was 0.53 mM, accounting for 90% of the nucleotide released from the adduct with protein.

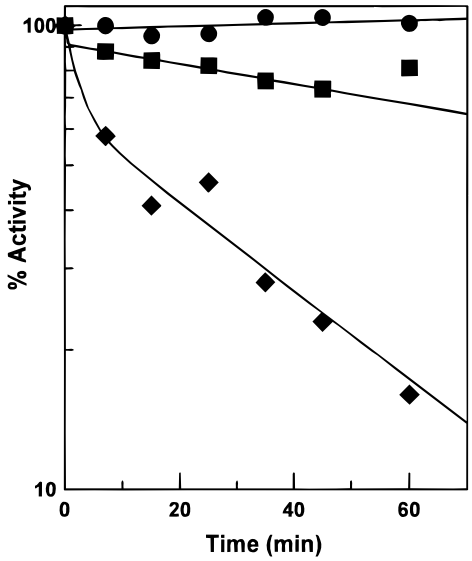


FIGURE 1: Time-dependent inactivation of HMG-CoA synthase and protection from inactivation by acetyl-CoA. HMG-CoA synthase (16 μM in 0.12 mL of Tris-HCl, 100 mM, pH 8.2) was incubated at room temperature in the absence of (●) or in the presence of 0.58 mM 3-oxobutylsulfoxyl-CoA (◆). A protection experiment was performed by including 4.3 mM acetyl-CoA with 0.58 mM 3-oxobutylsulfoxyl-CoA (■). At the indicated times aliquots were removed and assayed for activity as described in Methods.

A control experiment was conducted in which 1.7 μmol of 3-oxobutylsulfoxyl-CoA was incubated in the presence of 778 μmol of DTT (conditions identical to those employed to release nucleotide from the protein-containing sample). The nucleotide was purified as described above using DE52 anion exchange chromatography. The single nucleotide that eluted from the column was characterized by an HPLC retention time identical to that measured using 3-oxobutylsulfoxyl-CoA starting material. The phosphotransacetylase end point assay for CoASH revealed that less than 1% of the nucleotide in this control sample was attributable to CoASH.

RESULTS

Covalent Modification of HMG-CoA Synthase by Oxobutylsulfoxyl-CoA. When HMG-CoA synthase was incubated with oxobutylsulfoxyl-CoA, a time-dependent loss of activity resulted (Figure 1), clearly indicating a mode of inhibition that involved more than simple reversible binding of this analog. Inclusion of substrate acetyl-CoA in the incubation mix afforded considerable protection against activity loss (>10-fold increase in $t_{1/2}$), supporting the expectation that this substrate analog would bind to the enzyme's active site.

Table 1: Stoichiometry of HMG-CoA Synthase Active-Site Occupancy by Acetyl-CoA and 3-Oxobutylsulfoxyl-CoA

HMG-CoA synthase sample	% activity	mol of [^{14}C]acetyl-CoA/mol of subunit[a]	mol of [^{32}P]CoA/mol of subunit[b]
untreated prior to acetyl-CoA incubation	100	0.68	
inactivated prior to acetyl-CoA incubation[c]	9.5	0.016	
inactivated[d]	10		0.9

[a] Determined by rapid centrifugal gel filtration (Misra et al., 1993) after incubation of enzyme in 200 μM [^{14}C]acetyl-CoA (15 571 dpm/nmol). [b] Determined by trichloroacetic acid precipitation as described in Methods. [c] HMG-CoA synthase (5.2 nmol) was inactivated with 156 nmol of 3-oxobutylsulfoxyl-CoA in 0.3 mL of 100 mM Tris-HCl, pH 8.2. The inactivated protein was passed through a G-50 centrifugal gel filtration column to remove free inhibitor; aliquots were evaluated for acetyl-CoA binding. [d] HMG-CoA synthase (12 nmol) was inactivated with 400 nmol of 3-oxobutylsulfoxyl-[3′-^{32}P]CoA (10 241 dpm/nmol) in 0.66 mL of 100 mM Tris-HCl, pH 8.2.

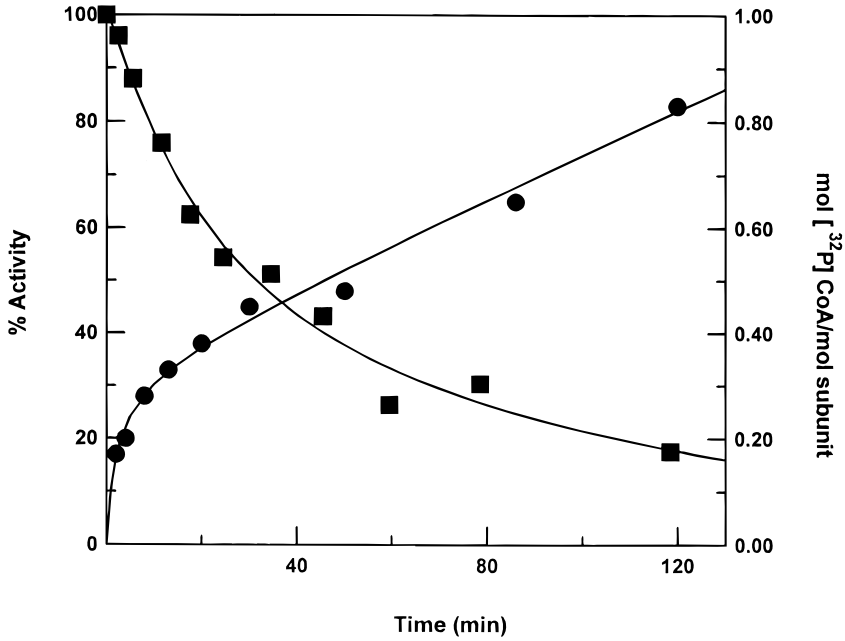


FIGURE 2: Correlation of incorporation of [3′-^{32}P]CoA with the extent of enzyme inactivation. HMG-CoA synthase (34.7 nmol) was incubated with 1.2 μmol of 3-oxobutylsulfoxyl-[3′-^{32}P]CoA (13 696 dpm/nmol) in 100 mM Tris-HCl, pH 8.2, at room temperature. At the indicated times aliquots were removed and assayed for enzyme activity (■). Protein-bound ^{32}P (●) was determined by trichloroacetic acid precipitation as described in Methods.

The prediction of specificity was supported by the observation (Table 1) that prior inactivation of the enzyme with oxobutylsulfoxyl-CoA blocked its binding of the substrate, [^{14}C]acetyl-CoA, to form the Michaelis complex and acetyl-S-enzyme intermediate.

Preparation of [^{32}P]-labeled oxobutylsulfoxyl-CoA allowed additional scrutiny of HMG-CoA synthase inactivation by this reagent. In a more direct labeling experiment, inactivation of enzyme with oxobutylsulfoxyl-[^{32}P]CoA resulted in stoichiometric labeling by the inhibitor (Table 1) to form an adduct that included the nucleotide moiety. The covalent nature of the modification, implied by the time dependence of inactivation, was demonstrated by the persistence of labeling after denaturation of protein by trichloroacetic acid. Furthermore, a close correlation between activity loss and the extent of covalent labeling was observed (Figure 2). The stoichiometric labeling of enzyme that occurred as inactivation by oxobutylsulfoxyl-CoA approached completion argued for the selectivity that would be expected for an active-site directed process.

Implication of a Cysteine in Adduct Formation. The nature of the adduct formed between oxobutylsulfoxyl-[^{32}P]CoA and HMG-CoA synthase was tested by measuring the stability of the preformed adduct under various chemical conditions (Table 2). In contrast to the inability of hydroxylamine or dilute acid to reverse the modification, a variety of mercaptans can disrupt the linkage between protein and a CoA nucleotide-containing portion of the inactivator. DTT was most effective in this respect, readily displacing >80% of the labeled nucleotide from the protein adduct and restoring enzyme activity. These observations suggested that the covalent adduct formed between inactivator and protein might involve a cysteine residue.

Table 2: Stability of the Adduct Formed upon 3-Oxobutylsulfoxyl-CoA Inactivation of HMG-CoA Synthase

experiment	incubation reagent	% label remaining[a]
A[b]	untreated	100
	hydroxylamine (1.5 M)	92
	HCl (375 mM)	108
	DTT (150 mM)	1.1
B[c]	untreated	100
	DTT (5 mM)	13
	2-mercaptoethanol (5 mM)	44
	cysteine (5 mM)	61
	glutathione (5 mM)	103

[a] Labeling stoichiometry determined by TCA precipitation as described in Methods. [b] Paired samples of inactivated HMG-CoA synthase (0.6 nmol) were incubated 1 h at 37 °C with the specified reagent prior to determining the stoichiometry of modification. [c] Paired samples of inactivated HMG-CoA synthase (0.6 nmol) were incubated 0.5 h at 25 °C with the specified reagent before determining the stoichiometry of modification.

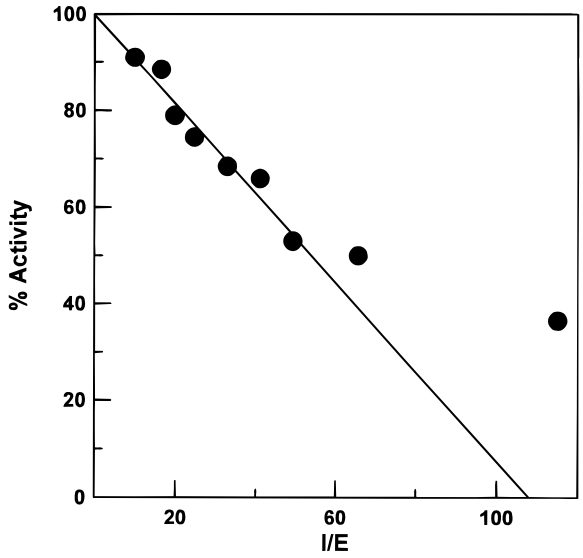


FIGURE 3: Inactivation of HMG-CoA synthase at differing ratios of 3-oxobutylsulfoxyl-CoA/enzyme. Estimates of residual activity were performed after a 2 min incubation with inhibitor. The extrapolated partition ratio is 107.

Evidence for Conversion of Oxobutylsulfoxyl-CoA into a Reactive Inactivator upon Incubation with HMG-CoA Synthase. The inactivation of HMG-CoA synthase by oxobutylsulfoxyl-CoA did not follow simple first-order kinetics (Figure 1), suggesting the possible depletion of inhibitor during the incubation (Silverman, 1995). In principle, this observation might be attributed to reagent lability, an explanation that seemed unlikely since control experiments involving incubation of the analog in the absence of enzyme indicated that no degradation of reagent occurred. Likewise, exposure of the analog to DTT or other mercaptans at levels that restored activity to oxobutylsulfoxyl-CoA-treated HMG-CoA synthase and reversed protein labeling did not alter the analog. Moreover, analog reisolated after such exposure to mercaptan remained effective as an inactivator. Another possible explanation for the observed kinetics involved an inactivation process in which enzyme catalyzed the formation from oxobutylsulfoxyl-CoA of a reactive species that modifies and inactivates the protein. If release of the enzyme-generated reactive species occurs, it either could rebind and covalently modify the protein or could be quenched in solution by reaction with other components of the incubation mixture. Any of these mechanisms would decrease the initial concentration of oxobutylsulfoxyl-CoA available for the inactivation. When enzyme activity was measured after incubation for a fixed time period with variable concentrations of oxobutylsulfoxyl-CoA, the resulting data (Figure 3) supported the possibility that enzyme produced a reactive species from this analog. While effective inactivation does occur at even modest inactivator/enzyme ratios, a partition ratio (rate of inactivator formation/rate of enzyme inactivation) in excess of 100 was indicated by extrapolation of the linear portion of the plot to the *x*-axis (Silverman, 1995). Such a partitioning of reagent would certainly deplete the initial concentration of oxobutylsulfoxyl-CoA and would account for the departure from first-order kinetics that becomes apparent from the data taken late in the time course of inactivation (Figure 1). Inclusion of DTT (2 mM) in the enzyme incubation mixtures resulted in complete protection against loss of activity. Glutathione (2 mM) also minimized loss of activity (<20% inactivation) when it was included in the incubation mixtures. In contrast to the stability of oxobutylsulfoxyl-CoA in buffered aqueous solutions containing mercaptans, this result was compatible with the reaction of DTT or glutathione to scavenge a more reactive species that was released from the enzyme. The possibility that these mercaptans reversed the adduct that resulted when the enzyme-generated reactive species modified the active site prior to dissociation seems less likely, since glutathione was not very effective in disrupting preformed enzyme-inactivator adducts (Table 2). The collected data suggested that HMG-CoA synthase could efficiently catalyze formation from 3-oxobutylsulfoxyl-CoA of a reactive species that was the actual inactivator of the enzyme.

Inactivation of Other Acyl-CoA-Utilizing Enzymes by Oxobutylsulfoxyl-CoA. Since a cysteine had been implicated (Table 2) in formation of the adduct that correlated with inactivation of HMG-CoA synthase, the efficacy of oxobutylsulfoxyl-CoA as an inactivator of other acyl-CoA-utilizing enzymes that contain reactive active site cysteines was evaluated. *Z. ramigera* β-ketothiolase metabolizes acetoacetyl-CoA and contains two active site cysteines; one forms an acetyl-S-enzyme intermediate (Thompson et al., 1989) while the other functions as a general base (Palmer et al., 1991). As observed with HMG-CoA synthase, incubation of thiolase with oxobutylsulfoxyl-CoA almost completely abolished activity (Table 3), a time-dependent effect that was reversed (~80% recovery of activity) upon subsequent treatment with DTT. *P. mevalonii* HMG-CoA lyase binds short chain, branched chain, and heterocyclic acyl-CoAs and also contains a reactive active site cysteine (Hruz et al., 1992; Narasimhan et al., 1995). This enzyme is highly sensitive to oxobutylsulfoxyl-CoA, since even at low temperatures (4 °C) and low concentrations of the analog, HMG-CoA lyase was inactivated in a time-dependent fashion. Subsequent incubation with DTT restored ~80% of the initial activity (Table 3). These collected results suggested that oxobutylsulfoxyl-CoA could be useful in inactivation of various enzymes that accommodate binding of short chain acyl-CoAs and contain an accessible, reactive cysteine situated near the bound nucleotide.

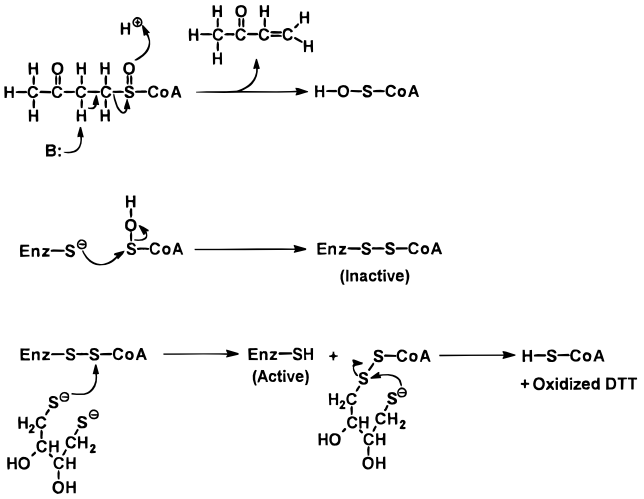
Release of CoASH upon Reversal of Inactivation by DTT. The preceding results implicated a cysteine residue in formation of an adduct between protein and inactivator. Additional insight into the nature of the adduct was obtained by analyzing the species produced when DTT was used to restore activity to oxobutylsulfoxyl-CoA-inactivated HMG-CoA synthase. A large sample of the synthase-inactivator adduct is available due to the efficiency in overexpression of the recombinant protein (Misra et al., 1993). In a larger scale inactivation, enzyme (1.7 μmol) was incubated with oxobutylsulfoxyl-CoA (24 μmol) until <10% activity remained. The sample was chromatographed using Sephadex G-50 to produce a sample of inactivated protein that was free of low molecular weight components. This sample was incubated with DTT (20 mM), restoring 74% of maximal activity. Ultrafiltration was used to separate protein from the low molecular weight fraction, which was chromatographed on a DEAE cellulose anion exchange column. Only one adenine nucleotide peak was recovered; HPLC analysis (reversed phase) indicated that elution of the nucleotide was coincident with that of CoASH. To verify the identity of the recovered nucleotide, the sample was analyzed using a

Table 3: 3-Oxobutylsulfoxyl-CoA Inactivation of Several Acyl-CoA-Utilizing Enzymes and Restoration of Activity by DTT

treatment	% activity		
	HMG-CoA synthase	β-ketothiolase	HMG-CoA lyase
no inhibitor	100	100	100
inactivation by 3-oxobutylsulfoxyl-CoA	10[a]	2.9[b]	4.5[c]
inactivation by 3-oxobutylsulfoxyl-CoA followed by incubation with DTT	78[d]	80[d]	80[e]

[a] HMG-CoA synthase (12 nmol) was reacted with 3-oxobutylsulfoxyl-CoA (400 nmol) in 100 mM Tris-HCl, pH 8.2; the activity was measured after 2.5 h incubation at room temperature. [b] β-Ketothiolase (0.14 nmol) was reacted with 3-oxobutylsulfoxyl-CoA (175 nmol) in 65 mM Tris-HCl, pH 8.2; the activity was measured after 1 h incubation at room temperature. [c] HMG-CoA lyase (0.22 nmol) was reacted with 3-oxobutylsulfoxyl-CoA (1.63 nmol) in 10 mM potassium phosphate, pH 7.2; the activity was measured after 1 h incubation at 4 °C. [d] Inactivated protein was incubated with 14.3 mM DTT for 25 min at 30 °C. [e] Inactivated protein was incubated with 14.3 mM DTT for 10 min at 30 °C.

Scheme 1



phosphotransacetylase-based end point assay for CoASH (Bergmeyer et al., 1985). The results indicated that 90% of the recovered nucleotide (concentration based on A_{257}) was attributable to CoASH. Thus, any adduct that is postulated to form when HMG-CoA synthase is inactivated by oxobutylsulfoxyl-CoA must have chemical properties that are compatible with the subsequent DTT-dependent production of free CoASH (Scheme 1). In control experiments performed in the absence of enzyme but under otherwise identical incubation and isolation conditions, no production of CoASH from oxobutylsulfoxyl-CoA was detectable.

The assignment of CoASH as the nucleotide released upon DTT treatment of inactivated enzyme was supported by coupling the enzymatic production of the reactive inactivator from oxobutylsulfoxyl-CoA to the phosphotransacetylase assay for CoASH. Assays were performed in the presence of DTT (0.83 mM) to reduce any inactivator released from enzyme and to reverse any formation of enzyme−inactivator adducts, allowing multiple turnover of oxobutylsulfoxyl-CoA. In such experiments, a direct correlation was observed between the rate of CoASH production and the amount of HMG-CoA synthase used for the turnover of oxobutylsulfoxyl-CoA (Table 4). Similar observations of CoASH production rates that were proportional to the amount of oxobutylsulfoxyl-CoA-targeted protein (Table 4) were made using β-ketothiolase and HMG-CoA lyase. These two enzymes exhibit high turnover numbers in catalyzing their physiological reaction, and, under the experimental conditions employed, they reacted with inhibitor to ultimately form CoASH at rates in excess of that observed with HMG-CoA synthase, an enzyme with a low turnover rate.

An extension of these studies involved a mutant HMG-CoA lyase (C237A) which lacks the active site cysteine but has been shown by biophysical and kinetic studies to contain a full complement of intact substrate binding sites (Narasimhan et al., 1995). C237A lyase catalyzes HMG-CoA cleavage at a rate 10^4-fold lower than wild-type enzyme. When screened for oxobutylsulfoxyl-CoA cleavage activity, this mutant was observed to produce CoASH at a slightly faster rate than that measured with wild-type lyase (Table 4). This observation suggested that, while inactivation of wild-type enzyme may be explained by disulfide formation between a protein's cysteinyl sulfhydryl and the inhibitor-derived CoASH, HMG-CoA lyase catalyzed the cleavage of oxobutylsulfoxyl-CoA to form a reactive nucleotide species in a reaction that does not require the intermediacy of an active site cysteine.

Table 4: Rates of Enzyme-Catalyzed Production of CoASH from 3-Oxobutylsulfoxyl-CoA

enzyme	rate (units/mg)[a]
HMG-CoA synthase	0.0050
β-ketothiolase	0.14
HMG-CoA lyase/wild-type	0.090
HMG-CoA lyase/C237A	0.23

[a] Initial rate of CoASH production was determined by a phosphotransacetylase coupled assay which measures the conversion of CoASH to acetyl-CoA (Bergmeyer, 1985). The reaction was started by the addition of enzyme to a 0.6 mL assay mix (30 °C) containing 10 milliunits of phosphotransacetylase in 70 mM Tris-HCl, pH 7.6, 10 mM acetyl phosphate, 0.337 mM 3-oxobutylsulfoxyl-CoA, and 0.83 mM DTT. The measured rates were linearly dependent upon the concentrations of enzyme employed in the assay.

DISCUSSION

It seems quite clear that modification of the acyl-CoA-utilizing enzymes by oxobutylsulfoxyl-CoA requires a *reactive* derivative of CoA. The production from the inhibitor of fully reduced CoASH, which is a product of the HMG-CoA synthase and β-ketothiolase reactions, would not account for inactivation of those enzymes. The formation of a CoA nucleotide with a highly oxidized sulfur (e.g., a sulfinic or sulfonic acid) upon enzymatic cleavage of the bond between the oxobutyl group and the sulfoxide does not have any strong chemical precedent. Moreover, any adduct that such an oxidized species would form with enzyme is not expected, upon subsequent reaction with DTT, to completely convert all of the nucleotide to free CoASH (Nishimura et al., 1982). In contrast, enzymes that contain an active site general base could react with oxobutylsulfoxyl-CoA to form a reactive sulfenic acid form of CoA (Scheme 1) that appears to account for all of our experimental observations. Cleavage of a carbon−sulfur bond adjacent to a sulfoxide with concomitant formation of a sulfenic acid

is well documented in organic chemistry (Shelton & Davis, 1967; Hogg, 1978) and has also been implicated in the enzyme-catalyzed cleavage of sulfoxide-containing compounds (Tomisawa et al., 1993). In the presence of a reactive sulfhydryl, the putative sulfenic acid can react with protein to form a disulfide (Allison, 1976; Prohaska, 1980). Based on these precedents, the enzymes inactivated by oxobutylsulfoxyl-CoA must not only cleave the substrate analog to produce a sulfenic acid form of CoA (Scheme 1) but must also contain a cysteinyl sulfhydryl appropriately juxtaposed to this reactive species. A disulfide-linked nucleotide forms, occupying the active site and accounting for loss of enzyme activity. Subsequent reaction of this disulfide with a mercaptan such as DTT will liberate CoASH and restore the enzyme's sulfhydryl group (Scheme 1) as well as its catalytic competency.

Analogs such as oxobutylsulfoxyl-CoA appear to offer some advantages over other CoA derivatives that are directed toward active site cysteines. A thiosulfonate containing reagent, reported by Nishimura et al. (1982), incorporated two CoA nucleotides and, due to steric considerations (i.e., a potential requirement for two nucleotide binding pockets), its general utility with many acyl-CoA utilizing enzymes might be expected to be limited. *S*-Methanesulfonyl-CoA, prepared by reaction of methanesulfonyl chloride with CoASH (Owens et al., 1981), proved effective as an inactivator of succinate thiokinase and β-hydroxyacyl-CoA dehydrogenase. However, it was also reported to function as a simple group specific reagent, presumably due to the poor binding specificity afforded by the methyl group. The selectivity demonstrated by oxobutylsulfoxyl-CoA in modifying HMG-CoA synthase argues that the reagent may be useful in future physical or structural studies that require an enzyme sample in which the CoA nucleotide binding pocket is stably occupied. Such a goal was not achieved when the enzyme was modified with the mechanism-based inhibitor 3-chloropropionyl-S-CoA, due to the lability of the thioester linkage in that compound (Miziorko & Behnke, 1985a).

Oxobutylsulfoxyl-CoA or related sulfoxyl-containing analogs may be useful in characterizing engineered mutant enzymes. If a wild-type protein is inactivated by such reagents with formation of a disulfide linkage to CoA, stoichiometric formation of such a linkage in mutant forms of the protein would argue for the retention of an active site tertiary structure in which an active site cysteine remains appropriately juxtaposed to the CoA binding site. Such an observation would represent a substantial argument for the structural integrity of the mutant.

Another potential use of this class of reagents derives from the enzyme catalyzed cleavage reaction that produces the putative sulfenic acid form of CoA. Chemical precedent suggests that elimination of a sulfenic acid from an alkylsulfoxyl-containing compound can proceed via deprotonation of the β-carbon (Durst, 1978; Hashmi et al., 1992). All three enzymes that are demonstrated in this report as oxobutylsulfoxyl-CoA targets require a base-mediated deprotonation to support catalysis. It is possible that a general base on the enzyme initiates cleavage of oxobutylsulfoxyl-CoA to form the sulfenic acid (Scheme 1). If this is the case, then cleavage of the inhibitor (and the resulting recovery, under reducing conditions, of CoASH) may represent a test for the identity and/or function of a general base. If this test is applied to the C237A form of HMG-CoA lyase, the ability of this mutant to catalyze analog cleavage and, in the presence of DTT, CoASH formation (Table 4) would argue that the sulfhydryl group of C237 does not function as the active site base. Future work involving additional comparisons of wild-type and mutant acyl-CoA metabolizing enzymes would, however, appear to be worthwhile in the context of evaluating alkylsulfoxyl-CoA cleavage as a potential test for the function of an active site base.

ACKNOWLEDGMENT

We thank Drs. Dale Drueckhammer and Ben Schwartz (Stanford University) for the generous gift of dephospho-CoA kinase and Dr. Vernon Anderson (Case Western Reserve University) for providing us with β-ketothiolase. Mr. Frank Laib performed the mass spectroscopic analyses of *S*-oxobutyl-CoA and oxobutylsulfoxyl-CoA. Dr. Jacqueline Roberts provided us with HMG CoA Lyase C237A. We thank Dr. Dennis Bennett and Bill Hodgeman (University of Wisconsin—Milwaukee) for help with FTIR measurements.

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BI962751C