Mechanism-Based Enzyme Inactivation Using an Allyl Sulfoxide–Allyl Sulfenate Ester Rearrangement

Michael Johnston, Ronald Raines, Christopher Walsh, and Raymond A. Firestone

Abstract: 2-Amino-4-chloro-5-(p-nitrophenylsulfinyl)pentanoic acid (1) has been synthesized and shown to induce mechanism-based inactivation of two pyridoxal phosphate dependent enzymes: (1) cystathionine γ-synthetase, which catalyzes a γ-replacement reaction in bacterial methionine biosynthesis; and (2) methionine γ-lyase, which catalyzes a γ-elimination reaction in bacterial methionine breakdown. The inactivations are irreversible and display saturation kinetics. Each enzyme inactivates p-nitrophenylthiolate anion, which forms, in the case of methionine γ-lyase, stoichiometrically with enzyme reactivated.

Introduction
Considerable interest has been generated in the last several years in mechanism-based enzyme inactivators, also called suicide substrates. Much of this interest results from the fact that the targeted enzyme uses some portion of its catalytic mechanism to “unmask”, from an otherwise chemically unreactive group in the inactivator, a functionality reactive for alkylation of the enzyme. The reactive species is generated only in the enzyme’s active site, and, thus, suicide substrates promise greater in vivo selectivity than do conventional affinity reagents.

A variety of functional groups have been used for mechanism-based inactivations, including acetylenes, olefins, nitriles, and β-halo substitutions, which become activated usually by rearrangement or elimination to generate electrophiles susceptible to Michael-type addition by an active-site nucleophile. Certain functionalized penicillins, such as the clavulanes and penicillin sulfones, cyclopropylamines, fluoro- and nitrodeoxyuridylates, and such drugs as allopurinol are all known to function as specific suicide substrates, exemplifying the rich chemical diversity of this class of reactions.

It has occurred to us that a novel strategy for the generation of an electrophile in situ might use a sigmatropic rearrangement to function as specific suicide substrates, exemplifying the rich chemical diversity of this class of reactions. In this regard, we have chosen the 2,3-sigmatropic rearrangement of allyl sulfoxide (1a) because it is highly reactive to nucleophilic addition as a likely mode of suicide-substrate inactivation (eq 1). A reagent of the type imagined will have greatest potential selectivity if designed such that the allyl sulfoxide is generated only within the active site of the targeted enzyme. Therefore, we have further con-
allyl allyl sulfoxide considered that a halo sulfoxide precursor might undergo enzyme-mediated loss of HX to "uncover" the allylic double bond required for rearrangement (eq 1).

Thus, the reaction strategy involves a minimal two-step pathway for activation, such that the suicide chemistry is accomplished by the operation of eq 2 and 1 in sequence.

Pyridoxal phosphate dependent enzymes which catalyze the elimination of good leaving groups at the γ carbon of their amino acid substrates immediately suggest themselves as likely prospects for the generation of an allyl sulfoxide by HX elimination. Two such enzymes (in bacterial metabolism) are methionine γ-lyase (catalyzing reaction 3) and cystathionine γ-synthetase (catalyzing reaction 4), both of which use bound pyridoxal phosphate cofactor to facilitate elimination of the γ substituent through stabilization of α- and β-carbanion equivalents (Scheme I). Thus, both methionine γ-lyase and cystathionine γ-synthetase might be expected to catalyze γ-chloro elimination from amino acids 1 and 2 to generate an enzyme-bound allyl sulfoxide. Rearrangement to the allyl sulfenate ester could, in turn, give the desired electrophile susceptible to attack by an active-site nucleophile.

In this paper we report the preparation of amino acids 1 and 2 and the inactivation of both methionine γ-lyase and cystathionine γ-synthetase, with evidence for the indicated 2,3-sigmatropic rearrangement as the operating mechanism in these inactivations.

Results

1. Kinetics of Inactivation. 2-Amino-4-chloro-5-(p-nitrophensulfinyl)pentanoic acid (1) is a time-dependent, irreversible inactivator of both cystathionine γ-synthetase and methionine γ-lyase. Time-dependent loss of catalytic activity ultimately results in fully inactivated enzyme. Typical semilog plots of remaining enzymatic activity vs. time are shown in Figure 1. Kinetic data for the process shown in eq 5 are given in Table I. Neither 2, the p-tolylsulfinyl compound, nor 3, the reverse regioisomer of 1, give inactivation of either enzyme.

\[
E + I \xrightleftharpoons[k_1]{k_2} EI \rightarrow E-I
\]

Scheme I

Compound 1 has three asymmetric centers (about the α and γ carbons and at sulfur). The efficacy of inactivation is certainly conditioned by differential binding of the α carbon D and L isomers and may be constrained further by the absolute stereochemistry at each of the other two chiral centers. The data of Table I may, therefore, represent potential maximal values for \( k_1 \) and minimal values for \( k_2 \). We have successfully separated \( 10 \) by preparative thin-layer chromatography, two sets of isomers of the inactivator. However, neither of these individual preparations gives kinetic parameters significantly different from those obtained with solutions which may contain all four diastereomeric pairs.

Both enzymes are specific for the L isomers at the α carbon. Both enzymes will probably eliminate chloride from both the 4R and 4S γ isomers by a two-step carbanion mechanism. The absolute stereochemistry at sulfur may also not determine
Table I. Kinetic Parameters for Inactivation of 2-Amino-4-chloro-5-(p-nitrophenylsulfinyl)pentanoic acid (I)

<table>
<thead>
<tr>
<th>enzyme</th>
<th>$K_1$, mM</th>
<th>$k_2 \times 10^4$, s$^{-1}$</th>
</tr>
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<tbody>
<tr>
<td>cystathionine γ-synthetase</td>
<td>0.38</td>
<td>6.5</td>
</tr>
<tr>
<td>methionine γ-lyase</td>
<td>1.50</td>
<td>5.1</td>
</tr>
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</table>

the overall observed rate of inactivation if the electrophilic
sulfenate is in rapid equilibrium with the sulfoxide; reversible
rearangements would effectively racemize the sulfoxide
sulfur prior to interception.

II. Stoichiometry of Labeling. A. Ring-Labeled Inactivator.

Figure 2 gives the elution profiles of the two enzymes inacti-

vated by the ring-labeled compound 1a and subsequently ex-
posed to Sephadex G25 gel filtration chromatography. A peak
of radioactivity coelutes in each case with the protein absorb-
ance at 280 nm, findings which confirm our expectation that
the inactivations are covalent and irreversible.

Table II. Thiol Reactivation of Cystathionine γ-Synthetase and Methionine γ-Lyase Inactivated by 2-Amino-4-chloro-5-(p-nitrophenylsulfinyl)pentanoic acid (I)

| thiol (10 mM) | % act. recovered | half-time for recovery of 100% catalytic act., min$^a$ | $p$-nitrophenyl thiol production, $^b$
<table>
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<tr>
<td></td>
<td>cystathionine γ-synthetase (~20 μM)</td>
<td>methionine γ-lyase (~50 μM)</td>
<td>$t_{1/2}$, min$^a$</td>
</tr>
<tr>
<td>dithiothreitol</td>
<td>25</td>
<td>100</td>
<td>2.5</td>
</tr>
<tr>
<td>2-mercaptoethanol</td>
<td>0</td>
<td>100</td>
<td>6.75</td>
</tr>
<tr>
<td>3-mercaptopropionate</td>
<td>0</td>
<td>100</td>
<td>8.2</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>0</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

$^a$ Data for methionine γ-lyase only. $^b$ For generation of $p$-nitrophenylthiolate stoichiometric with enzyme reactivated. n.d. = not determined.
to ethanol and mercaptopropionate, neither of which is a reactivating thiol of this enzyme (vide supra). Methionine γ-lyase is protected from inactivation by neutral thiols DTT and mercaptoethanol but is not at all protected by the charged (at physiological pH) thiols mercaptopropionate and glutathione (data not shown). The lack of protection by glutathione (present intracellularly in concentrations up to 8 mM) is probably a necessary condition for any potential utility of 1 as an in vivo inactivator.

Every inactivation carried out in the presence of added thiol (protection) gives catalytic formation of p-nitrophenylthiolate anion, and thus allows a measure of the rate of in vitro processing of 1 (vide infra).

IV. Identification of p-Nitrophenylthiol as Product from Thiol Reactivation of Inactivated Methionine γ-Lyase. A. Spectral and Chromatographic Analyses. Native methionine γ-lyase has two absorbance bands with \( \lambda_{\text{max}} \) at 280 and 418 nm; the \( A_{280}/A_{418} = 4.85 \) for native enzyme. \(^2\) Inactivation by 1 proceeds without change in either the 280- or 418-nm band. The fully inactivated enzyme has \( A_{280}/A_{418} = 4.9 \). The lack of any spectral change at 418 nm suggests that alkylation of the enzyme involves no net alteration in the oxidation state of the pyridoxal phosphate cofactor.

Thiol reactivation of the dead enzyme, by contrast, produces two notable spectral changes. As shown in Figure 5, the addition of dithiothreitol gives a dramatic absorbance enhancement of the visible band and a blue shift in its absorbance maximum to 408 nm. The rate of accumulation of the 408-nm absorbance was observed to be pseudo first order (inset) and synchronous with the regain of catalytic activity (see Table II). The end-point 408-nm absorbance (\( t_{30 \text{ min}} \), inset) corresponds to 100% recovery of catalytic activity.

We suspected that this spectral change resulted not from an alteration of either protein or cofactor absorbance but from the production of free p-nitrophenylthiolate anion \( \lambda_{\text{max}} = 408 \text{ nm at } \text{pH} 8.2; \epsilon = 10.5 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1} \) upon reactivation. To test this hypothesis, the fully reactivated enzyme of Figure 5 was subjected to Sephadex G25 gel filtration, and each column fraction was scanned for absorbance at 254, 280, and 408 nm. The elution profile is shown in Figure 6.

The gel filtration column resolved three sharply defined absorbance bands. The 280-nm absorbance, which emerges in the void volume (9.0 mL), represents elution of fully active methionine γ-lyase. The peak tube of 280-nm absorbance had a specific activity of 3.6 U/mg and an \( A_{280}/A_{418} \) ratio of 5.0. The peak tube of 254-nm absorbance gave an optical spectrum identical with that of 1, thus representing excess, unreacted inactivator.

The peak tube of 408-nm absorbance was recovered and gave a visible spectrum which was isospectral with authentic p-nitrophenylthiol. The total 408-nm absorbance recovered from the G25 column corresponds to a 46 \( \mu \text{M} \) solution of nitrophenylthiol, \(^4\) which is stoichiometric with enzyme originally inactivated.

On high-performance LC, the recovered 408-nm absorbance had a retention time of 6.5 min (conditions are described in the Experimental Section), identical with the retention time for authentic p-nitrophenylthiol.

B. Chromatographic Detection of Radiolabeled p-Nitrophenylthiol on Reactivation of \( ^3\text{H}-\text{Ring-Labeled Methionine} \gamma\text{-Lyase.} \) Methionine γ-lyase, which had been fully inactivated
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Figure 4. Kinetics of inactivation of cystathionine γ-synthetase by 1 mM 1 (○), pH 7.3, 37 °C, and by 1 mM 1 in the presence of 10 mM diithiothreitol (O), 10 mM 3-mercaptopropionate (▲), and 10 mM 2-mercaptoethanol (□).

Figure 5. Absorbance spectra of methionine γ-lyase obtained during reactivation by 10 mM dithiothreitol (solid line) of enzyme fully inactivated by 1 (dashed line).

Figure 6. Sephadex G25 elution profile of methionine γ-lyase which had been fully reactivated by 10 mM dithiothreitol. Experimental conditions in the text.

Figure 7. Rechromatography (Sephadex G25) of the radiolabeled protein recovered from the Sephadex column chromatography experiment described by Figure 2B. The enzyme (methionine γ-lyase) was incubated with 10 mM dithiothreitol, and full catalytic activity was regained prior to the second gel filtration. Experimental conditions are described in the text.

by ring-labeled inactivator (1a), was obtained by pooling the radiolabeled fractions from the gel filtration described by Figure 2B (fractions 12, 13, and 14). The pooled fractions were made 10 mM in dithiothreitol and, once full catalytic activity had been recovered, were rechromatographed on the G25 column. The elution profile is shown in Figure 7.

Note that the protein absorbance at 280 nm lacks a coincident band of radioactivity. All the radioactivity coelutes with a new band of absorbance at 408 nm. The radioactivity recovered under the 408-nm peak is stoichiometric with enzyme reactivated. Each of the 408-nm fractions was pooled, and the pooled fraction gave an absorbance spectrum isospectral with authentic p-nitrophenylthiolate. This radiolabeled solution was then subjected to high-performance LC and, like that for the previously chromatographed unlabeled 408-nm absorbance, cochromatographed with authentic p-nitrophenylthiol (retention time = 6.5 min).

These results, from both experiments with unlabeled and ring-labeled 1, show that the 3H-ring-labeled fragment of the inactivator incorporated into methionine γ-lyase is a quantitative precursor, under mild reductive conditions of RS− addition, of p-nitrophenylthiol anion.

C. Kinetics of p-Nitrophenylthiol Production. The production of p-nitrophenylthiolate from 1 in the presence of protecting thiols is a catalytic process which may be described by eq 6. Incubation of 1 with dithiothreitol generated no p-nitrophenylthiol in the absence of enzyme. The KM for 1 in eq 6 is 1.1 mM (compare KI = 1.5 mM for inactivation). The overall first-order rate constant, k2, for nitrophenylthiol production in eq 6 is 4.7 × 10^-3 s^-1 (compare k2 = 6.5 × 10^-4 s^-1 for pseudo-first-order inactivation). These results represent a steady state between rates of inactivation and of turnover to p-nitrophenylthiol during protection.

Discussion

I. Inactivation Mechanism. We propose that 2-amino-4-chloro-5-(p-nitrophenylsulfinyl)pentanoic acid (I) inactivates cystathionine γ-synthetase and methionine γ-lyase by a mechanism (Scheme II) which involves a key 2,3-sigmatropic rearrangement of an allyl sulfoxide (4) to an allyl sulfenate ester (5). Thermal rearrangements are uncommon in primary metabolism. A notable example, however, is the chorismate-prephenate conversion, a key reaction in bacterial aromatic
amino acid biosynthesis; this reaction has been well characterized as a 3,3-sigmatropic, Claisen rearrangement. Very recently, evidence has been given for the involvement of a 2,3-sigmatropic allyl sulfoxide-allyl sulfenate rearrangement in the in vivo conversion of the herbicide S-2,3-dichloroallyl diisopropylthiocarbamate (diallate) to the bacterial mutagen 2-chloroacrolein.

For the inactivations described here we suggest that the pyridoxal phosphate enzymes carry out the normal catalytic sequence on the substrate: that is, formation of stabilized \( \alpha \)-and then \( \beta \)-carbanion equivalents. \( \beta \)-Carbanion assisted halide elimination then generates the allyl sulfoxide adduct (4). Although we have not actually demonstrated that chloride ion is produced coincident with inactivation, the ability of each enzyme to support elimination of a good leaving group (-OR, -SR, or halide) from both \( \alpha \) and \( \gamma \) carbons of amino acid substrates is now well documented.

The reverse regioisomer (3) does not inactivate either enzyme, consistent with the process 4 → 5 during inactivation by 1. While 3 may form a PLP adduct and might also undergo enzymatic \( \alpha \)- and then \( \beta \)-carbanion equivalents, \( \beta \)-Carbanion assisted halide elimination then generates the allyl sulfoxide adduct (4). Although we have not actually demonstrated that chloride ion is produced coincident with inactivation, the ability of each enzyme to support elimination of a good leaving group (-OR, -SR, or halide) from both \( \beta \) and \( \gamma \) carbons of amino acid substrates is now well documented.

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In support of the proposed mechanism, the quantitative isolation of p-nitrophenylthiol upon thiol reactivation of the dead enzyme demonstrates a two-electron reduction of sulfur.
While our data argue for thiol reactivation by disulfide interchange, and thus point to a cysteinyl sulfur as the most likely alkylating nucleophile, they do not unequivocally rule out attack by an enzymatic nitrogen nucleophile (perhaps the ε-amino nitrogen of an enzymatic lysine). The resulting structure 6 could then be a sulfinamide; sulfinamides are known to form readily by attack of primary amines on sulfides (eq 7) and by reaction of amines with sulfenyl chlorides. The sulfinamide bond is labile to sodium iodide and to cleavage in acid-catalyzed reactions, but suffers attack by thiols only slowly. It seems less likely, therefore, that inactivated methionine γ-lyase, which is rapidly and completely reactivated by thiols, is formed by attack of an enzymatic nitrogen species on 5.

Cystathionine γ-synthetase, however, is only 25% reactivated by dithiothreitol, a finding which might be occasioned by competition between two active-site nucleophiles. One of these could be sulfur, giving the thiol-labile disulfide 6 25% of the time, and the other might be nitrogen, giving the more thiol-stable sulfinamide 75% of the time. Further structural studies will test these proposals. We have previously given evidence for the requirement for two distinct bases in the catalytic reactions of cystathionine γ-synthetase.

III. Thiol Protection. As shown in Scheme III, we argue that protection is afforded by capture of the electrophilic species 5 by the added thiol. The alternate proposition, wherein protection is merely apparent, achieved through reactivation by disulfide exchange on 6, is excluded by the observation that no thiol affords complete protection. Moreover, certain protecting thiols do not reactivate. These findings imply a partitioning on 5 between the inactivation pathway and the protection pathway (in the presence of added thiols). Thus, the degree to which any thiol offers protection is conditioned by its ability to compete kinetically with enzymatic thiophiles for the interception of 5, assuming that access to the active site is not itself a constraining parameter.

Dithiothreitol competes effectively with the enzymatic cysteinyl nucleophile for capture, as evidenced by the almost tenfold greater rate (4.7 × 10^{-3} s^{-1}) for nitrophenylthiol production (eq 6) than for inactivation (6.5 × 10^{-4} s^{-1}) in its absence. This rate difference reflects a partitioning ratio of 10:1 for turnover (to nitrophenylthiol in the presence of DTT) vs. inactivation, a disparity which itself implies that the key 2,3-sigmatropic rearrangement cannot be a slow step in the processing of 1. That is, were the allyl sulfide to allyl sulfenate conversion a rate-determining process, one would expect that the rate of inactivation would approximate the rate of p-nitrophenylthiol production during protection (partitioning ratio ≈ 1).

Protection (Scheme III) involves interception of 5 to give formation of an initial allylic alcohol–PLP p-quinoid adduct (9). We suspect that this carbanionic species undergoes normal reprotonation to 10 and processing to the dienamino PLP adduct (11). We have not verified this pathway experimentally. However, either this sequence or some mechanistic variant must operate in concert with the reactions of Scheme III to effect reactivation of the enzymes and to support turnover of 1 in the thiol-protection sequence where p-nitrophenylthiolate is formed catalytically.

IV. The Labeling Pattern. Upon inactivation by 1a, both cystathionine γ-synthetase and methionine γ-lase incorporate nitrophenyl ring-labeled tritium stoichiometrically. We argue from this finding that capture of 5 by an enzymatic nucleophile is the exclusive inactivating alkylation. The incorporation of a fractional amount of tritium label by methionine γ-lase when inactivated by 1b points toward a second alkylation. One possibility is that this secondary modification occurs by generation of a Michael-type acceptor from the PLP–allylic alcohol (9), formed after attack of the inactivating nucleophile on 5. These reactions are shown in Scheme IV.

The allylic alcohol–PLP adduct (9) formed by cleavage of the S–O bond of 5 may, as outlined above, isomerize by reprotonation at C1 to generate ultimately the product pentenolate. If, however, 9 loses water part of the time, the resulting dienamino PLP adduct (12) is a potential Michael acceptor for attack by a second enzymatic nucleophile. In support of the key elimination 9 → 12, we have determined that methionine γ-lase will catalyze an elimination sequence on the β-hydroxy amino acid L-threonine to generate the unsaturated four-carbon α-ketobutyrate. The pathway of Scheme IV must be only a minor mechanistic contributor in that only about 12% of the protein monomers incorporate tritium label from the carbon chain labeled compound. Further, such a minor alternate pathway cannot represent the “killing” alkylation since 1b affords complete inactivation but only fractional stoichiometry.

Conclusion

Two pyridoxal phosphate dependent enzymes are inactivated by nucleophilic capture following a 2,3-sigmatropic rearrangement of an allyl sulfide to an allyl sulfenate, confirming our expectation that an electrocyclic rearrangement is a rational strategy for the generation of a reactive electrophile. The rearrangement is preceded by an enzyme-catalyzed β,γ elimination of HCl, which “uncovers” the allylic double bond. These reactions operate in sequence, which constitutes...
a two-step activation pathway. This principle can be applied to a variety of enzymatic types and we are pursuing these objectives.

Synthesis

Synthetic routes leading to the arylsulfinylpentanoic acids are outlined in Scheme V. All products were isolated as the trifluoroacetyl (TFA) salts. Acid- and amine-blocking groups are always the expected, but undesired, a two-step activation pathway. This principle can be applied to the more thermodynamically stable, but the principle kinetic isomers were created two new stereoisomers at CHCl and CHS, as does the addition, via an episulfonium rearrangement. In this way, with a trace of added TFA to the aqueous solution. Removal of solvent in vacuo leads to partial loss of TFA, and a sample reconstituted in water without restoring the TFA slowly deposited insoluble material, presumably lactone.

Experimental Section

$^1$H NMR spectra were run, except as noted, on a Varian T-60. Several colleagues at Merck, Sharp and Dohme Research Laboratories were most helpful in the analysis of synthetic products. We thank Dr. B. Arison and H. Flynn for the 300-MHz spectra, and Dr. Arison for valuable interpretative assistance. $^{13}$C spectra were kindly run and interpreted for us by Dr. A. Douglas. Mass spectra were expertly determined by J. Smith. Particular thanks are owed to Dr. A. Rosegay and M. Walsh for their expert advice and assistance with the radiochemical preparations. Radiochemical assays were kindly done by H. Meriweither and N. Allen.

N-Boc-allylglycine (13). A mixture of 884 mg of allylglycine (7.68 mmol), 4.6 mL of water, 4.6 mL of dioxane, 1.16 mL of Et$_3$N (11.5 mmol), and 2.08 g of Boc-ON was stirred for 3.8 h at room temperature. Then 15 mL of water and 20 mL of ether were added. The aqueous layer was washed with ether, and acidified to pH 2.0 with 6 N HCl. The crystallized product was filtered, washed with water, and dried; yield, 1.41 g; 85.4%; mp 109-111 °C; NMR (6, CDCl$_3$) 1.45 (s, 9 H, t-Bu), 2.35 (m, 2 H, CH$_2$), 4.35 (br, 1 H, NH), 4.9-5.2 (m, 4 H, αH and vinyl), 7.17 (s, 1 H, COOH).

N-Boc-allylglycine Benzylhydryl Ester (14). To a solution of 269 mg (1.25 mmol) of N-Boc-allylglycine in 25 mL of MeCN at room temperature was added 243 mg (1.25 mmol) of diphenylazomethane. Most of the color faded during 1-h stirring. The solvent was evaporated in vacuo and was replaced with benzene. The solution was washed with aqueous NaHCO$_3$ and then, dried with MgSO$_4$ and evaporated. The crystalline product was washed with hexane and dried; yield, 380 mg; 80%; mp 79-80 °C; NMR (6, CDCl$_3$) 6.9 (s, 1 H, CHPh$_2$), 7.3 (s, 10 H, Ph$_2$); MS 381 (+M$^+$), 325 (M$^+$-t-Bu), 170 (M$^+$-COOCPh$_2$), 167 (CH$_2$Ph$_2^+$); TLC 50:1 CHCl$_3$-EtOAc, R$_f$ 0.3.

p-Tolyl- and $p$-Nitrobenzensulfenyl Chlorides. p-Tolylsulfenyl chloride was made according to ref 30. p-Nitrobenzensulfenyl chloride was also made by this procedure, adding the p-nitrothiophenol as a 5% solution from a heated dropping funnel to keep it in solution; mp 44-47 °C (lit. 50 °C; 52 °C.$^2$).

Tritiated p-nitrobenzensulfenyl chloride was prepared as follows. Pulverized p-nitrobenzene disulfide (50 mg, 0.162 mmol, recrystallized from benzene) was heated 4 days at 120 °C in CH$_3$SO$_3$H. cooled, diluted with water, filtered, and dried; yield, 40 mg of tritiated compound. This, with another 21-mg sample prepared similarly, was combined with 100 mg of proto compound and recrystallized together from benzene: yield, 168 mg of p-nitrobenzene disulfide (7.0 mCi/mg, 2.16 Ci/mol). It had been previously determined that this CH$_3$SO$_3$H procedure returned good quality disulfide.

The chlorination was performed in this way. Pulverized p-nitrobenzenesulfenyl chloride (1.001 g, 3.25 mmol) in 8 mL of N,N-dimethylacetamide was treated with 2.0 mL of a solution of 0.739 mL of liquefied Cl$_2$ in 10 mL of CCl$_4$ (3.25 mmol) in a sealed tube for 3 h at 60 °C with stirring. Gradually, almost all the starting material dissolved. The mixture cooled, filtered, and evaporated in vacuo; yield, 1.186 g (96%) of pure p-nitrobenzensulfenyl chloride; mp 48 °C. The radioactive sample was made in this way from 168 mg of radiolabeled disulfide; yield, 163.3 mg (90%) of ring-tritiated p-nitrobenzensulfenyl chloride.

Compounds 15 and 16. To 381 mg of 14 (1.00 mmol) in 1.5 mL of CH$_3$Cl$_2$ at -18 °C under N$_2$ was added over 30 min, 186.3 mg (0.98 mmol) of p-nitrobenzenesulfenyl chloride in 2 mL of CH$_3$Cl$_2$. The reaction mixture was left overnight at room temperature and evaporated in vacuo.

NMR showed a mixture of 15 and 16 in about a 1:10 ratio, but 16 could not be thermally rearranged into its regioisomer as 20 had been (vide infra). A partial separation was effected by PLC (50:1 CHCl$_3$-EtOAc, four 20 x 20 cm plates, 2-mm layer; R$_f$ 0.3-0.6) with 16 running faster than 20. Both compounds could be isolated pure by repeated chromatography, but the partially purified mixtures were generally carried forward because separation was easier at the sulfone stage.

NMR of 15 (6, CDCl$_3$) 1.37 (s, 9 H, t-Bu), 2.35 (m, 2 H, β-CH$_2$), 3.3-3.7 (m, CH$_2$S), 3.9-4.35 (m, 1 H, CHCl), 4.35-4.85 (m, 1 H, CO$_2$H).
alpha-(CH), 5.3 (m, 1 H, NH), 6.9 (s, 1 H, CH(Ph)), 7.3 s, 10 H, Ph(2)), 7.35 (d, 8.0, J = 7 Hz, 4 H, C6H4).

From 70 mg of 17-II was similarly obtained 14.6 mg (116%) of 11-I: NMR similar to that of 1-I, with CH3H2 at 1.3 ppm upfield.

Compounds 2-1 and 2-11. Compound 2-1 (47 mg, 0.085 mmol; three isomers) was dissolved in 0.25 mL of anisole and treated with 1.25 mL of TFA at 0°C for 10 min. Volatiles were removed at 30°C (0.1 Torr). Water and CH3Cl2 were added, and the aqueous layer was separated and evaporated in vacuo, leaving 32 mg (96%) of 2-11 as the TFA salt: NMR (D2O ppm from HDO) 2.4 (up field, 3 H, ArCH3), 2.3 (m up, 2 H, CH2=CH), 1.4 (m up, 1 H, CHCl2), 1.4 (d up, J = 7 Hz, 2 H, CHPh2), 0.45 (up, CH and CHCl) 3.1 (d down, 3.5 d down, J = 9 Hz, 4 H, C6H4).

NMR similar to that of 5-1 in 11-I, was similarly obtained 14.6 mg (116%) of 11-I: NMR similar to that of 1-I, with CH3H2 at 1.3 ppm upfield.

Compounds 3-1 and 3-11. Compound 3-1 (15 mg) was treated with TFA-anisole as above, yielding 9.0 mg of 3-11 (83%). Similarly, 7.5 mg (84%) of 3-11 was obtained from 12 mg of 18-II. Both had similar NMR spectra (D2O ppm from HDO): 2.4 (up field, 2 H, CH2=CH), 1.0 (m up, 0.6 (m up), and 0.3 (m up, CHs, CH2Cl and CH=CH). 3.2 (d, J = 7 Hz, 2 H, C6H4).

Radioiodinated Compounds 1a and 1b. Ring-labeled 1a was prepared according to the scheme outlined by reaction of 14 with ring-tritiated p-nitrobenzenesulfenyl chloride: yield 29.6 mg; 1.37 Ci/mmol, 0.59 Ci/mol.

C5 tritiated compound 1b was prepared by the standard synthetic route beginning with 5-1-[1H]allyglycine: yield, 20.8 mg; 1.39 Ci/mmol, 0.604 Ci/mol.

5-[3H]Propargylglycine. L-Propargylglycine (2-amino-4-butyonate; 400 mg, ca. 4 mmol) was dissolved in 0.5 mL of 1 N NaOH and then added dropwise to a solution of iodine (0.3 mmol) in 0.5 mL of CH2Cl2 at 0°C. The solution was stirred for 10 min and then lyophilized to dryness. The residue was neutralized with the addition of 1.0 mL of 2 N HCl and then lyophilized. The sample was dissolved in water and repeatedly lyophilized to constant specific activity; recrystallized from water–ethanol; specific activity, 8.0 Ci/mol. The synthesis of propargylglycine has been described elsewhere.

5-[3H]Allyglycine. 5-1-[3H]Allyglycine (114 mg, 1 mmol; 8.0 Ci/mol) was dissolved in 5 mL of H2O. Hydrogenation was carried out at room temperature, 1 atm, with an Adams catalyst (5 mg of PtO2). The reaction was stopped when 1.1 equiv of H2 had been taken up and the catalyst filtered away. Paper chromatography (butanol–acetic acid–water; 1:1:1:1) revealed allyglycine (80%; estimated by radioactivity scanning) and norvaline (20%). To the solution was added unlabeled allyglycine and recrystallization was accomplished twice from water–ethanol. The product was recrystallized with a small amount of norvaline (4% by weight, 10% by radioactivity; TLC analysis); specific activity, 3.3 Ci/mol.

Biochemical Analyses. I. Enzymes and Substrates. Methionine gamma-lyase (EC 4.4.1.1) was the generous gift of Professor Kenji Soda and was purified from Pseudomonas ovalis (IFO 3738) according to the method of Tanaka.2 The specific activity for gamma elimination on L-methionine (reaction 3, assay described below) was 3.5 U/mg. Homochiral used in these experiments had 96% of L-enantiomor at 20°C. Values for A349/A418 were obtained for enzyme stored for long periods. It seems likely that the enzyme suffers both autooxidation and loss of the pyridoxal cofactor, as we have had some success in regenerating fully active enzyme by dialysis against KF buffered (pH 7.3) containing 50 pL MP and 0.1 mL diithiothreitol. Dialysis also reduces the native A349/A418 ratio.

Cystathionine gamma-synthetase (EC 4.2.99.99) was purified from Salmonella typhimurium megA (ATCC 25241) as described previously.11,12 Homogeneous enzyme has a specific activity of 20 U/mg for the gamma elimination of succinate from O-succinyl-L-homoserine (half-life of eq 4, assay described below). The homozyme has a absorbance maxima at 280 and 422 nm; the A349/A418 was 3.90 for purified en-
zyme. O-Succinyl-l-homoserine (OSHS), L-methionine, L-cysteine hydrochloride, diithiothreitol (DTT), and glutathione were Sigma products. p-Nitrophenylthiol, 2-mercaptoethanol, and 3-mercapto-
propionate were obtained from Aldrich. Lactate dehydrogenase (LDH) and reduced nicotineamide adenine dinucleotide (NADH) were Boehringer products. All other reagents were of the best commercial grade.

II. Enzyme Assays. Each of the two enzymes was assayed for the ability for form α-ketobutyril by the continuous reduction of the keto acid product in the presence of LDH and NADH; product keto-
butyrate formation was monitored as the disappearance of the ab-
sorbance of NADH at 340 nm. Reaction conditions for each enzyme assay have been described.12

III. Reactions with Inhibitor and Inhibitor Analogues. A. Inactivation
Kinetics. The following general protocol was employed for the de-
termination of the rate of enzymatic inactivation using 1 and its
structural analogues. At time zero, enzyme was added to a solution of the putative inactivator in 50 mM KP buffer, pH 7.3,39 at 37 °C. Aliquots (usually 25 µL or less) were removed at intervals and assayed for remaining enzymatic activity by dilution to a 1:0.0 solution of the appropriate assay solution. The inactivation half-time was ob-
tained from semilog plots of percent activity remaining vs. time. Multiple inactivation experiments were conducted at varying con-
centrations of inactivator to determine values of k1 and k2 for the process described by eq 5.

For inactivation of cystathionine γ-synthetase, the inactivation system usually contained 15 µg of enzyme in 120-µL total volume (for methionine γ-lyase, 130 µg of enzyme in 500 µL).

B. Stoichiometry of Inactivation. The stoichiometry of inactivation was determined as follows. Each enzyme (0.45 mg of cystathionine γ-synthetase and 2.1 mg of methionine γ-lyase) was reacted in 1.0 mL with 0.5 mM 1a or 1b, and the loss of activity was monitored by dilution to a 1:0.0 solution assay system. Reactions were carried out in a 1-ml quartz cuvette at 37 °C. The UV-visible spectra were obtained periodically during inactivation. Spectra were obtained using the Perkin Elmer 544 spectrophotometer.

Inactivated enzyme (<10% residual activity) was loaded onto a Sephadex G25 column (1 × 33 cm, 25.9 mL) which had been previously calibrated for separation of bovine serum albumin (Sigma, 1.0 mg) and 0.19 mM [35S]proline (New England Nuclear, 270 Ci/mole). The column was eluted (0.5 mL/h) with 10 mM KP buffer, pH 7.3, 4 °C, and 0.5 or 1.0-mL fractions were collected. Each fraction was examined for absorbance and counted for 3H radioactivity.

V. High performance liquid chromatography was performed using a Waters Associates LC system. Chromatography of p-nitrophenyl-
thiolate was accomplished using a Waters μ-Bondapak C-18 column (0.4 × 30 cm) run with 10 mM NaP buffer, pH 7.0 in 10% ethanol, at a flow rate of 2.0 mL/min. Nitrophenylthiol was detected by absorbance at 405 nm.

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 Glycine.

References and Notes

(1) Abbreviations used are: Boc, tert-butoxyacarbonyl (Boc in structures); DTT, diithiothreitol; ELN, triethylamine; EDTAC, ethyl acetate; KP, potassium inorganic phosphate; KPP, potassium inorganic pyrophosphate; LDH, lactate dehydrogenase, mCPBA, m-chloroperbenzoic acid; NADH, reduced nicotineamide adenine dinucleotide; NaPF, sodium inorganic phosphate; OSSH, O-succinyl-l-homoserine; TFA, trifluoroacetic acid; MeSOI, tetra-

methylsilane; PLP, pyridoxal phosphate.


(10) A stoichiometric resolution may have been obtained based on the chirality of sulfur at the enzyme. See Experimental Section.


(14) Based on a determined extinction coefficient of 12.3 × 10 M⁻¹ cm⁻¹ for authentic p-nitrophenylthiol in 20 mM KP buffer, pH 7.3, 10 mM di-
thiothreitol.


(17) In addition to nitrogen and sulfur, oxygen is an available enzymic nucleophile for attack on the electrophiic sulfur of 5. The structure of the inactivated enzyme resulting in a sulfane ester, probably unstable to solvolysis or subsequent transfer to a secondary enzymic nucleophile (N or S) and, therefore, consistent with the formation of an irreversibly labeled protein.

(18) Or sulfenimide if the nucleophile is an imidazole secondary nitrogen provided by an enzymatic histidine.


(22) It is also possible that $S$ can collapse directly to 12 generating by $\alpha$-carbonization-assisted elimination, an electrophiic p-nitrophenylsulfenic acid. If the sulfenic acid were intercepted by an enzyme nucleophile before escape from the active site, apparent stoichiometric labeling by $1 \alpha$ would result. [See D. Barton, P. Sammes, M. Taylor, C. Cooper, G. Hewitt, B. Locket, and G. Underwood, Chem. Commun., 1137 (1971).]

(23) Enzymatic attack on $S$ at either the vinylogous $C(SO_{2})_{2}$ or the bisalicylic $C(SO_{2})_{2}$ could also generate a p-nitrophenylsulfenic acid.


(29) While both enzymes give maximal activity at about pH 7.0 and 7.3 to protect against base-catalyzed halide elimination upon the inactivator.