A. L.; Hruska, F. E.; Ogilvie, K. K.; Holy, A. *Can. J. Chem.* **1978**, *56*, 1170–1176.
(32) Son, T.-D.; Chachaty, C. *Biochim. Biophys. Acta* **1973**, *335*, 1–13.
(33) (a) Akasaka, K.; Imoto, T.; Shibata, S.; Hatano, H. *J. Magn. Reson.* **1975**, *18*, 328–343. (b) Zens, A. P.; Bryson, T. A.; Dunlap, R. B.; Fisher, R. R.; Ellis, P. D. *J. Am. Chem. Soc.* **1976**, *98*, 7559–7564.
(34) Davies, D. B.; Danyluk, S. S. *Biochemistry* **1975**, *14*, 543–554.
(35) For a recent review, see De Clercq, E.; Torrence, P. F. *J. Carbohydr. Nucleosides Nucleotides* **1978**, *5*, 187–224.
(36) Torrence, P. F.; Huang, G.-F.; Edwards, M. W.; Bhooshan, B.; Descamps, J.; De Clercq, E. *J. Med. Chem.* **1979**, *22*, 316–319.
(37) (a) Hillen, W.; Gassen, H. G. *Biochim. Biophys. Acta* **1978**, *518*, 7–16. (b) Mertes, M. P.; Chang, C. T.-C.; De Clercq, E.; Huang, G.-F.; Torrence, P. F. *Biochem. Biophys. Res. Commun.* **1978**, *84*, 1054–1059.
(38) Prusoff, W. H.; Ward, D. C. *Biochem. Pharmacol.* **1976**, *25*, 1233–1239.
(39) Freese, E. *J. Mol. Biol.* **1959**, *1*, 87–105.
(40) Wataya, Y.; Santi, D. V.; Hansch, C. *J. Med. Chem.* **1977**, *20*, 1469–1473.
(41) (a) Huheey, J. E. *J. Phys. Chem.* **1965**, *69*, 3284–3291. (b) *Ibid.* **1966**, *70*, 2086–2092.
(42) Hansch, C.; Leo, A.; Unger, S. H.; Kim, K. H.; Nikaitani, D.; Lien, E. J. *J. Med. Chem.* **1973**, *16*, 1207–1216.
(43) Baur, W. H. *Acta Crystallogr., Sect. B* **1972**, 1456–1465.
(44) Altona, C.; Sundaralingam, M. *J. Am. Chem. Soc.* **1973**, *95*, 2333–2344.
(45) Ekiel, I.; Remin, M.; Darzynkiewicz, E.; Shugar, D. *Biochim. Biophys. Acta* **1979**, *562*, 177–191.
(46) Ahmed, F. R.; Hall, S. R.; Pippy, M. E.; Huber, C. P. *J. Appl. Crystallogr.* **1973**, *6*, 309–346.
(47) Johnson, C. K. Oak Ridge, Tenn., 1970, ORTEP Report ORNL-3794 (2nd revision).

Mechanism-Based Enzyme Inactivation Using an Allyl Sulfoxide–Allyl Sulfenate Ester Rearrangement[1]

Michael Johnston,[†] Ronald Raines,[†] Christopher Walsh,*[†] and Raymond A. Firestone*[‡]

Contribution from the Departments of Chemistry and Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and the Membrane and Arthritis Research Department, Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065.
Received December 3, 1979

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 incorporates roughly 1 mol of tritium per mol of enzyme monomer when inactivated by 2-amino-4-chloro-5-(*p*-nitro[^{3}H]phenylsulfinyl)pentanoic acid (**1a**), confirming that the modification of each protein is covalent and stoichiometric. Substoichiometric labeling (0.12 mol of tritium per mol of enzyme monomer) is given when methionine γ-lyase is fully inactivated by 2-amino-4-chloro-5-[^{3}H]-5-*p*-nitrophenylsulfinyl)pentanoic acid (**1b**). Both enzymes, inactivated by **1**, are susceptible to reactivation by thiols. Inactivated cystathionine γ-synthetase recovers 25% of its catalytic activity upon incubation with excess dithiothreitol, while methinonine γ-lyase is 100% reactivated by dithiothreitol, mercaptoethanol, and mercaptopropionate. Reactivation generates *p*-nitrophenylthiolate anion, which forms, in the case of methionine γ-lyase, stoichiometrically with enzyme reactivated. Both enzymes are "protected" from inactivation by **1** in the presence of thiols, which simultaneously generates *p*-nitrophenylthiol. In the presence of dithiothreitol, the protection reaction gives *p*-nitrophenylthiol production with pseudo-first-order kinetics. 2-Amino-4-chloro-5-(*p*-tolylsulfinyl)pentanoic acid (**2**) and 2-amino-4-(*p*-nitrophenylsulfinyl)-5-chloropentanoic acid (**3**), the reverse regioisomer of **1**, have also been prepared and give no evidence of inactivation of either enzyme. The data are taken to indicate a novel form of suicide inactivation (Scheme II) wherein β-carbanion-assisted γ-halide elimination generates an allyl sulfoxide-enzyme-pyridoxal adduct (**4**) which undergoes spontaneous 2,3-sigmatropic rearrangement to an electrophilic allyl sulfenate ester (**5**). The latter is then captured by an enzymic nucleophile to give an inactive enzyme **6**, which may be a mixed disulfide or, less likely, a sulfenamide.

Introduction

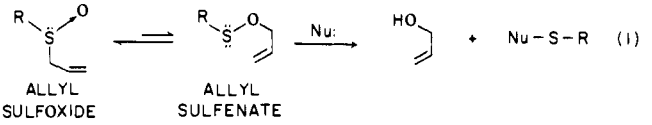
Considerable interest has been generated in the last several years in mechanism-based enzyme inactivators, also called suicide substrates.[2] Much of this interest results from the fact that the targeted enzyme uses some portion of its catalytic mechanism to "unmask", from an otherwise chemically *unreactive* 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,[2] which become activated usually by rearrangement or elimination to generate electrophiles susceptible to Michael-type addition by an active-site nucelophile. Certain functionalized penicillins, such as the clavulanates and penicillin sulfones,[3] cyclopropylamines,[4] fluoro- and nitrodeoxyuridylates,[5] and such drugs as allopurinol[6] 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, wherein the first partner in the rearrangement would be unreactive to nucleophilic addition but the second, rearranged partner would serve to derivatize the enzyme. In this regard, we have chosen the 2,3-sigmatropic rearrangement of allyl sulfoxides[7a,b] (unreactive to nucleophiles) to allyl sulfenate esters (highly reactive to nucleophilic addition)[8a,b] 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-

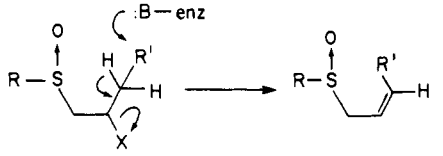
[†] Massachusetts Institute of Technology.
[‡] Merck, Sharp and Dohme Research Laboratories.

0002-7863/80/1502-4241$01.00/0



(1)

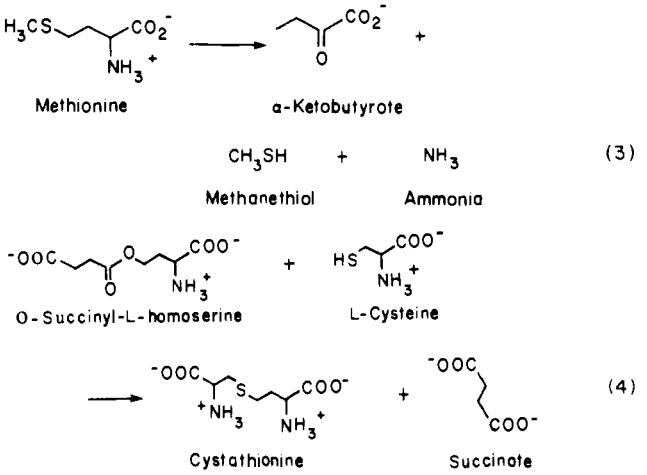
sidered that a halo sulfoxide precursor might undergo enzyme-mediated loss of HX to "uncover" the allylic double bond required for rearrangement (eq 2). Thus, the reaction strategy



(2)

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).[9] 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.

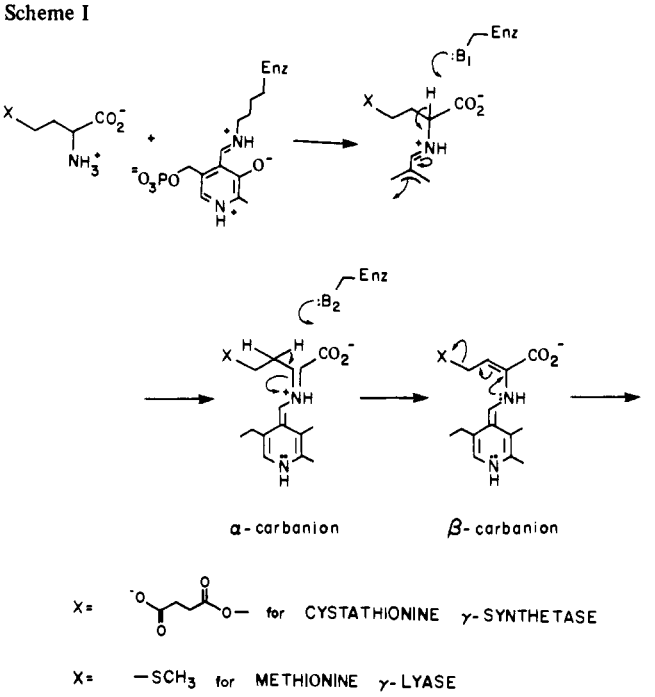
In this paper we report the preparation of amino acids **1** and **2** and the inactivation of both methionine γ-lyase and cystathionine γ-synthetase, with evidence for the indicated 2,3-sigmatropic rearrangement as the operating mechanism in these inactivations.

Results

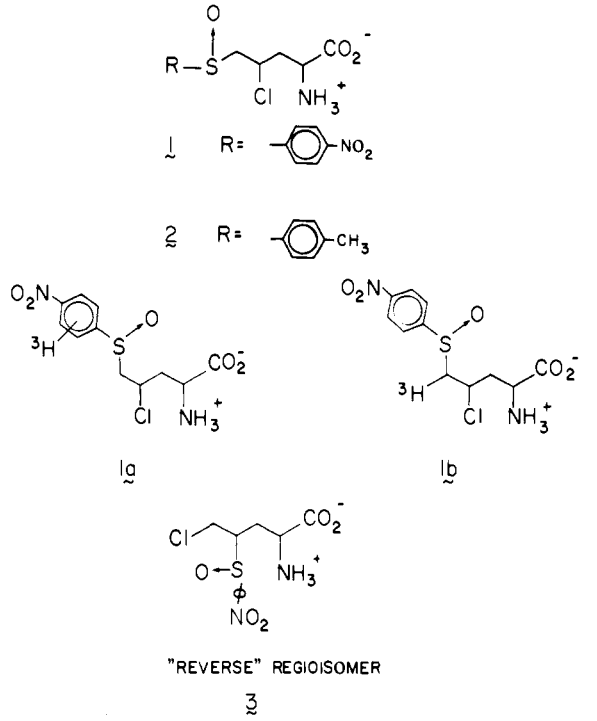
I. Kinetics of Inactivation. 2-Amino-4-chloro-5-(*p*-nitrophenylsulfinyl)pentanoic acid (**1**) is a time-dependent, irreversible inactivator of both cystathionine γ-synthetase and methionine γ-lyase. Time-dependent loss of catalytic activity ultimately results in fully inactivated enzyme. Typical semilog 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.

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E\text{-}I \qquad (5)$$

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_I and minimal values for k_2.



We have sucessfully 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 4*R* and 4*S* γ isomers by a two-step carbanion mechanism.[11] The absolute stereochemistry at sulfur may also not determine

Table I. Kinetic Parameters for Inactivation by 2-Amino-4-chloro-5-(*p*-nitrophenylsulfinyl)pentanoic acid (**1**)

enzyme	K_I, mM	$k_2 \times 10^4$, s^{-1}
cystathionine γ-synthetase	0.38	6.5
methionine γ-lyase	1.50	5.1

the overall observed rate of inactivation if the electrophilic sulfenate is in rapid equilibrium with the sulfoxide; reversible rearrangement would effectively racemize[7a,b] the sulfoxide sulfur prior to interception.

II. Stoichiometry of Labeling. A. Ring-Labeled Inactivator. Figure 2 gives the elution profiles of the two enzymes inactivated by the ring-labeled compound **1a** and subsequently exposed to Sephadex G25 gel filtration chromatography. A peak of radioactivity coelutes in each case with the protein absorbance at 280 nm, findings which confirm our expectation that the inactivations are covalent and irreversible. As seen in the figure, a second elution band of radioactivity coelutes with absorbance at 254 nm. This represents excess, unreacted compound **1a** which has a single ultraviolet absorbance band with λ_{max} at 254 nm ($\epsilon = 1.38 \times 10^3$ cm^{-1} M^{-1}).

The total radioactivity recovered under the protein peak in Figure 2A corresponds to a stoichiometry of labeling for cystathionine γ-synthetase of 1.08 molar equiv of ^3H per mol of enzyme monomer. The enzyme is an $\alpha_2\beta_2$ tetramer[12] (mol wt 160 000) which contains 1 mol of PLP per mol of enzyme monomer.[13] All subunits in the enzyme tetramer are modified on complete inactivation.

The total radioactivity recovered under the protein peak in Figure 2B corresponds to a stoichiometry of labeling for methionine γ-lyase of 1.18 molar equiv of ^3H per mol of enzyme monomer. Methinonine γ-lyase is also an $\alpha_2\beta_2$ tetramer (mol wt 180 000) which contains one tightly bound PLP per enzyme monomer.[12]

B. Carbon Chain-Labeled Inactivator. Figure 3 gives the Sephadex G25 elution profile of methinonine γ-lyase fully inactivated by the 5-^3H-labeled compound **1b**. The total radioactivity recovered under the protein peak corresponds to a substoichiometric labeling of only 0.12 equiv of ^3H per enzyme monomer. This label, like those obtained from the ring-labeled molecule, is stable to prolonged dialysis.

Differential labeling ($\simeq$1 label incorporated from **1a** and only $\simeq$0.1 label incorporated from **1b**) is consistent with fragmentation of the inactivator and suggests that the inactivating alkylation is characterized predominantly by incorporation of the *p*-nitrophenyl fragment.

III. Reactions with Thiols. A. Thiol Reactivation. Both cystathionine γ-synthetase and methionine γ-lyase, fully inactivated by **1**, are reactivated as catalysts by incubation with thiols. But note from Table II that while methinone γ-lyase is fully reactivated by both mono- and dithiols, cystathionine γ-synthetase is only partially reactivated, and then only by dithiothreitol.

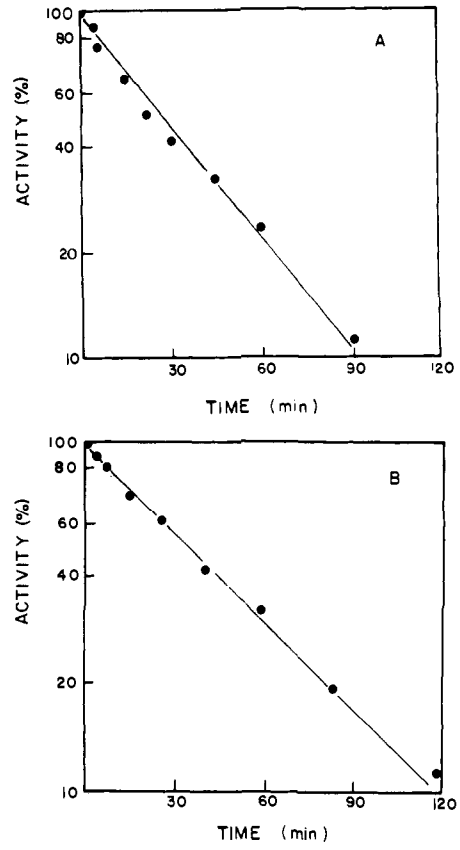


Figure 1. Kinetics of inactivation of cystathionine γ-synthetase (A) and methionine γ-lyase (B) by 1.0 mM **1**, pH 7.3, 37 °C.

Reactivation of methionine γ-lyase (and of cystathionine γ-synthetase by DTT) generated *p*-nitrophenylthiolate anion (vide infra), λ_{max} 408 nm at pH 7.3.

For methionine γ-lyase, the rate of recovery of catalytic activity is a function of the thiol used (see Table II); both monothiols reactivate at rates ($t_{1/2}$ = 6.75 and 8.2 min, respectively) slower than that given by dithiothreitol ($t_{1/2}$ = 2.5 min). Note also from Table II that *p*-nitrophenylthiol, in amounts stoichiometric with enzyme reactivated, accumulates in apparent synchrony with full recovery of catalytic activity when DTT is the reactivating thiol. However, for the two monothiols tested, stoichiometric quantities of *p*-nitrophenylthiol are generated *prior to* full reactivation of the enzyme.

B. Thiol Protection. Both enzymes are protected from loss of catalytic activity if the inactivation sequence is carried out in the presence of excess thiol. Figure 4 shows that the half-time for inactivation of cystathionine γ-synthetase is increased by added thiols, although none of the thiols tested affords complete protection. It is of interest to note that cystathionine γ-synthetase is protected from inactivation by both mercap-

Table II. Thiol Reactivation of Cystathionine γ-Synthetase and Methionine γ-Lyase Inactivated by 2-Amino-4-chloro-5-(*p*-nitrophenylsulfinyl)pentanoic acid (**1**)

thiol (10 mM)	% act. recovered: cystathionine γ-synthetase (~20 μM)	% act. recovered: methionine γ-lyase (~50 μM)	half-time for recovery of 100% catalytic act., min[a]	*p*-nitrophenyl thiol production, [a] $t_{1/2}$, min[b]
dithiothreitol	25	100	2.5	2.6
2-mercaptoethanol	0	100	6.75	4.3
3-mercaptopropionate	0	100	8.2	3.7
L-cysteine	0	n.d.	n.d.	n.d.

[a] Data for methionine γ-lyase only. [b] For generation of *p*-nitrophenylthiolate stoichiometric with enzyme reactivated. n.d. = not determined.

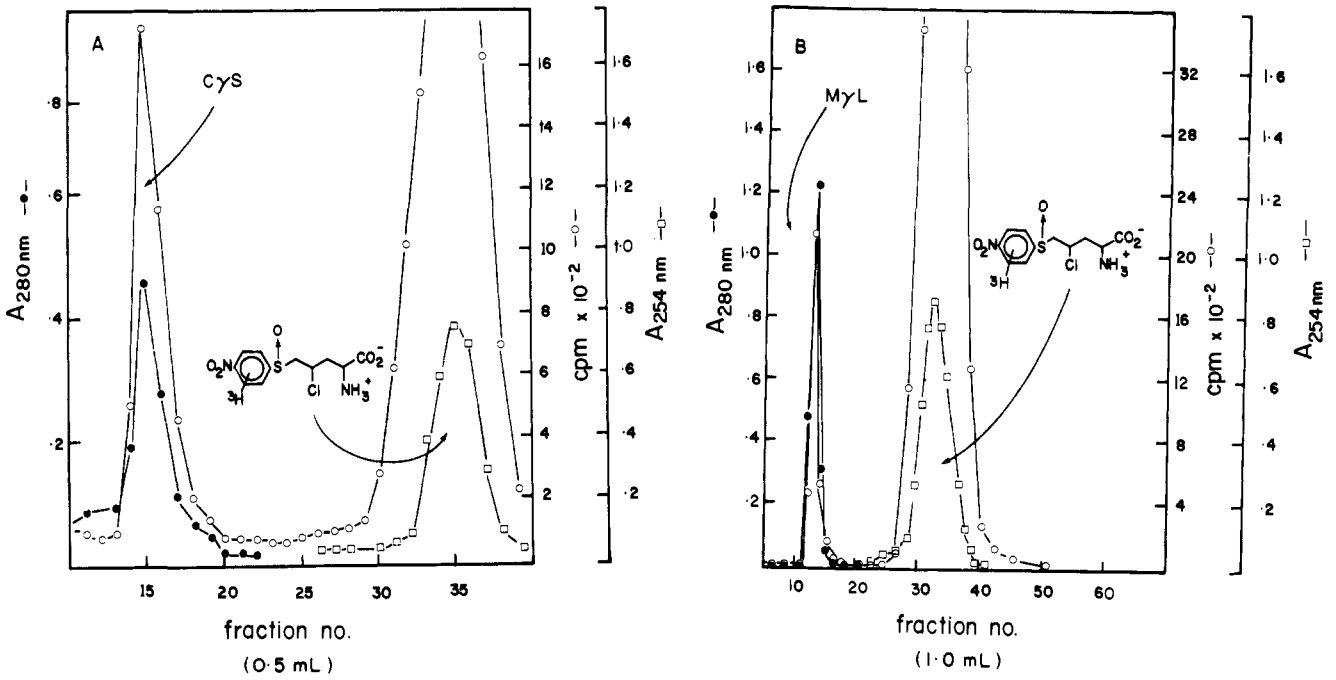


Figure 2. Sephadex G25 elution profiles of cystathionine γ-synthetase (A) and methionine γ-lyase (B) after inactivation by **1a**. Experimental conditions are given in the text.

toethanol 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 λ_{max} at 280 and 418 nm; the A_{280}/A_{418} = 4.85 for native enzyme.[12] 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 ($t_{30\ min}$, 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 (λ_{max} 408 nm at pH 8.2; $\epsilon = 10.5 \times 10^3\ cm^{-1}\ M^{-1}$) 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

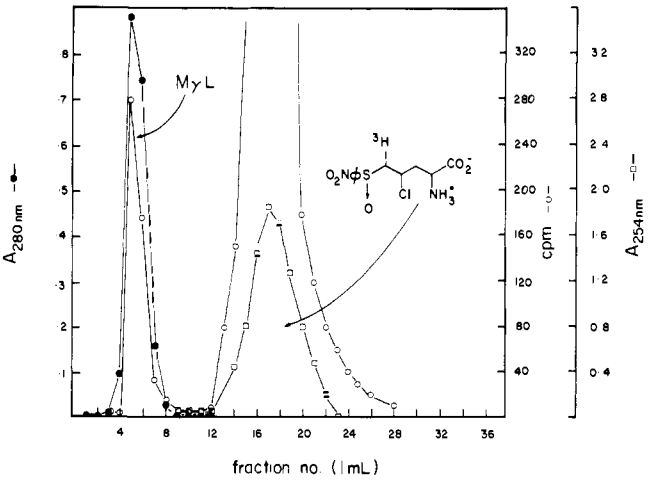


Figure 3. Sephadex G25 elution profile of methionine γ-lyase inactivated by **1b**. Experimental conditions are given in the text.

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 μM solution of nitrophenylthiol,[14] 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}H-Ring-Labeled Methionine γ-Lyase. Methionine γ-lyase, which had been fully inactivated

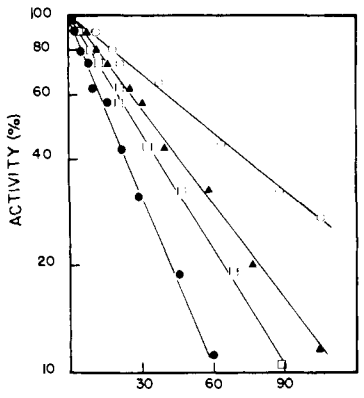


Figure 4. Kinetics of inactivation of cystathionine γ-synthetase by 1 mM **1** (●), pH 7.3, 37 °C, and by 1 mM **1** in the presence of 10 mM dithiothreitol (○), 10 mM 3-mercaptopropionate (▲), and 10 mM 2-mercaptoethanol (□).

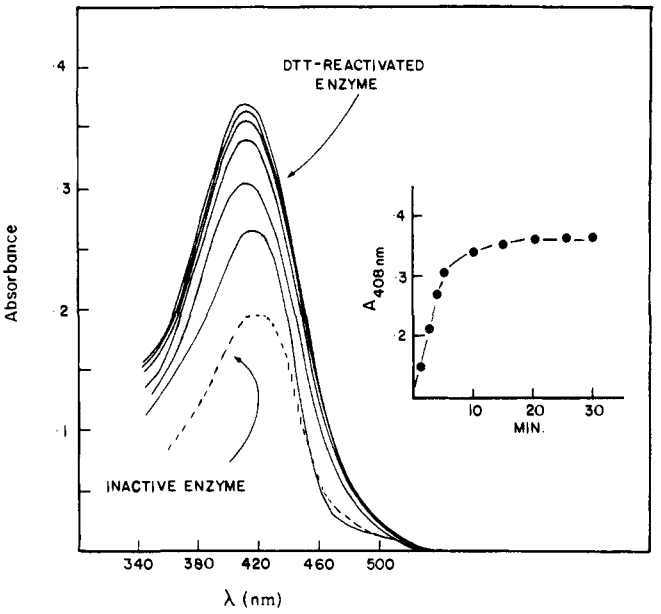


Figure 5. Absorbance spectra of methionine γ-lyase obtained during reactivation by 10 mM dithiothreitol (solid lines) of enzyme fully inactivated by **1** (dashed line).

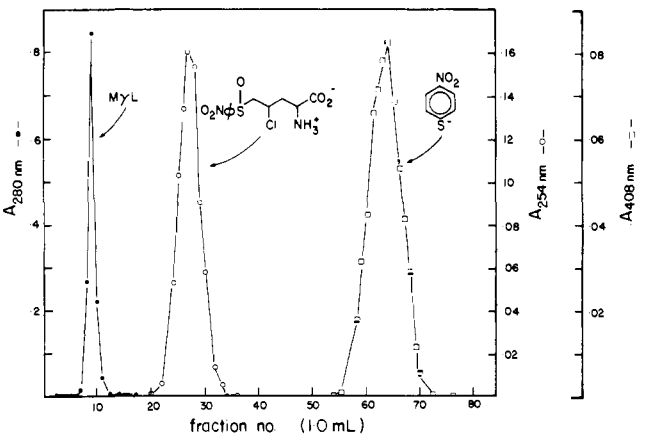


Figure 6. Sephadex G25 elution profile of methionine γ-lyase which had been fully reactivated by 10 mM dithiothreitol. Experimental conditions in the text.

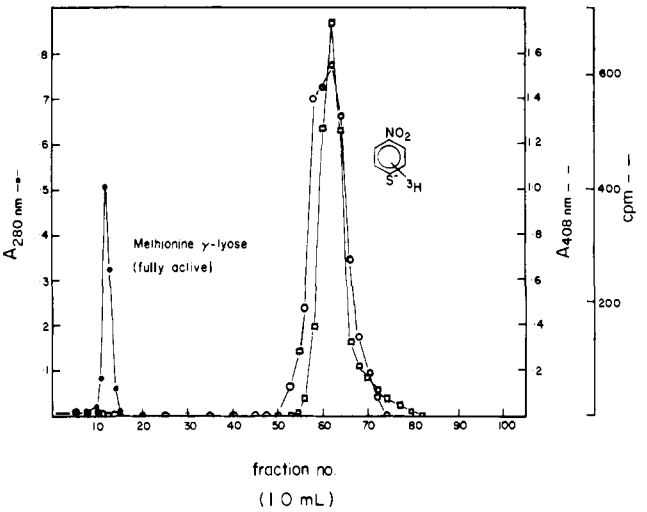


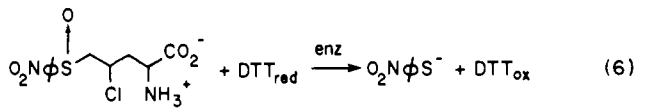
Figure 7. Rechromatography (Sephadex G25) of the radiolabeled protein recovered from the Sephadex column chromatography experiment described by Figure 2B. The enzyme (methionine γ-lyase) was incubated with 10 mM dithiothreitol, and full catalytic activity was regained prior to the second gel filtration. 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 ^{3}H-ring-labeled fragment of the inactivator incorporated into methionine γ-lyase is a quantitative precursor, under mild reductive conditions of RS^- addition, of *p*-nitrophenylthiolate 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*-ni-

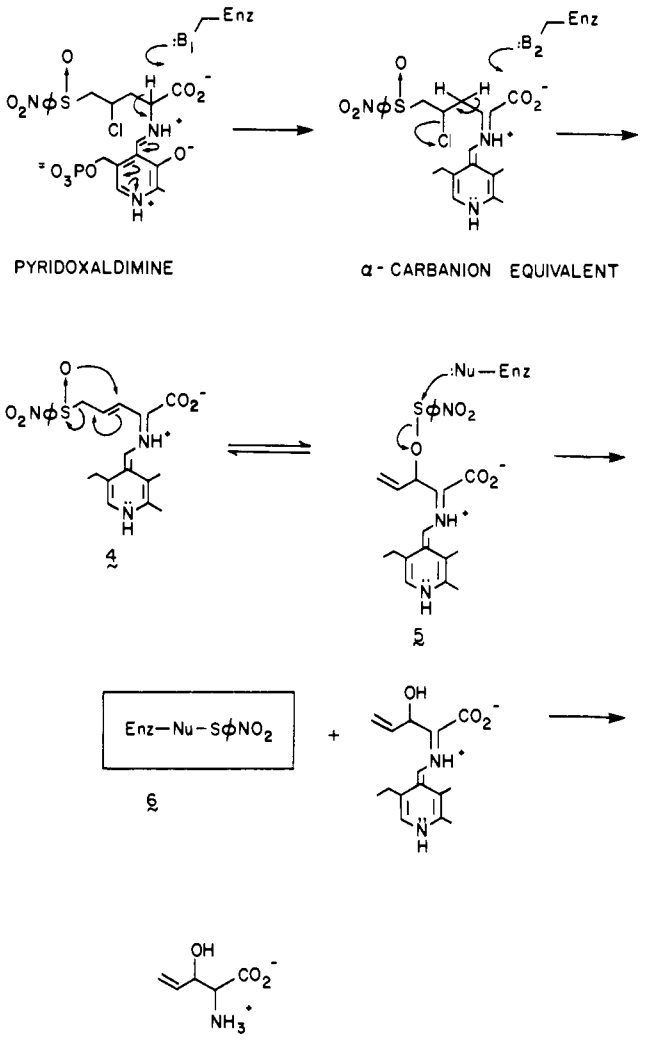


trophenylthiol in the absence of enzyme. The K_M for **1** in eq 6 is 1.1 mM (compare K_I = 1.5 mM for inactivation). The overall first-order rate constant, k_2, for nitrophenylthiol production in eq 6 is 4.7×10^{-3} s^{-1} (compare $k_2 = 6.5 \times 10^{-4}$ s^{-1} for pseudo-first-order inactivation). These results represent a steady state between rates of inactivation and of turnover to *p*-nitrophenylthiol during protection.

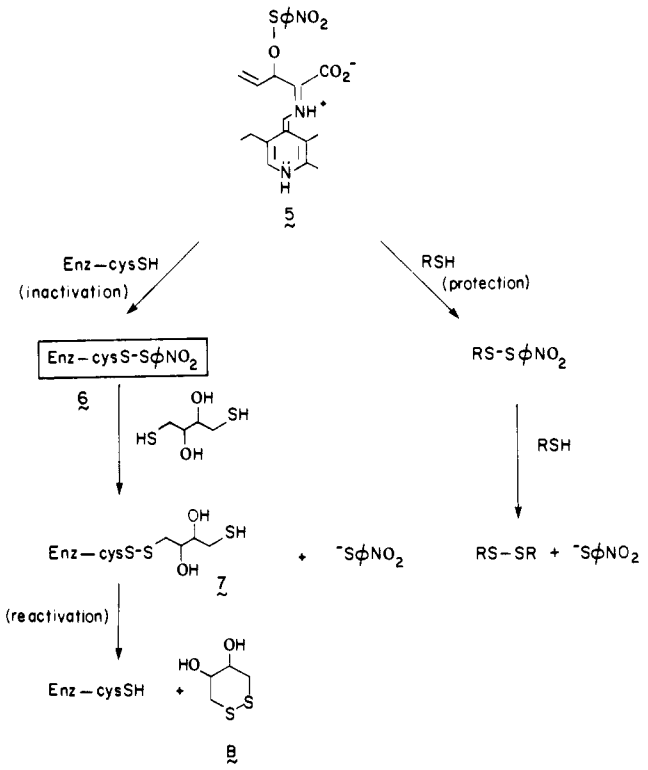
Discussion

I. Inactivation Mechanism. We propose that 2-amino-4-chloro-5-(*p*-nitrophenylsulfinyl)pentanoic acid (**1**) 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

Scheme II



Scheme III



amino acid biosynthesis; this reaction has been well characterized as a 3,3-sigmatropic, Claisen rearrangement,[15] 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.[16]

For the inactivations described here we suggest that the pyridoxal phosphate enzymes carry out the normal catalytic sequence on **1**: that is, formation of stabilized α- and then β-carbanion equivalents. β-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 β and γ carbons of amino acid substrates is now well documented.[9,11]

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 α- and β-carbanion formation, an allyl sulfoxide functionality cannot be uncovered.

Mislow and his colleagues observed that at equilibrium both *p*-tolyl and *p*-nitrophenyl allyl sulfoxides exist almost exclusively in the sulfoxide form (>99%), but that they undergo reversible sigmatropic rearrangement at very different rates,[7b] Equilibration of *p*-nitrophenyl allyl sulfoxide could be studied conveniently at 7 °C, while the *p*-tolyl analogue required 40–60 °C. Since our enzyme inhibition studies were conducted at 37 °C, it is easy to understand why the *p*-tolyl compound (**2**), in contrast with the *p*-nitrophenyl compound (**1**), is not an inactivator. It is possible that a shift in the equilibrium toward sulfenate also plays a role, since strongly electron-withdrawing groups such as trichloromethyl do cause a measurable shift.

Scheme II proposes that the uncovered electrophile reactive to enzymatic nucleophilic attack is **5**, the PLP-allyl sulfenate ester; attack on sulfur is expected from model chemistry[8] to be the predominant mode for cleavage of the S–O bond. The structure of the inactivated enzyme **6** is most probably a mixed disulfide between an enzymatic cysteine sulfhydryl (EnzNu of Scheme II is CysSH) and *p*-nitrophenylthiol. This structure best accommodates our results for thiol reactivation, thiol protection, and *p*-nitrophenylthiol production. These results are summarized by the mechanism proposed in Scheme III.

II. Thiol Reactivation. Methionine γ-lyase, fully inactivated, is fully reactivated by incubation with thiols. When dithiothreitol is the reactivating thiol, *p*-nitrophenylthiol is produced in synchrony with the recovery of enzyme activity and in amounts stoichiometric with enzyme initially inactivated (Table II). These results suggest that reactivation of the dead enzyme by dithiothreitol proceeds by disulfide interchange between **6** and the free sulfhydryls of DTT, displacing *p*-nitrophenylthiolate. The *new* disulfide resulting (**7**) may then undergo intramolecular thiol reduction of the disulfide to generate a free enzymatic cysteine and, thus, fully reactivated enzyme.

Monothiols will also undergo disulfide interchange with **6**, generating *p*-nitrophenylthiol by displacement. But the new mixed disulfide in this case (EnzCysS-SR) will generate a free enzymatic cysteine sulfhydryl only upon reaction with a *second* equivalent of RSH. It seems likely that this bimolecular disulfide interchange will be slower than the intramolecular disulfide reduction given from **7**, accounting for (1) the slower rates of enzymatic reactivation afforded by monothiols and (2) the lag in recovery of catalytic activity relative to *p*-nitrophenylthiol production (Table II).

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

(from S^0 of **1** to S^{2-} of *p*-nitrophenylthiol), as required by an allyl sulfoxide to allyl sulfenate (**4** → **5**) rearrangement.

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[17] (perhaps the ε-amino nitrogen of an enzymatic lysine). The resulting structure **6** could then be a sulfenamide;[18] sulfenamides are known to form readily by attack of primary amines on disulfides (eq 7)[19] and by reaction of amines with sulfenyl chlo-

$$RS-SR + H_2NR' \rightleftharpoons RS-NHR' + RSH \qquad (7)$$

rides.[20] The sulfenamide bond is labile to sodium iodide[20] and to cleavage in acid-catalyzed reactions,[21] but suffers attack by thiols only slowly.[20] 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**.

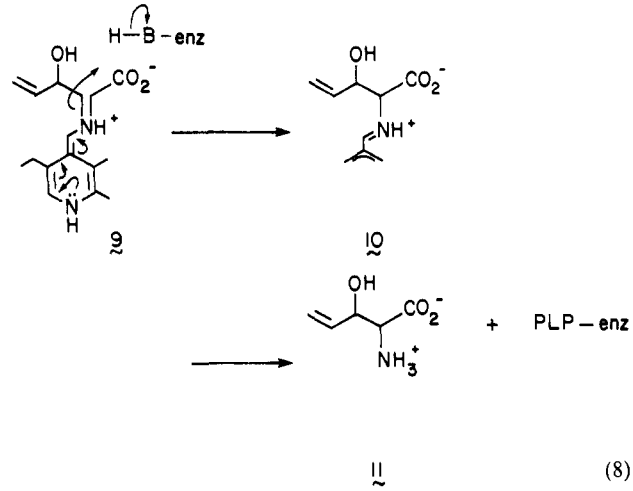
Cystathionine γ-synthetase, however, is only 25% reactivated by dithiothreitol, a finding which might be occasioned by competition between *two* active-site nucleophiles. One of these could be sulfur, giving the thiol-labile disulfide **6** 25% of the time, and the other might be nitrogen, giving the more thiol-stable sulfenamide 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.[11,12]

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 dispartiy 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 sulfoxide 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 2-amino-3-hydroxy-4-pentenoate (**11**). We have not verified this pathway (8) 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.[22] The incorporation of a fractional amount of tritium label by methionine γ-lyase

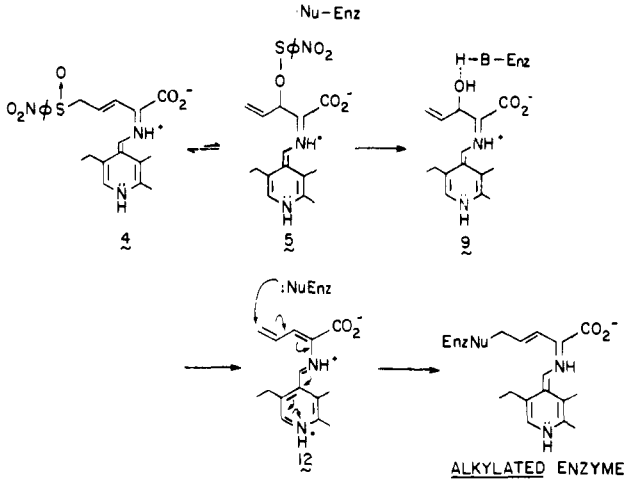


(8)

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 C_1 to generate ultimately the product pentenoate **11**. 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.[23] In support of the key elimination **9** → **12**, we have determined that methionine γ-lyase 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.

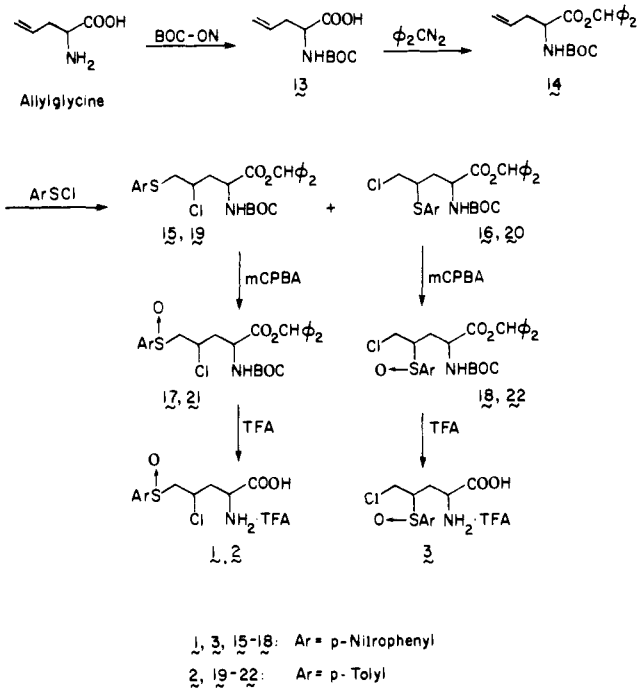
Scheme IV



Conclusion

Two pyridoxal phosphate dependent enzymes are inactivated by nucleophilic capture following a 2,3-sigmatropic rearrangement of an allyl sulfoxide 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

Scheme V



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 were chosen that could be removed simultaneously. At 0 °C in 5:1 TFA–anisole, the minimum reaction time for quantitative cleavage of *N-tert*-butoxycarbonyl (*N*-Boc) residues is 10–12 min; for cleavage of the benzyhydryl esters, 1–2 min was required.

Addition of arylsulfenyl chlorides to the double bond of **14** is fast and quantitative, but the principle kinetic isomers were always the expected,[24] but undesired, **16** and **20.** Thermal isomerization of **20** to the more thermodynamically stable **19** was realized (this transformation may be catalyzed by traces of acid[24]), but **16** could not be isomerized to **15** in this way because rearrangement of **16** was much slower than that of **20,** and was accompanied by degradation. Presumably this rearrangement occurs, as does the addition, via an episulfonium intermediate,[24] which is less easily re-formed from the nitro-substituted β-chloro sulfide **16.**

Compounds **15, 17, 19** and **21** were distinguished from their regioisomers **16, 18, 20,** and **22** by NMR. The CHCl and CHS protons are consistently more deshielded than the CH_2Cl and CH_2S protons, respectively, and have very similar chemical shifts to their counterparts in the benzenesulfenyl chloride–propylene adducts.[24]

Oxidation of the sulfides (**15, 16, 19,** and **20**) to the sulfoxides (**17, 18, 21,** and **22**) created two new stereoisomers at sulfur, which were separable by chromatography. In **1, 2, 17,** and **21,** stereoisomers at CHCl are also possible, but were not seen in the *p*-nitro series. In the *p*-tolyl series the isomers were seen but not separated. Combined sulfoxide stereoisomers were used in most enzyme inhibition experiments.

It was necessary to protect the final products against intramolecular displacement by both the amino group and the carboxylate anion. Therefore, compounds were stored as TFA salts and 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

^1H 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. Meriwether 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, 116 mL of Et_3N (11.5 mmol), and 2.08 g of Boc-ON (Aldrich; 8.45 mmol) 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 separated, 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 (δ, $CDCl_3$) 1.45 (s, 9 H, *t*-Bu), 2.55 (m, 2 H, CH_2 vinyl), 4.35 (br, 1 H, NH), 4.9–6.2 (m, 4 H, α-H and vinyl), 11.7 (s, 1 H, COOH).

***N*-Boc-allylglycine Benzhydryl 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 diphenyldiazomethane. 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 brine, dried with $MgSO_4$, filtered, and evaporated. The crystalline product was washed with hexane and dried: yield, 380 mg; 80%; mp 79–80 °C; NMR (δ, $CDCl_3$) 6.9 (s, 1 H, $CHPh_2$), 7.3 (s, 10 H, Ph_2); MS 381 (M^+), 325 ($M^+ - t$-Bu), 170 ($M^+ - COOCHPh_2$), 167 ($CHPh_2^+$); TLC 50:1 $CHCl_3$–EtOAc, R_f 0.3.

***p*-Tolyl- and *p*-Nitrobenzenesulfenyl Chlorides.** *p*-Tolylsulfenyl chloride was made according to ref 30. p-Nitrobenzenesulfenyl 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;[25] 52 °C[26]).

Tritiated *p*-nitrobenzenesulfenyl 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 $CH_3SO_3[^3H]$-CH_3SO_3H, 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 protio 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_3SO_3H procedure returned good quality disulfide.

The chlorination was performed in this way. Pulverized *p*-nitrobenzene disulfide (1.001 g, 3.25 mmol, in 8 mL of sieve-dried CCl_4) 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 was cooled, filtered, and evaporated in vacuo: yield, 1.186 g (96%) of pure *p*-nitrobenzenesulfenyl chloride; mp 48 °C. The radioactive sample was made in this way from 168 mg of radiolabeled disulfide: yield, 186.3 mg (90%) of ring-tritiated *p*-nitrobenzenesulfenyl chloride.

Compounds 15 and 16. To 381 mg of **14** (1.0 mmol) in 1.5 mL of CH_2Cl_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_2Cl_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 **15** as **20** had been (vide infra). A partial separation was effected by PLC (50:1 $CHCl_3$–EtOAc, four 20 × 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 sulfoxide stage.

NMR of **15** (δ, $CDCl_3$): 1.37 (s, 9 H, *t*-Bu), 2.35 (m, 2 H, β-CH_2), 3.3–3.7 (m, CH_2S), 3.9–4.35 (m, 1 H, CHCl), 4.35–4.85 (m, 1 H,

α-CH), 5.3 (m, 1 H, NH), 6.9 (s, 1 H, C*H*Ph_2), 7.3 s, 10 H, Ph_2), 7.35 (d), 8.0 (d, J = 9 Hz, 4 H, C_6H_4).

NMR of **16:** 2.1 (m, 1 H), 2.55 (m, 1 H, β-CH_2), 3.7 (m, 3 H, CH_2Cl and CHS), 4.5-4.95 (m, 1 H, α-CH).

MS: both compounds, 403 Cl_1 (M^+ − $CHPh_2$), 347 Cl_1 (M^+ − $CHPh_2$ and t-Bu). R_fs on TLC: **15,** 0.4; **16,** 0.5.

Compounds 19 and 20. To 114.3 mg of **14** (0.3 mmol) in 0.5 mL of CH_2Cl_2 at −20 °C under N_2 was added, over 20 min, 47.6 mg of p-tolylsulfenyl chloride (0.3 mmol) in 0.5 mL of CH_2Cl_2. After another 20 min at room temperature, the solvent was evaporated in vacuo. TLC (50:1 $CHCl_3$-EtOAc; R_f 0.5) and NMR showed neither starting compound left. The product was principally **20** by NMR (δ, $CDCl_3$): 1.5 (s, 9 H, t-Bu), 2.35 (s, 3 H, Ar*CH*$_3$), 1.8-2.5 (m, 2 H, β-CH_2), 3.0-3.8 (m, CHS and CH_2Cl), 4.6-5.2 (m, 2 H, α-CH and NH), 6.95 (s, 1 H, C*H*Ph_2), 7.35 (s, 10 H, Ph_2), 7.1 (d), 7.35 (d, 4 H, J = 9 Hz, C_6H_4). During 17-h refluxing in $CHCl_3$ there was a gradual rearrangement to **19,** which was isolated from PLC (50:1 $CHCl_3$-EtOAc, 20 × 20 cm plate, 2-mm layer; R_f 0.5), 105 mg (65%). NMR showed now 3.1-3.4 (m, 2 H, CH_2S), 3.8-4.3 (m, 1 H, CHCl), 4.5-4.9 (m, 1 H, α-CH), 4.9-5.4 (m, 1 H, NH). At 300 MHz, CH_2Cl is at 3.7-3.8 in **20,** and CHCl is at 3.9-4.1 in **19,** both isolated tight multiplets with no overlap.

Compounds 17 and 18. To 128 mg (0.244 mmol) of **15** and **16** (enriched with **15** by PLC) in 4 mL of CH_2Cl_2 at 0 °C under N_2 was added, over 1 h, a solution of 45.5 mg (0.244 mmol) of mCPBA (85% pure) in 4 mL of CH_2Cl_2. After 30 min at room temperature the solution was washed with aqueous $NaHCO_3$, dried over $MgSO_4$, filtered, and evaporated in vacuo: yield, 131 mg. From PLC (4:1 $CHCl_3$-EtOAc, two 20 × 20 cm plates, 1.5-mm layer) was obtained two isomers of **18: 18-I** (35 mg, R_f 0.7) and **18-II** (36 mg, R_f 0.6). Compound **17** (40 mg; R_f 0.3-0.5 in 4:1 $CHCl_3$-EtOAc) also separated on PLC into two isomers: **17-I** and **17-II** (R_fs 0.5 and 0.4, respectively).

Separation between **17** and **18** was usually very good, and overall yields of **17** from **14,** combining fractions from chromatograms of **15** and **17,** were usually 9-10%.

The T-60 NMRs of **17-I** and **17-II**, though discernible, are very similar, so data from 300-MHz spectra are given. For **17-I** (δ, $CDCl_3$): 1.45 (s, 9 H, t-Bu), 2.15 (m), 2.35 (m, 2 H, β-CH_2), 3.1 (m, CH_2S), 4.65 (m, CHCl and α-CH), 5.3 (d, 1 H, J = 8 Hz, NH), 6.9 (s, 1 H, C*H*Ph_2), 7.35 (s, 10 H, Ph_2), 7.85 (d, 8.4 d, 4 H, J = 9 Hz, C_6H_4). For **17-II**: 2.45 (m, 2 H, β-CH_2), 3.25 (m), 3.4 (m, 2 H, CH_2S), 4.3 (m, CHCl), 4.65 (m, α-CH), 5.4 (d, 1 H, J = 8 Hz, NH). NMR (T-60) of **18-I**: 1.5 (s, 9 H, t-Bu), 2.4 (m, 2 H, β-CH_2), 3.1 (m, 1 H, CHS), 3.6 (m, 2 H, CH_2Cl), 4.8 (m, 1 H, α-CH), 5.5 (d, 1 H, J = 8 Hz, NH), 6.95 (s, 1 H, C*H*Ph_2), 7.35 (s, 10 H, Ph_2), 7.9 (d), 8.3 (d, 4 H, J = 9 Hz, C_6H_4). The NMR of **18-II** is similar but with β-CH_2 at 2.25, CHS at 3.25, CH_2Cl at 3.75, and α-CH at 4.1.

The MS of both **17-I** and **17-II** have 529 Cl_1 (M^+ − t-Bu), 419 Cl_1 (M^+ − $CHPh_2$), and 362 (M^+ − t-Bu and $CHPh_2$).

Compound 21. To 95 mg of **19** (0.176 mmol) in 2.5 mL of CH_2Cl_2 at 0 °C under N_2 was added a solution of 36 mg of mCPBA (0.177 mmol; 85% pure) in 2.5 mL of CH_2Cl_2 over 1.7 h. The reaction was aged 50 h at room temperature, washed twice with aqueous $NaHCO_3$, dried over $MgSO_4$, filtered, and subjected to PLC (4:1 $CHCl_3$-EtOAc, two 20 × 20 cm plates, 1-mm layer).

Compound **21** was isolated from two bands: **21-I** (19 mg, 19%, R_f 0.5), which was one isomer by NMR, and **21-II** (47 mg, 48%, R_f 0.35), which was three isomers. Up to four isomers of **21** are possible.

NMR of **21-I** (δ, $CDCl_3$, 300 MHz): 1.45 (s, 9 H, t-Bu), 2.45 (s, 3 H, Ar*CH*$_3$), 2.4 (m, 2 H, β-CH_2), 2.95 (m, 1 H) and 3.2 (m, 1 H, CH_2S), 4.65 (m, 2 H, α-CH and CHCl), 5.35 (m, 1 H, NH), 6.95 (s, 1 H, C*H*Ph_2), 7.35 (s, 10 H, Ph_2), 7.35 (d), 7.55 (d, J = 9 Hz, 4 H, C_6H_4); MS 388 Cl_1 (M^+ − $CHPh_2$), 332 Cl_1 (M^+ − $CHPh_2$ and t-Bu). NMR of **21-II**: 3.0-3.2 (m) and 3.35 (m, 2 H, CH_2S), 4.2 (m, <1 H) and 4.6 (m, >1 H (2 H together), α-CH and CHCl), NH at 5.05, 5.25, and 5.45 (1 H together), t-Bu two unequal lines, 9 H together, C*H*Ph_2 three unequal lines, 1 H together, and at 7.55 there are six lines, 2 H together; MS 388, 332.

Compounds II and III. Compound **17-I** (37 mg) was treated with 0.3 mL of anisole and 1.5 mL of TFA for 11 min at 0 °C by the method outlined for **21** (vide infra), affording 29.6 mg of **1-I** (108%; weight yields sometimes exceeded 100% because of incomplete drying of the glassy product): NMR (D_2O, ppm from HDO) 2.3 (m upfield, 2 H, β-CH_2), 1.4 (d up, J = 7 Hz, 2 H, CH_2S), ~0.5 (m up, α-CH and CHCl), 3.1 (d down), 3.5 (d down, J = 9 Hz, 4 H, C_6H_4.

From 70 mg of **17-II** was similarly obtained 14.6 mg (116%) of **1-II**: NMR similar to that of **1-I**, with CH_2S at 1.3 d upfield.

Compounds 2-I and 2-II. Compound **21-II** (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 CH_2Cl_2 were added, and the aqueous layer was separated and evaporated in vacuo, leaving 32 mg (94%) of **2-II** as the TFA salt: NMR (D_2O, ppm from HDO) 2.4 (m upfield, 3 H, Ar*CH*$_3$), 2.3 (m up, 2 H, β-CH_2), 1.4 (m up, 2 H, CH_2S), 0.4 (m up, ca. 2 H, CHCl and α-CH), 2.65 (d down), 2.8 (d down, 4 H, J = 9 Hz, C_6H_4). ^{13}C NMR (D_2O, broad band decoupled, ppm from Me_4Si using dioxane at 67.40 as secondary standard): 171.7 and 171.6 (COOH), 145.05, 145.0, and 144.8 (C-1′ ArS-O), 131.0, 125.9, 125.8, and 125.6 (C-2′, -3′, -5′, -6′ Ar), 64.1 and 63.1 (CH_2S), 53.3 and 52.5 (CCl), 51.2, 51.0, and 50.8 (α-C), 38.5, 38.3, and 38.2 (β-C), 21.5 (Ar*CH*$_3$).

17-I (18 mg, one isomer) was similarly converted to **2-I** using 0.1 mL of anisole and 0.5 mL of TFA, providing 7.5 mg (56%): NMR same as above, except 0.5 (m up, 1 H, CHCl), ~0.3 (m up, α-CH). ^{13}C NMR was the same as above but only one line per carbon atom.

Compounds 3-I and 3-II. Compound **18-I** (15 mg) was treated with TFA-anisole as above, yielding 9.0 mg of **3-I** (83%). Similarly, 7.5 mg (84%) of **3-II** was obtained from 12 mg of **18-II**. Both had similar NMR spectra (D_2O, ppm from HDO): 2.4 (m upfield, 2 H, β-CH_2), 1.0 (m up), 0.6 (m up), and ~0.3 (m up, CHS, CH_2Cl and α-CH), 3.2 (d) and 3.7 (d down, J = 9 Hz, 4 H, C_6H_4).

Radiolabeled 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 μCi/mg, 0.595 Ci/mol.

C_5 tritiated compound **1b** was prepared by the standard synthetic route beginning with C_5-[^{3}H]allylglycine: yield, 20.8 mg; 1.39 μCi/mg, 0.604 Ci/mol.

5-[^{3}H]Propargylglycine. L-Propargylglycine (2-amino-4-butynoate; 400 mg, ca. 4 mmol) was dissolved in 0.5 mL of 1 N NaOH and evaporated to dryness in vacuo. The solid residue was dissolved in 0.5 mL of ^3H_2O (5 Ci/mL), stirred at room temperature for 30 min, and then lyophilized to dryness. The glassy residue was neutralized by the addition of 1.0 mL of 2 N HCl and then lyophilized. The sample was then 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.[27]

***C*$_5$-[^{3}H]Allylglycine.** 5-L[^{3}H]Propargylglycine (114 mg, 1 mmol; 8.0 Ci/mol) was dissolved in 5 mL of H_2O. Hydrogenation was carried out at room temperature, 1 atm, with an Adams catalyst (5 mg of PtO_2). The reaction was stopped when 1.1 equiv of H_2 had been taken up and the catalyst filtered away. Paper chromatography (butanol-acetic acid-water, 4:1:1) revealed allylglycine (80%; estimated by radioactive scanning) and norvaline (20%). To the solution was added unlabeled allylglycine and recrystallization was accomplished twice from water-ethanol. The off-white crystalline product was contaminated 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 γ-lyase (EC 4.4.1.11) was the generous gift of Professor Kenji Soda and was purified from *Pseudomonas ovalis* (IFO 3738) according to the method of Tanaka.[9] The specific activity for γ elimination on L-methionine (reaction 3, assay described below) was 3.5 U/mg. Holoenzyme used in these experiments had absorbance maxima at 280 and 418 nm; the A_{280}/A_{418} was 4.85. In our hands, methionine γ-lyase is somewhat unstable to storage. As much as 50% of the catalytic activity is lost after 6 weeks at −20 °C. Values for A_{280}/A_{418} as high as 6.5 have been obtained for enzyme stored for long periods. It seems likely that the enzyme suffers both autoxidation and loss of the pyridoxal cofactor, as we have had some success in regenerating fully active enzyme by dialysis against KP_i buffers (pH 7.3) containing 50 μM PLP and 0.1 mM dithiothreitol. Dialysis also recovers the native A_{280}/A_{418} ratio.

Cystathionine γ-synthetase (EC 4.2.99.9) was purified from *Salmonella typhimurium* meA (ATCC 25241) as described previously.[11,28] Homogeneous enzyme has a specific activity of 20 U/mg for the γ elimination of succinate from *O*-succinyl-L-homoserine (half-reaction of eq 4, assay described below). Holoenzyme has absorbance maxima at 280 and 422 nm; the A_{280}/A_{422} was 3.90 for purified en-

zyme.

O-Succinyl-L-homoserine (OSHS), L-methionine, L-cysteine hydrochloride, dithiothreitol (DDT), and glutathione were Sigma products. *p*-Nitrophenylthiol, 2-mercaptoethanol, and 3-mercaptopropionate were obtained from Aldrich. Lactate dehydrogenase (LDH) and reduced nicotinamide 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-ketobutyrate by the continuous reduction of the keto acid product in the presence of LDH and NADH; product ketobutyrate formation was monitored as the disappearance of the absorbance 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 determination 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_i buffer, pH 7.3,[29] 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-mL solution of the appropriate standard assay. The inactivation half-time was obtained from semilog plots of percent activity remaining vs. time. Multiple inactivation experiments were conducted at varying concentrations of inactivator so to determine values of K_1 and k_2 for the process described by eq 5.

For inactivation of cystathionine γ-synthetase, the inactivation system usually contained 18 μg of enzyme in 120-μL total volume (for methionine γ-lyase, 130 μg of enzyme in 500 μL).

B. Thiol Reactivation. Each of the two enzymes was inactivated as described above. Once an end-point residual activity had been obtained, the solution was made 10 mM in one of the following thiols: dithiothreitol, glutathione, mercaptoethanol, or mercaptopropionate. These mixtures were then assayed for recovery of catalytic activity by dilution to an appropriate ketobutyrate-forming reaction.

C. Thiol Protection. For experiments wherein thiol protection was evaluated, the standard inactivation mixture was made 10 mM in one of the above thiols *prior to* the addition of enzyme. Dilution assays for remaining enzymatic activity were made in the standard way.

IV. Inactivation Stoichiometry. 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 standard assay solution. Reactions were carried out in a 1-cm quartz cuvette at 37 °C, so that the UV-visible spectra could be obtained periodically during inactivation. Spectra were obtained using the Perkin Elmer 554 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 [^{14}C]proline (New England Nuclear, 270 Ci/mol). The column was eluted ($\simeq$6 mL/h) with 10 mM KP_i 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 ^{3}H radioactivity.

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

Acknowledgments. Support for this research was provided in part by National Institutes of Health Grant No. GM 20011 and by an NIH Postdoctoral Fellowship, GM 06430-01, to one of us (M.J.). R. Raines is an MIT Undergraduate Research Opportunities Participant. We thank especially Professor Kenji Soda and his colleagues at the University of Kyoto who have provided us generously with samples of methionine γ-lyase. We also express our appreciation to Dr. Michael Chang of our laboratory who carried out the reduction of 5-[^{3}H]propargylglycine.

References and Notes

(1) Abbreviations used are: *Boc*, *tert*-butoxycarbonyl (BOC in structures); DTT, dithiothreitol; EtN_3, triethylamine; EtOAc, ethyl acetate; KP_i, potassium inorganic phosphate; KPP_i, potassium inorganic pyrophosphate; LDH, lactate dehydrogenase, mCPBA, *m*-chloroperbenzoic acid; NADH, reduced nicotinamide adenine dinucleotide; NaP_i, sodium inorganic phosphate; OSHS, *O*-succinyl-L-homoserine; TFA, trifluoroacetic acid; Me_4Si, tetramethylsilane; PLP, pyridoxal phosphate.
(2) K. Bloch, *Enzymes, 3rd Ed.*, **V**, 441 (1972); R. Rando, *Sceince*, **185**, 320 (1974); A. Maycock and R. Abeles, *Acc. Chem. Res.*, **9**, 313 (1976); C. Walsh, *Horiz. Biochem.* **3**, 36 (1977).
(3) J. Fisher, R. Charnas, and J. Knowles, *Biochemistry*, **17**, 2180 (1978); R. Charnas, J. Fisher, and J. Knowles, *Ibid.*, **17**, 2185 (1978); S. Cartwright and A. Coulson, *Nature (London)*, **278**, 360 (1979); A. English, J. Retsema, A. Girard, J. Lynch, and W. Barth, *Antimicrob. Agents Chemother.*, **14**, 414 (1978).
(4) R. Abeles, "Enzyme-Activated Irreversible Inhibitors", N. Seiler, M. J. Jung, and J. Koch-Weser, Eds., Elsevier/North Holland, Amsterdam, 1978, p 1; J. Wiseman and R. Abeles, *Biochemistry*, **18**, 427 (1979).
(5) A. Pogolotti and D. Santi, *Bioorg. Chem.*, **1**, 227 (1977); D. Santi, Y. Wataya, and A. Matsuda, "Enzyme-Activated Irreversible Inhibitors", N. Sieler, M. J. Jung, and J. Koch-Weser, Eds., Elsevier/North Holland, Amsterdam, 1978, p 291.
(6) V. Massey, H. Komai, G. Palmer, and G. Elion, *J. Biol. Chem.*, **245**, 2387 (1970).
(7) (a) P. Bickart, F. Carson, J. Jacobus, E. Miller, and K. Mislow, *J. Am. Chem. Soc.*, **90**, 4869 (1968); (b) R. Tang and K. Mislow, *J. Am. Chem. Soc.*, **92**, 2100 (1970).
(8) (a) D. J. Abbott and C. J. M. Stirling, *J. Chem. Soc. C*, 818 (1969); (b) D. Evans and G. Andrews, *J. Am. Chem. Soc.* **94**, 3672 (1972).
(9) L. Davis and D. Metzler, *Enzymes, 3rd Ed.*, **VII**, 33 (1972); H. Tanaka, N. Esaki, and K. Soda, *Biochemistry*, **16**, 100 (1977); N. Esaki, T. Suzuki, H. Tanaka, K. Soda, and R. Rando, *FEBS Lett.*, **84**, 309 (1977).
(10) The resolution may have been obtained based on the chirality at sulfur. See Experimental Section.
(11) M. Johnston, P. Marcotte, J. Donovan, and C. Walsh, *Biochemistry*, **18**, 1729 (1979).
(12) M. Johnston, D. Jankowski, P. Marcotte, H. Tanaka, J. Esaki, K. Soda, and C. Walsh, *Biochemistry*, **18**, 4690 (1979).
(13) M. Kaplan and M. Flavin, *J. Biol. Chem.*, **241**, 5781 (1966).
(14) Based on a determined extinction coefficient of 12.9×10^3 M^{-1} cm^{-1} for authentic *p*-nitrophenylthiol in 20 mM KP_i buffer, pH 7.3, 10 mM dithiothreitol.
(15) C. Walsh, "Enzyme Reaction Mechanisms", W. H. Freeman, San Francisco, 1979, pp 634–635.
(16) I. Schuphan, J. Rosen, and J. Casida, *Science*, **205**, 1013 (1979).
(17) In addition to nitrogen and sulfur, oxygen is an available enzymic nucleophile for attack on the electrophilic sulfur of **5**. The structure of the inactivated enzyme resulting would be itself a sulfenate ester, probably unstable to solvolysis or subsequent transfer to a secondary enzymic nucleophile (N or S) and, thereby, inconsistent with the formation of an irreversibly labeled protein.
(18) Or a sulfenimide if the nucleophile is an imidazole secondary nitrogen provided by an enzymatic histidine.
(19) H. Al-Rawi, K. Stacey, R. Weatherhead, and A. Williams, *J. Chem. Soc., Perkin Trans. 2*, 663 (1978).
(20) T. Kobayashi, K. Iino, and T. Hiraska, *J. Am. Chem. Soc.*, **99**, 5505 (1977).
(21) O. Foss, *Acta Chem. Scand.*, **1**, 307 (1947).
(22) It is also possible that **5** may collapse directly to **12** generating, by α-carbanion-assisted elimination, an electrophilic *p*-nitrophenylsulfenic acid. If the sulfenic acid were intercepted by an enzyme nucleophile before escape from the active site, apparent stoichiometric labeling by **1a** would result. [See D. Barton, P. Sammes, M. Taylor, C. Cooper, G. Hewitt, B. Looker, and G. Underwood, *Chem. Commun.*, 1137 (1971).]
(23) Enzymic attack on **5** at either the vinylogous $C_5(S_N2')$ or the bisallylic $C_3(S_N2)$ could also generate a *p*-nitrophenylsulfenic acid.
(24) W. H. Mueller and P. E. Butler, *J. Am. Chem. Soc.*, **90**, 2075 (1968).
(25) F. Montanari, *Gazz. Chim. Ital.*, **86**, 406 (1956).
(26) M. Raban and F. B. Jones, Jr., *J. Am. Chem. Soc.*, **93**, 2692 (1971).
(27) R. Abeles and C. Walsh, *J. Am. Chem. Soc.*, **95**, 6124 (1973).
(28) S. Guggenheim and M. Flavin, *J. Biol. Chem.*, **244**, 3722 (1969).
(29) While both enzymes give maximal activity at about pH 8.3 (and are standardly assayed at this pH), inactivation reactions were always conducted at pH 7.0–7.3 to protect against base-catalyzed halide elimination upon the inactivator.
(30) F. Kerzer and J. R. Powell, "Organic Syntheses", Collect. Vol. IV, Wiley, New York, 1900, p 934.