Mechanism-Based Inactivation of *E.coli* γ-Glutamylcysteine Synthetase by Phosphinic Acid- and Sulfoximine-Based Transition-State Analogues

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On the basis of the proposed reaction mechanisms of γ-glutamylcysteine synthetase, tetrahedral phosphinic acid- and sulfoximine-based compounds **1** and **2** were synthesized and evaluated as inhibitors of *E. coli* γ-glutamylcysteine synthetase. Both compounds inactivated the enzyme in a time-dependent manner with an overall inhibition constant K_i of 4.95 and 0.39 μM, respectively. The enzyme inactivation was observed only when ATP was present, suggesting that the inhibitors are phosphorylated in the enzyme active site, serving as a mechanism-based inactivator. The inhibition potency was 10 and 100 times, respectively, higher than L-buthionine-*SR*-sulfoximine, a well known inhibitor of this enzyme. In particular, the sulfoximine **2** served as an almost irreversible enzyme inactivator with a very slow regain of enzyme activity ($t_{1/2}$ = 3.9 day). This compound may find a potential use as chemotherapeutic agent.

keywords: γ-glutamylcysteine synthetase/ transition-state analogue/ phosphinic acid/ sulfoximine/ time-dependent inactivation/ mechanism-based phosphorylation

Glutathione biosynthesis is mediated consecutively by two mechanistically related ligases, γ-glutamylcysteine synthetase (γ-GCase, EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3). The design and synthesis of specific inhibitors of these physiologically important enzymes are of critical importance for the development of therapeutic agents as well as for use as mechanistic and physiological probes in glutathione metabolism (1). Following the success in determining the three dimensional structure of glutathione synthetase (2) and in developing and characterizing a specific inhibitor of this enzyme (3), we started the program for the design and synthesis of transition-state analogue inhibitors of γ-GCase, which is a physiologically more relevant enzyme regulating the glutathione biosynthesis. The reaction catalyzed by γ-GCS is thought to proceed *via* the initial formation of an acyl phosphate intermediate followed by nucleophilic attack of cysteine to yield γ-Glu-Cys, ADP and
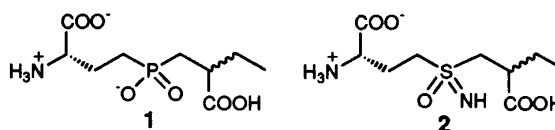
MOLECULAR BIOFUNCTION —Functional Molecular Conversion—

Scope of research
Our research aims are to elucidate structure-function relationships of biocatalysts in combination with organic chemistry, molecular biology and X-ray crystallographic technique, and to design and generate a novel biocatalysts for use as a tailor-made catalyst for organic reactions. Major subjects are (1) X-Ray diffraction analysis of asparagine synthetase, (2) Design and synthesis of transition-state analogue inhibitors of ATP-dependent synthetases, (3) Time-resolved X-ray crystallographic study of glutathione synthetase, (4) Characterization of an activation protein of Pseudomonas lipase, and (5) Design and preparation of catalytic antibodies for chemiluminescence.

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inorganic phosphate. According to this proposed reaction mechanism, we designed the phosphinic acid- and sulfoximine-based transition-state analogues **1** and **2**, in which the γ-carboxyl group of L-Glu was replaced by tetrahedral phosphorus and sulfur atom, respectively, and the attacking cysteine moiety was mimicked by α-ethylpropionic acid. Diastereomeric mixture of **1** and **2** were synthesized from a vinylglycine derivative and homocystine, respectively (4).

Both phosphinate **1** and sulfoximine **2** were found to serve as potent inactivators of *E. coli* γ-GCS. Treatment of γ-GCS with varying concentration of **1** or **2** in the presence of ATP caused a time-dependent inactivation of the enzyme (Figure 1).
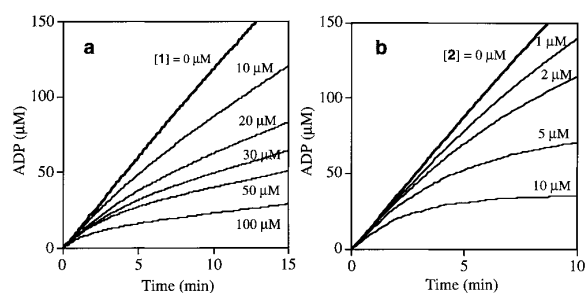
Figure 1. Progress curves for the inactivation of γ-GCS by (a) phosphinate **1**, and (b) sulfoximine **2**. The reaction was initiated by adding the enzyme to an assay mixture containing L-Glu (0.75 mM), L-2-aminobutyric acid (150 mM), ATP (1 mM), $MgSO_4$ (10 mM) and **1** (10-100 μM) or **2** (1-10 μM) in 0.1 M Tris-HCl (pH 7.5) at 37°C.

The shape of the progress curves represents a typical slow binding inhibition as observed with the inhibition of glutathione synthetase by a phosphinic acid transition-state analogue (3). Table 1 depicts the extent of inhibition, the second order rate constants for time-dependent inhibition (k_{inact}/K_i), and the overall dissociation constants (K_i^*) obtained from the steady-state reaction rates after the binding equilibrium was reached. For comparison, the inhibition by sulfone **3** and L-buthionine-*SR*-sulfoximine (L-BSO), a well

known inhibitor of this enzyme (5), was also examined.

The overall binding of phosphinate **1** and sulfoximine **2** is one and two orders of magnitude greater than that of L-BSO, respectively. In particular, sulfoximine **2** acted as an extremely powerful inactivator: although the sulfoximine **2** is a mixture of eight diastereomers, it inhibited γ-GCS about 126 times more effectively than did L-BSO. Assuming that the sulfoximine **2** contains equal amounts of diastereomers and only one stereoisomer inhibits the enzyme, it could be more than 500 times as effective as an active diastereomer of L-BSO. Both phosphinate **1** and sulfoximine **2** required ATP for enzyme inactivation: Non-hydrolyzable ATP analogue such as 5'-adenylylimidodiphosphate (AMPPNP) failed to cause the inactivation of the enzyme, suggesting a mechanistic scheme involving phosphorylation of the inhibitors by ATP within the enzyme active site (3, 5). This is also supported by the fact that sulfone **3** served as a simple reversible inhibitor. Although sulfone **3** did not cause ATP-dependent inactivation of the enzyme, its inhibition potency was more than five times higher than that of L-BSO as measured by the inhibition constant. The difference is still underestimated because the initial inhibition constant of sulfone **3** ($K_i = 9.2 \mu M$) is compared with the overall inhibition constant of L-BSO ($K_i^* = 49 \mu M$) where the ATP-dependent tight binding equilibrium was established.

Another criterion relevant to inhibitor potency is the duration of enzyme inactivation. We therefore measured the extent of recovery of enzyme activity upon 1000-fold dilution after the enzyme was completely inactivated with sufficient concentrations of **1** or **2** and ATP. Under a standard assay condition, no regain of enzyme activity was observed with sulfoximine **2**, whereas a significant enzyme reactivation ($t_{1/2} < 1$ min) was noted with phosphinate **1**. Although the inhibition by sulfoximine **2** was virtually irreversible within the time scale of assay, a very slow regain of enzyme activity was observed ($t_{1/2} = 3.9$ day) when the inactivated enzyme was gel filtered and incubated in the absence of ATP. Under the same conditions, the enzyme inactivated by L-BSO regained almost 40% of activity immediately after gel filtration. Thus, the inhibition potency of sulfoximine **2** is much higher than L-BSO in terms of both binding affinity and duration of enzyme inactivation. The sulfoximine **2** is a promising candidate for use as a physiological probe and chemotherapeutic agent to cause a long-lasting glutathione depletion in the living organisms.

Table 1. Inhibition of *E. coli* γ-glutamylcysteine synthetase

Compound	Conc. [μM]	% Inhibition[a]	k_{inact}/K_i [$M^{-1}sec^{-1}$]	K_i^* [μM]
Phosphinate **1**	5.7	35	436 ± 33	4.95 ± 0.27
Sulfoximine **2**	5.1	98	1206 ± 117	0.39 ± 0.11
L-BSO[b]	25.0	9.7	ND[c]	49.3 ± 7.40
Sulfone **3**	16.3	32	—[d]	9.23 ± 1.76[e]

[a] As measured by steady state inhibited velocities (v_s);
[b] L-Buthionine-*SR*-sulfoximine (Sigma); [c] Not determined;
[d] No time-dependent inhibition was observed; [e] Initial inhibition constant (K_i).

3   **L-BSO**
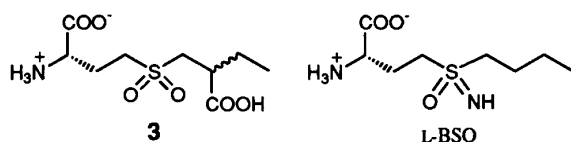
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