

Title	Mechanism-Based Inactivation of Glutathione Synthetase by Phosphinic Acid Transition-State Analogue (MOLECULAR BIOFUNCTION - Functional Molecular Conversion)
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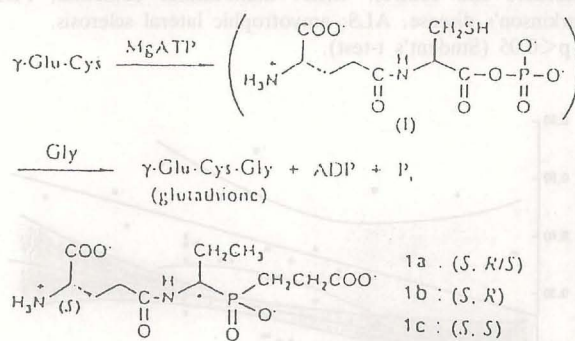
Mechanism-Based Inactivation of Glutathione Synthetase by Phosphinic Acid Transition-State Analogue

Jun Hiratake, Hiroaki Kato, and Jun'ichi Oda

A potent and time-dependent inactivator of glutathione synthetase was synthesized. According to the proposed reaction mechanisms of glutathione synthetase, we designed and synthesized the phosphinate-type transition-state analogue **1**. The (*S*, *R*)-isomer of **1** having the same relative configurations as γ -Glu-L-Cys was found to be a potent and time-dependent inactivator of this enzyme with an inhibition constant K_i of 21 nM, while the (*S*, *S*)-**1** related to γ -Glu-D-Cys was practically inactive. The time-dependent inactivation occurred only when ATP was present, with the onset rate of inactivation ($k_{on} = 8.29 \text{ sec}^{-1} \text{ mM}^{-1}$) being 75-times slower than the enzymatic reaction, and the inactivated enzyme slowly regained its activity ($t_{1/2} = 53 \text{ hr}$). The molecular basis for this inactivation was probed by an X-ray crystallography of the enzyme complexed with (*S*, *R*)-**1** and ATP. The X-ray diffraction analysis has shown that a mechanism-based inactivation was operative in which the phosphinate oxygen (P-O⁻) of **1** was phosphorylated by ATP within the enzyme active site to form ADP and a phosphorylated **1** which is highly analogous to the proposed transition state.

Keywords: Glutathione synthetase/ Transition-state analogue/ Phosphinate/ Time-dependent inactivation/ Mechanism-based phosphorylation

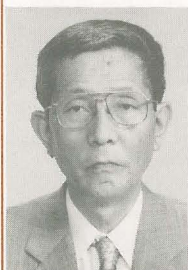
Glutathione synthetase (GSHase, EC 6.3.2.3), catalyzing the ligation of γ -Glu-Cys and glycine with the aid of ATP, is a key enzyme in glutathione biosynthesis. The detailed reaction mechanisms, however, are yet to be well understood, although the X-ray crystal structure of this enzyme has recently been defined [1]. In the light of mechanistically well-studied other ligases such as glutamine synthetase [2] or D-Ala-D-Ala ligase [3], the reaction catalyzed by GSHase is thought to proceed through the initial formation of a putative acyl phosphate intermediate (I), followed by nucleophilic attack of glycine to yield glutathione, ADP and inorganic phosphate (Scheme 1).



MOLECULAR BIOFUNCTION —Functional Molecular Conversion—

Scope of research

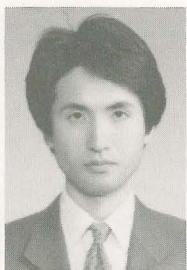
Our research aims are to analyze structure-function relationships of biocatalysts in combination with organic chemistry, structural biology and computer science, and to apply biocatalysts to stereospecific organic synthesis. Major subjects are the design and preparation of antibodies catalyzing stereoselective reactions, the reaction mechanisms of glutathione synthetase from *E. coli* with static and time-resolved X-ray crystallography, the mode of action of lipase-activating protein, crystallographic analysis of asparagine synthetase and γ -L-glutamyl-L-cystein synthetase, and creation of enzymatic reaction database and development of algorithms for protein sequence analysis.



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We therefore designed the transition-state analogue **1** in which the C-terminal carboxyl group is replaced by a tetrahedral phosphinyl group with a 2-carboxyethyl moiety mimicking the incoming glycine, and used the phosphinate **1** to probe the reaction mechanisms of this enzyme. Starting with racemic, (*R*)-, and (*S*)-1-(amino-propyl)phosphinic acid derivative [4], the diastereomeric mixture (*S*, *R/S*)-**1a** and each diastereomer (*S*, *R*)-**1b** and (*S*, *S*)-**1c** were synthesized.

The phosphinate **1a** was found to be a remarkably potent inactivator of GSHase. Treatment of GSHase with **1a** resulted in time-dependent inactivation of the enzyme as shown by the progress curves (Figure 1). Interestingly, no inactivation was observed without ATP, whereas complete inactivation resulted when both **1a** and ATP were present.

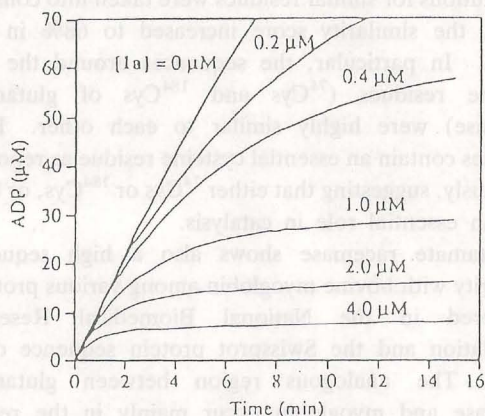
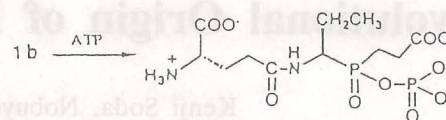


Figure 1. Progress curves for the inactivation of GSHase by **1a**. The reaction was initiated by adding enzyme to an assay mixture containing γ -Glu-Cys (0.2 mM), Gly (15 mM), ATP (5 mM) and **1a** (0.2–4 μ M) in 50 mM Tris-HCl (pH 7.5) at 37°C.

The inactivated enzyme slowly regained its activity ($t_{1/2}$ = 53 hr) when incubated for several days, but not within a steady-state time scale, thus the inhibition being practically irreversible. The onset rate of inactivation (k_{on}) [5] was calculated from the progress curves and was found to be $8.29 \text{ s}^{-1} \text{ mM}^{-1}$ with **1a**. This value, compared with the kinetic constants for γ -Glu-Cys [$k_0/K_m = 625 \text{ s}^{-1} \text{ mM}^{-1}$], means that the inactivation process is 75 times slower than the enzymatic reaction. Steady-state kinetic analysis revealed that the inhibition was competitive with γ -Glu-Cys, with the inhibition constant K_i being 53 nM with **1a**. As expected, the (*S*, *R*)-**1b** having the same relative configuration as γ -Glu-L-Cys was found to be an extremely potent and time-dependent inactivator ($K_i = 21 \text{ nM}$), whereas the (*S*, *S*)-isomer **1c** related to D-Cys showed only 18% inhibition at 39 mM.

The molecular basis for the time- and ATP-dependent inactivation by **1** was examined. Considering that the enzyme inactivation was observed only when both ATP and phosphinate **1** were present, the inhibition pattern is



most likely to reflect a mechanism-based phosphorylation of the phosphorus oxyanion (PO^-) of **1b** by ATP within the enzyme active site.

If this is the case, then the tightly bound phosphorylated inhibitor **1b** and ADP should be visible with an X-ray diffraction analysis of GSHase complexed with **1b** and ATP. This in fact proved to be the case: the electron density map around the active site has clearly shown that the γ -phosphate of ATP has been transferred to the inhibitor phosphorus oxyanion (PO^-) to form a phosphorylated **1b** and ADP within the enzyme active site (Figure 2). Since the phosphorylated **1b** is highly analogous to the proposed transition state, the phosphinate **1** serves as an excellent probe for defining what the real transition state is like and how this enzyme stabilizes it to facilitate the reaction.

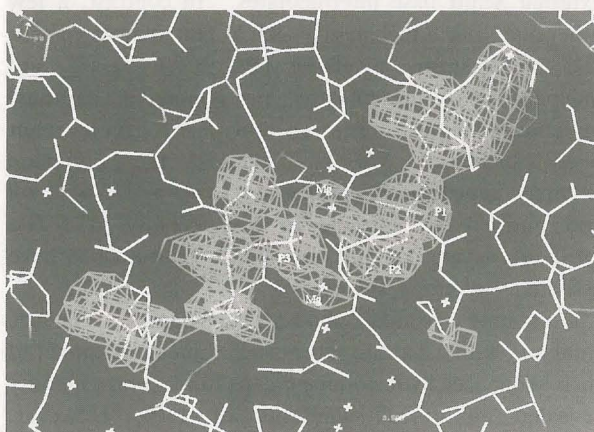


Figure 2. The F_0 - F_c electron density surface of the phosphorylated phosphinate **1b** and MgADP, superimposed with refined structure of the active site region. The contributions of the phosphorylated **1b** and MgADP were omitted from the F_c calculation. The contour level is 3.5σ .

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