Mechanism-Based Inactivation of Glutathione Synthetase by Phosphinic Acid Transition-State Analogue

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Glutathione synthetase (GSHase, EC 6.3.2.3) is the enzyme involved in the final stage of glutathione biosynthesis and catalyzes the ligation of γ-L-glutamyl–L-cysteine (γ-Glu-Cys) and glycine with the aid of ATP. We have reported mutagenesis and X-ray structural studies on the *Escherichia coli* B enzyme.[1] Although some mechanistic similarities with other ligases are suggested,[2] the detailed reaction mechanisms of this enzyme are yet to be defined. We therefore decided to synthesize a transition-state analogue inhibitor of this enzyme to probe the reaction mechanisms from a structural as well as a kinetic point of view. We now describe a potent and time-dependent inactivation of GSHase with a phosphinic acid transition-state analogue (**1**) and ATP. An X-ray crystal structure of GSHase complexed with **1** and ATP revealed that a mechanism-based inactivation is operative in which the phosphorus oxyanion of **1** was phosphorylated by ATP to yield a tight binding species within the enzyme active site.

In the light of mechanistically well-defined ligases such as glutamine synthetase[3] or D-Ala–D-Ala ligase,[4] 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).[5] We therefore designed 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. Starting with racemic, (*R*)-, and (*S*)-(1-aminopropyl)phosphinic acid derivative **2**,[6] the diastereomeric mixture (*S,R/S*)-**1a** and each diastereomer (*S,R*)-**1b** and (*S,S*)-**1c** were synthesized as depicted in Scheme 2.[7]

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).[8] Interestingly, no inactivation was observed without ATP, whereas complete inactivation resulted when both **1a** and ATP were present.[9] Regain of activity was not observed within a steady-state time scale, indicating that the inhibition was practically irreversible.[10] The onset rate of inactivation (k_{on})[11] was calculated from the progress curves and was found to be 8.29 s^{-1} mM^{-1} with **1a**. This value, compared with the kinetic constants for γ-Glu-Cys [k_0 = 150 s^{-1}, K_m = 0.24 mM], indicates that the inactivation process is 75 times slower than the enzymatic reaction. Steady-state kinetic analysis[12] revealed that the inhibition was competitive with γ-Glu-Cys, and the inhibition constant K_i was found to be 53 nM with **1a**. As expected, the (*S,R*)-**1b** having the same configuration as L-Cys at the stereocenter α to phosphorus was proven to be an extremely potent and time-dependent inactivator (K_i = 21 nM), whereas the (*S,S*)-isomer **1c** related to D-Cys showed only 18% inhibition at 39 μM.

The molecular basis for the time- and ATP-dependent inactivation by **1** was examined. With analogy drawn by specific

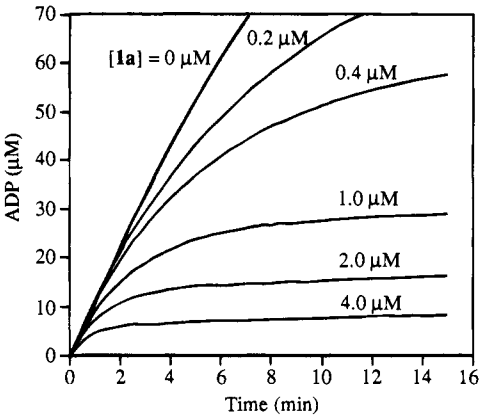
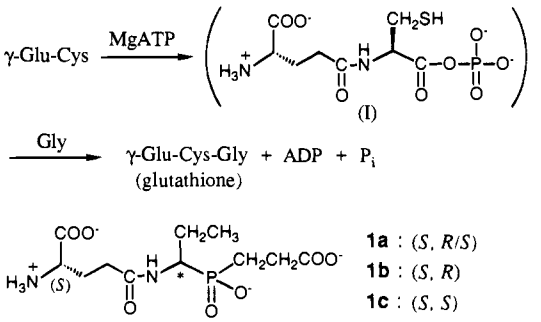
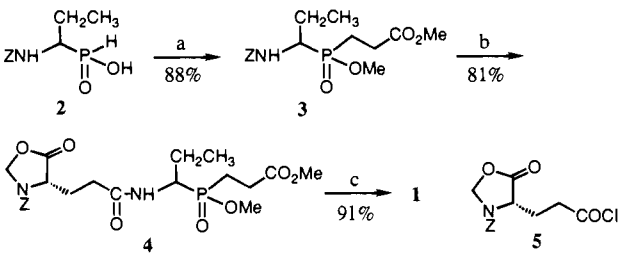


Figure 1. Progress curves for the inactivation of GSHase by **1a**. The reaction was initiated by adding enzyme to an assay mixture containing γ-Glu-Cys (0.2 mM), Gly (15 mM), ATP (5 mM), and **1a** (0.2–4 μM) in 50 mM Tris-HCl (pH 7.5) at 37 °C.

Scheme 1



Scheme 2[a]



a (a) (i) CH_2N_2, Et_2O; (ii) NaOMe, methyl acrylate, MeOH, 0 °C → 25 °C. (b) (i) H_2, 10% Pd–C, MeOH; (ii) **5**, HOBt, NEt_3, dry CH_2Cl_2, 0 °C → 25 °C. (c) (i) NaOH, THF–H_2O (1:1), 25 °C; (ii) H_2, 10% Pd–C, MeOH–AcOH–H_2O; (iii) Dowex 50W X-8 (H^+), eluted with H_2O.

(1) (a) Kato, H.; Tanaka, T.; Yamaguchi, H.; Hara, T.; Nishioka, T.; Katsube, Y.; Oda, J. *Biochemistry* **1994**, *33*, 4995–4999. (b) Tanaka, T.; Yamaguchi, H.; Kato, H.; Nishioka, T.; Katsube, Y.; Oda, J. *Ibid.* **1993**, *32*, 12398–12404. (c) Tanaka, T.; Kato, H.; Nishioka, T.; Oda, J. *Ibid.* **1992**, *31*, 2259–2265.

(2) Meister, A. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1974; Vol. 10, pp 671–697.

(3) (a) Meister, A. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1974; Vol. 10, pp 699–754. (b) Meek, T. D.; Villafranca, J. J. *Biochemistry* **1980**, *19*, 5513–5519. (c) Meek, T. D.; Johnson, K. A.; Villafranca, J. J. *Biochemistry* **1982**, *21*, 2158–2167. (d) Wedler, F. C.; Horn, B. R. *J. Biol. Chem.* **1976**, *251*, 7530–7538.

(4) Mullins, L. S.; Zawadzke, L. E.; Walsh, C. T.; Raushel, F. M. *J. Biol. Chem.* **1990**, *265*, 8993–8998.

(5) Steady-state kinetics of GSHase obeyed a sequential but not a ping-pong mechanism (unpublished results).

(6) Baylis, E. K.; Campbell, C. D.; Dingwall, J. G. *J. Chem. Soc., Perkin Trans. 1* **1984**, 2845–2853.

(7) The phosphinate **1** was obtained as a nonhygroscopic solid on a gram scale. The stereochemical assignment of **1** was determined by X-ray crystallography of the enzyme complexed with **1b** and ATP (Figure 2). The crystalline acid chloride **5** was prepared from (*S*)-3-(benzyloxycarbonyl)-5-oxo-4-oxazolidinepropanoic acid (Itoh, M. *Chem. Pharm. Bull.* **1969**, *17*, 1679–1686) [$SOCl_2$ (1.5 equiv), dry CH_2Cl_2, catalyst DMF, reflux 2 h, 92%].

(8) Enzymatic reaction was monitored by measuring ADP formation with a pyruvate kinase (PK)–lactate dehydrogenase (LDH)-coupled enzyme assay (ref 1c).

(9) 5′-Adenylylimidodiphosphate (AMPPNP) was not effective in enzyme inactivation.

(10) A very slow regain of enzyme activity was observed ($t_{1/2}$ = 53 h) at 25 °C in 50 mM Tris-HCl (pH 7.5).

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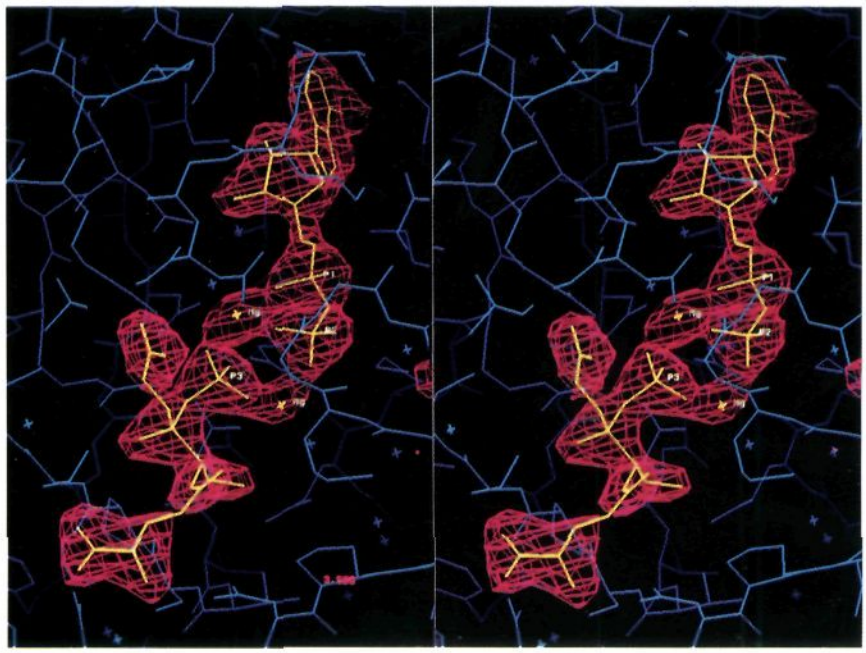
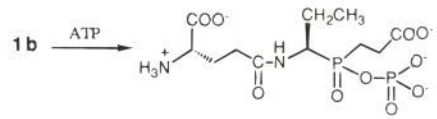
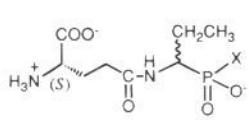


Figure 2. Stereoview of the F_o-F_c electron density surface (red) of the phosphorylated inhibitor **1b** and MgADP, superimposed with refined structure of the active site region. P1 and P2 are the α- and β-phosphates of ADP, respectively. P3 is the transferred phosphate. The contributions of the phosphorylated inhibitor **1b** and MgADP were omitted from the F_c calculation. The contour level is 3.5σ.



inhibitors of glutamine synthetase[13,14] or D-Ala−D-Ala ligase,[15] the inhibition pattern of **1** is most likely to reflect a mechanism-based phosphorylation of the phosphorus oxyanion (PO^-) by ATP within the enzyme active site. If this is the case, then the tightly bound phosphorylated inhibitor **1** and ADP should be visible with an X-ray diffraction analysis of GSHase complexed with **1b** and ATP. In fact, the electron density map around the active site has clearly shown that the γ-phosphate of ATP was transferred to the inhibitor phosphorus oxyanion (PO^-) to form a phosphorylated **1b** and ADP within the enzyme active site (Figure 2). To the best of our knowledge, this is the first direct demonstration of mechanism-based phosphorylation by X-ray crystallography.[16]

A related set of α-aminophosphorus analogues **6**−**9** were synthesized and evaluated as inhibitors of GSHase. Comparison



	% Inhibition (conc.)
6 : X = OH	70% (40 μM)
7 : X = OCH_2COO^-	42% (40 μM)
8 : X = O-n-C_4H_9	7 % (40 μM)
9 : X = n-C_4H_9	9 % (39 μM)

of the inhibition potencies of these analogues revealed some interesting facets. First, a structurally analogous phosphonate **7** was found to be a simple competitive-type inhibitor with significantly lower potency than phosphinate **1**. Second, removal of the terminal carboxyl group from the glycine moiety (**8** and **9**) also resulted in a marked decrease in inhibition potency with concomitant loss of time dependence in their inhibition behavior. These results are interpretable in terms of the inactivation mechanism mentioned above. The introduction of an electronegative oxygen atom as in **6** and **7** is most likely to reduce the nucleophilicity of the attacking oxyanion (PO^-),[17] thereby impairing the phosphorylation. The lack of the terminal carboxyl group as in **8** and **9** probably results in an unfavorable mode of inhibitor binding,[18] where the nucleophilic oxygen is no longer oriented properly to undergo phosphorylation. The phosphinate **1**, on the other hand, meets both criteria for effective phosphorylation, thus serving as a potent mechanism-based inactivator of GSHase.

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Supplementary Material Available: Text describing experimental details (10 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(11) The inhibition was analyzed with the following rate expression (Morrison, J. F.; Walsh, C. T. In *Advances in Enzymology*; Meister, A., Ed.; John Wiley: New York, 1988; Vol. 61, pp 201−301):

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} E\cdot S \xrightarrow{k_{cat}} E + P$$

$$k_{off} \uparrow\downarrow k_{on}[\mathbf{I}] \qquad E\cdot I$$

$$v_s = \frac{k_{cat}[E][S]}{K_m(1+[\mathbf{I}]/K_i)+[S]}$$

$$K_i = k_{off}/k_{on}$$

$$k_{obsd} = k_{on}[\mathbf{I}]/(1+[S]/K_m) + k_{off}$$

(12) A mixture of enzyme (1.08 nM), **1a** (16−400 nM), ATP (5 mM), and Gly (15 mM) was preincubated for 10 min at 37 °C to establish binding equilibrium. The assay was then started by adding an assay solution containing γ-Glu-Cys (final concentration, 0.2 mM).

(13) Methionine sulfoximine: (a) Ronzio, R. A.; Rowe, W. B.; Meister, A. *Biochemistry* **1969**, *8*, 1066−1075. (b) Rowe, W. B.; Ronzio, R. A.; Meister, A. *Biochemistry* **1969**, *8*, 2674−2680.

(14) Phosphinothricin: (a) Colanduoni, J. A.; Villafranca, J. J. *Bioorg. Chem.* **1986**, *14*, 163−169. (b) Abell, L. M.; Villafranca, J. J. *Biochemistry* **1991**, *30*, 6135−6141.

(15) (a) Duncan, K.; Walsh, C. T. *Biochemistry* **1988**, *27*, 3709−3714. (b) Parsons, W. H.; Patchett, A. A.; Bull, H. G.; Schoen, W. R.; Taub, D.; Davidson, J.; Combs, P. L.; Springer, J. P.; Gadebusch, H.; Weissberger, B.; Valiant, M. E.; Mellin, T. N.; Busch, R. D. *J. Med. Chem.* **1988**, *31*, 1772−1778. (c) McDermott, A. E.; Creuzet, F.; Griffin, R. G.; Zawadzke, L. E.; Ye, Q.-Z.; Walsh, C. T. *Biochemistry* **1990**, *29*, 5767−5775.

(16) Just after our manuscript was accepted, an X-ray crystal structure of D-Ala-D-Ala ligase complexed with a phosphinate analogue inhibitor was reported, in which the formation of phosphinophosphate was demonstrated (Fan, C.; Moews, P. C.; Walsh, C. T.; Knox, J. R. *Science* **1994**, *266*, 439−443).

(17) A similar bias of inhibitor potency was reported with phosphorus-containing peptide analogues as inhibitors of thermolysin, in which a phosphonate-type inhibitor was much inferior to the corresponding phosphinate or phosphonamidate analogues (Grobelny, D.; Goli, U. B.; Galardy, E. *Biochemistry* **1989**, *28*, 4948−4951).

(18) The terminal carboxyl group of the glycine moiety of **1** gave particularly high electron density in the electron density map (Figure 2), suggesting that this region is a major recognition site by the enzyme.