Identification of Catalytic Nucleophile of *Escherichia coli* γ-Glutamyltranspeptidase by Mechanism-Based Affinity Label

Jun Hiratake, Makoto Inoue, Hideyuki Suzuki, Hidehiko Kumagai and Kanzo Sakata

γ-Glutamyltranspeptidase (EC 2.3.2.2) is the enzyme involved in glutathione metabolism and catalyzes the hydrolysis and transpeptidation of γ-glutamyl compounds such as glutathione and its derivatives. The reaction is thought to proceed via a γ-glutamyl-enzyme intermediate where a hitherto unknown catalytic nucleophile is γ-glutamylated. Neither affinity labeling nor site-directed mutagenesis of conserved amino acids has succeeded so far in identifying the catalytic nucleophile. We describe here the identification of the catalytic nucleophile of *Escherichia coli* γ-glutamyltranspeptidase by a novel mechanism-based affinity labeling agent, 2-amino-4-(fluorophosphono)butanoic acid (**1**), a γ-phosphonic acid monofluoride derivative of glutamic acid. Compound **1** rapidly inactivated the enzyme in a time-dependent manner (k_{on} = 4.83 × 10^4 $M^{-1}sec^{-1}$). The inactivation was competitive with respect to the substrate. The inactivated enzyme did not regain its activity after prolonged dialysis, suggesting that **1** served as an active-site-directed affinity label by phosphonylating the putative catalytic nucleophile. Ion-spray mass spectrometric analyses revealed that one molecule of **1** phosphonylated the one molecule of small subunit. LC/MS experiments of the proteolytic digests of the phosphonylated small subunit identified the N-terminal peptide Thr391-Lys399 as the phosphonylation site. Subsequent MS/MS experiments of this peptide revealed that the phosphonylated residue was Thr-391, the N-terminal residue of the small subunit. We conclude that the N-terminal Thr-391 is the catalytic nucleophile of *E. coli* γ-glutamyltranspeptidase. This result strongly suggests that γ-glutamyltranspeptidase is a new member of N-terminal nucleophile hydrolase family.

Keywords: *E. coli* γ-Glutamyltranspeptidase/ Glutathione metabolism/ Mechanism-based affinity labeling/ Phosphonofluoridate/ Catalytic nucleophile/ Phosphonylation/ Ion-spray MS/ N-Terminal nucleophile hydrolase family/

γ-Glutamyltranspeptidase (γ-GGT, EC 2.3.2.2) catalyzes the cleavage of γ-glutamyl bond of glutathione and related γ-glutamyl compounds to transfer the γ-glutamyl group either to water or to amino acids and peptides to complete hydrolysis or transpeptidation, respectively. This enzyme is widely distributed among the living organisms from bacteria to mammals and plays important roles in glutathione metabolism. Despite the physiological importance of this enzyme, details of the catalytic mechanism still remain unclear. The reaction catalyzed by γ-GGT is thought to proceed via a γ-glutamyl-enzyme intermediate followed by nucleophilic substitution by water, amino acids, or peptides (Scheme 1). Although a hydroxy group of a Ser or Thr is proposed as the γ-glutamylation site, the catalytic nucleophile has remained to be identified either by chemical modification or by site-directed mutagenesis. Here we designed 2-amino-4-(fluorophosphono)butanoic acid **1**, a γ-phosphonic acid monofluoride derivative of glutamic acid, as a novel affinity labeling agent to trap the catalytic nucleophile of *E. coli* γ-GGT. Compound **1** is expected to bind covalently to the catalytic nucleophile in a mechanism-based manner, forming a transition-state like adduct in the enzyme active site (Scheme 1).

Compound **1**, sysnthesized from 2-amino-4-phosphonobutanoic acid in four steps, was relatively stable under acidic conditions (pH 5.5, $t_{1/2}$ = 21.6 h) at which the enzyme exhibited the maximal activity.

MOLECULAR BIOFUNCTION — Chemistry of Molecular Biocatalysts —

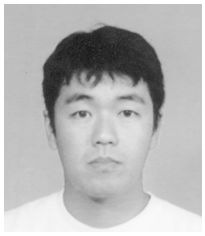
Scope of research

Our research aims are to elucidate the chemistry-function relationships of various biocatalysts (enzymes) in combination with organic chemistry, molecular biology and X-ray crystallography. The biochemical and physiological roles of enzymes and hormone receptors are also studied from the chemical point of view. Main subjects are (1) Chemical, biochemical and molecular biological studies on β-primeverosidase, a major tea aroma-producing β-glycosidase concerned with tea-manufacturing process, and on its original physiological roles in tea plants, (2) Design and synthesis of transition-state analogues and mechanism-based inhibitors of ATP-dependent ligases and glycosidases to probe the enzyme mechanisms, (3) Development of a new method for functional cloning of plant hormone receptors and biochemical studies on plant hormone biosynthesis, (4) X-Ray crystallography of firefly luciferase and Maize pyruvate phosphate dikinase, (5) Development of a novel lipase with altered reaction specificity by directed evolution.

Prof
SAKATA, Kanzo
(D Agr)

Assoc Prof
HIRATAKE, Jun
(D Agr)

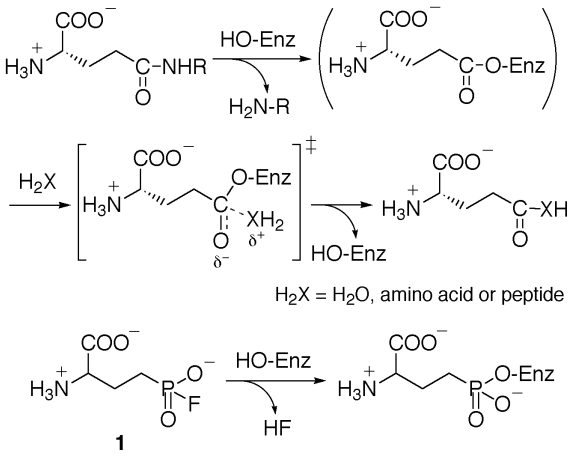
Instr
MIZUTANI, Masaharu
(D Agr)



Instr
SHIMIZU, Bun-ichi
(D Agr)

INOUE, Makoto (DC), FUJII, Ryota (DC), MA, Seung-Jin (DC), AHN, Young-Ock (DC), EMA, Jun-ichi (MC), NAKANISHI, Tsugumi (MC), SATO, Tadashi (MC), INOUE, Kazuko (MC), INOUE, Toshiki (MC), KATO, Masahiro (MC), SAITO, Shigeki (MC), UTSUNOMIYA, Yuji (MC), OHNISHI, Toshiyuki (RS), ASADA, Junko (UG, RS), UEMURA, Miyuki (secretary)

Scheme 1



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The enzyme was rapidly and competitively inhibited by **1** in a time-dependent manner (Figure 1). No regain of enzyme activity was observed after prolonged dialysis (10 days), suggesting that the enzyme active site was covalently modified with **1.**

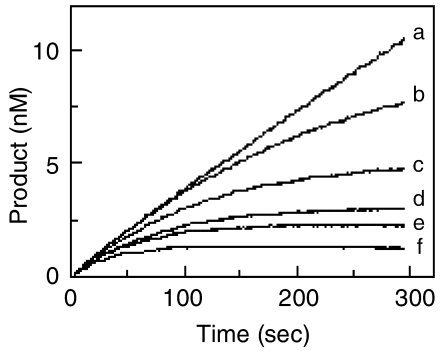

Figure 1. Time-dependent inhibition of *E. coli* γ-glutamyltranspeptidase by **1.** The enzymatic reaction was carried out in the presence of the following concentrations of **1**: (a) 0, (b) 0.17, (c) 0.33, (d) 0.5, (e) 0.67 and (f) 1.0 μM.

The inactivated enzyme was analyzed by ion-spray mass spectrometry. The *E. coli* γ-GGT is composed of two subunits with a molecular mass of 20,010 and 39,196 Da for the small and large subunit, respectively. Mass analyses revealed that the molecular mass of the small subunit increased from 20,014 Da (unmodified enzyme) to 20,178 Da (modified enzyme) (Figure 2). The observed mass increase of 164 Da corresponded well to the expected mass increase of 165 Da caused by the phosphonylation by **1**, indicating that one molecule of **1** was attached covalently to the small subunit.

The small subunit of each unmodified and modified enzyme was digested by lysyl endopeptidase C, and the resulting proteolytic digests were analyzed by LC/MS to find that the N-terminal peptide of the small subunit (Thr391-Lys399, 1049.7 Da) was phosphonylated to increase its molecular mass by 165 Da.

Finally, the phosphonylated residue in the N-terminal peptide was identified by MS/MS analysis. The $(M + 3H)^{3+}$ ions at *m/z* 350.7 (unmodified peptide) and 405.9 (phosphonylated peptide) were subjected to collision-induced decay (CID). The resulting CID spectrum and the amino acid sequence of the N-terminal peptide Thr391-Lys399 are depicted in Figure 3. The CID spectrum of the $(M + 3H)^{3+}$ precursor ion at *m/z* 405.8

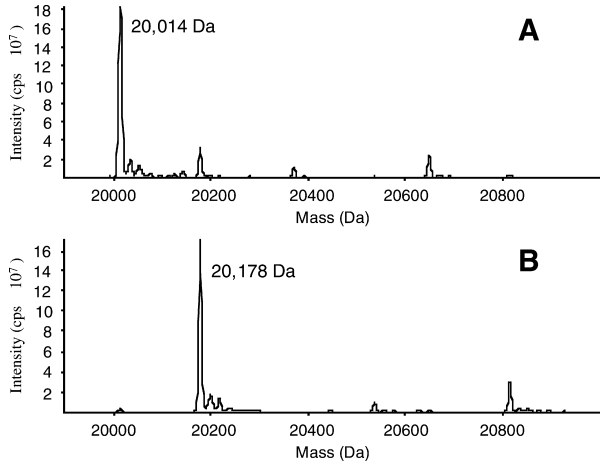

Figure 2. Reconstructed mass spectra of the small subunit. (A) Small subunit from the unmodified enzyme. (B) Small subunit from the modified enzyme.

(phosphonylated peptide) was the same as that of the unmodified peptide, except for the precursor ion (Figure 3C). Thus, the mass increase of 165 Da was observed only in the precursor ion. This result has clearly shown that compound **1** phosphonylated the N-terminal residue of the small subunit, that is, Thr-391. In addition, two fragment ions (*m/z* 184.1 and 516.4) served as a good indication of β-elimination of 2-amino-4-phosphonobutanoic acid (m/z 183) from the phosphonylated N-terminal peptide Thr 391-Lys399.

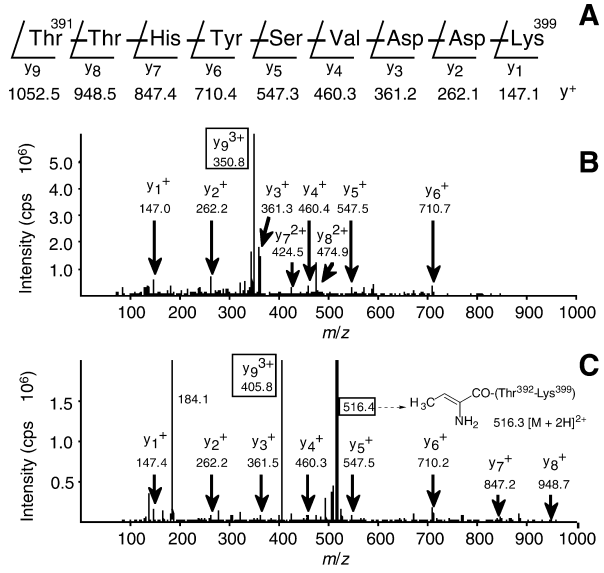

Figure 3. MS/MS analysis of the peptide Thr391-Lys399. (A) Predicted product ions of type y. (B) CID spectrum of the *m/z* 350.7 precursor ions (Thr391-Lys399) from the unmodified enzyme. (C) CID spectrum of the *m/z* 405.9 precursor ions (Thr391-Lys399) from the modified enzyme.

Thus, the N-terminal Thr 391 was identified as the catalytic nucleophile of *E. coli* γ-GGT. This result, along with sequence similarity and a chracteristic post-translational processing of this enzyme, strongly suggested that *E. coli* γ-GGT is a member of N-terminal nucleophile hydrolases, a recently recognized new hydrolase family.

Referencce: M. Inoue, J. Hiratake, H. Suzuki, H. Kumagai, K. Sakata *Biochemistry* **2000**, *39*, 7764-7771.