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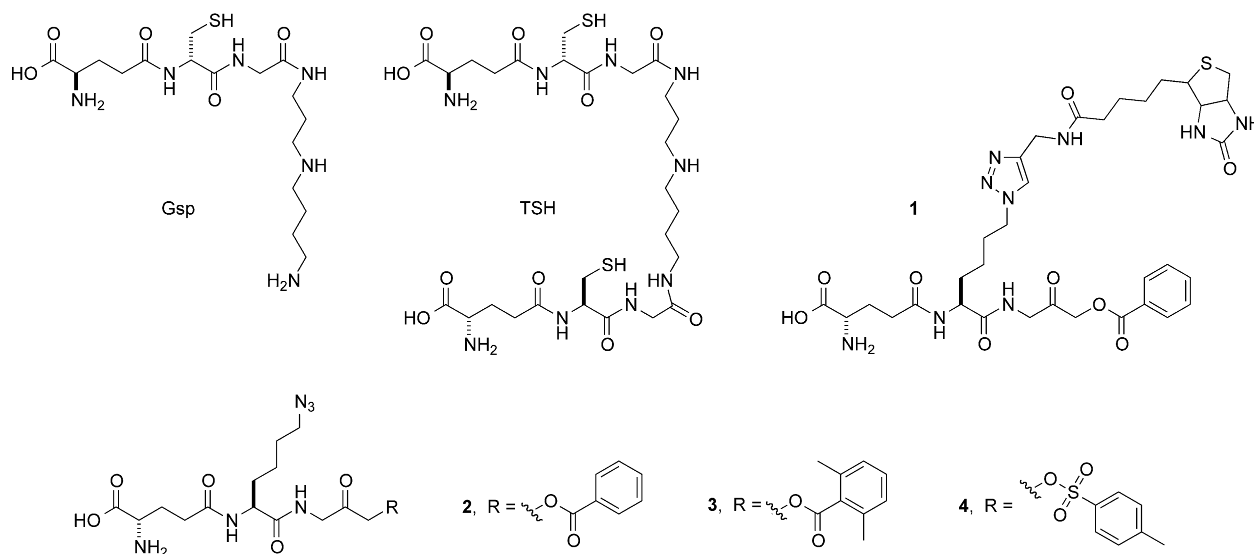
An Acyloxymethyl Ketone-Based Probe to Monitor the Activity of Glutathionylspermidine Amidase in *Escherichia coli*

Cheong-Meng Chong,[a] Shijay Gao,[a] Bing-Yu Chiang,[a] Wen-Hung Hsu ,[a, b] Ting-Chien Lin,[c] Tzu-Chieh Chen ,[a, b] and Chun-Hung Lin *[a, b, c]

The two conjugates of glutathione (GSH) and spermidine (Spd), glutathionylspermidine (Gsp) and trypanothione (TSH) are essential for redox regulation in Gram-negative bacteria and protozoa, respectively. In *Escherichia coli*, Gsp synthetase catalyzes the ATP-dependent formation of an amide bond between the glycine carboxylate of glutathione and N[1] of spermidine (see Figure S1 in the Supporting Information).[1] Protozoal parasites have a TSH synthetase for the synthesis of TSH from Gsp and GSH or a Gsp/TSH synthetase that is responsible for both synthetic steps (Figure S1).[2] The *E. coli* Gsp synthetase and *Leishmania major* TSH synthetase are similar in both amino acid sequence (51.5% similarity) and structures.[3,4] In trypanosomatids, TSH deficiency decreases viability, suggesting that the enzymes involved in TSH synthesis are a potential drug target for parasitic diseases, such as, African sleeping disease and Chagas disease.[5] Intriguingly, in *E. coli* GspSA, the amidase domain (GspA), which is upstream of the synthetase domain, hydrolytically cleaves Gsp to GSH and Spd.[1] Given that two functionally opposing domains reside in one protein, how both functions are regulated to avert futile ATP consumption has attracted much attention.[1] The concentrations of GSH, Spd and Gsp alter rapidly as growth conditions of *E. coli* change.[6] We previously reported that the activity of GspA was selectively inactivated by H_2O_2, whereas H_2O_2 hardly affected Gsp synthetase activity.[7] In addition, the GspA activity in GspSA is negatively regulated by the synthetase activity, although this depends on the combination of synthetase substrates/analogues.[8] The aforementioned observations are indications of a rather complicated regulation of GspSA activity in response to environmental pressures.



GspA belongs to the CHAP (cysteine, histidine-dependent amidohydrolases/peptidases) domain superfamily.[9] The catalytic triad of GspA, Cys59, His131, and Glu147, which is completely conserved in CHAP-domain proteins, forms a hydrogen-bond network that enhances the nucleophilicity of Cys59.[7] Previous results indicated that the inactivation of amidase activity increases Gsp level in *E. coli*, which then protects against harmful oxidants as follows.[7] Oxidants are consumed when Gsp–Gsp disulfides (or other mixed disulfides) are formed. Gsp also prevents the oxidation of protein cysteine thiols by Gsp-protein cysteine disulfide formation.[5] Gsp accumulates because Cys59 is transiently oxidized under oxidative stress to a sulfenic acid. Therefore, the regulation of GspA activity is possi-

[a] C.-M. Chong,[+] Dr. S. Gao,[+] Dr. B.-Y. Chiang,[+] W.-H. Hsu , T.-C. Chen , Prof. C.-H. Lin
Institute of Biological Chemistry, Academia Sinica
No. 128 Academia Road Section 2, Nan-Kang, Taipei 11529 (Taiwan)
E-mail: chunhung@gate.sinica.edu.tw

[b] W.-H. Hsu , T.-C. Chen , Prof. C.-H. Lin
Institude of Biochemical Sciences, National Taiwan University
Taipei 10617 (Taiwan)

[c] T.-C. Lin, Prof. C.-H. Lin
Department of Chemistry, National Taiwan University
Taipei 10617 (Taiwan)

[+] *These authors contributed equally to this work.*

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bly linked to the redox state of Cys59, and consequently the ability to sensitively and specifically detect GspA activity in vivo would be a useful probe of the redox-mediated regulatory mechanism(s) of Gsp and the physiological roles of the enzymes involved.

Although several methods are available to detect protein expression levels in vivo, most do not measure the variations in the enzyme activities. Enzyme-activity profiling using a synthetic activity-based probe (ABP) has emerged as an approach that complements conventional methods.[10–12] For example, the cysteine protease domain of *Clostridium difficile* toxin B auto-proteolytically releases a cytotoxic effecter. Bogyo, Shen, and co-workers have developed ABPs to dissect the activation mechanism of the toxin and have screened for molecules that can inhibit autoprocessing.[13]

In general, the structures of ABPs are designed to include a reactive group that can covalently bind to an enzyme active-site residue, a recognition group that selectively targets the desired protein(s), and a reporter group to detect the ABP/protein complex. Herein, we report the design and synthesis of an ABP (**1**) that targets GspA. Given the selectivity for cysteine proteases and inertness to intracellular nucleophiles such as glutathione,[14] we chose acyloxymethyl ketone (AOMK) as the reactive group. We predicted that the AOMK would react with the nucleophilic Cys59 of GspA to form a thiohemiketal intermediate (Figure S2) for which the tetrahedral oxyanion would be stabilized by hydrogen bonds with the side-chain amide nitrogens of Gln58 and Asn149 (Figure S2). After release of the benzoic acid, the remainder of the probe would most probably form a thioether adduct with Cys59 (Figure S2). The sulfonate ester analogue **4** was also prepared to contain a better leaving group. In the X-ray structure of the GspA/Gsp complex,[15] the GspA binding site is a crevice. Gsp binds GspA mainly through interactions involving its GSH moiety—the γ-glutamyl oxygens and nitrogen of Gsp form ion pairs with Arg64 and Asp77 of GspA,[15] respectively (Figure 1). Conversely, the cysteine side chain of Gsp protrudes into the solvent and does not participate in complex formation. We therefore designed compounds **2**, **3** and **4** that contain a lysine with an ε-azide-modified side chain in place of the cysteine because the azide can be further reacted with a biotin or a fluorescent tag that would then serve as the reporter.

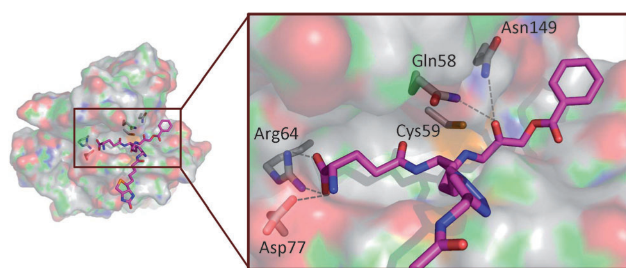
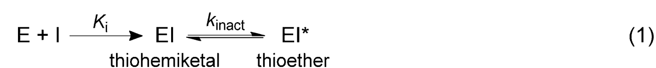


Figure 1. A stick model of **1** docked into the active site of GspA (PDB ID: 3A2Y). The model was created using PyMOL. The carbon, oxygen and nitrogens atoms of **1** are represented in magenta, red and blue, respectively. The side chains of GspA residues that probably interact with **1** are shown as sticks. Protein carbon, hydrogen, oxygen, and nitrogen atoms, are shown in grey, green, red, and blue, respectively.

After synthesizing **2**, **3**, and **4** (Scheme S1), we measured their binding affinities with GspA and found that **2** (IC_{50} = 0.68 μм) bound three times more tightly to GspA than did **3** (IC_{50} = 2.17 μм). Despite the affinity of **4** for GspA (IC_{50} = 0.75 μм), its development was abandoned because it was unstable in aqueous solution. Furthermore, **2** was able to covalently label Cys59 of the recombinant GspA, as shown by matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-MS/MS) analysis, which required prior incubation of GspA with **2** for 2 h and subsequent trypsin digestion. As shown in Figure S3, one of the peptides has a molecular weight of 1392.5, which is expected for the thioether adduct formed by the Cys59-containing sequence ^{57}WQCVEFAR^{64} (*Mr* = 1037) and the remainder of **2** (M_r = 355; Figure S3). The covalent modification of Cys59 is evidenced by the detection of y- and b-ions. As a consequence, **2** was transformed to **1** by incorporation of a biotin group and utilized for further studies (Scheme S1). Computational modeling (Discovery Studio 2.5) indicated that the modified lysine side chain should extend into the solvent, which could be expected given the solvent-accessible position of the Gsp cysteine in the GspA/Gsp complex (Figure 1).

Brady et al. reported that activated ketones (including AOMKs) inhibit cysteine proteases by three different mechanisms.[16] The progression curves of **1** followed a bimodal inhibition—rapid inhibition was initially observed, followed by slow inactivation. GspA and compound **1** thus reversibly form a thiohemiketal complex that then slowly coverts irreversibly into a thioether adduct [Eq. (1)]. Under these circumstances, the faster reversible inhibition reaction can be separated from the slower irreversible reaction. Following the reported procedure for the analysis of the bimodal inhibition model, the values of K_i and k_{inact} were determined to be 0.5 μм and 2.5 × 10^{-4} s^{-1}, respectively (Figure S4). Moreover, **1** (10 μм) covalently labeled recombinant GspSA, as shown by immunoblotting with anti-biotin (Figure 2A), but did not label the inactive GspSA mutant C59A, which affirmed the conclusion that **1** specifically targeted Cys59. As little as 0.1 μg of GspSA could be detected by immunoblotting with anti-biotin (Figure S5). Compound **1** was also able to distinguish between active GspA and GspA that had been inactivated by prior treatment with H_2O_2[17] in a dose-dependent manner (Figure 2B), which was consistent with the results of the colorimetric assay (Figures 2B and S6).



$$E + I \xrightarrow{K_i} \underset{\text{thiohemiketal}}{EI} \xrightleftharpoons{k_{inact}} \underset{\text{thioether}}{EI^*} \quad (1)$$

To examine if **1** specifically targeted GspSA in *E. coli*, **1** was incubated with an *E. coli* lysate for 3 h after first treating the bacteria with H_2O_2 for 10 min and further culturing the bacteria for 0 to 4 h in the absence of H_2O_2. Immunoblotting revealed an intense band corresponding to the control sample at ~70 kDa (expected MW for GspSA) and a clear membrane background, which suggested that **1** targeted only GspSA (Fig-

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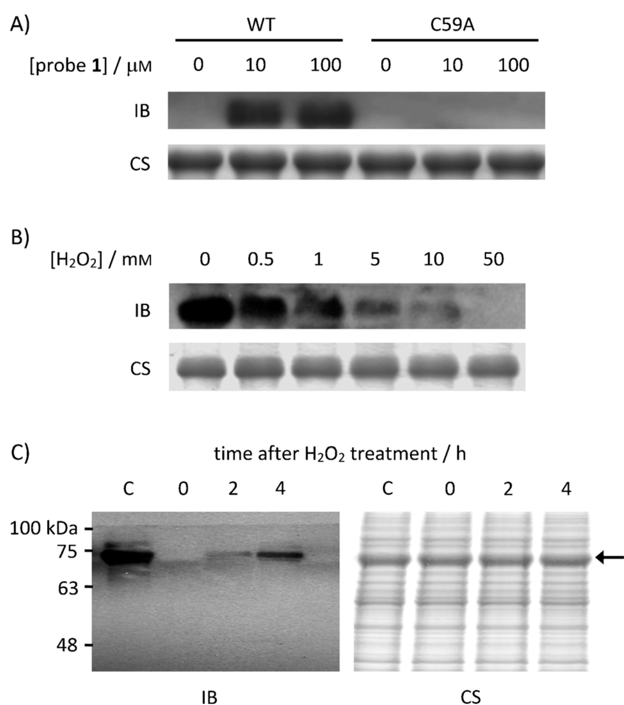

Figure 2. Activity-dependent labeling of Gsp amidase by **1**. A) Recombinant Gsp synthetase/amidase (GspSA) and the mutant C59A (1 μg each) were individually incubated with **1** (0, 10, or 100 μM) for 3 h at 37 °C. The mixtures were then subjected to SDS-PAGE followed by immunoblotting. B) GspSA was treated with various concentrations of H_2O_2 (0 to 50 mM), incubated with **1** (10 μM), and subjected to SDS-PAGE and immunoblotting. C) *E. coli* cultures were treated with H_2O_2 (10 mM) for 10 min that was then removed by centrifugation and replacing the culture medium. After additional incubation for 0, 2, or 4 h, the cells were collected by centrifugation, lysed, and treated with **1** (10 μM) to determine the amount of active Gsp amidase. The arrow identifies the expected position of GspSA (M_W = 70 kDa). WT: wild-type GspSA; IB: immunoblotting using anti-biotin; CS: Coomassie Blue staining; C: negative control, no H_2O_2 treatment.

ure 2C). Interestingly, the time-course indicated that GspA activity had significantly recovered within 4 h after removal of H_2O_2. In addition to reaffirming the finding that inactivated amidase activity was restored after removing H_2O_2, the result also strengthened the idea that **1** can be used to assess cellular redox conditions. We also assessed the ability of **1** to detect GspA activity in *E. coli* almost immediately after the cells had been individually exposed to H_2O_2, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP, a donor of nitric oxide), or methylglyoxal (a reactive aldehyde/ketone and also a side-product of several metabolic pathways, which is toxic as it reacts with proteins and nucleic acids).[18] It should be noted that GSH and TSH protect cells against methylglyoxal toxicity.[19] When added to *E. coli* lysates prepared immediately after treatment with an oxidant or a toxin, **1** did not label GspSA or did so to a lesser extent than it did GspSA from untreated *E. coli* (Figure 3). GspSA appears to be sensitive to oxidants, including reactive oxygen and nitrogen species, and methylglyoxal in its local environment, as H_2O_2, SNAP, and methylglyoxal had each diffused into the cells and suppressed GspA activity. Temporary inactivation of GspA would assist the rapid accumulation of Gsp that would then defend against the oxidative threat.

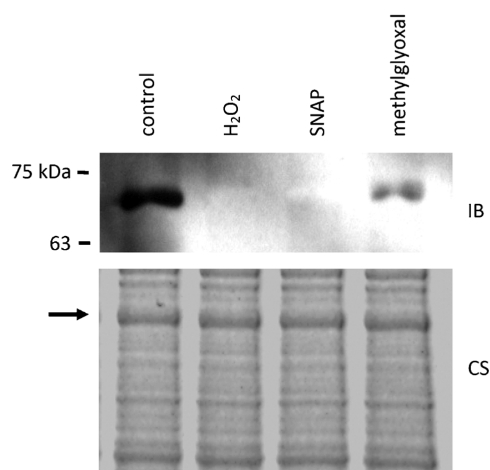

Figure 3. Detection of Gsp amidase activity in *E. coli* after exposure to H_2O_2, SNAP, or methylglyoxal. *E. coli* cultures were untreated (control), treated with H_2O_2, SNAP, or methylglyoxal (10 mM each), then lysed, treated with compound **1** (10 μM), and subjected to immunoblotting to determine Gsp amidase activity. IB: immunoblotting with anti-biotin; CS: Coomassie Blue staining.

In summary, we have designed and synthesized an AOMK-based probe for the detection of GspA activity. Compound **1** was used to measure the amount of active GspA under different conditions. Because *gspSA* is found in several bacterial pathogens, including *Salmonella enterica*, *Klebsiella pneumoniae*, and *Shigella flexneri* (Figure S7), GspSA and Gsp probably are found in these bacteria as well as in *E. coli*. Our probe can be used to detect active GspA in these bacteria and in protozoa, and to determine how amidase activity is regulated in vivo in response to reactive oxygen and nitrogen species and to methylglyoxal. Moreover, because all CHAP domain family proteins contain the same catalytic residues,[9] these proteins probably share the same catalytic mechanism. Our AOMK-based probe should therefore be useful for investigating other members of CHAP domain family.

Experimental Section

The following experiments are described in the Supporting Information: synthesis of compounds **1**–**17** and their structural characterization by ^{1}H and ^{13}C NMR spectroscopy, optical rotation, and HRMS; the MALDI-MS/MS analysis to identify the Cys59-thioether adduct peptides; the procedure to determine the K_i and k_{inact} of **1** for GspA; immunoblotting to determine the lower limit of GspSA that could be detected by **1**.

Labeling of recombinant GspSA: The cloning, overexpression, and purification of recombinant GspSA, C59A and GspA fragment (that corresponds to the N-terminal 197 residues in GspSA) have been previously described.[3] GspSA and C59A were individually incubated with **1** (10 μM) in HEPES buffer (100 mM, pH 8.0) for 3 h at 37 °C. After adding SDS-PAGE loading buffer, the samples were heated at 100 °C for 10 min to stop the reactions. The resulting mixtures were subjected to SDS-PAGE. The GspSA/**1** complex was detected by immunoblotting with anti-biotin (Sigma–Aldrich).

Labeling of GspA in *E. coli*: *E. coli* BL21 (DE3) cells were transformed with pET28-GspSA, cultured in LB medium until the A_{600}

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value of the culture was 0.7, induced with isopropyl-thio-β-D-galactopyranoside (1 mM) for 10 min, and treated with H_2O_2 (10 mM) for 10 min. Alternatively the cells were treated with SNAP or methylglyoxal (10 mM each, both from Sigma–Aldrich) or left untreated. Cells were then collected by centrifugation and lysed by sonication. The lysates were centrifuged at 18000 *g* at 4 °C for 20 min. The supernatants were incubated with **1** in HEPES buffer (100 mM, pH 8.0) for 3 h at 37 °C and subjected to SDS-PAGE and immunoblotting with anti-biotin.

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Keywords: activity-based probes · acyloxymethyl ketone · chap domain · glutathionylspermidine amidase · redox chemistry

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