Methylglyoxal Detoxification Revisited: Role of Glutathione Transferase in Model Cyanobacterium Synechocystis sp. Strain PCC 6803

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ABSTRACT Methylglyoxal (MG) is a detrimental metabolic by-product that threatens most organisms (in humans MG causes diabetes). MG is predominantly detoxified by the glyoxalase pathway. This process begins with the conjugation of MG with glutathione (GSH), yielding a hemithioacetal product that is subsequently transformed by the glyoxalase enzymes into D-lactate and GSH. MG has been overlooked in photosynthetic organisms, although they inevitably produce it not only by the catabolism of sugars, lipids, and amino acids, as do heterotrophic organisms, but also by their active photoautotrophic metabolism. This is especially true for cyanobacteria that are regarded as having developed photosynthesis and GSH-dependent enzymes to detoxify the reactive oxygen species produced by their photosynthesis (CO₂ assimilation) and respiration (glucose catabolism), which they perform in the same cell compartment. In this study, we used a combination of in vivo and in vitro approaches to characterize a logical, but as yet never described, link between MG detoxification and a (prokaryotic) representative of the evolutionarily conserved glutathione transferase (GST) detoxification enzymes. We show that the Sll0067 GST of the model cyanobacterium Synechocystis sp. strain PCC 6803 plays a prominent role in MG tolerance and detoxification, unlike the other five GSTs of this organism. Sll0067 catalyzes the conjugation of MG with GSH to initiate its elimination driven by glyoxalases. These results are novel because the conjugation of MG with GSH is always described as nonenzymatic. They will certainly stimulate the analysis of Sll0067 orthologs from other organisms with possible impacts on human health (development of biomarkers or drugs) and/or agriculture.

IMPORTANCE In most organisms, methylglyoxal (MG), a toxic metabolite by-product that causes diabetes in humans, is predominantly detoxified by the glyoxalase enzymes. This process begins with the so-called “spontaneous” conjugation of MG with the cytoprotectant metabolite glutathione (GSH). In this study, we unravel a logical, but as yet unsuspected, link between MG detoxification and a (prokaryotic) representative of the ubiquitous glutathione transferase (GST) enzymes. We show that the Sll0067 GST of the model cyanobacterium Synechocystis sp. strain PCC 6803 plays a prominent role in MG tolerance and detoxification, unlike the other five GSTs of this organism. Sll0067 catalyzes the conjugation of MG with GSH to initiate its elimination driven by glyoxalases. This finding is important because this reaction, always regarded as nonenzymatic, could exist in plants and/or human and thus have an impact on agriculture and/or human health.

KEYWORDS cyanobacteria, detoxification, enzyme assay, glutathione transferase, glyoxalase pathway, in vivo analysis, methylglyoxal, oxidative stress, sugar metabolism

Methylglyoxal (MG) is a very dangerous dicarbonyl compound that strongly interacts with lipids, nucleic acids, and the lysine and arginine residues of proteins, generating advanced glycation end products (AGEs) that strongly disturb cell metab-
olism in prokaryotes (1) and eukaryotes (2, 3). In fact, MG has a dual nature depending on its concentrations within the cells, acting in signaling at low concentrations while provoking detrimental effects at high concentrations (2, 4). In humans, MG is implicated in diabetes and age-related disorders, such as retinopathy, nephropathy, cancer, and Parkinson’s and Alzheimer’s diseases (3), and MG is increasingly regarded as a marker of diabetes-related diseases. In plants, MG is thought to play signaling roles via Ca^{2+}, reactive oxygen species (ROS), K^+ and abscisic acid, and these processes are thought to provide the foundation for developing stress-resilient crops capable of coping with rapidly changing environments (2).

MG is predominantly detoxified by the glyoxalase pathway (Fig. 1), which starts by the so-called “spontaneous” (nonenzymatic) conjugation of MG with glutathione (GSH) to form a hemithioacetal (HTA). Then, HTA is isomerized by glyoxalase I (GlxI; S-D-lactoylglutathione lyase; EC 4.4.1.5) to S-D-lactoylglutathione (S-lactoylGSH) that is hydrolyzed by glyoxalase II (GlxII) to release GSH and D-lactate.

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MG dehydrogenase, aldehyde dehydrogenases, aldo-keto reductases, α-dicarbonyl/L-xylulose reductase, and the MG reductase (3, 4).

Little attention has been paid so far to MG metabolism in photosynthetic organisms, even though they inevitably produce MG by their active photosynthetic assimilation of CO₂ (2, 5, 6), explaining why plant genomes possess multiple *glxI* and *glxII* genes (2). In contrast, heterotrophic organisms from *Escherichia coli* to humans possess only one copy of each *glxI* or *glxII* gene (7). MG production, signaling, and detoxification systems are of primary importance in cyanobacteria, the environmentally important prokaryotes (8), because they perform the two MG-producing pathways, photosynthesis (CO₂ fixation and gluconeogenesis) and respiration (glucose catabolism), in the same cell compartment (6). Furthermore, cyanobacteria are regarded as the inventor of oxygenic photosynthesis (9, 10), and GSH and GSH-utilizing enzymes such as glutathione transferases (GSTs), to cope with the ROS often produced by their active photosynthesis (11). Attesting to the importance of GST in cyanobacteria, the well-studied unicellular model *Synechocystis* sp. strain PCC 6803 endowed with a small genome (about 4 Mb), possesses six GST (Sll0067, Sll1147, Sll1545, Sll1902, Slr0236, and Slr0605). We previously showed that Sll1545 and Slr0236 operate in the protection against photo-oxidative stress triggered by high light or H₂O₂ (12) and that Sll1147 and its human orthologs play a prominent role in the tolerance to membrane stresses triggered by heat, cold, and lipid peroxidation (13). Concerning Sll0067, we report here that it operates in the protection against MG, unlike the other five GSTs. Consistently, we show that Sll0067 catalyzes the conjugation of GSH with MG, the first step in MG detoxification by the Glx enzymes. These findings are important because the conjugation of GSH with MG is always described as spontaneous (nonenzymatic) in all organisms (2–4). Our report will likely stimulate research on MG signaling and detoxification in humans and animals (with a possible influence on the identification of biomarkers and drugs), plants (with possible influence on agriculture), and cyanobacteria (with influence on the production of carbon-based chemicals, such as lactate) (14).

**RESULTS**

Sll0067 is dispensable to the photoautotrophic growth of *Synechocystis* sp. strain PCC 6803, but it operates in the protection against methylglyoxal. To analyze the role of Sll0067 in *Synechocystis* sp. strain PCC 6803, we constructed a Δsll0067::Kmr deletion mutant and verified by PCR (see Tables S1 and S2 and Fig. S1 in the supplemental material) that the kanamycin resistance gene (Kmr) marker had properly replaced *sll0067* in all 10 copies of the polyploid chromosome (15). All Δsll0067::Kmr mutants grew as healthy as the wild-type (WT) strain (Fig. 1) and possessed only Δsll0067::Kmr chromosomes (Fig. S1). The absence of WT chromosomes in the Δsll0067::Kmr mutant (here called Δsll0067) was confirmed by studying cells grown for multiple generations in the absence of Km (absence of counterselection of WT, i.e., Km⁺ chromosome copies). Collectively, these data demonstrate that *sll0067* is not essential for the standard photoautotrophic growth of *Synechocystis* sp. strain PCC 6803.

Next, the influence of various stresses on the growth and survival of the Δsll0067 mutant and the WT strain were tested. The Δsll0067 mutant was not affected by either photo-oxidative stresses (high light, H₂O₂, menadione, or methylene blue), unlike the Δsll1545, Δslr0236, and Δsll1545-Δslr0236 mutants (12), or temperature stresses (heat or cold) or n-tert-butyl hydroperoxide, unlike the Δsll1147 mutant (13).

Very interestingly, the Δsll0067 mutant appeared to be sensitive to exogenous MG (Fig. 2), unlike the other five GST-lacking mutants (Δsll1147, Δsll1545, Δsll1902, Δslr0236, and Δslr0605), the construction and analysis of which have been already reported (12, 13) or will be published elsewhere (in the case of the Δsll1902 and Δslr0605 mutants). Collectively, these findings indicate that Sll0067 is specifically involved in the protection against MG.

The MG-sensitive Δsll0067 mutant exposed to exogenous MG (or glucose) accumulates MG. The role of Sll0067 in MG resistance was studied by measuring the
intracellular content of MG in the Δsll0067 and WT strains incubated with or without MG, using a standard assay based on the derivatization of MG with the 5,6-diamino-2,4-dihydroxypyrimidine sulfate dihydrate (DDP) chemical that generates the 2-methylumazine fluorescent product (16, 17). To validate this assay in Synechocystis sp. strain PCC 6803, we verified that it could measure the difference in the intracellular MG content of relevant strains altered in MG production or elimination. These control strains were the GSH-depleted mutant (ΔgshB) previously reported (18) and the MG synthase deletion mutant (Δmgs) presently constructed (Fig. S2); both strains grow well under standard conditions (18; see also Fig. S2).

In the absence of exogenous MG, no MG was observed in the WT, Δmgs, and Δsll0067 strains (Fig. 3), whereas MG was abundant in the ΔgshB mutant (Fig. 3) lacking GSH that is required for MG removal (3, 4, 6). In response to exogenous MG all studied strains WT, Δmgs, ΔgshB, and Δsll0067 accumulated MG (Fig. 3). The levels were similar in WT and Δmgs strains, in agreement with MG synthase playing no role in MG uptake or elimination. MG accumulation was higher in the ΔgshB and Δsll0067 mutants that are hypersensitive to MG (18; see also Fig. 2). These data indicate that, similar to GSH, Sll0067 is required for MG removal (Fig. 3).

MG accumulation was also observed in all WT, Δmgs, ΔgshB, and Δsll0067 strains incubated with glucose, which stimulates glucose catabolism (19), a process generating MG. As anticipated, the MG levels were low in the Δmgs mutant (Fig. 3) and higher in the ΔgshB and Δsll0067 mutants, confirming that Sll0067 operates in MG removal, like GSH (Fig. 3).

The Δsll0067 mutant exposed to exogenous MG accumulates GSH in addition to MG. Since the main MG detoxification pathway is catalyzed by the GSH-requiring glyoxalase pathway (20), we measured the kinetics of the MG-triggered accumulation of MG and the possible changes in GSH abundance in the WT and Δsll0067 strains...
In response to MG, the Δsll0067 mutant transiently accumulated more MG and GSH than did the WT strain. These data indicate that Δsll0067 cells are sensitive to MG because they have a reduced capability of using GSH to eliminate MG. By extension, these data suggest that Sll0067 normally operates in a GSH-dependent MG removal process such as the glyoxalase system.

Sll0067 catalyzes the conjugation of GSH with MG. The above-mentioned in vivo findings that Sll0067 operates in resistance to MG and the removal of MG and GSH (Fig. 2 and 4) prompted us to analyze in vitro the influence of Sll0067 on MG and GSH (Fig. 5A). A His-tagged recombinant Sll0067 protein was purified as a homodimer product of about 43kDa (Fig. S3) and found to have a good GSH transferase activity (Table S3) toward the classical GST substrates phenetyl isothiocyanate (PITC), benzyl isothiocyanate (BITC), and 1-chloro-2,4-dinitrobenzene (CDNB) with measured catalytic efficiencies (kcat/Km) of 6.7 × 10^5 M^-1 s^-1, 5.7 × 10^5 M^-1 s^-1, and 112.5 M^-1 s^-1, respectively. The lower catalytic efficiency observed for CDNB is due to the lower Sll0067 affinity observed for this substrate (3,800.0 µM) compared to the values observed for BITC (82.0 µM) and PITC (31.4 µM). The comparable kinetic parameters obtained for BITC and PITC indicate that the modulation in the aromatic group (benzyl versus phenethyl) does not affect substrate recognition (Table S3).

The capacity of Sll0067 to catalyze the conjugation of GSH with MG was then assayed by measuring the levels of free (unconjugated) GSH and MG as a function of incubation times in the presence or absence of Sll0067. Sll0067 appeared to accelerate the disappearance of free MG and GSH (the conjugation of MG with GSH) over the spontaneous (nonenzymatic) levels observed in the absence of Sll0067 or after heat inactivation of Sll0067 (Fig. 5B to D). This finding is important because the conjugation of GSH with MG, the first step of MG detoxification by the glyoxalase system, is always presented as being spontaneous (nonenzymatic) in both prokaryotes (1) and eukaryotes (2, 3).

The Sll0067-driven disappearance of free GSH and MG was further studied (Fig. 6) using classical two-substrate steady-state kinetic analysis (21). Hence, we measured the initial velocity of MG transformation (conjugation with GSH) at fixed MG levels for various concentrations of GSH and vice versa, yielding the primary plots $1/V_{\text{MG}} = \ldots$
f(1/[GSH]) and 1/V_{\text{app}}[MG] values of each curve (i.e., each fixed MG concentrations) from the primary double-reciprocal pattern and vice versa for GSH (Fig. 6B and E). The V_{\text{app}}[MG] pattern revealed the allosteric behavior of Sll0067 toward MG (Fig. 6D) and suggests a positive K-type cooperativity. The corresponding Hill number $n_h$ (1.873 ± 0.261; Table 1) is consistent with the dimeric nature of Sll0067 (22; see also Fig. S3 in the supplemental material), and it validates the cooperative
fixation of MG onto SII0067 \((n_h > 1)\). The \(V_{max}^{app}[GSH] = f([GSH])\) pattern revealed the Michaelian behavior of SII0067 toward GSH (Fig. 6E). These secondary plots were also used to calculate the SII0067 apparent \(K_m\) and \(k_{cat}\) for GSH, and Hill number \(n_h\) and \(K_{0.5}\) for MG (Table 1). The reasonably good catalytic efficiency of SII0067 for GSH (355.6 M\(^{-1}\) s\(^{-1}\)) suggests that the \(K_m\) value for GSH (421.7 ± 70.5 μM; Table 1) is consistent with the high intracellular content in GSH (5 to 20 mM [12, 13]). Furthermore, the kinetic data represented in Fig. 6D suggests that the (allosteric) fixation of MG on SII0067 increases its affinity for GSH.

The relatively high \(K_{0.5}\) value for MG (221.7 ± 26.3 μM; Table 1) indicates that the positive (K\(^{+}\)) cooperative fixation of one MG molecule on the first SII0067 monomer stimulates the fixation of a second MG molecule on the second SII0067 monomer and the subsequent catalysis. Furthermore, SII0067 appeared to be more active on MG
FIG 6 SI0067 catalyzes the conjugation of MG with GSH by a steady-state sequential mechanism. (A) Schematic representation of the SI0067-accelerated conjugation of GSH with MG. (B) Initial velocity (primary) plot of SI0067 reaction with GSH (variable concentrations) and MG (fixed concentrations) with double-reciprocal plots of $1/V_i [MG]_x = f(1/[GSH])$, and the corresponding $V_i$ shown in the upper right corner. (C) Initial velocity plot of SI0067 activity with GSH (fixed concentrations) and MG (variable concentrations) with double-reciprocal plots of $1/V_i [GSH]_x = f(1/[MG])$, and the corresponding $V_i$ shown in the upper right corner. (D) Initial velocity (secondary plot) of SI0067 activity as a function of MG concentration. (E) Initial velocity (secondary plot) of SI0067 activity as a function of GSH concentration. The data are presented as means ± the SD of three experiments.
The present study reports two-substrate kinetic analysis of SII0067 (Fig. 6B to E) consistent with the steady-state sequential kinetic mechanism observed for other GSTs acting on other substrates (23). SII0067 activity is stimulated by S-D-lactoylglutathione, the intermediate product in MG detoxification. Our evidence that SII0067 catalyzes the conjugation of GSH with MG, likely yielding the hemithioacetal (HTA) subsequently transformed by GlxI into S-D-lactoylglutathione (S-lactoylGSH) (6), together with the crystallization of a Phi-class GST (similar to SII0067) in the presence of S-lactoylGSH (24), prompted us to test the influence of S-lactoylGSH on the SII0067-driven conjugation of GSH with MG. All measured initial velocities of SII0067 activity were increased by S-lactoylGSH (Fig. 7B and C). These data indicate that S-lactoylGSH stimulates the SII0067-catalyzed conjugation of GSH with MG to facilitate MG detoxification by the glyoxalase pathway.

**DISCUSSION**

Glutathione transferases (GSTs) are widespread enzymes known to use glutathione (GSH) for the detoxification of ROS, metabolite by-products, xenobiotics, and/or heavy metals. The present study reports two-substrate kinetic analysis of SII0067 (Fig. 6B to E) consistent with the steady-state sequential kinetic mechanism observed for other GSTs acting on other substrates (23). SII0067 activity is stimulated by S-D-lactoylglutathione, the intermediate product in MG detoxification. Our evidence that SII0067 catalyzes the conjugation of GSH with MG, likely yielding the hemithioacetal (HTA) subsequently transformed by GlxI into S-D-lactoylglutathione (S-lactoylGSH) (6), together with the crystallization of a Phi-class GST (similar to SII0067) in the presence of S-lactoylGSH (24), prompted us to test the influence of S-lactoylGSH on the SII0067-driven conjugation of GSH with MG. All measured initial velocities of SII0067 activity were increased by S-lactoylGSH (Fig. 7B and C). These data indicate that S-lactoylGSH stimulates the SII0067-catalyzed conjugation of GSH with MG to facilitate MG detoxification by the glyoxalase pathway.

**TABLE 1 Kinetic parameters of SII0067 activity on both MG and GSH substrates**

| Parameter                  | Mean ± SD<sup>a</sup> |
|----------------------------|------------------------|
|                           | MG                     | GSH                    |
| k<sub>cat</sub> (s<sup>-1</sup>) | NA                     | 0.15 ± 0.02            |
| K<sub>m</sub> (μM)         | NA                     | 421.70 ± 70.50         |
| k<sub>cat</sub>/K<sub>m</sub> (M<sup>-1</sup> s<sup>-1</sup>) | NA                     | 355.60 ± 0.12         |
| k<sub>0.5</sub> (μM)      | 221.7 ± 26.3           | NA                     |
| n<sub>h</sub>             | 1.873 ± 0.261          | NA                     |

<sup>a</sup>Enzymatic activities were measured as described in Materials and Methods using various concentrations of the following substrates MG and GSH. The results are presented as the means of three independent measurements. NA, not applicable.
metals. Attesting to the importance of GSTs, higher organisms possess many GSTs (20, 25), making it difficult to analyze their selectivity or redundancy.

The analysis of GSTs is easier in cyanobacteria, the environmentally crucial prokaryotes (8) regarded as the originators of photosynthesis (10) and GSH-dependent enzymes (11), because they possess few GSTs. For example, the model cyanobacterium Synechocystis sp. strain PCC 6803 has only six GSTs (SIL0067, SIL1147, SIL1545, SIL1902, Slr0236, and Slr0605). Furthermore, cyanobacteria have a great potential for an ecological production of industrially interesting chemicals that is often hampered by our limited knowledge of cyanobacterial responses to different stresses (26).

In this study, we analyzed the role of SIL0067 in Synechocystis sp. strain PCC 6803. Therefore, a Δsll0067::Kmr (Δsll0067) deletion mutant was constructed and appeared to grow as fit as the WT strain in standard (photoautotrophic) conditions. Furthermore, we found that SIL0067 is not involved in the tolerance to either photo-oxidative stress, unlike SIL1545 and Slr0236 (12), or to heat, cold, or lipid peroxidation, unlike SIL1147 (13). Very interestingly, the Δsll0067 deletion mutant appeared to be hypersensitive to exogenous MG (Fig. 2), a toxic metabolite by-product (it causes diabetes in humans) (1, 3), unlike all other GST deletion mutants (Δsll1147, Δsll1545, Δsll1902, Δslr0236, and Δslr0605).

The MG-sensitive Δsll0067 mutant exposed to MG (or glucose) accumulated MG (Fig. 3), indicating that SIL0067 plays a significant role in MG removal. These findings are welcome because MG has been overlooked in photosynthetic organisms, even though they inevitably produce MG not only by the catabolism of sugars, amino acids, and lipids, like heterotrophic organisms (from E. coli to humans) but also by their active photosynthetic assimilation of CO2 (2). This issue is even more acute in cyanobacteria that perform photosynthesis (fixation of CO2 and gluconeogenesis) and respiration (glucose catabolism) in the same cell compartment (6).

The Δsll0067 mutant challenged by MG accumulated not only MG but also GSH (Fig. 4), indicating that SIL0067 operates in a MG elimination process that requires GSH, similarly to the GSH-dependent detoxification of MG catalyzed by the glyoxalase system. This interpretation was confirmed by in vitro tests showing that SIL0067 catalyzes the conjugation of GSH with MG (Fig. 5). This finding is interesting because in most organisms MG is mainly detoxified by the GSH-dependent glyoxalase pathway that begins with the conjugation of MG and GSH, a reaction always presented as spontaneous (nonenzymatic). The resulting hemithioacetal metabolite is then isomerized by GlxI and hydrolyzed by GlxII to release D-lactate and GSH (3, 4).

Our data also indicate that the cooperative fixation of one MG molecule on the first subunit of the SIL0067 dimeric enzyme stimulates the fixation of a second MG molecule on the second SIL0067 monomer (Fig. 6), thereby increasing SIL0067 activity. We also found that the fixation of MG on SIL0067 enhances its affinity for GSH (Fig. 6) and that SIL0067 is also activated by S-D-lactoylGSH (Fig. 7), the intermediate product in MG detoxification. All of these findings indicate that MG enhances the SIL0067-driven conjugation of GSH and MG to promote MG detoxification by the glyoxalase pathway. They will undoubtedly stimulate research on MG signaling and detoxification in animals and humans (with possible implications on identification of biomarkers and drugs), plants (with possible influence on agriculture), and cyanobacteria (with probable implications on the production of industrially interesting carbon-based chemicals). Last, but not least, our evidence that SIL0067 acts in the detoxification of MG, involved in diabetes in humans, is consistent with the existence of a correlation between the occurrence of diabetes and the (poor) activity of a human GST homologous to SIL0067 (27).

MATERIALS AND METHODS

Bacterial strains, growth, and stress assays. Escherichia coli strains used for gene manipulations were grown at 37°C in LB culture medium containing the selective antibiotics: ampicillin (Amp) ay 100 μg ml⁻¹ and kanamycin (Km) at 50 μg ml⁻¹ (Top10 strain; Invitrogen) or Km at 50 μg ml⁻¹ and chloramphenicol at 34 μg ml⁻¹ [Rosetta2(DE3)/pLysS strain; Novagen].

July/August 2020 Volume 11 Issue 4 e00882-20 mbio.asm.org 10
Synechocystis sp. strain PCC 6803 was routinely grown at 30°C in liquid mineral medium (MM) under white light (2,500 lx; 31.25 μE m⁻² s⁻¹) as described previously (13). The deletion mutants were grown in the presence of the selective antibiotic (Km, 50 μg ml⁻¹). For growth analysis of the MG effect, mid-exponential-phase cultures (optical density at 580 nm [OD₅₈₀] = 0.3 to 0.8) adjusted to an OD₅₈₀ of 0.02 (5 × 10⁶ cells ml⁻¹) were incubated in MG-containing liquid MM prior to measuring the OD₅₈₀ or photographing the culture flasks. For survival analyses, 10-ml portions of mid-exponential-phase cultures (adjusted to OD₅₈₀ 0.1) were challenged with MG, serially diluted in MM, spread on MM solidified with 1% agar (Difco), and incubated (for 5 to 7 days) under standard conditions before counting the colonies generated by viable cells.

Targeted deletion of the sll0067 gene. The Δsll0067::Kmr deletion cassette was constructed by replacing the full sll0067 coding sequence (CS) by a transcription-terminator-less kanamycin resistance gene (Kmr) for selection, while preserving the sll0067/CS flanking DNA regions for homologous recombination mediating targeted gene replacement in Synechocystis sp. strain PCC 6803 (15). These DNA regions (about 300 bp) amplified by PCR, using specific primers (Table S2), were joined by PCR-driven overlap extension on both sides of a SmaI restriction site and cloned in pGEMT (Table S1). The resulting regions (about 300bp) amplified by PCR, using specific primers (Table S2), were joined by PCR-driven overlap extension on both sides of a SmaI restriction site and cloned in pGEMT (Table S1). The resulting deletion cassette Δsll0067::Kmr was verified by PCR and DNA sequencing (Mix2Seq kit; Eurofins Genomics) before and after transformation (15) to Synechocystis sp. strain PCC 6803.

Glutathione assay. This assay was performed strictly as previously described (12, 13). Cells were rapidly collected by filtration, resuspended in an acidic phosphate buffer and disrupted by freezing-thawing cycles. Cell extracts were purified by centrifugation through an Amicon filter and stored at −80°C. Cell extracts treated with 2-vinylpyridine and triethanolamine to block reduced glutathione (GSH) from assaying (total glutathione minus GSSG) assays were incubated with yeast glutathione reductase, NADPH, and DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] prior to measuring the absorption at 412 nm of TNB (5′-thio-2-nitrobenzoic acid). Standard curves prepared with various concentrations of GSH or GSSG were used to calculate the cell content in GSSG, total glutathione, and GSH (total glutathione minus GSSG) using the Synechocystis sp. strain PCC 6803 cell volume of 1.2 × 10⁻¹⁵ ml (28).

Methylglyoxal assay. Next, 100 ml of exponentially growing cultures were diluted to an OD₅₈₀ of 0.6 and incubated under white light (2,500 lx) in the presence of 200 μM MG (Sigma-Aldrich). Cells were rapidly collected by filtration, resuspended in an acidic phosphate buffer and disrupted by freezing-thawing cycles. Cell extracts were purified by centrifugation through an Amicon filter and stored at −80°C. Cell extracts treated with 2-vinylpyridine and triethanolamine to block reduced glutathione (GSH) from assaying (total glutathione minus GSSG) assays were incubated with yeast glutathione reductase, NADPH, and DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] prior to measuring the absorption at 412 nm of TNB (5′-thio-2-nitrobenzoic acid). Standard curves prepared with various concentrations of GSH or GSSG were used to calculate the cell content in GSSG, total glutathione, and GSH (total glutathione minus GSSG) using the Synechocystis sp. strain PCC 6803 cell volume of 1.2 × 10⁻¹⁵ ml (28).

Production and purification of the His-tagged Sll0067 recombinant protein. The sll0067/CS was cloned into pE-PET, transformed into E. coli Rosetta2(DE3)/pLysS cells (Table S1). The production of Sll0067 was induced in cells grown at 37°C in LB + Km + Cm at an OD₅₈₀ of 0.7 to 0.8 with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h. Cells were harvested by centrifugation, resuspended in lysis buffer (30 mM Tris-HCl [pH 8.0], 200 mM NaCl), and stored at −20°C prior to sonication and centrifugation (35,000 × g, 25 min, 4°C) to collect the supernatant. The (C-terminal) His-tagged Sll0067 protein was purified by affinity chromatography on a nickel nitrotriacetate (Ni-NTA) agarose resin (Qiagen), followed by size exclusion chromatography on a preparative Superdex 200 16/600 column (equilibrated in 30 mM Tris-HCl [pH 8.0], 200 mM NaCl) connected to an AKTA purifier (GE Healthcare). The protein concentration was determined by measuring the absorbance at 280 nm and by using a specific extinction coefficient of 28,420 M⁻¹ cm⁻¹.

To determine the oligomerization state of Sll0067, 300 μg of purified protein in 300 μl was injected at a flow rate of 0.5 ml/min on an analytical Superdex 200 10/300 column (equilibrated in 30 mM Tris-HCl [pH 8.0]–200 mM NaCl connected to an AKTA purifier equipped with a multiangle light scattering detector (miniDAWN TREOS; Wyatt Technology) and a refractometer (T-rEX; Wyatt Technology). Data were processed using Astra 7 software (Wyatt Technology).

Enzymatic activity of Sll0067 GST. GSH-conjugations on benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PITC), or 1-chloro-2,4-dinitrobenzene (CDNB) were assayed by monitoring the absorbance at 274 nm (BITC or PITC) or 340 nm (CDNB) as described previously (29). Reactions were carried out at 25°C in 500 μl of 100 mM phosphate buffer (pH 6.5; BITC or PITC) or 30 mM Tris-HCl (pH 8.0)–1 mM EDTA (CDNB). Various concentrations of BITC, PITC, and CDNB were tested at a fixed 1 mM GSH concentration. Reactions were started by the addition of a 2.21 μM Sll0067 concentration, yielding a linear response range. Measured velocities were corrected by subtracting the rate of the spontaneous reaction (absence of Sll0067). Three independent experiments were performed at each substrate concentration. The kinetic parameters (kₘ and apparent Kₘ) were obtained by fitting the data to the
nonlinear regression Michaelis-Menten model in Prism 8 software (GraphPad). The $k_{cat}$ values are expressed as μmol of substrate oxidized per s per μmol of enzyme, using specific molar absorption coefficients of 6,220 M$^{-1}$ cm$^{-1}$ at 340 nm for NADPH, 8,890 M$^{-1}$ cm$^{-1}$ at 274 nm for BITC, and 9,600 M$^{-1}$ cm$^{-1}$ at 340 nm for CDNB.

**Enzymatic assays of GSH and MG depletions.** The disappearance of MG (50 μM) and GSH (1000 μM) catalyzed by Sll0067 (2.21 μM) was measured at 30°C in 100 mM KH$_2$PO$_4$/K$_2$HPO$_4$–1 mM EDTA (pH 7.5). Three independent experiments were performed for each time of reaction, which were started by the addition of both MG and Sll0067 and stopped by the addition of ice-cold 0.5 ml of 1.0 M NH$_4$Cl/ NH$_3$, (pH 10.0; Merck). Samples were stored, prior to measuring the remaining MG as described above. GSH-consuming reactions were stopped by the addition of 1 ml of 100 mM KH$_2$PO$_4$/K$_2$HPO$_4$, 1 mM EDTA, and 1.2% (wt/vol) 5-sulfosalicylic acid. Then, 5 μl of the reaction mixture was loaded onto a microplate, and the remaining concentration of GSH was measured as described above. The initial velocity of Sll0067 reaction was expressed as μM s$^{-1}$ mg$^{-1}$ of enzyme.

**Two-substrate kinetic analysis of Sll0067 GST.** Steady-state kinetic of Sll0067-driven consumption of GSH and MG were performed at 30°C in 1 ml of 100 mM KH$_2$PO$_4$/K$_2$HPO$_4$, and 1 mM EDTA (pH 7.5). Initial velocities were determined by assaying variable MG concentrations at fixed GSH concentrations and vice versa. The reactions were started by adding both MG and Sll0067 (2.21 μM), and three independent experiments were performed at each substrate concentration. The measured velocities were corrected by subtracting the rate of the spontaneous reactions (absence of Sll0067). The kinetic parameters ($k_{in}$ and apparent $k_{cat}$) and allosteric parameters (Hill number $n_h$ and $K_d$) were obtained by fitting the data to the nonlinear regression Michaelis-Menten model and to the allosteric sigmoidal model in GraphPad Prism 6 software, respectively.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**TABLE S1**, DOCX file, 0.01 MB.

**TABLE S2**, DOCX file, 0.01 MB.

**TABLE S3**, DOCX file, 0.01 MB.

**FIG S1**, TIF file, 0.4 MB.

**FIG S2**, TIF file, 0.3 MB.

**FIG S3**, TIF file, 0.3 MB.

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X.K., A.H., N.R., F.C., and C.C.-C. designed the experiments. X.K. and A.H. performed the experiments. X.K., F.C., and C.C.-C. wrote the draft manuscript. X.K., A.H., N.R., F.C., and C.C.-C. wrote the paper. We declare there are no conflicts of interest.

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