ORIGINAL RESEARCH COMMUNICATION

A Glutathione-Dependent Detoxification System Is Required for Formaldehyde Resistance and Optimal Survival of *Neisseria meningitidis* in Biofilms

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Abstract

Aim: The glutathione-dependent AdhC-EstD formaldehyde detoxification system is found in eukaryotes and prokaryotes. It is established that it confers protection against formaldehyde that is produced from environmental sources or methanol metabolism. Thus, its presence in the human host-adapted bacterial pathogen *Neisseria meningitidis* is intriguing. This work defined the biological function of this system in the meningococcus using phenotypic analyses of mutants linked to biochemical and structural characterization of purified enzymes. **Results:** We demonstrated that mutants in the *adhC* and/or *estD* were sensitive to killing by formaldehyde. Inactivation of *adhC* and/or *estD* also led to a loss of viability in biofilm communities, even in the absence of exogenous formaldehyde. Detailed biochemical and structural analyses of the esterase component demonstrated that *S*-formylglutathione was the only biologically relevant substrate for EstD. We further showed that an absolutely conserved cysteine residue was covalently modified by *S*-glutathionylation. This leads to inactivation of EstD. **Innovation:** The results provide several conceptual innovations. They provide a new insight into formaldehyde detoxification in bacteria that do not generate formaldehyde during the catabolism of methanol. Our results also indicate that the conserved cysteine, found in all EstD enzymes from humans to microbes, is a site of enzyme regulation, probably *via S*-glutathionylation. **Conclusion:** The *adhC-estD* system protects against formaldehyde produced during endogenous metabolism. *Antioxid. Redox Signal.* 18, 743–755.

Introduction

THE HIGHLY CYTOTOXIC compound formaldehyde is a by-product of numerous environmental processes (16). This strong electrophile undergoes indiscriminate, nonenzymatic, and rapid reactions with biological macromolecules, leading to irreversible alkylations and cross-linkages, with resulting damage to proteins and DNA (7). Thus, it comes as no surprise that formaldehyde detoxification systems are diverse and widespread across the biological world. These systems often depend on the glutathione (GSH) pool, as it reacts spontaneously with formaldehyde to form *S*-hydroxymethylglutathione (HMGS, Fig. 1) (7). This adduct is in turn oxidized by a class

Innovation

The *adhC-estD* detoxification system for formaldehyde is commonly understood to remove formaldehyde generated from exogenous sources or as a by-product of methanol metabolism. Our results suggest that the *adhC-estD* system is required for protection against formaldehyde that is generated during endogenous metabolism. They provide a new insight into formaldehyde detoxification in bacteria that do not generate formaldehyde during the catabolism of methanol.

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FIG. 1. Proposed reaction mechanism of formaldehyde detoxification by AdhC and EstD.

III, zinc-containing, NAD^+ -dependent alcohol dehydrogenase (AdhC) to generate *S*-formylglutathione (SFG) (48). GSH is regenerated *via* subsequent hydrolysis of SFG by a thioesterase, which also generates formate as a by-product (Fig. 1) (49).

It has been known for decades that formaldehyde is also produced endogenously during cellular metabolism of ethanol and methanol. As a result, there is a wealth of literature on this GSH-dependent detoxification system from the mammalian liver and plants (12, 48). More recently, it was recognized that Gram-negative methylotrophic bacteria also employ a class-III alcohol dehydrogenase (AdhC) and a thioesterase (EstD) to detoxify formaldehyde during methanol metabolism (5, 6, 14, 50). Intriguingly, analysis of bacterial genomes has revealed that *adhC* and *estD* genes are well distributed in bacteria that do not oxidize methanol. An example is *Escherichia coli*, where formaldehyde induces the expression of the operon consisting of *frmR*, encoding a formaldehyde-responsive repressor, *frmA* (*adhC*), and *frmB* (*estD*) (13).

The bacterial pathogen *Neisseria meningitidis* is the etiological agent of meningococcal meningitis. It is closely related to *Neisseria gonorrhoeae*, the causative agent of gonorrhoea. In both organisms, *adhC* and *estD* are found as an operon that is controlled by NmlR, a MerR-like regulator that was recently characterized in the gonococcus (24). While *adhC* is not functional in all strains of *N. gonorrhoeae* due to a frameshift mutation in the gene, the *adhC* gene in *N. meningitidis* has remained intact (36). Like *E. coli*, these pathogenic *Neisseria* species are not able to use methanol as a carbon source, and thus the presence and function of these formaldehyde detoxification genes in their genome remain unclear. In this study, we report the effect of mutation of *adhC* and *estD* on the ability of *N. meningitidis* to defend against reactive aldehyde stress. To understand the molecular basis of formaldehyde defense in *N. meningitidis*, we carried out a biochemical characterization of the esterase encoded by the *adhC-estD* operon. We also report the crystal structure of EstD and describe its role in formaldehyde detoxification. Our studies provide new insights into the covalent modification of a conserved cysteine present at the enzyme active site of EstD. Our results also provide an understanding of the possible biological role of *adhC* and *estD* in defense against endogenously produced formaldehyde.

Results

NMB1304 (*adhC*) and *NMB1305* (*estD*) are required for resistance to formaldehyde in *N. meningitidis*

The operon consisting of NMB1304 and NMB1305 within the *N. meningitidis* MC58 genome (44) contains genes that were annotated as a *adhC* and an *S*-formylglutathione hydrolase (SFGH, EstD), respectively (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/ars). This operon is also present in the meningococcal C311#3 strain. We have sequenced these genes in both strains

and found them to be identical (data not shown). To assess the functional role of this operon in the meningococcus, we constructed mutant strains in which the entire bicistron or each of the individual genes was replaced with a kanamycin-resistance cassette (Supplementary Fig. S1), creating C311#3*adhC*, C311#3*estD*, and C311#3*adhC-estD*. These engineered strains were subsequently challenged with a range of reactive aldehydes: formaldehyde as well as glutaraldehyde, glycolaldehyde, glyoxal, and methylglyoxal. Figure 2A shows the results from a disc-diffusion killing assay, where the wild-type and mutant strains displayed similar zones of growth inhibition for all chemical challenges, with the exception of formaldehyde. In this case, a significant increase in the zone of inhibition was observed for the single and double NMB1304 and NMB1305 mutant strains when compared to wild-type meningococci. Additional short-chain aldehydes, such as acetaldehyde and propionaldehyde, were also tested, but no difference between the mutant strains was observed (Supplementary Fig. S2).

To confirm this formaldehyde-sensitive phenotype, we developed a killing assay where serial dilutions of meningococci were plated on a solid medium containing increasing concentrations of formaldehyde (0–1.6 mM). Figure 2B confirmed that mutants of the NMB1304-1305 operon were more sensitive to killing by formaldehyde than wild-type cells. The data also indicated that this sensitivity was most pronounced for the NMB1305 single mutant. This observation was confirmed when the colony counts of cells grown in the presence of formaldehyde were determined. Figure 2C shows the recovery of colony-forming units (CFUs) after incubation with decreasing concentrations of formaldehyde for the wild-type cells. Compared to the wild type, there was a decline in CFU recovery as a function of formaldehyde concentration for the NMB1304 single and NMB1304-1305 double mutants that followed an almost identical pattern. However, the CFU recovery for the NMB1305 single mutant was notably lower than for the other two mutants, and this loss of CFU also began at lower formaldehyde concentrations (Fig. 2C). Complementation of each mutant by insertion of a single copy of the entire NMB1304-1305 operon into the meningococcal chromosome led to a complete recovery of the wild-type formaldehyde-resistant phenotype (Fig. 2D). Taken together, these results confirmed the annotation of NMB1304 and NMB1305 as *adhC* and *estD*, respectively, and confirmed the role of these gene products in the protection against formaldehyde toxicity in *N. meningitidis*.

adhC and *estD* are required for survival in biofilms

N. meningitidis is an obligate human pathogen that frequently colonizes the nasopharynx, and formation of meningococcal biofilms on the nasopharyngeal epithelial cells is a key virulence factor (33). We examined biofilm formation by wild-type meningococci and *adhC*, *estD*, and *adhC-estD* mutant strains on glass coverslips over a period of 24 h. No exogenous formaldehyde was added in these experiments. COMSTAT analyses found no significant difference in the abilities of these strains to form biofilms, as indicated by biofilm thickness and average biomass (Fig. 3A). However, when we evaluated the survival of meningococci within the biofilm matrix using a viability stain, we observed that while wild-type cells survived, and the *adhC*, *estD*, and *adhC-estD* mutant strains were nonviable (Fig. 3B and Supplementary

FIG. 2. The *adhC-estD* operon in *Neisseria meningitidis* confers resistance to formaldehyde. (A) Sensitivity of meningococci to reactive aldehydes, as determined by disc diffusion assay. Black bars, wild type; white bars, *adhC* mutant; dark gray bars, *estD* mutant; light gray bars, *adhC-estD* mutant. MG, methylglyoxal; GTA, glutaraldehyde; GCA, glycoaldehyde; FA, formaldehyde. Six independent experiments were performed. Y-error bars indicate $\pm$ standard error from the mean. **p* < 0.0002; ***p* < 0.0005; ****p* < 0.0007. (B) Growth of meningococci on a solid medium containing increasing concentrations of formaldehyde as indicated. The experiment was repeated at least three times, and representative results are shown. Number of cells inoculated is shown on the Y-axis. (C) Bacterial count after overnight growth on a formaldehyde-containing medium as in panel B. Filled circles (●), wild type; empty circles (○), *adhC* mutant; filled triangles (▼), *estD* mutant; empty triangles (△), *adhC-estD* mutant. Three independent experiments were performed. Y-error bars indicate $\pm$ standard error from the mean. (D) Recovery of resistance to formaldehyde by chromosomal complementation with an intact *adhC-estD* operon. Filled circles (●), wild type; empty circles (○), *estD* mutant; filled triangles (▼), *estD* *adhC-estD*⁺ complemented mutant; empty triangles (△) *adhC-estD* mutant; filled squares (■), *adhC-estD* *adhC-estD*⁺ complemented mutant.

Fig. S3). This apparent aging of biofilms points to a possible role of the *adhC-estD* operon in the survival of *N. meningitidis*.

NmEstD is an SFGH

The gene product of *estD* (herein referred to as NmEstD) exhibits high sequence identity (40%–60%) with several SFGHs from human (ESD), *Saccharomyces cerevisiae* (YJG8), and *E. coli* (FrmB and YeiG). In particular, the serine catalytic triad (Ser145-Asp221-His254) in NmEstD is fully conserved (Supplementary Fig. S4). To confirm the expectation that NmEstD functions as a typical SFGH, it was expressed as a recombinant protein in *E. coli* with a hexa-His epitope at the N-terminus. The enzyme was obtained routinely in high yield (~ 300 mg/L culture) and purity ($>95\%$ pure) in a dimeric and fully reduced form (Supplementary Fig. S5 and Supplementary Table S1, respectively). The purified enzyme was subsequently assayed for activity against SFG. We also tested

S-lactoylglutathione (SLG), a naturally occurring thioester that is produced during the removal of methylglyoxal using the glyoxalase system. The synthetic carboxyl ester *p*-nitrophenyl acetate (pNPAC) was used as a positive control for the reaction. NmEstD was active toward all tested substrates, and Table 1 lists the kinetic parameters obtained for the NmEstD-catalyzed hydrolysis of these substrates under the same conditions. The catalytic efficiencies toward pNPAC (k_{cat}/K_m $3.2 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) and SLG (k_{cat}/K_m $2.90 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) were comparable to those reported previously for other SFGHs (Table 2). However, the highest activity of NmEstD was obtained with SFG (k_{cat}/K_m $1.95 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$) (Fig. 4), with a catalytic efficiency of at least three orders of magnitude higher than those obtained for pNPAC and SLG. It was also noted that the specificity rate constant toward SFG was at least 25-fold higher than those reported previously for other SFGHs (Table 2). These results are consistent with the conclusion that SFG is the likely physiological substrate of NmEstD.

FIG. 3. The *adhC-estD* operon is required for biofilm viability. (A) COMSTAT analysis of biofilm biomass (black bars) and average thickness (white bars). The error bars represent $\pm$ standard error of the mean. No statistical difference ($p \leq 0.05$) was observed in either the biomass or thickness of the biofilms formed by the *adhC*, *estD*, and *adhC-estD* mutants relative to the wild-type strain. These results are the combination of three independent experiments. (B) Three-dimensional reconstructions of stacked z-series of meningococcal wild-type, *adhC*, *estD*, and *adhC-estD* mutants grown over glass taken at 200 $\times$ magnification and rendered using EZ-C1 viewer (Nikon). Cells staining green represent viable cells, while cells staining red represent dead cells. These experiments were performed three times, and representative results are shown. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars.)

Despite the presence of a serine catalytic triad, the absolute conservation of a cysteine residue (Cys54 in NmEstD; Supplementary Fig. S4) among various SFGH homologs and the sensitivity of these enzymes to thiol-modifying reagents led to their initial misclassification as cysteine hydrolases (28). In

this study, we confirmed that NmEstD functions using a typical serine hydrolase active site. Substitution of the active-site serine (Ser145 in NmEstD) with alanine completely abolished enzyme activity toward all tested substrates (Table 1). We also tested the effect of replacing Cys54 with alanine on enzyme activity. The C54A mutant had K_m values comparable to those of the wild-type enzyme toward all tested substrates (Table 1). However, we noted that this mutant exhibited a small (19%) decrease in its k_{cat} value toward SFG compared to wild-type NmEstD. Similarly, alanine substitution of a second, solvent-exposed cysteine (Cys26) did not affect enzyme activity toward SLG or pNPAC, and only a minimal decrease in k_{cat} (1.7-fold) was detected for SFG (Table 1). These results confirmed that Cys54 is not involved in a direct catalytic role.

Structural characterization of NmEstD

To characterize the molecular basis of the apparent specificity for SFG, we determined the crystal structure of NmEstD. Crystals of NmEstD had the symmetry of the hexagonal space group ($P6_522$), two molecules per asymmetric unit, and they diffracted to 1.3 $\text{\AA}$ resolution. Statistics for data processing and structure refinement are listed in Table 3. The NmEstD structure reveals a typical α/β hydrolase fold displayed by other members of the SFGH family (35). Nine central mixed β -strands ($\beta1-\beta9$) form a twisted β -sheet that is surrounded by nine α -helices [$\alpha A-\alpha I$; secondary structure notation as in (35)] and three 3_{10} helices (Fig. 5A). There is one large dimer interface in the NmEstD crystals (occluding $\sim 900 \text{ \AA}^2$ of solvent accessible area per monomer), and it involves polar and nonpolar interactions (Fig. 5D) between residues in $\beta1-\beta2$, $\alpha A-\alpha B$, and αI . Electrostatic interactions involve the side chains of Asp253 and His7 in one monomer and the hydroxyl group of Tyr252 and the main-chain N-atom of Gly11 in the other monomer. Further contacts involve cation- π interaction between Arg67 in one monomer and Phe10 and Tyr68 in the other (Fig. 5C). These residues are conserved among other bacterial SFGHs, but have been substituted in eukaryotic proteins (Supplementary Fig. S4).

The structure further revealed that the substrate-binding pocket of NmEstD displayed an overall positive charge due to residues Asn58, Lys62, and His144 (Fig. 5C). These residues are conserved among NmEstD homologs and may be important for orienting the GSH moiety within the active site. Indeed, the dimensions of this site (roughly $14 \times 8 \times 6 \text{ \AA}$) can easily accommodate the GSH moiety in both SFG and SLG substrates. However, despite various attempts, we were unable to cocrystallize NmEstD with its substrates. Thus, we employed a simple docking protocol to model binding of SFG in this substrate pocket. These *in silico* analyses confirmed that atoms from residues Asn58 and Lys62 are well positioned to form hydrogen bonds to charged groups from the GSH moiety (Fig. 6A, B). These residues are likely to be important for the proper orientation of the substrate into the active site. However, the size of the side chain attached to GSH is central in defining substrate specificity. Previous studies have shown that the size of the substrate that can be accommodated in the active site is defined by the acyl pocket (9, 10, 29). The acyl pocket of NmEstD is highly similar to those previously seen for other SFGHs and is formed by residues Leu52, Tyr94, Ile171, Trp179, Phe223, and Leu228 (Fig. 5B). It is likely that this shallow acyl pocket of NmEstD is responsible for its

TABLE 1. KINETIC PARAMETERS OF NmEstD AND ITS VARIANTS

Protein	Substrate	K_m (mM)	V_{max} ($\mu\text{mol}\cdot\text{min}^{-1}\cdot\mu\text{mol protein}^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
WT	SFG	2.21 ± 0.35	$2.6 \times 10^5 \pm 1.8 \times 10^4$	4300	1.95×10^6
	SLG	2.69 ± 0.47	$4.8 \times 10^2 \pm 0.3 \times 10^2$	7.90	2.90×10^3
	pNPAC	0.62 ± 0.06	$1.2 \times 10^2 \pm 0.3 \times 10^2$	2.00	3.20×10^3
C54A	SFG	2.77 ± 0.26	$2.1 \times 10^5 \pm 9.4 \times 10^3$	3500	1.26×10^6
	SLG	2.08 ± 0.52	$1.6 \times 10^2 \pm 0.1 \times 10^2$	2.70	1.30×10^3
	pNPAC	0.79 ± 0.06	$7.8 \times 10^1 \pm 2.7 \times 10^1$	1.30	1.65×10^3
C26A	SFG	4.23 ± 0.66	$1.6 \times 10^5 \pm 1.4 \times 10^4$	2600	6.15×10^5
	SLG	3.00 ± 0.90	$5.2 \times 10^2 \pm 0.6 \times 10^2$	8.70	2.90×10^3
	pNPAC	0.48 ± 0.05	$9.5 \times 10^1 \pm 2.9 \times 10^1$	1.60	3.30×10^3
S145A	SFG	ND	ND	ND	ND
	SLG	ND	ND	ND	ND
	pNPAC	ND	ND	ND	ND

ND, not detectable; pNPAC, *p*-nitrophenyl acetate; SFG, *S*-formylglutathione; SLG, *S*-lactoylglutathione.

preference for SFG over bulkier substrates such as SLG, or those with aromatic moieties like pNPAC. The presence of an ordered water molecule (B factor $12.4 \text{ \AA}^2$) in close proximity to the enzyme active site further allowed us to identify the oxyanion hole in NmEstD. Based on the proposed reaction mechanism for SFGHs (9, 29), the O atom from the formate moiety in SFG interacts with backbone amide groups from the oxyanion hole. In the NmEstD structure, the O atom from the water molecule is within $3.1 \text{ \AA}$ of the backbone amide groups of Leu52 and Met146, and therefore these residues are the most likely candidates to for the oxyanion hole in NmEstD.

Cys54 is *S*-glutathionylated by reaction with *S*-nitrosoglutathione

There is increasing evidence that cysteine residues can function as redox switches and control the catalytic activity of enzymes (26). Thiol-modifying compounds have been shown previously to target the conserved cysteine residue in SFGH homologs from *S. cerevisiae*, *Arabidopsis thaliana*, and *E. coli*, which has led to the suggestion that this residue (Cys54 in NmEstD) may play a role in the secondary regulation of enzyme activity (9, 13, 29). In this work, we tested the reactivity of the cysteines in NmEstD toward synthetic alkylating agents and analyzed the reaction products by electrospray ionization mass spectrometry (ESI MS). Incubation with a large excess of iodoacetamide (IA; 50 molar equivalents) led to a mass increase of +57 Da compared to the untreated reduced protein

(Fig. 7A). These results are consistent with the alkylation of only one of the two solvent-exposed Cys residues in NmEstD. We extended this work to determine if NmEstD could be covalently modified *via S*-glutathionylation. Incubation with various molar ratios of the naturally occurring reagent *S*-nitrosoglutathione (GSNO) increased the mass of NmEstD by +305 Da, corresponding to the attachment of a single GSH moiety to one Cys residue (Fig. 7B). A second biologically relevant *S*-glutathionylation agent, oxidized glutathione (GSSG), was also used in these experiments, but it did not modify the enzyme with the same efficiency as GSNO (data not shown). To identify the reactive cysteine in NmEstD, two protein variants were produced in which each of the surface Cys residues was replaced with an alanine. In the C26A variant, only Cys54 is accessible to the solvent. Likewise, in the C54A variant, only Cys26 is present at the surface. GSNO treatment followed by ESI MS analyses revealed that the C26A variant was modified almost to completion, while only a third of the C54A mutant was modified under the same conditions (Fig. 7B). In addition, the C54A mutant was also resistant to *S*-alkylation by IA (Fig. 7A). Taken together, the above results suggest that Cys54 is in fact the reactive Cys residue in NmEstD.

We further examined the effect of Cys54 modification on the thioesterase activity of NmEstD. For simplicity, we used the C26A and C54A mutants of NmEstD in these studies, as each mutant only contained one solvent-accessible Cys residue. Figure 7C shows that activity of the C54A mutant

TABLE 2. KINETIC PARAMETERS OF EstD HOMOLOGS

Organism	SFG			SLG			<i>p</i> NPAC			Ref
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	
<i>Neisseria meningitidis</i>	2.21	4300	2.0×10^6	2.69	7.9	2900	0.62	2	3200	This study
<i>Saccharomyces cerevisiae</i>	ND	ND	ND	>1.25	ND	ND	0.4	1.8 ^a	4500 ^a	(29)
<i>Escherichia coli</i> FrmB	0.41	28.5	6.9×10^4	0.6	0.5	800	0.29	0.3	1030	(13)
<i>E. coli</i> YeiG	0.43	6.51	1.5×10^{4b}	0.58	1.02	1750	0.45	0.26	570	(13)
<i>Arabidopsis thaliana</i>	0.13	10.4 ^c	4.6×10^{4c}	ND	ND	ND	1.02	2.9 ^c	2900 ^c	(27)
<i>Agrobacterium tumefaciens</i>	ND	ND	ND	ND	ND	ND	0.48	1.95	4070	(51)

^aConverted from min^{-1} .

^bCorrected value obtained from the data published.

^cConverted from $\text{mM}\cdot\text{s}^{-1}$ and k_{cat} value as published.

FIG. 4. NmEstD is a SFGH. Activity of NmEstD was assayed against SFG (filled circles, ●), SLG (empty circles, ○), and pNPAC (filled triangles, ▼). The results were plotted as a function of substrate concentration and fitted to a rectangular hyperbola. All assays were performed in HEPES buffer (50 mM, pH 7.0) at 37°C using 0.4 μ M EstD for SLG and pNPAC or 4 nM EstD for SFG. Units = μ mol.min⁻¹. pNPAC, *p*-nitrophenyl acetate; SFGH, *S*-formylglutathione hydrolase; SLG, *S*-lactoylglutathione.

remained relatively unaffected by GSNO. In contrast, the C26A mutant was inhibited under the same conditions. The loss of activity was proportional to the amount of GSNO added, and the activity was recovered by incubation with dithiothreitol (DTT) (Fig. 7C). These results supported the proposal that *S*-glutathionylation of Cys54 in NmEstD could modulate the activity of this enzyme. However, this covalent modification of Cys54 appeared to have no effect on its secondary structure (Supplementary Fig. S6) or dimeric state (Supplementary Fig. S5). To this end, we have not been able to crystallize the GSNO-modified form of NmEstD. Nevertheless, examination of the crystal structure showed that Cys54 is located in a loop between β 4 and α A and sits at the entrance to the catalytic site of NmEstD (Fig. 5C). We were able to model the impact of *S*-glutathionylation using a docking protocol as described in the Materials and Methods section. These *in silico* results suggest that this modification would in fact block access to the substrate-binding pocket of NmEstD, thus providing a rationale behind the inhibition observed above (Fig. 6C).

To further characterize the role of Cys54 in the NmEstD-mediated formaldehyde detoxification *in vivo*, we constructed a C54AestD mutant strain by complementing the *estD* deletion strain with a single copy of the same operon expressing a C54A mutant of NmEstD. Our killing assay showed that the C54AestD mutant was resistant to formaldehyde to the level seen in wild-type meningococci (Fig. 8). The same was observed when a C26A mutation was introduced. By contrast, the S145AestD mutant was sensitive to killing by formaldehyde, consistent with our observation that the same mutation in the NmEstD enzyme led to a complete loss of SFGH activity (cf. Table 1).

TABLE 3. DATA COLLECTION AND REFINEMENT STATISTICS FOR NmEstD

<i>Data collection and processing</i>	
X-ray source	MX-2 Australian Synchrotron
Wavelength (Å)	0.954
Space group	P6 ₁ 22
Cell dimensions [a, b, c (Å)]	111.3, 111.3, 169.9
Resolution range (Å) ^a	19.84–1.30 (1.30–1.37)
Rmerge (%) ^a	6.7 (80.8)
<I/σI> ^a	10.0 (1.0)
Completeness (%) ^a	99.3 (97.0)
Multiplicity ^a	5.2 (5.0)
Number of total reflections ^a	783,482 (106,886)
Number of unique reflections ^a	150,461 (21,149)
<i>Refinement</i>	
Space group	P6 ₅ 22
Resolution range (Å) ^a	19.84–1.40 (1.42–1.40)
R _{work} /R _{free} (%) ^b	14.34/15.39
Average B-factor protein/solvent (Å ²)	20.16/31.46
r.m.s. deviation bonds (lengths/angles)	0.011 Å/1.22°
Ramachandran (%) favored/allowed	96.8/100.0

^aNumbers in parentheses refer to the statistics for the highest resolution shell.

^b $R = \sum_{hkl} (|F_{obs,hkl}| - |F_{calc,hkl}|) / \sum_{hkl} |F_{obs,hkl}|$, where $|F_{obs,hkl}|$ and $|F_{calc,hkl}|$ represent the observed and calculated structure factor amplitudes, respectively.

Discussion

The combination of an alcohol dehydrogenase and a serine thioesterase represents an established system for the GSH-dependent detoxification of formaldehyde across the biological world. Although the first bacterial systems to be described were those in gram-negative methylotrophs (5, 6, 14, 50), it has become clear that *adhC* and *estD* are present in a wide variety of bacteria, the majority of which do not generate formaldehyde *via* methanol oxidation. *N. meningitidis* is one such bacterium, and our *in vitro* killing assays demonstrate that the primary function of the meningococcal *adhC-estD* system is in fact to protect this bacterium against the toxic effects of formaldehyde. This observation is similar to those made for *E. coli* (13), a bacterium that can survive in a variety of environments where it might be expected to encounter formaldehyde. By contrast, *N. meningitidis* is an obligate human host-adapted bacterium, and this raises the question about the circumstances under which this bacterium would encounter this toxic aldehyde.

It is known that formaldehyde is produced endogenously in host tissues from methylamine (8, 55). In addition, it also appears to be formed during lipid peroxidation and hydroxyl radical formation (40, 45), two processes that occur during inflammation (32). Inflammation of the meninges of the brain or spinal cord (meningitis) is associated with meningococcal infection, and so it may be the case that the *adhC-estD* system has a protective role during the systemic dissemination of *N. meningitidis*. It is notable that a highly similar NmLR-regulated *adhC-estD* system is found in *Haemophilus influenzae*, a bacterium that can also spread systemically and also causes

FIG. 5. X-ray crystal structure of NmEstD. (A) Cartoon representation of the NmEstD dimer. Active site residues and those involved in the dimer interface are shown as ball-and-stick models. (B) Organization of the active site. Zoom-in shows residues making up the catalytic triad (S145, H254, and D221) and acyl pocket as highlighted in panel A. Hydrogen bonds between residues in the catalytic triad are shown as dashed lines. (C) Surface representation of the active site as colored by electrostatic potential (negative [red], to positive [blue]). Residues contributing to the overall positive charge are shown as ball-and-stick models together with those in the catalytic triad. Primed numbers indicate residues from the second monomer. (D) Details of the dimer interface. The protein backbone is shown as a ribbon, and residues taking part in cation- π interactions are shown as ball-and-stick models. Individual NmEstD monomers are shown in different colors (green and blue). Primed numbers indicate residues from the second monomer. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars.)

FIG. 6. *In silico* models of NmEstD with its SFG substrate and S-glutathionylated Cys54. (A) Surface as colored by electrostatic potential (negative [red], to positive [blue]) showing a cross-section of the substrate-binding pocket with the docked substrate SFG. Residues making up the oxyanion hole are shown as ball-and-stick representation. Atoms from the substrate are also shown as ball-and-stick representation. Potential hydrogen bonds are indicated by dashed lines. (B) Top view of the substrate-binding pocket (as shown in A) with docked SFG. Potential hydrogen bonds between substrate and enzyme atoms as suggested by the docking analysis are shown as dashed lines. (C) Modification of Cys54 by GSH blocks the substrate-binding pocket as suggested by the docking analysis. GSH is shown as a stick model; SFG is also shown in the background as a semitransparent ball-and-stick model. Docking of GSH was restrained by enforcing a disulfide bond between the thiol groups from Cys54 and GSH (dark-yellow dashed line). GSH, glutathione. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars.)

FIG. 7. S-Glutathionylation of Cys54 by GSNO. (A) Mass spectra of NmEstD wild-type and C54A variant before and after treatment with 50 equivalents of IA. (B) Mass spectra of NmEstD wild-type, C26A, and C54A variants after treatment with 0 or 15 molar equivalents of GSNO. S-glutathionylated forms (-SSG) are indicated on top of each spectrum. The mass spectrum of the C26A mutant after additional treatment with DTT is shown. The peak indicated by an asterisk (+178 Da) represents a modified form of NmEstD due to the spontaneous α -N-6-phosphogluconoylation of the hexa-His epitope. (C) Effect of GSNO treatment on the SFGH activity of NmEstD. The C26A and C54A variants from panel (A) were assayed for activity using 1 mM SFG as substrate. Black bars, C26A mutant protein; white bars, C54A mutant protein. (D) Recovery of activity by DTT. Black bars, wild-type protein; white bars, C26A mutant. DTT, dithiothreitol; GSNO, S-nitrosoglutathione; IA, iodoacetamide. The error bars represent $\pm$ standard error of the mean. These experiments were performed three times and representative results are shown.

inflammation and meningitis (23). A number of reactive aldehydes are produced as part of the innate immune response to infection. Methylglyoxal and glycoaldehyde are produced by neutrophils as a consequence of the myeloperoxidase-driven oxidation of threonine and serine, respectively (4). However, as observed for *E. coli*, mutation of *estD* (and *adhC*) did not alter the susceptibility of *N. meningitidis* to methylglyoxal. Furthermore, in the case of the meningococcal mutant, we observed no change in sensitivity to these other reactive aldehydes. This observation may also be explained by the low specificity displayed by NmEstD toward SLG, a product of methylglyoxal detoxification. It is likely that the GloA/GloB system that is widespread among bacteria (46), including *N. meningitidis*, is responsible for defense against methylglyoxal toxicity.

Another possibility is that formaldehyde is generated endogenously within *N. meningitidis* during carbon metabolism. In *H. influenzae*, it is known that AdhC is most highly expressed under highly aerobic conditions (11), and we have

previously observed that an *adhC* mutant of this bacterium grows more slowly under conditions of high oxygen tension than wild-type cells (23). Under these conditions, intermediary carbon metabolism may generate higher internal levels of sugar phosphates as a consequence of a switch to the pentose phosphate cycle. The short-chain sugars of the pentose phosphate cycle (erythrose and glyceraldehyde) have the potential to form toxic dicarbonyl compounds (34), which can drive formation of aldehydes from α -amino acids via a process known as Strecker degradation (53). In the case of glycine, the product formed would be formaldehyde. If this is the case, then the *adhC-estD* system may be considered a detoxification system that is linked more closely to intermediary metabolism rather than a defense system associated with protection against formaldehyde generated from exogenous sources. That the former might be true comes from our observation that the viability of *N. meningitidis* cells within biofilm communities was reduced in the *adhC* and *estD* mutants compared

FIG. 8. A C54A*estD* mutant, but not S145A*estD*, is resistant to formaldehyde. Bacterial counts after overnight growth on a solid medium containing formaldehyde (0–1.6 mM). Filled circles (●), wild type; empty circles (○), *estD* mutant; filled triangles (▼), *estD adhC-estD* C26A+ complemented mutant; empty triangles (△), *estD adhC-estD* C54A+ complemented mutant; filled squares (■), *estD adhC-estD* S145A+ complemented mutant.

to wild-type cells. This reduced viability or premature aging of biofilms may be a consequence of the accumulation of the toxic metabolites due to the inactivation of the *adhC-estD* formaldehyde detoxification system. This would be consistent with endogenous formaldehyde stress in this bacterium.

Although EstD is recognized as a part of the GSH-dependent formaldehyde detoxification system, most studies have focused on AdhC, based perhaps on the assumption that this enzyme would be the more critical component of the defense system. However, our observation that the *estD* mutant was more sensitive to formaldehyde than the *adhC* single mutant or even the *adhC-estD* double mutant may suggest the following: (i) SFG itself is toxic, and/or (ii) the failure to regenerate reduced GSH in the *estD* mutant leads to GSH depletion and cell death as a consequence of loss of intracellular thiol-redox poise. That the latter might be the case is supported by observations that mutations in systems that fail to generate NADPH and thereby were unable to maintain the GSH pool in a reduced state lead to increased sensitivity to formaldehyde (19). Further work is needed to test if this is the case in *N. meningitidis*.

The biochemistry of NmEstD described in this study has revealed features that were previously unrecognized. NmEstD displayed a dramatic preference (50-fold) for SFG compared to other possible substrates, including SLG and pNPAC, which is presumably dictated by its small catalytic site. Yet, despite the high conservation of residues that make up the active site, such a high specificity for SFG by a prokaryotic or eukaryotic thioesterase has not been previously reported. It is tempting to suggest that this feature is a part of an adaptation mechanism by *N. meningitidis*, which thrives under only a limited range of environment when compared to that of other less-fastidious bacteria such as *E. coli*. The same

may be true for other host-adapted pathogens that carry this thioesterase, such as *N. gonorrhoeae* and *H. influenzae*.

Previous reports identifying the sensitivity of EstD toward cysteine-modifying agents lead to the incorrect suggestion that the conserved Cys54 in NmEstD and its homologs played an important role in catalysis (28, 49). This idea has been contradicted by a substantial body of evidence that SFGs are serine hydrolases (3, 9, 13, 51, 54), and our observation that mutation of Ser154 inactivates NmEstD is entirely consistent with the present models for SFGH mechanism. Nevertheless, the possibility that Cys54 might have a biological function is intriguing. The previous observation that modification of Cys54 with thiol-specific reagents resulted in enzyme inactivation led to the suggestion that Cys54 may act as a gatekeeper (9). Such a model is consistent with the structure of the active site and the position of Cys54. We have extended this line of thinking and demonstrated that NmEstD was inactivated *via* S-glutathionylation by a naturally occurring reagent GSNO. This covalent modification is becoming increasingly recognized to play a key regulatory role in biology (25). Unfortunately, we were not able to generate diffracting crystals of the GSH-modified form of NmEstD. However, our modeling of the NmEstD structure with a GSH-modified Cys54 indicates that the active site would indeed be blocked by such a modification.

It is possible that this modulation of activity *via* S-glutathionylation may be linked to complex metabolic signaling associated with GSNO. It has been suggested that the interplay between the metabolism of GSH and its derivatives, including GSNO and HMGSH, could have a role in regulation of NO signaling *via* S-nitrosylation of proteins (41). In fact, in mammals, yeast, and *E. coli*, AdhC is known to catalyze the NADH-dependent reduction of GSNO (30), and it has been proposed that this enzyme protects against nitrosative stress (17, 30, 42). Interestingly, EstD is not generally considered to play a role in the removal of the products of GSNO reduction (41). In the present study, we found that compared to the wild-type strain, neither the *adhC* nor the *estD* mutants of *N. meningitidis* were any more sensitive to killing by generators of nitrosative stress such as nitrite and GSNO (Chen, Djoko, and McEwan, unpublished observations). In addition, purified NmEstD was unable to remove the products of AdhC-catalyzed detoxification of GSNO (Chen, Djoko, and McEwan, unpublished observations). Our observation that NmEstD is highly specific for SFG supports the idea that the AdhC-EstD system in the meningococcus is dedicated solely to the removal of formaldehyde and not any other toxic species. Our biofilm results further suggest that this pathway represents an innate mechanism for survival in the human host.

Materials and Methods

Chemicals and reagents

Unless otherwise specified, chemicals were obtained from commercial suppliers. GSNO (15) and SFG (47) were synthesized as described previously.

Bacterial strains and culture conditions

Uncapsulated *N. meningitidis* strain C311#3 (52) was propagated on a solid brain-heart infusion (BHI) medium (Oxoid, Adelaide, South Australia) supplemented with 10% (v/v) Levinthal's base (2), and grown by incubation at 37 °C and 5% CO₂. *E. coli* strains DH5 α and BL21(DE3) were

cultured on a Luria-Bertani (LB) medium. Where appropriate, kanamycin (100 µg/ml), spectinomycin (50 µg/ml), or ampicillin (100 µg/ml) was used.

Construction of mutant meningococcal strains

Meningococcal *adhC* (NMB1304) gene, *estD* (NMB1305) gene, and the *adhC-estD* operon were inactivated by deletion and subsequent insertion of a kanamycin-resistance cassette as illustrated in Supplementary Figure S1. Briefly, the upstream and downstream flanking regions of the target gene (H1 and H2, respectively) were amplified separately by polymerase chain reaction (PCR) using primers listed in Supplementary Table S2 and combined with the kanamycin cassette *via* splice-overlap PCR. The resulting constructs were cloned into the *SmaI* site of pUC19 to generate pUC19::*adhC*::kan, pUC19::*estD*::kan, and pUC19::*adhC-estD*::kan. Plasmids were linearized with *EcoRI* and transformed into *N. meningitidis* C311#3 wild-type strain. Kanamycin-resistant transformants were selected, and correct insertion of the kanamycin cassette was confirmed by PCR using primers that annealed to regions flanking each insertion (Supplementary Table S2).

Mutant meningococci were complemented by inserting a wild-type copy of the entire *adhC-estD* operon and a spectinomycin-resistance cassette into the *proB* gene in the *N. meningitidis* chromosome (43). Briefly, a region containing the *adhC-estD* operon was amplified by PCR using the primers AEnm_comp_XmaI_F and AEnm_comp_AflII_R (Supplementary Table S2), and the product was inserted between the *XmaI* and *AflII* sites of pCTS32. The resulting plasmid pCTS32::*adhC-estD* was linearized with *NdeI* before transformation. Positive transformants were checked with PCR using the primer pair proBnm-F/R (Supplementary Table S2).

To generate site-directed variants of the *estD* gene, the *estD* single-mutant strain was complemented as described above with the C26A*estD*, C54A*estD*, or S145A*estD* gene. The desired mutation was introduced by splice-overlap PCR using primers as listed in Supplementary Table S2. All mutant strains constructed in this study were confirmed through DNA sequencing.

Bacterial sensitivity assays

Sensitivity of *N. meningitidis* C311#3 to reactive aldehydes was tested using methylglyoxal (40% w/v), glyoxal (40% w/v), glutaraldehyde (25% w/v), glycoaldehyde (1 M), and formaldehyde (35%–40% w/v). Disc diffusion susceptibility assays were performed by placing paper discs containing 10 µl of each stress reagent onto a lawn of meningococci on a BHI medium. After overnight incubation, the zone of clearing around the disc was measured. Sensitivity to formaldehyde was also examined by a modified killing assay. Briefly, fresh lawns of meningococci from an overnight agar plate were re-suspended in phosphate-buffered saline (PBS) to an OD₆₀₀ of 0.4 (~8 × 10⁷ CFU/ml). Serial dilutions (5 µl each) were plated on a BHI solid medium containing increasing concentrations of formaldehyde (0–1.6 mM). Survival was assessed by counting the number of visible colonies after overnight growth.

Growth of biofilms in continuous-flow chambers over glass

N. meningitidis C311#3 wild-type, *adhC*, *estD*, and *adhC-estD* mutants were assayed for their abilities to form biofilms over

glass coverslips as described previously (39). The strains were grown to an OD₆₀₀ of 0.25 in a GC medium supplemented with 1% IsoVitalEx (Becton Dickinson, North Ryde, NSW, Australia), Kellogg's supplements (22), and 0.042% w/v sodium bicarbonate. This cell suspension was used to inoculate a continuous-flow chamber. Cells were allowed to adhere to the glass surface for 1 h at 37°C before the chambers were incubated for 24 h under a flow rate of 180 µl biofilm medium (a GC medium diluted 1:4 in PBS with 1% IsoVitalEx and Kellogg's supplements added) per minute. At the end of the experiment, the effluent was cultured to check for contamination, and the biofilm was stained using LIVE/DEAD BacLight bacterial viability stain (Invitrogen, Mulgrave, VIC, Australia). Z-series photomicrographs were scanned with a Nikon C1 confocal microscope, and three-dimensional images were rendered using EZ-C1 software (Nikon, Lidcombe, NSW, Australia). COMSTAT analyses were performed on each z-series to quantify the average biomass and thickness (18).

Expression and purification of NmEstD

The *estD* gene was amplified from the chromosomal DNA of *N. meningitidis* C311#3 using the cloning primer pair estDnm_NdeI_F/R (Supplementary Table S2). Site-directed Cys variants were constructed by splice-overlap PCR using primers carrying the desired mutation (Supplementary Table S2). The resulting PCR products were inserted between *NdeI* and *BamHI* sites of plasmid pET-15b (Novagen, Kilsyth, VIC, Australia) and transformed into *E. coli* strain BL21(DE3) for protein overexpression. Bacteria were grown in an LB medium (1 L) containing ampicillin at 37°C with vigorous shaking at 200 rpm. When the OD₆₀₀ reached 0.4, isopropyl 1-thio-β-D-galactopyranoside (0.4 mM) was added. Cells were harvested after 5 h of further growth. Cell pellets were re-suspended in 50 ml of Buffer A (25 mM Tris-HCl, 15 mM imidazole, 300 mM NaCl, and 5 mM β-mercaptoethanol, pH 8.0) and lysed by three passages through a French pressure cell (16,000 psi) (Thermo Fisher, Scorseby, VIC, Australia).

NmEstD proteins were purified by metal ion affinity chromatography using a HiTrap Chelating Sepharose column (5 ml; GE Healthcare, Rydalmere, NSW, Australia) in Buffer A. Briefly, crude lysates were cleared by centrifugation and loaded on the column. Unbound proteins were removed by washing with Buffer A, and bound proteins were eluted using a linear gradient of 0–500 mM imidazole in the same buffer. Fractions containing NmEstD were pooled, and dialyzed into the Tris-HCl buffer (20 mM, 1 mM DTT, pH 8.0). The purity of each protein preparation was checked routinely by SDS-PAGE. The identity of each protein was confirmed by ESI MS as described below (Supplementary Table S3). Protein concentrations were quantified using solution absorbances at 280 nm (ϵ_{280} , 51,300 M⁻¹cm⁻¹).

Generation of S-glutathionylated forms of NmEstD

Reduced forms of NmEstD (50 µM) were prepared by incubation with excess DTT (100 molar equivalents) for 30 min, followed by desalting into the HEPES buffer (50 mM, pH 7.0). To generate S-glutathionylated forms, GSNO was added, and the mixture was incubated for 1 h at 37°C in the dark. Unreacted GSNO was removed by elution on a PD-10 (GE Healthcare) column. Each modified form of NmEstD was

identified using ESI MS. The esterase activity was also assayed immediately using 2 mM pNPAC and 1 mM SFG as described below.

Measurement of esterase activity

Carboxylesterase activities of wild-type Cys variants and S-glutathionylated forms of NmEstD were assayed using pNPAC as a substrate. Briefly, to a solution of pNPAC (0–4 mM) in the HEPES buffer (50 mM, pH 7), NmEstD (0.4 μ M) was added, and generation of *p*-nitrophenol was monitored at 37°C at 400 nm (ϵ , 16,400 M⁻¹ cm⁻¹). Initial rates of reactions were calculated from the rate obtained 30 s after the addition of NmEstD, and the results were plotted as a function of substrate concentration. Michaelis–Menten parameters were obtained by nonlinear regression to a rectangular hyperbola.

Thioesterase activity was measured as above, but using SLG (0–10 mM) or SFG (0–4 mM) as substrates. Hydrolysis of the thioester bond was monitored at 240 nm (ϵ , 3100 and 3000 M⁻¹ cm⁻¹ for SLG and SFG, respectively) (47), except when higher concentrations of SLG were used (5–10 mM), in which case the solution absorbance at 247 nm was monitored (ϵ , 1675 M⁻¹ cm⁻¹). Due to the high activity of EstD toward SFG, only 4 nM of NmEstD was used in these assays.

Electrospray ionization mass spectrometry

NmEstD samples (10–20 μ M) were prepared in 50% acetonitrile containing 0.01% formic acid. Spectral data were collected on QSTAR Pulsar ESI-QqTOF (Applied Biosystems, Mulgrave, VIC, Australia) using the positive ion (ESI+) mode with a cone voltage 25–35 V and a flow rate of 5 μ l acetonitrile per min. The average molar mass of each sample was obtained by applying a deconvolution algorithm to the recorded spectrum, which was calibrated with horse heart myoglobin (16,951 Da).

Crystal structure determination and structural analysis

NmEstD was crystallized by hanging-drop vapor diffusion in 25 mM bis-Tris (pH 6.5), 200 mM lithium sulfate, and 25% PEG 3350. Crystallization drops consisted of 2 μ l of purified EstD at 20 mg/ml in 10 mM HEPES, pH 7.0, 1 mM DTT, and 2.0 μ l reservoir solution. Large (0.3 $\times$ 0.1 $\times$ 0.1 mm), hexagonal crystals were grown at 293 K. Before X-ray diffraction analysis, crystals were mounted on nylon loops (Hampton Research, Aliso Viejo, CA) and flash-cooled by plunging into a liquid nitrogen bath. To preserve the diffraction quality of the crystals after flash cooling, 1 μ l of a solution containing the reservoir solution supplemented with 20% glycerol was added directly to the crystallization drop. A complete diffraction dataset from the EstD crystals was collected using a frame width of 0.5° at the Australian Synchrotron (beamline MX2) at 0.954 Å wavelength, using Blu-Ice software (31). Images were processed using XDS (21), and scaled and merged using software within the CCP4 suite (37). The crystallographic phase problem was solved by molecular replacement using the human EstD as the search model (PDB ID 3FCX). Molecular replacement and structure refinement were performed using the programs within PHENIX (1). Data processing and structure refinement statistics can be found in Table 3. The structure has been deposited in the Protein Data Bank (PDB ID 4B6G).

In silico modeling of SFG binding to NmEstD and S-glutathionylation of Cys54

Models for SFG and GSH were generated using the Dundee PRODRUG server (38). All water molecules and ligands were removed from the coordinate file of NmEstD before docking. The program GOLD (20) was used for all docking calculations. All hydrogen atoms were added to the protein within GOLD. For docking of SFG, the coordinates of the oxygen atom from Ser145 defined a search radius of 15 Å. Docking of SFG to NmEstD was restrained based on ordered solvent molecules found within hydrogen bond distances of the main-chain nitrogen atoms from Met144 and Leu52 (oxyanion hole) and the oxygen atom from Ser145. A similar protocol was employed for the *in silico* modeling of S-glutathionylated Cys54, but in this case, a disulfide bond was introduced between the sulfur atoms from the protein residue and that from GSH. All other bonds in the GSH ligand were kept unrestrained. For each ligand, 20 independent docking searches were undertaken and scored with the default setting, ChemPLP.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ADH = alcohol dehydrogenase
CFU = colony-forming unit
DTT = dithiothreitol
ESI MS = electrospray ionization mass spectrometry
FA = formaldehyde
GCA = glycoaldehyde
GSH = glutathione
GSNO = S-nitrosoglutathione
GSSG = oxidized glutathione
GTA = glutaraldehyde
HMGSH = S-hydroxymethylglutathione
IA = iodoacetamide
LB = Luria-Bertani
MG = methylglyoxal
PBS = phosphate-buffered saline
PCR = polymerase chain reaction
pNPAC = <i>p</i> -nitrophenyl acetate
SFG = S-formylglutathione
SFGH = S-formylglutathione hydrolase
SLG = S-lactoylglutathione