Molecular and structural basis of glutathione import in Gram-positive bacteria via GshT and the cystine ABC importer TcyBC of *Streptococcus mutans*

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/mmi.12274

Summary

Reduced glutathione (GSH) protects cells against oxidative injury and maintains a range of vital functions across all branches of life. Despite recent advances in our understanding of the transport mechanisms responsible for maintaining the spatiotemporal homeostasis of GSH and its conjugates in eukaryotes and Gram-negative bacteria, the molecular and structural basis of GSH import into Gram-positive bacteria has remained largely uncharacterized. Here, we employ genetic, biochemical and structural studies to investigate a possible glutathione import axis in *Streptococcus mutans*, an organism that has hitherto served as a model system. We show that GshT, a type 3 solute binding protein, displays physiologically relevant affinity for GSH and glutathione disulfide (GSSG). The crystal structure of GshT in complex with GSSG reveals a collapsed structure whereby the GS-I-leg of GSSG is accommodated tightly via extensive interactions contributed by the N- and C-terminal lobes of GshT, while the GS-II leg extends to the solvent. This can explain the ligand promiscuity of GshT in terms of binding glutathione analogues with substitutions at the cysteine-sulfur or the glycine-carboxylate. Finally, we show that GshT primes glutathione import via the L-cystine ABC transporter TcyBC, a membrane permease, which had previously exclusively been associated with the transport of L-cystine.

Introduction

Glutathione (GSH), short for γ-L-glutamyl-L-cysteinylglycine, is integral to cellular physiology across all kingdoms of life mediating a diversity of cellular functions, such as protection against oxidative, xenobiotic, and metal ion stresses, the control of intracellular redox homeostasis, cell signaling, and salvage of the essential amino acid cysteine (Bachhawat *et al.*, 2013, Franco *et al.*, 2007). Intracellular glutathione concentrations typically reach mM levels, manifested predominantly (>98%) in the thiol-reduced form (GSH) with the remaining amounts undergoing thiol oxidation to form glutathione disulfide (GSSG) and mixed disulfides with target proteins (Dalle-Donne *et al.*, 2009) or other biological organothiols such as cysteine and coenzyme A, and thioetherifications or -esterifications to form glutathione S conjugates. These conjugates may be salvaged intracellularly to recover glutathione, or may be excreted to detoxify the intracellular milieu. Our understanding of extracellular and intracellular homeostasis of glutathione and its conjugates in prokaryotes and eukaryotes has in recent years taken an unexpected twist with the discovery of transport mechanisms that operate along or against concentration gradients of glutathione and its conjugates (Bachhawat et al., 2013, Desai *et al.*, 2011, Franco & Cidlowski, 2012, Vergauwen *et al.*, 2010). While the existence of export mechanisms can be readily rationalized in terms of their link to obligate extracellular glutathione catabolism and the need to excrete cytotoxic glutathione S conjugates (Ballatori *et al.*, 2009, Keppler, 1999), the necessity for import mechanisms of glutathione has not been entirely clear in the case of animal cells, although glutathione import into bacterial and unicellular yeasts was shown to serve as a supply for organic sulfur (Brown, 1974, Elskens *et al.*, 1991, Prescott, 1961, Suzuki *et al.*, 2005, Vergauwen *et al.*, 2003b).

In 1978, the remarkable finding was reported by the Fahey group that glutathione was not synthesized by most Gram-positive bacteria (Fahey *et al.*, 1978). Presently, we know that the majority of these bacteria are loaded with functionally analogous cysteine derivatives or redox active organothiols (e.g. mycothiol, bacillithiol, coenzyme A) (Fahey, 2013), and that within a certain phylum, i.e. the Firmicutes, quite a number of genera (e.g. *Listeria*, *Streptococcus*, *Lactococcus*, *Lactobacillus*, *Clostridium*) harbor strains that are even able to acquire genuine glutathione (Newton *et al.*, 1996, Sherrill & Fahey, 1998). At the same time, the possible physiological role of glutathione in Gram-positive bacteria has been described in a variety of settings. For instance, as a medium-supplement supporting growth in *L. lactis*, *L.*

casei, *S. agalactiae*, *S. bovis*, *S. pyogenes*, and *S. mutans* (Brown, 1974, Pophaly *et al.*, 2012, Prescott, 1961, Sherrill & Fahey, 1998, Slade *et al.*, 1951), as a protective agent for oxidative-, acid-, and osmotic stress in *L. lactis* and *S. mutans* (Korithoski *et al.*, 2007, Zhang *et al.*, 2010), and as a molecular factor contributing to virulence in *L. monocytogenes*, *S. pyogenes*, and *S. pneumoniae* (Brenot *et al.*, 2004, Gopal *et al.*, 2005, Stroeher *et al.*, 2007).

Despite the evidence for the importance of glutathione-accumulation in some Firmicutes, its underlying biochemical principles are poorly understood and are based primarily from studies on *S. mutans*, a normal inhabitant of dental plaque and the microorganism most closely associated with human dental caries (Smith & Spatafora, 2012). *S. mutans* can carry out *de novo* synthesis of intracellular glutathione (Newton et al., 1996, Sherrill & Fahey, 1998). In this regard, *S. mutans* likely employs a unique hybrid glutathione synthase designated GshF (formerly SMU_267c), which is 83% similar - 0% gap-score - to the well-characterized GshF of *S. agalactiae* (Janowiak & Griffith, 2005, Stout *et al.*, 2012, Vergauwen *et al.*, 2006). Furthermore, *S. mutans* also imports the tripeptide from the growth medium leading to intracellular concentrations up to 25-fold higher than the levels achieved by *de novo* synthesis alone (Sherrill & Fahey, 1998). A comparison of import kinetics for GSH, GSSG, and cysteine showed that these molecules can compete with each other to establish reducing intra- and extracellular environments (Sherrill & Fahey, 1998, Thomas, 1984). Mixed disulfides of glutathione, glutathione thioethers, and the glutathione breakdown product γ-glutamylcysteine were additionally shown to become allocrites for the putative glutathione transporter in *S. mutans* (Sherrill & Fahey, 1998).

A key recent advance in our understanding of the molecular and structural basis of glutathione import in Gram-negative bacteria has been the discovery of a dedicated solute-binding protein (SBP), designated GbpA, which specifically binds glutathione to prime the ATP-binding cassette (ABC) dipeptide transporter DppBCDF, a permease residing in the bacterial inner-membrane (Vergauwen et al., 2010). ABC transport systems are powered by the hydrolysis of ATP, and canonical bacterial ABC-type importers are composed of two transmembrane domains/subunits forming the translocation pathway of the permease, two nucleotide binding domains/subunits that bind and hydrolyze ATP, and an SBP receptor that is, in the case of Gram-positive bacteria, generally lipid-anchored to the cell membrane or fused to the permease. SBPs capture the substrate to prime the ABC transport complex and are the main determinants of substrate specificity of their cognate transporter. GbpA is a type 5 SBP, a cluster of big SBPs poised to bind rather large allocrites such as peptides (Tam & Saier, 1993). Subsequent characterization of GbpA-like proteins allowed the delineation of a

GbpA-family of proteins exclusively found in the *Pasteurellaceae*, which is thought to have been derived from canonical DppA sequences via gene duplication (Vergauwen *et al.*, 2011). However, there is still a lack of knowledge regarding the molecular and structural details of glutathione import in Gram-positive bacteria despite the overwhelming evidence of its importance for cellular fitness. GbpA/DppBCDF is not a valid candidate for mediating glutathione import in *S. mutans* because: i) no type 5 SBP homologs other than the oligopeptide-specific OppA are apparent from the available *S. mutans* genomes (Nepomuceno *et al.*, 2007), and ii) the *H. influenzae* transporter and the *S. mutans* transporter obviously differ in terms of specificity: the GbpA-dependent system is specific for GSH and GSSG (Vergauwen et al., 2010, Vergauwen et al., 2011), while the *S. mutans* transporter is biochemically much more promiscuous (Sherrill & Fahey, 1998, Thomas, 1984). In a recent study of LysR-type transcriptional regulators that control sulfur amino acid supply, a putative SBP (SMU_1942c) required for glutathione assimilation in *S. mutans* was identified (Sperandio *et al.*, 2010). SMU_1942c is a type 3 SBP (Tam & Saier, 1993), recently reclassified as cluster F SBPs (Berntsson *et al.*, 2010) that bind diverse small molecule substrates, ranging from trigonal planar anions (e.g. nitrate, sulfate), amino acids and derivatives thereof (e.g. glutamine, glycyl betaine), to, at least in one case, the somewhat larger dipeptide, glycyl-L-methionine (Williams *et al.*, 2004). SMU_1942c is mostly similar to SBPs belonging to the FliY family, so-called after the cystine-binding protein FliY from *Escherichia coli* (SMU_1942c and FliY share 26% sequence identity and a gap-score of 6%).

In this report, we use a combination of genetic, biochemical and structural studies to investigate glutathione import in the Gram-positive bacterium *S. mutans*, by specifically asking whether a type 3 SBP protein that is thought to have been evolutionary tailored to prime import of amino acids by ABC permeases can bind different oxidation states of the much larger glutathione with physiologically relevant affinity. We show that SMU_1942c displays binding characteristics that are compatible with its predicted biochemical function, and provide the structural basis of glutathione binding by SMU_1942c via the crystal structure of its complex with GSSG. Finally, gene mutation studies enabled us to identify an ABC permease that works in concert with SMU_1942c to enable uptake of glutathione by *S. mutans*. Together, our studies define the functional annotation of SMU_1942c as a glutathione binding SBP operating in conjunction with its dedicated membrane permease to transport glutathione. This justifies a name change of SMU_1942c to GshT per the usage in Sperandio *et al.* (2010) to reflect the biological function established in the current report and to enable more accurate annotation of homologous proteins.

RESULTS

Expression and purification of recombinant GshT

Most Gram-positive SBPs are lipoproteins due to a N-acyl diacylglyceryl moiety added posttranslationally to a cysteine residue in their N-terminal LipoBox (consensus sequence ([LVI] [ASTVI] [GAS] [C]), to mediate their anchoring to the cell surface (Sutcliffe & Harrington, 2002). Locus tag SMU_1942c, herein referred to as GshT, carries the LipoBox LTAC at positions 20-23. Our construct for expression of GshT in *E. coli* covers residues 29-267 of GshT fused to the PelB signal sequence of the pET-20b(+) expression vector for periplasmic localization. This strategy allowed production of mature GshT starting with a Met-Asp fusion at its N-terminus and ending with a 6xHis-tag at its C-terminus. Purification of recombinant GshT by immobilized-metal affinity chromatography, cation-exchange chromatography, and size-exclusion chromatography resulted in highly pure protein as judged by its electrophoretic mobility as a single band at around 30 kDa on Coommassie-stained SDS-PAGE. This is consistent with the predicted molecular mass for monomeric GshT (28,4 kDa), the elution profile of GshT on SEC, and the consensus monomeric state of SBP superfamily proteins (Tam & Saier, 1993).

GshT preferentially binds GSH and to a broad range of GSH derivatives

To characterize the ligand binding preferences of GshT, we employed two complementary lines of experimentation to probe ligand preferences of our *S. mutans* GshT construct. In the first instance, we used thermal denaturation assays based on the well-established thermofluor protocol (Matulis *et al.*, 2005) to obtain an approximation of the spectrum of ligand preferences. In a subsequent step, we quantified the thermodynamics, stoichiometry and affinity of binding of the identified candidate ligands by isothermal titration calorimetry (ITC).

At the outset, our thermal denaturation assays revealed that glutathione induced the largest shift in melting temperature suggesting that it might be the preferred ligand (Fig. 1). Remarkably, all of the tested glutathione analogues carrying a γ-glutamyl group and modifications at the Sγ position of cysteine and/or the C-terminal carboxylate group also induced significant shifts in melting temperature hinting that GshT might exhibit an unusual degree of binding promiscuity for a type 3 SBP. The smallest transition was seen for γ-

glutamylcysteine. GshT did not appear to associate at all with di- and tri-peptide ligands lacking a γ-glutamyl moiety (Gly-Cys-Gly, Cys-Gly, Gly-Cys) (Fig. 1) or all twenty L-amino acids.

We subsequently selected a range of ligands identified from our thermal denaturation assays (GSH, GSSG, S-methyl-GSH, homoglutathione, GSH-monoethylester, γ-glutamylcysteine) to derive the thermodynamic fingerprints of GshT complexes and to quantify the equilibrium dissociation constants (K_d) (Fig. 2). Indeed, determination of such K_d-values can be especially informative in terms of the physiological relevance of the relevant binding events, because K_d-values of ligand-SBP complexes are typically of the same order of magnitude as the Michaelis-Menten constants (K_m) for allocrite transport through the cognate ABC permeases (Eitinger *et al.*, 2011). Except for γ-glutamylcysteine, which did not show heats above those associated with the injection process, titration of all other selected ligands to purified recombinant GshT resulted in interpretable thermograms (Fig. S1). The derived K_d values were consistent with our results from thermal denaturation assays, such that GSH emerged as the superior GshT ligand (K_d of 0.47 ± 0.06 μM), followed by S-me-GSH (K_d = 2.3 ± 0.3 μM), GSSG (K_d = 12.2 ± 0.6 μM), GSH-monoethylester (K_d = 16.1 ± 0.8 μM), and homoglutathione (K_d = 20.7 ± 1.2 μM). We note that such low micromolar K_d-values are consistent with the physiological concentrations of GSH and GSSG in human saliva (2-5 μM; the average GSH/GSSG ratio being 0.72 ± 0.54) (Iwasaki *et al.*, 2006).

To investigate the possibility that binding of γ-glutamylcysteine to GshT is enthalpically neutral, we set up a competition ITC experiment (Velazquez-Campoy & Freire, 2006) probing GSH binding to GshT in the presence of 1.9 mM γ-glutamylcysteine. The apparent K_d for GSH shifted to 1.3 μM which allowed calculation of a K_d of 1.1 mM for the γ-Glu-Cys:GshT interaction. Thus, although the C-terminal glycine is not essential for GSH to interact with GshT, its removal decreased affinity by more than 3 orders of magnitude.

Interestingly, the thermodynamic signatures of our binders series (Fig. 2), exemplify the phenomenon of enthalpy/entropy compensation whereby enthalpic gains are counteracted by entropic losses and vice versa (Freire, 2008).

Crystal structure of GshT and structural basis of glutathione binding

In an effort to provide a structural framework for our findings, we pursued crystallographic studies of GshT in complex with the best binders. Our aim was two-fold: (1) to establish a

structural prototype for type 3 SBPs in Gram-positive bacteria, and (2) to obtain insights into the structural basis of ligand binding by GshT and the broad ligand preferences of GshT.

Crystallization trials using our post-ITC GshT solutions led to crystals of the GshT:GSSG complex that diffracted to high resolution (Table S1). Until now, we were not able to grow crystals of GshT in complex with GSH. The structure of GshT features a bean-shaped bilobal structure (N-terminal lobe: residues 3-89 and 189-240; C-terminal lobe: residues 90-188) separated by a deep cleft (Fig. 3). Each of the two lobes harbors a central 5-stranded β-sheet core flanked by six and four α-helices. The GshT structure determined in the presence of GSSG adopts a closed conformation of type 3 SBPs reflecting the so-called 'Venus-flytrap' conformational change typically observed for ligand-bound SBPs (Felder *et al.*, 1999). The asymmetric unit of the crystal contained two molecules of GshT related by a non-crystallographic (NCS) 2-fold axis resulting in the substrate binding pockets facing each other. The two GshT molecules are decorated by a total of 63 cadmium ions deriving from the crystallization condition, and which mediate a variety of crystal contacts. While the two GshT protomers superpose very well (r.m.s.d of 0.23 Å for 138 C_{α}-atoms), significant structural variability can be observed in the regions covered by residues 3-5, 19-23, and 37-44, which are stretches of amino acids thatalso exhibit high atomic displacement parameters.

Fourier difference electron density maps revealed clear evidence for the GS-I leg of GSSG bound to the inter-domain cleft of GshT (Fig. 3B). The GS-II leg of GSSG protrudes out of the binding cleft and reaches out to the NCS-related GS-II of GSSG bound to the second GshT molecule in the crystal asymmetric unit. The apparent electrostatic incompatibility of this arrangement is quenched by a Cd^{2+}-ion providing coordination for the carboxylate groups of the γ-glutamate and glycine components in each of the GS-II legs. The extensive GshT:GS-I interaction network reflects the poise of GshT to serve as a glutathione binding platform, and is mediated by a wide array of polar interactions contributed roughly equally by the two lobes of the GshT structure including several ordered water molecules (Fig. 4). In contrast, GS-II makes only three specific interactions with GshT. In addition to the multitude of polar interactions, hydrophobic interactions can also be observed. Most notably, the disulfide bond of GSSG is buried in a hydrophobic pocket defined by residues Val10, Val115 and Trp51. Furthermore, the side chains of Val115, Leu145 and Phe162 sandwich the Gly-I backbone, which likely decreases the pK_a of the Gly-I carboxylic acid group to enhance the strength of its contacts with nearby polar groups (N^{ζ} of Lys160, $N^{\varepsilon 2}$ of Gln148, and w11). The observed closed conformation for GshT in the GSSG-bound form also features three polar contacts mediated by residues from each structural lobe of GshT, one at the bottom of

the cleft (Trp51(N^{ε}):Gln116(O)) and three near the exit of the pocket (Asn14(N^{δ}):Glu173(O^{ε}); Asn14(O):Ser166(O^{γ})). Together with the interactions established upon glutathione binding these interactions might contribute additionally to transition from the open state to the closed Venus flytrap conformational state of GshT.

Type 3 SBPs that serve as binding platforms for amino acids display two main binding features towards reaching their ligand-bound forms according to the Venus-flytrap modalities. First, every atom in the bound ligand compatible with hydrogen-bonding is engaged by type 3 SBPs in such interactions. Second, charges in the ligand at the N- and C-terminal groups are sequestered by conserved residues contributed by the two structural lobes in SBPs offering complementary charges, e.g. D161 and R77 in the histidine-binding protein HisJ of *Salmonella enterica* (Oh *et al.*, 1994, Yao *et al.*, 1994), D177 and R96 in the arginine-binding protein of *Salmonella typhimurium* (Stamp *et al.*, 2011), and D180 and R98 in the glutamine-binding protein of *Burkholderia pseudomallei* (PDB code: 4F3P). The GshT:GSSG complex shows that GshT follows these consensus binding modalities closely and identifies the employment of E163 and R76 in sequestering complementary charges in glutathione's γ-Glu-I moiety (compare panel C and D of Fig. 3).

An intriguing aspect of ligand binding preferences of GshT has been the apparent tolerance for S-substituted glutathione derivatives, which can now be traced to the observed binding mode of GSSG in the GshT ligand-binding cleft. Our structural studies establish that GshT provides an extensive interaction epitope only with GS-I thus allowing GS-II to emanate outwards, which is in sharp contrast to what we have observed for GSSG accommodation by the Gram-negative GbpA receptor (Vergauwen et al., 2010). Thus, since the entrance of the binding site is open and solvent exposed, disulfide substituents other than a second glutathione molecule could probably fill this cavity with only minor penalties in the free energy of binding. This is consistent with a comparison of the thermodynamic binding fingerprints of GSH and GSSG, revealing that GSSG binding to GshT is less favorable by 1.9 kcal/mol (Fig. 2). We further note that accommodation of the GSSG disulfide in the binding cleft appears to be rather atypical. This is because the Sγ1 of GS-I hovers perpendicularly above the indole ring of Trp51 making tight van der Waals contacts with an GS-I($S^{\gamma 1}$):Trp51($C^{\delta 2}$) distance of 3.7 Å. This deviates strikingly from the consensus positioning of sulfhydryls and disulfides edge on to the plane of aromatic rings at ~5.5 Å from the ring centroid (Reid *et al.*, 1985**,** Zauhar *et al.*, 2000). This may explain the differences in the observed thermodynamic parameters. Whereas binding of GSSG is entropically more favored when compared to GSH (Fig. 2), likely due to the hydrophobic cushion against its disulfide, it

ends up being a weaker binder, likely because of the very tight, and therefore unfavorable, van der Waals contact between GS-I($S^{\gamma 1}$) and Trp51($C^{\delta 2}$). Even though we were not able to obtain a structure of a GshT:GSH complex, we propose that the GS-I($S^{\gamma 1}$) in such a complex would be free to adopt a new rotamer, thereby being poised to donate a hydrogen-bond to the $O^{\gamma 1}$-atom of the nearby Thr12 and to interact more favorably with the indole ring of Trp51. Such reshuffling in the interaction network mediated by GS-I($S^{\gamma 1}$) would contribute to the more enthalpically favored GshT:GSH interaction.

Along the same line of reasoning one may explain the more entropically driven interaction of GshT with the C-terminally esterified glutathione ligand, glutathione monoethyl ester. Such modification of glutathione by esterification would eliminate three polar contacts, the salt bridge with Lys160, the interaction with Gln148, and hydrogen-bonding with ordered water w11, thereby contributing unfavorably to enthalpy. At the same, the binding event would become entropically favored due to water displacement from the binding site by the extra ethyl group in the esterified ligand and hydrophobic interactions with Phe162.

Finally, superposition of the GshT:GSSG complex with other protein:ligand complexes involving amino acids, reveals that the γ-glutamyl moiety of GS-I holds an equivalent position in the binding pocket of SBP (Fig. 3C and D), thereby explaining the inability of glutathione breakdown products that lack the γ-glutamyl group to bind GshT.

GshT mediates import of glutathione- and glutathione variants in *S. mutans*

We sought to determine the physiological relevance of GshT in glutathione acquisition and to interrogate our *in vitro* specificity studies *in vivo*, by comparing growth of a *gshT* mutant in *S. mutans* UA159 to its isogenic parent under cysteine-starvation conditions. Our experimental approach is based on the observation that inoculation of sulfur-free minimal medium with *S. mutans* leads to cysteine starvation and metabolic stalling (Kim *et al.*, 2012, Sperandio et al., 2010). This type of cysteine-auxotrophy can be relieved by supplementing the medium with sulfur-containing organic molecules such as cystine, and glutathione (Kim et al., 2012, Sperandio et al., 2010). Our results show that all tested molecules except for the thioether conjugates of glutathione, S-dicarboxyethylglutathione, S-methylglutathione, and S-hexylglutathione permitted growth of wild-type *S. mutans* (Table 1). GSSG, S-lactoylglutathione, cysteine-glutathione disulfide, homoglutathione, and glutathione monoethylester, all shown to bind GshT by our *in vitro* studies, supported growth of wild-type *S. mutans* but not of the *gshT* mutant. On the other hand, γ-glutamylcysteine sustained growth

of both strains. Although not a possible source of cysteine, the thioether glutathione conjugates, S-methylglutathione, and S-hexylglutathione, at 1.25 mM, impaired growth of wild-type *S. mutans* UA159 cells in minimal medium supplemented with 20 μM GSSG, while their growth is unaffected in cystine-supplemented minimal medium (Table 1). This suggests that *S. mutans* is not equipped to catalyze cleavage of the thioether linkage in glutathione conjugates of different compositions. The thioether S-dicarboxyethylglutathione had no such effect probably reflecting the poor K_d for binding GshT which can be inferred from the minimal shift in melting temperature in our thermofluor experiments (T_m-shift comparable to the one of γ-glutamylcysteine for which we measured a K_d of 1.1 mM using ITC (Fig. 1 and 2)). Thus, every high-affinity ligand identified via our binding studies (Fig. 1 and 2) appears to be recognized and likely transported into the cytoplasm of *S. mutans*, where the thioester of glutathione (S-lactoylglutathione) – and possibly the C-terminally esterified (glutathione monomethylester) glutathione derivative, undergo hydrolysis and disulfide reduction in order to be catabolized as a source of cysteine. In the event that the glutathione breakdown product γ-glutamylcysteine is transported by the GshT-dependent system, our results suggest that a redundant transporter may also be at play.

GshT-mediated glutathione import in *S. mutans* proceeds via a shared membrane permease

In planning our experimental approach to identify the permease associated with the apparent function of GshT as a solute binding protein involved in the import of glutathione and derivatives thereof in *S. mutans*, we wondered whether GshT would be encoded in an operon together with its partner permease and partner ABC proteins. This expectation was based on the observation that solute binding proteins are generally encoded together with their partner ABC proteins and permeases on the same operon. At first glance, the *S. mutans gshT* gene ends 167 basepairs upstream of the start codon of the *atmBCDE* gene cluster, which codes for the functionally characterized high affinity methionine uptake ABC-type transporter (MUT) (Basavanna *et al.*, 2013). While this would be compatible with an operon-like architecture, we concluded against the possibility of a transcription to a single *gshT-atmBCDE* mRNA due to the following reasons: i) expression of *gshT* and *atmBCDE* is triggered by distinct promoters, which are regulated by different transcription factors, the LysR transcriptional regulators CysR (Sperandio et al., 2010) and MetR (Sperandio *et al.*, 2007), respectively, that bind cognate recognition sequences upstream of the respective genes, and ii) a predicted

transcription termination site (http://transterm.cbcb.umd.edu/) begins 10 basepairs after the *gshT* stop codon. In line with this, we found that an *S. mutans* UA159 mutant lacking the entire *atmBCDE* locus grows equally well in glutathione-supplemented minimal medium as the wild-type (data not shown), in contrast to the *gshT* mutant strain which is unable to grow in a medium containing glutathione as the sole sulfur source (Table 1 and Fig. 5).

Thus, we were led to consider the possibility that GshT might share a permease with a second SBP, the gene of which might exist in an operon structure together with the genes for its cognate ABC apparatus. Such scenario would not be uncommon among bacterial ABC systems. For instance, the ABC transporter CysATW is associated with the transport of two different substrates due to its coupling to two distinct binding proteins, SbpA priming sulfate transport and CysP mediating thiosulfate translocation (Sirko *et al.*, 1995). The *cysP* gene is part of the *cysPTWA* operon that encodes the complete ABC transporter, while the gene encoding SbpA stands alone in the *E. coli* genome. Based on the expectation that such duality in permease activity would be based on conserved structure-sequence features on the cognate SBPs, for example CysP and SbpA are 47% identical, we identified, employing BLAST searches (http://blast.ncbi.nlm.nih.gov/), all type 3 SBPs present in *S. mutans* UA159. Of the six homologs identified (the cystine-binding proteins Smu.459 (*tcyA*) and Smu.933 (*tcyE*), the glutamine-binding proteins Smu.1520 (*glnH*) and Smu.1177c, the putative arginine-binding protein Smu.817 and the SBP of unknown function Smu.1217c, the cystine-specific TcyA protein emerged as the closest homologue to GshT sharing a 40% sequence identity. The *tcyA* gene is part of the *tcyABC* operon encoding all components for a cystine-specific ABC transporter: the cystine-specific SBP, TcyA, the transmembrane permease, TcyB, and the ATPase, TcyC.

To probe the involvement of the Tcy-transporter in glutathione acquisition, we investigated the ability of *S. mutans* UA159 strains carrying a non-polar deletion in either *tcyB* or *tcyC* to grow on minimal sulfur-free medium supplemented with either cystine or glutathione. In agreement with a recent report (Kim et al., 2012), our *tcy* mutants grew wild-type-like in high (1 mM), but barely in low (0.02 mM) concentrations of cystine by virtue of the redundant low-affinity cystine-tranporter TcyDEFGH (data not shown). While the *tcyC* mutant strain was able to grow in glutathione-supplemented minimal medium, albeit at a slower pace than observed for the wild-type, our *tcyB* mutant culture remained clear (Fig. 5). Together, these results suggest that GshT primes the Tcy permease/ATPase machinery for translocation of glutathione across the cytoplasmic membrane and that the function of the ATPase TcyC is fulfilled by a redundant ABC importer ATPase. Functional ATPase-

exchange between permeases has been documented for e.g. sn-glycerol-3-phosphate and maltose transport in *E. coli* (Hekstra & Tommassen, 1993) and disaccharides and/or oligosaccharides in *S. mutans* (Webb et al., 2008), and is proposed to be a widespread characteristic of ABC transporters in bacteria (Webb et al., 2008). To cross-validate this result, intracellular and spent medium glutathione concentrations were measured for cells that were incubated in a rich broth containing about 10 μM of reducible glutathione conjugates (GSX) spiked with another 20 μM glutathione (Table 2). The mutant *tcyC* cells stored a significant intracellular pool of glutathione (25.1 ± 4.2 nmol per mg protein) at the expense of the extracellular supply, albeit to lower levels than the wild-type (118 ± 8 nmol per mg protein) or the *S. mutans* UA159 mutant lacking the entire *atmBCDE* locus (127 ± 11 nmol per mg protein). On the other hand, while cells devoid of GshT or TcyB accumulated intracellular glutathione levels to about 1/50-fold the amounts in wild-type *S. mutans* under these conditions (4.16 ± 1.23 nmol per mg protein and 2.73 ± 0.89 nmol per mg protein, respectively), they did not deplete the growth medium of the tripeptide. It thus appears that GshT and TcyB:TcyC work in concert to harness a surplus of glutathione as a backup of cysteine for growth under sulfur-limited conditions, while the GshF homolog can synthesize basal levels of glutathione to secure bacterial metabolism.

DISCUSSION

The molecular mechanisms of glutathione transport and import in Gram-positive bacteria had remained largely uncharacterized despite considerable progress in our understanding of such processes in Gram-negative bacteria and eukaryotes. Nearly three decades after the reporting of glutathione import in the Gram-positive bacterium *S. mutans* (Thomas, 1984, Thomas *et al.*, 1983), we present here the molecular and structural basis of how glutathione is bound by GshT, a type 3 solute binding protein, to prime import mechanisms in the Gram-positive *S. mutans* UA159 via the L-cystine ABC transporter TcyABC, a bacterial permease hitherto associated with the transport of L-cystine (Kim et al., 2012, Sperandio et al., 2010). Our work has led to two surprising findings: (1) GshT displays quite broad substrate specificity in that it not only binds the reduced and symmetrically oxidized forms of glutathione, but also recognizes derivatives of glutathione that are modified at either the cysteine-sulfur or the glycine-carboxylate, and (2) GshT shares the TcyABC ABC transporter with TcyA, thereby identifying TcyABC as a permease with a dual function. Our current findings can now explain

previous work focusing on the physiology of glutathione import in *S. mutans* and to facilitate further work on glutathione homeostasis in Gram-positive bacteria. In this regard, recent studies on the molecular response of the Gram-positive pathogen *Streptococcus pneumoniae* against oxidative stress and metal toxicity showed that *S. pneumoniae* imports extracellular glutathione via GshT, and that glutathione utilization may be key to host invasion and tissue colonization (Potter *et al.*, 2012).

Evolutionary relationships among SBPs binding amino acids and glutathione

Our structural studies of GshT from *S. mutans* in complex with GSSG and the availability of structural information on SBPs serving as cargo-carriers of diverse amino-acids, peptides, and glutathione in Gram-negative and Gram-positive bacteria (Berntsson et al., 2010, Vergauwen et al., 2010), provides opportunities to trace potential evolutionary relationships. GshT displays the highest structural similarity with the cystine-binding protein CysCysRec of *Neisseria gonorrhoeae* (pdb code 2YLN; rmsd: 1.54 Å for 138 Cα-atoms) (Bulut *et al.*, 2012). Comparison of ligand binding modes in the two proteins shows that the cystine backbone closely follows the Cα-trace of the GS-I moiety of GSSG. Although likely having served as the evolutionary predecessor of GshT, cystine-binding proteins, here structurally represented by CysCysRec, stabilize the functional groups of the cystine ligand in a totally different way, based on an alternative strategy that allows stabilization of the amine group to enable accommodation of the cystine disulfide against a cushion of hydrophobic interactions. Instead of having an Asp or Glu to stabilize the NH_3^+-group of the amino acid-ligand in type 3 amino acid-binding SBPs or the γ-glutamyl moiety of GS-I in GshT (cfr. Fig. 3C and D), CysCysRec uses two tyrosines and a bridging water molecule (w2029) to stabilize the amino group of Cys-I (w2029) and to provide favorable interactions with the cystine's disulfide bridge (Tyr59 and Tyr167) (Bulut et al., 2012). Hence, our structural studies suggest an evolutionary history of GshT as a type 3 SBP serving as a binding platform for amino acids. This underscores the versatility of the SBP fold as a protein scaffold that can adopt specificities towards new ligands stemming from a modest number of substitutions within the binding cleft.

GshT of *S. mutans*, and GbpA, the prototypic glutathione-binding protein from Gram-negative *Haemophilus* species (Vergauwen et al., 2010, Vergauwen et al., 2011), differ significantly from each other in terms of structure and function. GbpA, a type 5 SBP, is specific for GSH and GSSG, while GshT, a much smaller type 3 SBP, recognizes several forms of glutathione derviatives including reduced and oxidized glutathione and S-substituted

glutathione adducts. This functional difference may reflect differences in biological niche: *Haemophilus* species can thrive in the blood stream of mammals where reduced glutathione forms and excreted glutathione-S-conjugates are abundantly present (Keppler, 1999, Sharma *et al.*, 2000). On the other hand, the biological niche of *S. mutans* is the oral cavity, where oxidizing conditions prevail promoting the formation of various biologically useful mixed glutathione disulfides (Iwasaki et al., 2006). It is also unlikely that glutathione-S-conjugates reach appreciable levels in the oral cavity. Nonetheless, GshT and GbpA do share some evolutionary characteristics. In each case, the two proteins are confined to a single genus, *Streptococcus* in the case of GshT (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and *Haemophilus* in the case of GbpA (Vergauwen et al., 2011). GshT homologs in which the key residues for glutathione accommodation are conserved can be found in most published streptococcal genomes, and are annotated as "Bacterial extracellular solute-binding protein, family 3". However, GshT homologues in *S. parasanguinis* FW213, *S. sanguinis* SK36, *S. mitis* SK321, and *S. oralis* ATCC 35037, are designated as TcyA. Our work herein and elsewhere (Potter et al., 2012) calls for a rectification of databases accordingly. The rarity of finding the genes encoding for GshT and GbpA suggests that they are evolutionarily recent proteins, implying that glutathione acquisition by bacteria coding for such proteins has become of importance to their physiology only late in evolution.

The notion of GshT and GbpA proteins being evolutionarily young proteins is reinforced by the view that GshT proteins descended from a member of the omnipresent cystine-specific FliY SBP family (Ohtsu *et al.*, 2010), while GbpA proteins evolved from a member of the omnipresent dipeptide-specific DppA SBP-family (Vergauwen et al., 2011). Furthermore, the two SBP proteins belong to different families and are encoded by stand-alone genes, and yet they share omnipresent ABC-importer membrane permeases with other SBP proteins: GshT shares the Tcy importer with TcyA, GbpA uses a canonical Dpp ABC importer.

Functional and mechanistic considerations

The identification of the TcyABC permease as a shared membrane permease operating in conjunction with dedicated SBPs for the import of cystine and glutathione provides the necessary molecular and structural framework to explain diverse genetic and biochemical data that has existed for nearly three decades.

According to Sherrill and Fahey (Sherrill & Fahey, 1998), glutathione, GSSG, certain S-substituted and glutathione mixed disulfides are imported by *S. mutans* at comparable rates

and with comparable apparent K_m values of about 18 μM, very much akin the K_d-values reported here for the interaction between GshT and these allocrites. Particularly interesting, however, are earlier studies (Thomas, 1984, Thomas et al., 1983) showing that GSSG, GSH, and cystine compete for uptake by a common transporter in *S. mutans*, as GSSG inhibited the uptake of label from [^{3}H]cystine, and cystine inhibited the uptake of label from [^{3}H]GSSG. Our results now explain this early observation: molecular competition in this case of allocrite transport does not arise due to utilization of a common SBP but rather due to employment of a shared membrane permease. This molecular "ménage a trois" is reinforced at the transcriptional level where CysR, short for cysteine synthesis regulator, activates transcription of both *gshT* and *tcyABC* under conditions of limited cystine (Kim et al., 2012, Sperandio et al., 2010). Another identified target for CysR is the promoter region of *cysK (Sperandio et al., 2010)*, the gene encoding for cysteine synthase A, suggesting that CysR controls a regulon involved in the supply of steady-state levels of cysteine. This further suggests that glutathione import might merely serve a role in cysteine acquisition. These insights may now provide clues for why *S. mutans* acquire glutathione via at least two different avenues. According to one such scenario, *de novo* synthesis mediated by the bifunctional glutathione synthetase GshF establishes a basal level of intracellular glutathione, which is necessary to support a number of central biochemical processes. In parallel, import mechanisms through the GshT/TcyABC transporter provide an easy way to acquire enough cysteine for luxurious growth. Furthermore, the linkage between glutathione and cystine import may also have a functional origin as intracellular cystine reduction may depend on an operating glutathione redox cycle. As a corollary of this linkage, a TcyABC knockout not only imports cystine at a noticeably reduced rate (Kim et al., 2012), but also displays an impairment to acquire a surplus of glutathione from the growth medium as shown here. We note that cystine import is not abolished completely in this case likely because of the activity of the redundant low-affinity TcyDEFGH cystine transporter (Sperandio et al., 2010). Together these data now explain the unexpected growth phenotypes of non-polar *tcyA, tcyB* and *tcyC* mutants observed by Kim *et al.* (Kim et al., 2012); whereas non-polar *tcyA*, *tcyB* and *tcyC* mutants grow well in complex Todd-Hewitt yeast extract broth naturally containing both glutathione (disulfide) and cyst(e)ine, only the *tcyA* and *tcyC* mutants (albeit to only half the density of wild-type cultures), and not the tcyB mutant show growth when 1:20 inoculated from rich broth into minimal medium lacking a source of cysteine. Our explanation is that *tcyA* and *tcyC* mutant cells were able to acquire an effective storage of glutathione during growth in rich broth that they could hydrolyze to obtain the essential amino acid cysteine under sulfur-limited

conditions. By knocking out the permease TcyB, on the other hand, cells are made incompetent to store glutathione for later salvage of cysteine. Our results on intracellular glutathione accumulation from rich broth support this idea as we have observed equal amounts of intracellular glutathione for *gshT* and *tcyB* mutant cells, which are about 6-fold and 30-fold lower than those measured for *tcyC* mutant and wild-type cells, respectively (Table 2).

The concept of glutathione serving as a nutrient for *S. mutans* was previously introduced based on the observation that glutathione becomes rapidly degraded once imported (Sherrill & Fahey, 1998). However, as demonstrated in this study and by others, intracellular glutathione pools accumulate (Newton et al., 1996, Sherrill & Fahey, 1998), suggesting a more central role for glutathione in the physiology of *S. mutans*. Indeed, glutathione protected against growth-inhibition by the thiol-oxidizing agent diamide, and expression of its functionally associated oxidoreductase (Gor) is increased several folds in response to oxygen and acidic stress (Sherrill & Fahey, 1998, Yamamoto *et al.*, 1999). Moreover, the bacterial-type glutathione-dependent glyoxalase system has been found to be involved in the detoxification of the intracellular toxic electrophilic glycolytic byproduct methylglyoxal thereby supporting the acidogenic properties of *S. mutans* (Korithoski et al., 2007). To conclude, besides being an important store of cysteine, two of the foremost cariogenic determinants of *S. mutans*, acidogenicity and aciduricity (Banas, 2004), depend on an operative glutathione importer which may thus prove a valuable target for therapeutic interference to modulate *S. mutans* central metabolism and hence virulence.

Experimental procedures

Materials and reagents

GSH, GSSG, S-methyl-GSH, S-hexyl-GSH, S-(1,2-dicarboxyethyl)glutathione, S-lactoyl-glutathione, glutathione monoethyl ester, homoglutathione, γ-glutamylcysteine, cystine, and all the ingredients of the cdMIc minimal medium (Vergauwen *et al.*, 2003a, Vergauwen et al., 2003b) were purchased from Sigma-Aldrich. cdMIcGlucose medium was prepared as in ref. (Vergauwen et al., 2003a, Vergauwen et al., 2003b) with the exception that sodium lactate was replaced by 20 mM glucose and that hemin was left out of the recipe. The peptides Cys-Gly, Gly-Cys, Gly-Cys-Gly and cysteine glutathione disulfide were obtained from Bachem AG. Restriction endonucleases were from New England Biolabs, T4 DNA ligase from

Promega, plasmid purification kits from Qiagen, pET20b plasmid and *Escherichia coli* BL21(DE3) from Novagen, Sypro Orange, TOPO cloning kits and TOP10 cells from Invitrogen, and brain heart infusion (BHI) broth from Difco.

Cellular growth experiments

S. mutans strains, wild-type UA159, and its derivatives deficient in GshT, TcyB, TcyC*, (Kim et al., 2012)* and AtmBCDE *(Sperandio et al., 2010)* were used in this study. To examine the specificity of the GshT binding protein, wild-type and *gshT* knockout cells were grown overnight in BHI broth and subsequently diluted 1:20 in sulfur source-free cdMIcGlucose medium supplemented with 50 μM of test chemicals (i.e. potential cysteine sources). Growth was monitored (A_{600}) relative to a negative control without supplementation for 24 h of incubation in a candle extinction jar at 37°C. In the presence of glutathione, cells grew to a density of A_{600} ~ 0.75 under the applied conditions. In order to identify GshT's associated ABC transporter, glutathione import was probed in terms of cysteine salvage by the cystine ABC transporter mutants deficient in TcyB and TcyC. Thus, *S. mutans* UA159, *gshT*, *tcyB* and *tcyC* knockout cells were grown as overnight cultures in Todd-Hewitt broth (Becton Dickinson, MD) containing 0.3% yeast extract (Difco Laboratories) (THYE). Cells were pelleted and washed in phosphate-buffered saline, and diluted 1:20 in pre-warmed cyst(e)ine-free minimal medium with or without supplementation with 0.5 mM glutathione (Kim et al., 2012). Growth was monitored using a Bioscreen C automated growth reader (Labsystems, Finland) as described previously (Senadheera *et al.*, 2007).

Determination of Intracellular and spent medium glutathione

By use of the total glutathione (GSX) (i.e., reducible symmetrical or mixed disulfide forms of glutathione) quantification method described by Tietze (Tietze, 1969), BHI broth was found to contain approximately 10 μM GSX. Overnight cultures of wild-type *S. mutans* and its derivatives deficient in functional *gshT*, *atmBCDE*, *tcyB*, and *tcyC* expression were diluted 1:50 in BHI-medium supplemented with 20 μM glutathione and then grown to an A_{600} of 0.25 (exponential phase) or A_{600} of 0.75 (stationary phase) to determine intracellular and spent-medium GSX concentrations, respectively. Cells were harvested by centrifugation (7,000 × g, 5 min, 4 °C) and washed once with phosphate-buffered saline before being suspended in the same buffer. Cells were subsequently disrupted via sonication (Branson sonicator; four 30-s bursts of 45 watts with 30-s intervals), and cell-free extracts were prepared by centrifugation

(15,000 × g, 15 min, 25°C). After determination of total protein content, cell-free extracts were incubated at 95 °C for 15 min, and precipitated protein was removed by centrifugation (15,000 × g, 15 min, 25 °C). The supernatants were subsequently sampled for GSX determination. Spent-medium was sampled from the supernatants of pelleted stationary-phase cultures. The sample GSX concentrations were determined according to the GSSG reductase-based quantification assay described by Tietze using a glutathione standard curve (Tietze, 1969). The results were expressed as nmol of intracellular glutathione/mg of total protein or as μM GSX present in the spent-medium. The experiments were performed in triplicate; mean values are reported with errors representing S.E.

Production and purification of recombinant GshT

Nucleotides 88 to 804 of the *S. mutans gshT* gene were PCR-amplified using primers JVB520 (5′-gCCATGGaaaacagtaacccttgcg-3′), and JVB521 (5′-gCTCGAGtttcatatctttttttatctgg-3′), thereby modifying the amplicon with an end-standing *Nco*I and *Xho*I restriction site, respectively. The PCR fragments were purified and subcloned into the pTOPO-XL vector prior to NcoI/XhoI digestion. The digested fragments were then cloned into an NcoI/XhoI digested pET20b expression vector, thereby producing pET20gshT.

An overnight culture of *E. coli* strain BL21(DE3) bearing pET20gshT was used to inoculate 10 L of Cb-supplemented LB-medium at a ratio of 10 mL/L. This culture was incubated overnight at 37°C under vigorous shaking, without the addition of isopropyl-β-D-thiogalactoside. The cells were harvested by centrifugation at 4,000 × *g* for 20 min, at 4 °C, suspended in 20 mM Tris-HCl, pH 8.0 (Buffer A) (5 mL per liter original culture), and sonicated. The suspension was centrifuged at 15,000 × *g* for 20 min at 4°C to produce cell-free extract, which was centrifuged again at 100,000 × *g* for 20 min prior to the transfer onto a nickel-affinity chromatography column connected to an Äkta- Purifier FPLC system, pre-equilibrated with 20 mM imidazole-supplemented Buffer A. The column was then washed with 10 column volumes (100 mL) of the equilibration buffer, followed by elution using an elution buffer containing 500 mM imidazole in Buffer A. The collected 280-nm-absorbing material was immediately applied onto a desalting column [HiPrepTm 26/10 Desalting Column (Amersham Pharmacia Biotech)], preequilibrated with 20 mM HEPES, pH 7.0 (Buffer B). The eluate was subsequently loaded onto 1 mL of Source S cation-exchange resin (Amersham Pharmacia Biotech), which had been pre-equilibrated with Buffer B. The column was washed with 20 mL of Buffer B, and GshT protein was eluted with 100 mM NaCl in

Buffer B. Concentrated GshT (2 mL) and loaded onto a Superdex G-75 (16⁄60) gel-filtration column (Amersham Pharmacia Biotech), preequilibrated with 20 mM HEPES, 150 mM NaCl, pH 7.0. This buffer was used for down-flow elution at a rate of 1 mL⁄ min. GshT eluted as a single peak and was purified to electrophoretic homogeneity, as determined by SDS-PAGE. This polishing step also allowed a native molecular mass estimation when making a comparison to the elution volumes of the following molecular size standards: β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). Blue dextran was included to identify the void volume. The concentration of purified protein was determined by the method of Bradford using the Bio-Rad Protein Assay with bovine serum albumin as the standard. Ten liters of expression culture yielded about 10 mg of pure GshT protein.

Thermal denaturation assays

Thermofluor thermal shift assays were conducted in a C1000 thermal cycler equipped with a CFX96 optical reaction module (Bio-Rad). The microplate wells were loaded with 25-μL solutions, containing 35 μg GshT, 2x Sypro orange, and 1 mM of the test chemicals in 20 mM HEPES, pH 7.0. The plates were sealed with Microseal B film (Bio-Rad) and heated from 30 to 90 °C at a rate of 2 °C per min. The unfolding reactions were followed by simultaneously monitoring the relative fluorescence (FRET settings) using the charge-coupled device camera. Melting temperatures (T_m values) were those temperatures at which the first derivatives (rate of melting) of the melting peaks went through a minimum.

Isothermal titration calorimetry

Experiments were carried out using a VP-ITC Micro Calorimeter at 25°C, and data were analyzed using the Origin ITC analysis software package supplied by MicroCal. Purified GshT samples were dialyzed overnight against 20 mM HEPES, 150 mM NaCl, pH 7.0, at 4°C. The resultant dialysis buffer was then used to dissolve the test compounds. GshT concentrations were measured spectrophotometrically using an extinction coefficient of 27,850 M^{-1} cm^{-1} at 280 nm. GSSG concentrations were determined by the absorbance change at 340 nm resulting from the glutathione reductase-catalyzed NADPH-dependent conversion of GSSG to 2GSH (ε_{340} = 6,200 M^{-1} cm^{-1}). GSH concentrations were determined by the reaction with Ellman's reagent (ε_{412} = 14,000 M^{-1} cm^{-1}). All solutions were degassed prior to use. Titrations were always preceded by an initial injection of 2 μL and were carried out using

10-μL injections applied 200 s apart. The sample was stirred at a speed of 400 rpm throughout. Test compounds were always injected into the GshT-containing sample cell. The heats of dilution were negligibly small for the titration of each ligand into buffer; hence the raw data needed no correction. The thermal titration data were fit to the one binding site model, and apparent molar reaction enthalpy (ΔH°; in units of kcal mol^{-1}), apparent entropy (ΔS°), association constant (K_a), dissociation constant ($K_d = 1/K_a$) and stoichiometry of binding (n) were determined. Several titrations were performed to evaluate reproducibility. For low affinity binders or zero-enthalpy binders competition ITC could prove useful in which a thermodynamically characterized ligand (GSH) is titrated in the sample cell containing a mixture of target (GshT) and low affinity or zero-enthalpy binder (γ-glutamylcysteine). Displacement binding isotherms can then be fitted to the expression for a direct titration, whereby the apparent binding constant relates to the real K_d as is given by the single site competitive inhibition equation: $K_{d,GSH,app} = K_{d,GSH}$ (1+[γ-glutamylcysteine]/$K_{d,\gamma\text{-glutamylcysteine}}$), in which $K_{d,GSH,app}$ is the apparent dissociation constant for GSH, calculated from the displacement titration; $K_{d,GSH}$, the dissociation constant for GSH, calculated from direct titration; [γ-glutamylcysteine], the concentration of γ-glutamylcysteine included in the sample cell in the displacement titration; and $K_{d,\gamma\text{-glutamylcysteine}}$, the dissociation constant for γ-glutamylcysteine.

Crystallographic studies of GshT from S. mutans in complex with GSSG

We used purified recombinant GshT (10 mg⁄mL in 20 mM HEPES, 150 mM NaCl, pH 7.0) to carry out an extensive crystallization screen (using the following commercially available sparse-matrix screens: Hampton Crystal Screen 1 & 2, Hampton Index Screen, and Hampton PEG/Ion Screen 1 & 2 (Hampton Research)) in the presence of 1 mM GSSG using a Mosquito crystallization robot (TTP LabTech) based on 100-nL crystallization droplets (50-nL protein sample and 50-nL crystallization condition) equilibrated in sitting-drop geometry over 25-μL reservoirs containing a given crystallization condition. This led to the identification of two lead conditions (1: 0.1 M cadmium chloride hydrate, 0.1 M sodium acetate trihydrate pH 4.6, 30% v/v polyethylene glycol 400, and 2: 0.2 M Sodium chloride, 20% w/v Polyethylene glycol 3,350, pH 6.9) of which the former already gave diffraction quality crystals within 2 days. For data collection under cryogenic conditions (100 K), single crystals were flash cooled in liquid nitrogen after a short incubation (< 1 min) in cryoprotecting solution (mother liquor to which polyethylene glycol 400 was added to a

concentration of 20% v/v). Crystals grown in the condition containing $CdCl_2$ displayed the best X-ray diffraction characteristics and led to a native dataset (λ_1 = 1.00 Å) with a resolution of 1.55 Å. From the same crystal, a dataset at longer wavelength (λ_2 = 1.55 Å), with an anomalous signal up to 2.5 Å resolution was collected. The structure of GshT in complex with GSSG from *S. mutans* was determined by maximum-likelihood molecular replacement as implemented in the program suite PHASER (McCoy *et al.*, 2007). The search model was prepared from the structure of the arginine-, lysine-, histidine-binding protein ArtJ from the thermophilic bacterium *Geobacillus stearothermophilus* in complex with histidine (Vahedi-Faridi *et al.*, 2008) using the program Chainsaw (Stein, 2008), based on structure-based sequence alignments. Inspection of electron density maps confirmed the solution as evidenced by extra density for missing structural elements and side chains, as well as strong electron density in a large cavity sandwiched between the two domains indicating the presence of a bound part (GS-I; see Results and Discussion) of the GSSG molecule at high occupancy. Model (re)building was carried out via a combination of automated methods as implemented in the PHENIX suite (Adams *et al.*, 2010) and manual adjustments using the program COOT (Emsley *et al.*, 2010). An anomalous difference map was calculated in Phenix to identify the location of cadmium and chloride ions. Crystallographic refinement and structure validation was carried out using PHENIX.

Acknowledgements

BV and KV were supported by research fellowships from the Research Foundation Flanders, Belgium (FWO). This research was supported by a Ghent University GOA grant to SNS. We gratefully acknowledge beam-time allocation and technical support provided at the French National Synchrotron Facility (SOLEIL) and the Swiss Light Source (SLS). We are also thankful to M. Cordova for technical assistance and grants NIH R01DE013230-03 and CIHR-MT15431 to DGC.

Table legends

Table 1. Cell growth assays to probe GshT-dependent allocrite import in *S. mutans* UA159.

Table 2. *S. mutans* UA159, but not its mutant derivatives defective in either *gshT* or *tcyB* functional expression, accumulate glutathione at the expense of exogenous useful forms of glutathione (GSX).

Figures legends

Fig. 1. Thermal denaturation assays to probe binding specificity of *S. mutans* GshT. The temperature-induced changes in relative fluorescence of 50 μM GshT are shown as a function of oxidized and reduced forms of glutathione and type of glutathione analogues and glutathione breakdown products. The measurements reflect the temperature-induced change in relative fluorescence of Sypro orange (2x)/GshT (50 μM or 35 μg) mixtures, either in the absence (control, black dashed curve) or in the presence of 1.0 mM of the test compounds that did not shift the apo-form T_m value (represented by black curves): CysCys (solid line), Gly-Cys-Gly (dashed line), and Cys-Gly (dotted line), or that did shift the T_m value: GSH, and its C-terminally modified derivatives homo-GSH, and GSH-monoethyl-ester (solid, dashed, and dottedblue lines, respectively), GSSG (brown curve) and the mixed GSH disulfide cysteine-glutathione disulfide (red curve), the thioethers of GSH, S-dicarboxyethyl-GSH, S-me-GSH, and S-hexyl-GSH (solid, dashed, and dotted green lines, respectively), the thioester of GSH, S-lactoyl-GSH (purple curve) and the GSH breakdown product, γ-Glu-Cys (grey curve). (Inset) The first derivative fluorescence values of the raw data (identically color-coded) to facilitate comparison of T_m-values (refer to the positive peak maxima). The data for the 20 amino acids were omitted for clarity reasons.

Fig. 2. Thermodynamic signatures of physiologically relevant allocrites of *S. mutans* GshT. The plotted and processed thermodynamic data were obtained from ITC experiments performed at 25°C. Compared to the best binder, glutathione (GSH), which releases the largest amount of Gibbs free energy and enthalpy upon binding to GshT, an increase in entropic contribution for the other ligands is in most cases associated with an enthalpic penalty, a process which is referred to as enthalpy/entropy compensation and which has been observed for a number of enzyme inhibitors in drug discovery (Freire, 2008).

Fig. 3. Crystal structure of *S. mutans* GshT in complex with GSSG. (A) Overall structure of the two GshT molecules present in the crystallographic asymmetric unit (top molecule: N-terminal lobe, dark grey; C-terminal lobe, metallic green. Bottom molecule, light grey), decorated by a total of 63 cadmium ions (yellow spheres). The cadmium ion stabilizing the carboxylates of the GS-II moieties of the two bound GSSG ligands (purple sticks) is depicted as an orange sphere. (B) Zoom-in view of GSSG (stick representation) bound to the GshT binding cleft colored according to the encoded thermal factors in (hot) ROYGBIV (cold) gradient and modeled into the $2|F_o|-|F_c|$ and $|F_o|-|F_c|$ difference Fourier electron densities

contoured at 1 σ and 3 σ, respectively. Of note is the lack of density for reliably modeling the γ-glutamyl moiety of GS-II. (C) and (D) Similar zoom-in views to compare allocrite binding within type 3 SBPs: hydrogen-bonding pattern of the N- and C-terminal groups of the glutamine allocrite bound to the glutamine-binding protein of *B. pseudomallei* (C) and stabilization of the γ-glutamyl moiety of GS-I of the GSSG allocrite bound to GshT (D).

Fig. 4. Interaction landscape of the GSSG binding site of *S. mutans* GshT. Ligplot (http://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/) representation of the hydrogen bonding network, hydrophobic and van der Waals interactions involving the GSSG allocrite bound to GshT. Contacting residues originating from the N- and C-terminal lobes of GshT are labeled black and white, respectively. The GS-I and GS-II moieties of the bound GSSG are depicted and colored purple.

Fig. 5. GshT and TcyB are required for growth of *S. mutans* on glutathione as the sole source of sulfur. Overnight cultures of wild-type UA159 and its *gshT*$^-$, *tcyB*$^-$, and *tcyC*$^-$ mutants were grown in rich THYE-medium, diluted 1:20 in a pre-warmed minimal medium (sulfur-source-free) and growth was recorded in the presence or absence of 0.5 mM glutathione. Results are representative of at least three independent experiments, each conducted in quadruplicate.

Table 1. Cell growth assays to probe GshT-dependent allocrite import in *S. mutans* UA159.

Compound	Sulfur source for growth of WT *S. mutans* UA159 cells in cdMIcGlucose*	Sulfur source for growth of *gshT*⁻ *S. mutans* UA159 cells in cdMIcGlucose*	Growth inhibition of WT *S. mutans* cells in cdMicGlucoseGSSG‡
GSH	+	–	–
GSSG	+	–	–
S-methylglutathione	–	–	+
S-hexylglutathione	–	–	+
S-(1,2-dicarboxyethyl)glutathione	–	–	–
S-lactoylglutathione	+	–	–
Cysteine glutathione disulfide	+	–	–
glutathione monoethyl ester	+	–	–
homoglutathione	+	–	–
γ-glutamylcysteine	+	+	-
glycylcysteinylglycine	+	+	-
glycylcysteine	+	+	-

* Growth of wild-type (WT) or *gshT*⁻ *S. mutans* UA159 cells in the presence of the indicated glutathione derivatives (at 50 μM) as sole sources of sulfur. Growth was measured spectrophotometrically in cdMIcGlucose-medium supplemented with 50 μM of the candidate ligands. A plus sign and a minus sign indicate "growth" and "no growth," respectively. Growth of wild-type cells and lack of growth for *gshT* mutants identifies the molecule as a potential cargo for the GshT-dependent glutathione importer.

‡ Scoring of the ability of the indicated glutathione derivatives (at 1.25 mM) to inhibit growth of wild-type cells in cdMIcGlucose-medium supplemented with 20 μM GSSG. A minus sign indicates no differences in growth characteristics, while a plus sign indicates inhibition of growth in the ligand-supplemented culture. The positively scored derivatives at 1.25 mM inhibit growth completely and are nontoxic in growth experiments using cystine-supplemented cdMIcGlucose medium. Inhibition of growth possibly indicates a direct interaction of the tested molecule with the GshT-dependent glutathione importer.

Table 2. *S. mutans* UA159, but not its mutant derivatives defective in either *gshT* or *tcyB* functional expression, accumulate glutathione at the expense of exogenous useful forms of glutathione (GSX).

	medium	wild-type	*gshT*$^-$	*atmBCDE*$^-$	*tcyB*$^-$	*tcyC*$^-$
intracellular GSX (nmol/mg)	NA	118 ± 8	4.16 ± 1.23	127 ± 11	2.73 ± 0.89	25.1 ± 4.2
spent medium GSX (μM)	30.1 ± 2.0	22.7 ± 2.7	30.2 ± 1.8	16.4 ± 3.9	31.5 ± 3.7	26.3 ± 2.5

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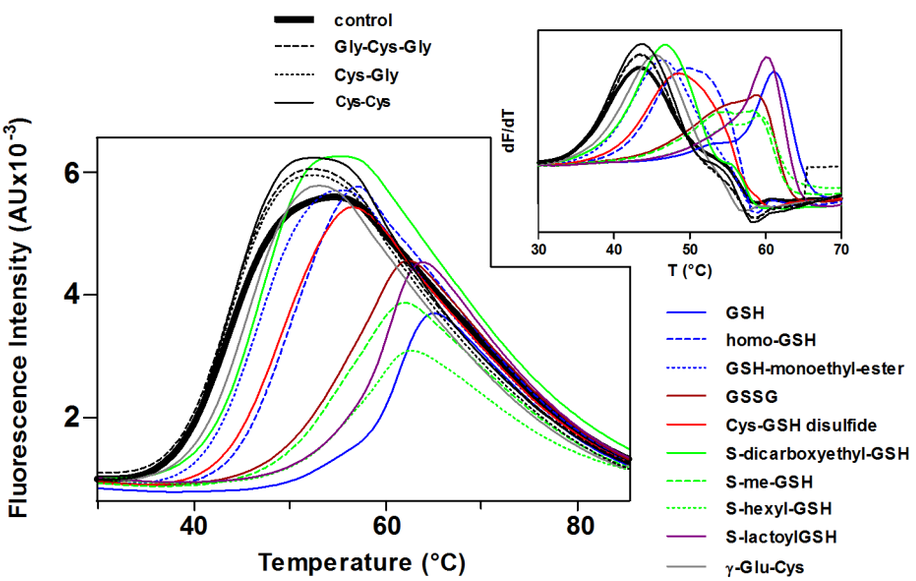

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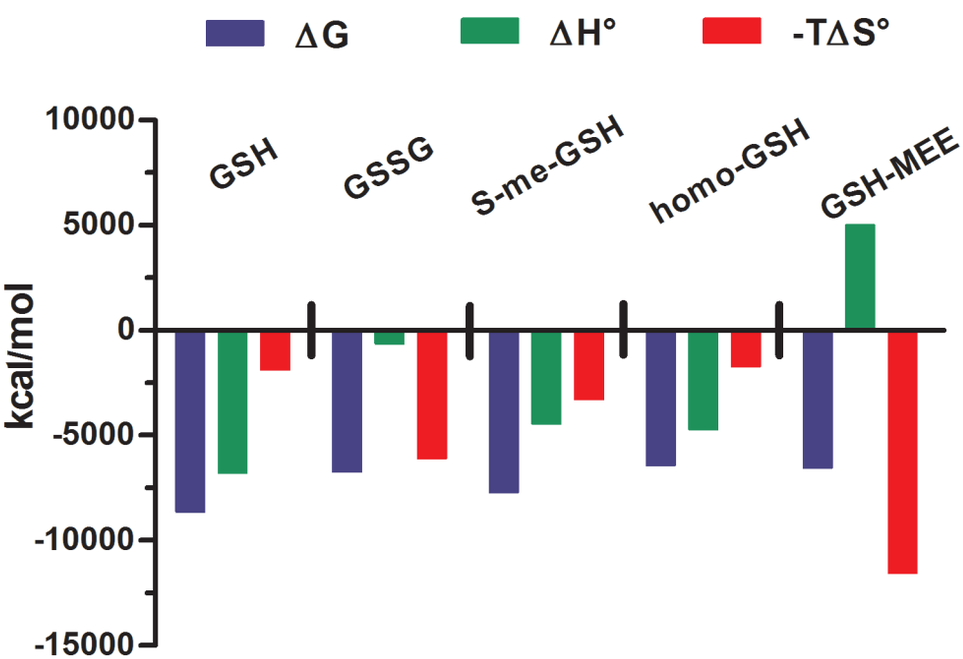

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K_d (μM)	0,47	12,2	2,33	20,7	16,1
ΔG (kcal/mol)	-8,63	-6,71	-7,7	-6,4	-6,52
ΔH° (kcal/mol)	-6,77	-0,63	-4,42	-4,69	5,01
TΔS° (kcal/mol)	1,86	6,08	3,28	1,7	11,5

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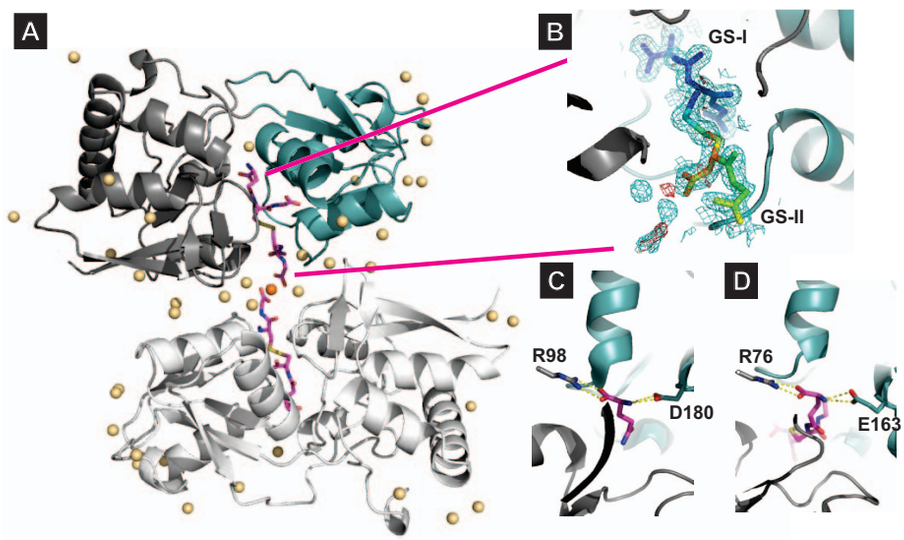

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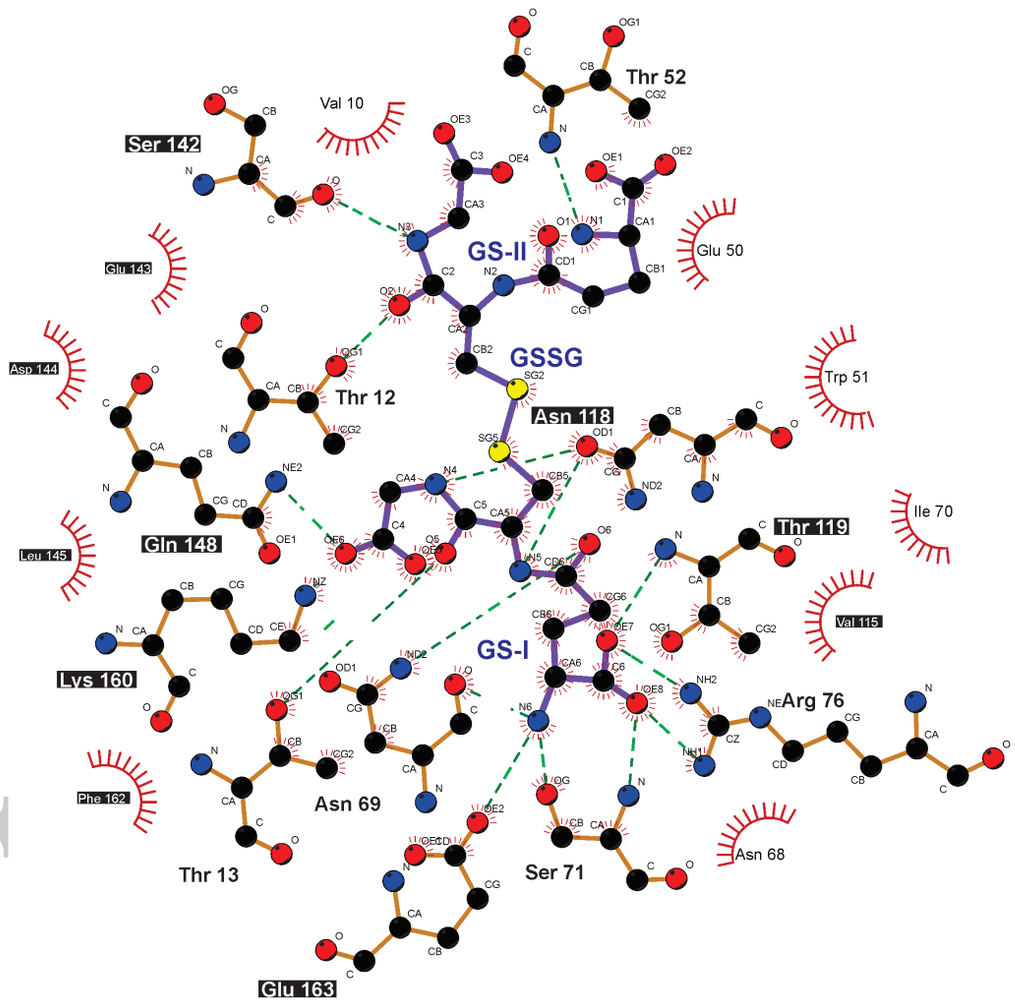

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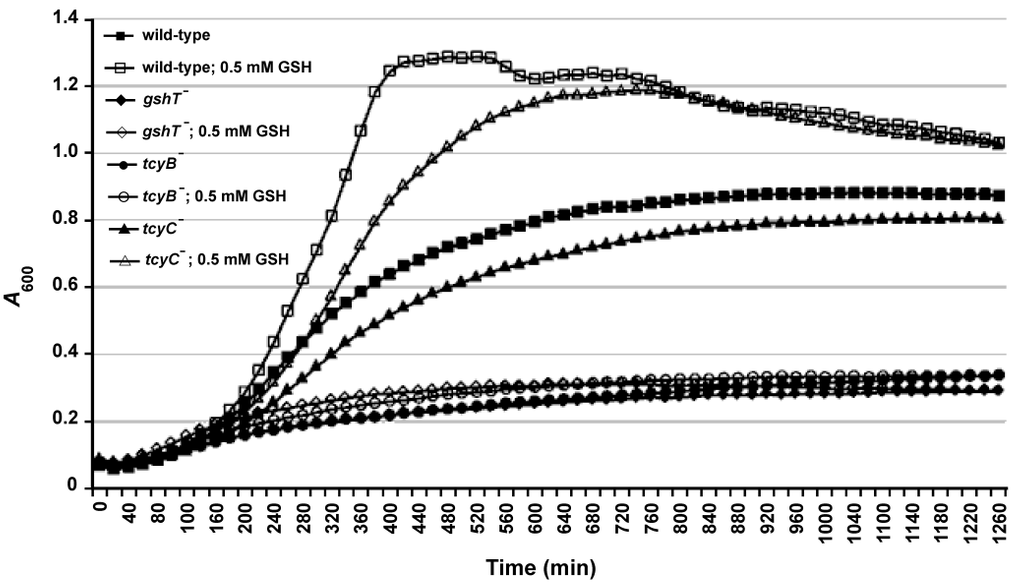

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