Characterization of the Bifunctional ɣ-Glutamate-cysteine Ligase/Glutathione Synthetase (GshF) of Pasteurella multocida

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Glutamate-cysteine ligase (ɣ-ECL) and glutathione synthetase (GS) are the two unrelated ligases that constitute the glutathione biosynthesis pathway in most eukaryotes, purple bacteria, and cyanobacteria. ɣ-ECL is a member of the glutathione synthetase family, whereas GS enzymes group together with highly diverse carboxyl-to-amine/thiol ligases, all characterized by the so-called two-domain ATP-grasp fold. This generalized scheme toward the formation of glutathione, however, is incomplete, as functional steady-state levels of intracellular glutathione may also accumulate solely by import, as has been reported for the Pasteurellaceae member Haemophilus influenzae, as well as for certain Gram-positive enterococci and streptococci, or by the action of a bifunctional fusion protein (termed GshF), as has been reported recently for the Gram-positive firmicutes Streptococcus agalactiae and Listeria monocytogenes. Here, we show that yet another member of the Pasteurellaceae family, Pasteurella multocida, acquires glutathione both by import and GshF-driven biosynthesis. Domain architecture analysis shows that this P. multocida GshF bifunctional ligase contains an N-terminal ɣ-proteobacterial ɣ-ECL-like domain followed by a typical ATP-grasp domain, which most closely resembles that of cyanophycin synthetases, although it has no significant homology with known GS ligases. Recombinant P. multocida GshF overexpresses as an ~85-kDa protein, which, on the basis of gel-sieving chromatography, forms dimers in solution. The ɣ-ECL activity of GshF is regulated by an allosteric type of glutathione feedback inhibition (Kᵢ = 13.6 mM). Furthermore, steady-state kinetics, on the basis of which we present a novel variant of half-of-the-sites reactivity, indicate intimate domain-domain interactions, which may explain the bifunctionality of GshF proteins.

Glutathione (GSH; L-γ-glutamyl-L-cysteinylglycine) is the predominant low molecular weight thiol present in many Gram-negative bacteria and in virtually all eukaryotes, except those that lack mitochondria (1, 2). Glutathione is made in a highly conserved two-step ATP-dependent biosynthesis pathway by two unrelated peptide bond-forming enzymes (3). In the first and rate-limiting reaction, γ-glutamate-cysteine ligase (γ-ECL) (EC 6.3.2.2) condenses the γ-carboxylate of l-glutamic acid with l-cysteine to form the dipeptide γ-glutamylcysteine (γ-EC), according to

$$\text{Me}^{2+} + \text{L-Glu + L-Cys + ATP} \rightarrow \gamma\text{-EC} + \text{ADP} + \text{P}_{\text{i}}$$

**REACTION 1**

where Me²⁺ can be magnesium or manganese (4, 5). In the second step, γ-EC is condensed with glycine in a reaction catalyzed by glutathione synthetase (GS; EC 6.3.2.3), according to

$$\gamma\text{-EC + Gly + ATP} \rightarrow \text{GSH} + \text{ADP} + \text{P}_{\text{i}}$$

**REACTION 2**

where Me²⁺ again can be magnesium or manganese.

The activity of eukaryotic γ-ECL is precisely controlled by nonallosteric glutathione feedback inhibition, the limited availability of cellular l-Cys, and the transcriptional and posttranslational regulation of the expression and activity of the enzyme under various physiological conditions (6). Bacterial glutathione homeostasis is less well characterized, although glutathione feedback inhibition appears to be of major importance, as Escherichia coli expresses its γ-ECL constitutively (7) and because the E. coli γ-ECL Kᵢ for glutathione is about 3 mM (8), a value consistent with feedback regulation at physiological glutathione levels.

Recently, the structure of the monomeric γ-ECL of E. coli has become available (9), which confirms its predicted structural homology with glutamine synthetases (5). By comparing the crystal structures of unliganded ligase and ligase complexed with a sulfoximine-based transition-state inhibitor, the cysteine binding site was identified to be formed inductively at the transition state (9). The basis for this induced fit mechanism is a conformational change of a switch loop consisting of residues 240–249. Moreover, this loop forms the starting point of a fit mechanism is a conformational change of a switch loop consisting of residues 240–249. Moreover, this loop forms the starting point of a
GS ligases can be grouped into bacterial and eukaryotic protein families, for which pairwise sequence comparisons indicate no significant relationship (12). Crystal structures of the E. coli (13, 14), human (15), and yeast (10) GS ligases, however, adopt a similar fold, which extends across two domains, collectively referred to as the ATP-grasp fold (16). Other members of the ATP-grasp enzyme superfamily include, among others, biotin carboxylase α-chain (17), succinate-CoA ligase (18), carboxamidophosphatase synthetase (19), cyanophycin synthetase (CphA (20)), and D-Ala-D-Ala ligase (DdB (21)), from which it can be appreciated that ATP-grasp enzymes carry out ATP-dependent carboxylation to amine/thiol ligase reactions in a number of unrelated biosynthetic pathways, accepting a wide variety of donor and acceptor substrates. In the course of evolution, ATP-grasp folds have therefore been independently recruited to provide the catalytic scaffold for specialized non-ribosomally encoded peptide bond-forming reactions.

Glutathione is considered to play a key role in protecting cells against oxidative toxicity, yet its production among prokaryotes appears to be largely confined to the Gram-negative cyanobacteria and proteobacteria (22). However, a number of Gram-positive bacteria, such as streptococci, enterococci, and clostridiales, collectively classified as firmicutes, have been found to accumulate glutathione (1), whereas other aerobic Gram-positive genera may produce alternative cysteine derivatives to functionally substitute for glutathione, such as streptomycetes, which accumulate the cysteine/sugar condensate mycothiol (23). Recently, Janowiak and Griffith (24) reported that glutathione synthesis occurs atypically in Streptococcus agalactiae, as they identified the functional gene responsible for glutathione accumulation as coding for a bifunctional protein catalyzing both γ-ECL and GS reactions. Shortly after the Janowiak and Griffith report (24), Gopal et al. (25) ascribed glutathione synthesis in Listeria monocytogenes to a similar bifunctional protein, termed GshF, which, by genetic inactivation, was shown to be essential for aerobic growth and virulence. The GshF fusions consist of a γ-ECL-like N-terminal domain fused to an ATP-grasp domain. Interestingly, inspection of the genome data base of lactococci, streptococci, and enterococci revealed that not all glutathione-accumulating strains possess a gshF-like sequence in their genomes (such as Lactococcus lactis and Streptococcus thermophilus) and, conversely, that Streptococcus mutans, which possesses a gshF-like gene, takes up rather than synthesizes glutathione (1, 24).

The present study originated from our interest into the role and metabolism of glutathione in Gram-negative Pasteurellaceae, which, as a consequence of their adaptation to a parasitic life style, are characterized by a reduction in genome information compared with their free-living ancestors (26). In an earlier report, we have shown that Haemophilus influenzae does not accumulate glutathione by biosynthesis but efficiently imports the tripeptide from the growth medium (27). In contrast to other Gram-negatives such as E. coli, the imported glutathione was found to be crucial for H. influenzae, as it scavenges its interior from a wide variety of peroxide chemicals (28, 29). Here, we report on the study of glutathione accumulation by Pasteurella multocida, which forms part of the normal flora in the nasopharynx of many domestic and wild animals. Most human P. multocida infections are soft tissue infections caused by animal bites (30). We show that P. multocida accumulates glutathione both by import and biosynthesis. Inspection of the genome data base of P. multocida revealed the presence of a gshF homologous sequence, which was probed as a candidate for glutathione biosynthesis. We show that the P. multocida gshF gene codes for a bifunctional ligase that is functional inside E. coli and carries out complete synthesis of glutathione. Finally, purified recombinant GshF was subjected to steady-state kinetic studies to provide insights into the significance of the bifunctionality of GshF proteins.

**MATERIALS AND METHODS**

**Biochemical Reagents**—All biochemical reagents used in this study were from Sigma-Aldrich unless indicated otherwise.

**Strains and Media**—E. coli strain K12 (wild type) was obtained from the E. coli Genetic Stock Center (New Haven, CT). E. coli strains BL21(DE3) (Novagen, Madison, WI) and TOP10 (Invitrogen) were used for the overexpression of recombinant P. multocida GshF and as host for cloning, respectively. The E. coli strains were routinely grown in 37 °C Luria-Bertani (LB) medium, supplemented with either, or a combination of, 100 μg/ml carbenicillin, 50 μg/ml kanamycin or 12.5 μg/ml chloramphenicol, as appropriate, on an orbital shaker rotating at 200 rpm. The P. multocida and S. agalactiae clinical isolates used in this study were kind gifts of Dr. Mario Vaneechoutte (Dept. of Clinical Chemistry, Microbiology, and Immunology, University Hospital, Ghent, Belgium). P. multocida were routinely grown aerobically in BHI (brain-heart infusion broth), which was prepared from a dehydrate (BD Biosciences). To study glutathione accumulation by P. multocida, cells were grown in chemically defined Mlc medium, prepared as described by Herriott et al. (31). This medium contains 100 μM cysteine as the source of organic sulfur. S. agalactiae was grown without agitation at 37 °C in Todd Hewitt broth (Invitrogen) supplemented with 2% yeast extract (THY media). To prepare agar plates, 1.8% agar was added to the BHI or Mlc liquid growth media before autoclaving.

**DNA Manipulations**—All nucleic acid-modifying enzymes were obtained from New England Biolabs (Beverly, MA) unless indicated otherwise. Plasmid isolations, ligations, and transformations were performed as described (32). Plasmid DNA for ligation experiments was prepared on a 30-ml scale using the Qiagen (Ghent, Belgium). P. multocida and S. agalactiae genomic DNA was prepared using the Qiagen genomic tip-100/G protocol according to the manufacturer’s instructions.

To verify the in vivo function of the γ-ECL-ATP-grasp fusion protein (referred to as GshF in this study), a 2,628-bp subgenomic fragment spanning the promoter and terminator regions of the P. multocida gshF gene (see Fig. 1) was amplified via PCR using P. multocida genomic DNA, Taq GoldStar® DNA polymerase (Eurogentec Ltd., Southamp-ton, UK), and the following primer pair: forward primer 5′-GTGAT-TCAATATTAGTGCC-3′ and reverse primer 5′-GTGTTCTCGTGCAATTGC-3′. The PCR fragments were ligated into the pGEM-T vector (Promega, Madison, WI), thereby constructing pGEM-gshFSCG.

To overexpress P. multocida GshF in an E. coli background, we amplified the gshF gene (open reading frame Pm1048) by PCR using P. multocida genomic DNA as a template, the forward primer 5′-TTCATATGAAAATTCAACATATC-3′, and the reverse primer 5′-TTACTCCAGTTCAGGAA-3′, thereby introducing an NdeI site (underlined) at the 5′ end. The 2,280-bp amplified product was cloned into the pGEM-T vector, and the resulting plasmid was digested with PstI. The linearized plasmid was made blunt-ended with Vent® DNA polymerase and was subsequently digested with Ndel. Similarly, the expression plasmid PET 11a (Novagen, Madison, WI) was digested with BamHI, blunt-ended, and digested with NdeI. Then vector and insert were ligated to each other, thereby constructing expression plasmid PET-GshFcow.

To overexpress S. agalactiae GshF in an E. coli background, we amplified the gshF gene (open reading frame SAG1821 and 71 bp of terminator region) by PCR using S. agalactiae genomic DNA as a template, the forward primer 5′-CAGCATATGATTATCGACGTGTAC-3′, and the reverse primer 5′-GCCAAGCTTCTAGTGAT-
Characterization of Pasteurella multocida GshF

GACAAGGGAT-3', hereby introducing an NdeI site (underlined) at the 5' end and a HindIII (underlined) site at the 3' end. The 2,339-bp amplified product was cloned into the pGEM-T vector, and the resulting plasmid was double digested with NdeI/HindIII. Similarly, the expression plasmid pET-GshF<sub>575</sub> was NdeI/HindIII double digested, and vector and insert were ligated to each other, thereby constructing expression plasmid pET-GshF<sub>575</sub>.

Construction of a gshA<sup>−</sup> gshB<sup>−</sup> E. coli K12 Double Mutant—In E. coli, γ-ECL and GS are encoded by the genes gshA and gshB, respectively. The gshA<sup>−</sup> gshB<sup>−</sup> E. coli K12 double mutant was constructed according to the "lambda Red" method (described in Ref. 33). Briefly, PCR fragments of the kanamycin-resistance gene from the pACYC177 plasmid (Fermentas GmbH, St. Leon-Rot, Germany) were amplified with primers that contained a 50-bp 5'-end extension, identical or complementary, respectively, to nucleotides 50 to 1 or 1558 to 1608 of the E. coli K12 1557-bp gshA gene (forward primer, 5'-ACCATTACAGTTATGCTAATTAACAGATTTGGAAGGCGGAGGTCAATATGAGCCATATCTACGCGG-3' primer, 5'-ATTCCAGAGTGAAATTGGCCACTCAGACTGGCTTTCCTTGTGAAGAAAACCTACTCAGCA-3'). K12 E. coli strain, harboring plasmid pKD46, which encodes arabinose-inducible Λ-phase Red recombinase, and grown in medium containing 100 mg/liter ampicillin and 0.04% L-arabinose, was transformed by electroporation with the purified PCR product. Kanamycin-resistant clones, which had integrated the PCR product by homologous recombination in frame to the gshA gene promoter, were selected on LB agar plates supplemented with the appropriate antibiotic, grown at 37 °C, and purified at 42 °C to cure the pKD46 plasmid of the strain. The in-frame replacement of the gshA gene by the PCR product was confirmed by PCR using primers that flank the replaced gshA gene (forward primer, 5'-TGTCGATAGGCGGAGG-3'; reverse primer, 5'-CATCCGGGATGATGAGTGCAACATC-3') (data not shown). The final gshA<sup>−</sup> gshB<sup>−</sup> E. coli K12 double mutant was then constructed applying an identical methodology, now using the gshA<sup>−</sup> E. coli K12 mutant as a host for the electroporation of gshB-interrupting PCR fragments. These PCR fragments contained the chloramphenicol-resistance cassette of the pACYC184 plasmid (Fermentas GmbH) and were amplified with primers that contained a 50-bp 5'-end extension, identical or complementary, respectively, to nucleotides 1–50 or 901–951 of the E. coli 951-bp K12 gshB gene (forward primer, 5'-ATGAACTAGCTGGCGATCGG-3'; reverse primer, 5'-TTACGCTTGGAAAGCTCGGTGGCGATCGG-3') (data not shown). The insert of the PCR product into the gshB gene was confirmed by PCR using primers that flank the interrupted gshB gene (forward primer, 5'-GAGACAACTGCGCACC-3'; reverse primer, 5'-GCTCGTGCATCAGATGTC-3') (data not shown).

Recombinant Expression and Purification of P. multocida and S. agalactiae GshF—An overnight culture of E. coli strain BL21(DE3) bearing pET-GshF<sub>1557</sub> was used to inoculate 5 liters of carbenicillin-supplemented LB medium at a ratio of 10 ml/liter. The expression culture was incubated at 37 °C under vigorous shaking to an optical density at 600 nm (OD<sub>600</sub>) of 0.7–0.9, after which isopropyl-β-D-thiogalactoside was added to a final concentration of 1 mM. After being cultured for another 20 min, at 4 °C. The cell pellets were suspended in 50 mM Tris-HCl, pH 7.4 (5 ml/liter original culture) and sonicated. The suspension was centrifuged at 15,000 × g for 20 min at 4 ºC to produce cell-free extract. An identical procedure was applied to obtain recombinant S. agalactiae GshF. To purify either P. multocida or S. agalactiae recombinant GshF, crude protein solution was subjected to medium pressure chromatography using an Äkta-design fast protein liquid chromatography system (FPLC, Amersham Biosciences). Other chromatographic equipment was also purchased from Amersham Biosciences. The cell-free extract was filtered through a 0.22-μm filter, diluted 3-fold with 50 mM Tris-HCl, pH 7.4, containing 10 mM NaCl (Buffer A), and applied onto a Q-Sepharose FF packed HR 16/10 column pre-equilibrated with Buffer A. The column was then washed with 100 ml of Buffer A followed by elution using an increasing step gradient of 5 mM NaCl increments in Buffer A, each increment generating a 50-ml elution fraction. To fractionate the fractions containing the highest amount of GshF, NH<sub>4</sub>(SO<sub>4</sub>)<sub>2</sub> was added to a final concentration of 1 M, after which precipitated protein was removed by centrifugation (15,000 × g for 20 min at 4 °C). The supernatant was applied onto a butyl-Sepharose FF packed HR 16/10 column that had been equilibrated with Buffer C (Buffer A, with 1 M NH<sub>4</sub>(SO<sub>4</sub>)<sub>2</sub>). The column was washed with Buffer C, and eluate was obtained using a linear gradient of Buffer A from a flow rate of 3 ml/min. GshF started to elute at 75% Buffer A. The fractions containing purest GshF were then applied on a desalting column (HiPrep<sup>TM</sup> 26/10 desalting column; 10 ml/min) pre-equilibrated with Buffer A. Eluate was subsequently applied onto 0.5 ml of Source Q anion-exchange resin that had been equilibrated with Buffer A, and GshF was concentrated by a single peak and was purified to electrophoretic homogeneity as determined by nonreducing SDS-PAGE. GshF proteins were stored without supplements at −80 °C until use.

The native molecular masses of either P. multocida or S. agalactiae recombinant GshF were determined by gel filtration on a Superdex G-200 (16/60) column with 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl (1 ml/min). The molecular size standards for gel filtration were β-amylose (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 (34) using the Bio-Rad protein assay with bovine serum albumin as the standard.

Agar Disk Diffusion Susceptibility Assays—E. coli (supplemented with the appropriate antibiotics) or P. multocida precultures, grown overnight, were diluted 1:50 to 1:100 in LB or Mlc medium, respectively, to an OD<sub>600</sub> of ~0.005 and then grown to an OD<sub>600</sub> of 0.75 (mid-exponential phase). Using sterile cotton swabs, cells were confluently inoculated onto LB agar plates (E. coli) or Mlc agar plates, either supplemented or not with 100 μM GSSG (P. multocida). Round sterile filters (5.2-mm diameter) were placed in the center of the plates and spotted with 5 μl of 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), 0.5 M β-butyrylhydroperoxide, 2.78 M methylglyoxal, or 2.0 mM diamin. Zones of growth inhibition were measured (in millimeters) after 24 h of incubation at 37 °C (E. coli) in a candle extinction jar (P. multocida). The experiments were performed in triplicate; mean values are reported, with error bars representing the standard error of the mean (S.E.), and were analyzed using the unpaired t test (Graphpad Instat Software, San Diego, CA) with p < 0.05 considered significant.

Determination of Intracellular Glutathione—Overnight grown E. coli (supplemented with the appropriate antibiotics) or P. multocida precultures were diluted 1:50 to 1:100 in LB or Mlc medium (either supplemented or not with 100 μM GSSG), respectively, to an OD<sub>600</sub> of ~0.005.
The proteins that are related to either the "ECL-like and ATP-grasp-like domains of GshF are represented as black arrows, and protein regions that exhibit pairwise sequence similarities, according to Altschul et al. (37), are indicated by boxes. The sequence identities of these regions with the corresponding GshF domains are depicted above the boxes as % id. The total length of the protein sequences is given in the left column, aa, amino acids.

and then grown to an A560 of 0.25 (early exponential phase). Cells were harvested by centrifugation (7,000 × g, 5 min, 4 °C) and were washed once with phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) before being suspended in the same buffer. Cells were subsequently disrupted via sonication, and cell-free extracts were prepared by centrifugation (15,000 × g, 15 min, 25 °C). After determination of total protein, 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 sample glutathione concentrations were determined according to the GSSG reductase-based quantification assay described by Tietze (35) using a glutathione standard curve. The amount of glutathione formed was recalculated to micromolar levels proportional to the amount of total glutathione present in the sample.

Enzyme Assays—The γ-ECL and GS activities of recombinant P. multocida GshF were determined by a recognized ATPase assay, which couples ADP to ATP recycling to the oxidation of NADH (using the coupled activities of pyruvate kinase (PK) and lactate dehydrogenase (LDH)), which can be monitored continuously at 340 nm (PK-LDH method (36)). A Uvikon 943 UV-visible double beam spectrophotometer (Kontron Instruments, Watford, UK) was used for all kinetic experiments, which were routinely performed at 25 °C. To record γ-ECL activity, the standard assay reaction mixtures contained Tris-HCl buffer (200 mM, pH 8.2), sodium L-Glu (50 mM), L-Cys (2.0 mM), MgCl2 (20 mM), and GshF (added last to start the reaction) in a final volume of 0.50 ml. Background rates were determined in the absence of L-Cys. To show feedback inhibition by glutathione, the standard assay reaction mixtures contained Tris-HCl buffer (200 mM, pH 8.2), sodium L-Glu (50 mM), L-Cys (2.0 mM), MgCl2 (20 mM), disodium ATP (5 mM), NaCl (100 mM), NADH (0.25 mM), PEP (2 mM), LDH (20 units/ml), PK (7 units/ml), and GshF (added last to start the reaction) in a final volume of 0.50 ml. Background rates were determined in the absence of γ-ECL.

To record the rate of coupled glutathione formation, the standard assay reaction mixtures contained Tris-HCl buffer (200 mM, pH 8.2), sodium L-Glu (50 mM), L-Cys (2.0 mM), MgCl2 (20 mM), disodium ATP (5 mM), NaCl (100 mM), and GshF (added last to start the reaction) in a final volume of 1.0 ml. At 1-min intervals, 50 μl of reaction mixture was transferred to a 950-μl 200 mM phosphate-buffered solution (pH 7.5) containing 1 mM diethionitrobenzoic acid, 500 μM NADPH, and 1 unit of glutathione reductase. As these phosphate concentrations completely inhibit GshF activity (data not shown), mixing instantly blocks the peptide bond forming reactions. The increase in 412 nm absorbance was recorded for 40 s and was, according to Tietze (35), proportional to the amount of total glutathione present in the sample. The amount of glutathione formed was recalculated to micromolar levels. Curve fitting and modeling of kinetic data were performed using the Graphpad PRISM 4.0 software package (GraphPad Software for Science).

RESULTS

Probing Glutathione Biosynthesis among Pasteurellaceae by Identification and Sequence Analysis of the P. multocida γ-ECL-ATP-grasp Fusion Protein GshF—In an earlier report, we showed that H. influenzae is not able to synthesize glutathione but instead acquires the thiol-tripeptide by import (27). To determine whether this type of glutathione acquisition is common among other Pasteurellaceae members, we analyzed the translated genome sequences (BLAST search at NCBI (37)) of Haemophilus somnus 129PT, H. somnus 2323, Haemophilus ducreyi 35000HP, Actinobacillus pleuropneumoniae serovar 1 strain 4074, Mannheimia succiniciproducens MBEL55E, and P. multocida subsp. multocida strain Pm70 for homologs of the E. coli K12 glutathione biosynthetic machinery. Although this search produced no significant hits for the GS query, γ-ECL homologous sequences were apparent from all but the H. ducreyi 35000HP genome. Strikingly, these identified Pasteurellaceae γ-ECL homologs all combine with an ATP-grasp superfamily sequence to form a fusion protein of ~755 amino acids. The P. multocida γ-ECL-ATP-grasp fusion (Pm1048) was then BLAST-searched against the NCBI microbial genome data base and was found to be homologous to a novel class of bifunctional glutathione biosynthesis.
FIGURE 2. Multiple amino acid sequence alignment of residues 1–480 of *P. multocida* GshF with monofunctional γ-ECL proteins. EC, *Escherichia coli*; Vc, *Vibrio cholerae*; Ca, *Clostridium acetobutylicum*; Pm, *Pasteurella multocida*. The alignment was generated using T-coffee (68) and was printed using the ESPript 2.1 software package. Conserved residues are depicted in white on a red background. Physicochemically conserved residues are depicted in red. Overall conserved regions are framed in blue. Top, sequence secondary structures correspond to the *E. coli* γ-ECL crystal structure (Protein Data Bank accession code 1V4G). Bottom, secondary structures are predicted from the GshF amino acid sequence using the PSIPRED Protein Structure Prediction Server (69). The *E. coli* γ-ECL crystal structure (9) comprises two structural domains: a catalytic domain (residues 18–387 and 442–518) and a small domain (residues 1–16 and 388–441; helices α1–α14, and α15, colored brown). These domains are linked through a disulfide bridge (C372–C396), which is absent in the *C. acetobutylicum* and *P. multocida* sequences. Two highly variable arms cover the active site (boxed green), the N-terminal variable arm (residues 105–144) and the central variable arm (residues 240–298), both of which exhibit deletions only in the *P. multocida* sequence. Twelve amino acids, probably involved in substrate binding (depicted in white on a purple background), are found to be highly conserved among the aligned γ-ECL sequences. The residues depicted in brown at the end of the *P. multocida* GshF sequence form the starting amino acid stretch of the ATP-grasp-like domain shown in Fig. 3.
proteins, found mostly in low GC Gram-positive bacteria, which catalyze the ATP-dependent formation of glutathione from the constituent amino acids L-Glu, L-Cys, and Gly, as demonstrated recently for the \( \gamma \)-ECL-ATP-grasp fusions from *Streptococcus agalactiae* (24) and *L. monocytogenes* (25). As proposed for the *L. monocytogenes* \( \gamma \)-ECL-ATP-grasp fusion (25), we refer to the *P. multocida* \( \gamma \)-ECL-ATP-grasp fusion protein as GshF. *P. multocida* GshF shares 47 and 39% amino acid identity with the GshF proteins of *S. agalactiae* and *L. monocytogenes*, respectively.

Fig. 1 shows the domain structure of the putative *P. multocida* 757-amino acid GshF. The N-terminal \( \gamma \)-ECL-like domain is fused to an amino acid stretch that, according to the 3D-PSSM (three-dimensional position-specific scoring matrix) fold recognition program, structurally belongs to the ATP-grasp superfamily of ATP-dependent carboxyl-to-amine/thiol ligases. With one exception, a BLAST search against the NCBI microbial genome database revealed no freely existing C-terminal domain. In the genome of the low GC Gram-positive bacterium *Clostridium acetobutylicum*, in which the gene encoding the ATP-grasp domain lies immediately downstream from a gene that, when translated, shares 35% identity with the \( \gamma \)-ECL domain of the *P. multocida* GshF, both genes are separated by an intergenic region of about 80 base pairs, strongly suggesting that they are organized in an operon.

On the basis of PSI-BLAST sequence similarity analysis, Copley and Dhillon (12) classified \( \gamma \)-ECL proteins into three groups. The first group consists primarily of sequences from \( \gamma \)-proteobacteria, the second from sequences from non-plant eukaryotes, and the third primarily of sequences from flowering plants and \( \alpha \)-proteobacteria. The N-terminal \( \gamma \)-ECL domain of GshF proteins falls within the first group of \( \gamma \)-proteobacterial sequences, although sequence similarities are rather low (ranging from 27 to 32% sequence identity) and cover only the central part of the catalytic domain of the group 1 \( \gamma \)-ECL proteins (see legend for Fig. 2). This figure shows a multiple sequence alignment of the typical group 1 \( \gamma \)-ECL sequences of *E. coli* and *Vibrio cholera*, together with the N-terminal portions of the *P. multocida* and *S. agalactiae* GshF fusions and the \( \gamma \)-ECL sequence of *C. acetobutylicum*. Interestingly, the nonfused *C. acetobutylicum* \( \gamma \)-ECL protein exhibits a higher and more overall similarity to the freely existing \( \gamma \)-proteobacterial \( \gamma \)-ECL sequences (36% overall sequence identity) than to the N-terminal \( \gamma \)-ECL-like portions of GshF proteins. On the basis of the recent crystal structure of *E. coli* \( \gamma \)-ECL (9), substrate-binding residues were identified of which all but one (which is physicochemically conserved) are strictly conserved in the \( \gamma \)-ECL domain of *P. multocida* GshF. Although the C-terminal 150 amino acid residues of the *E. coli* \( \gamma \)-ECL protein do not align significantly using the BLAST algorithm, complete structural conservation appears on the basis of a comparison between the observed and predicted secondary structures of the *E. coli* \( \gamma \)-ECL and the N-terminal \( \gamma \)-ECL domain of the *P. multocida* GshF, respectively (Fig. 2). On the basis of this comparison, the N-terminal 462 amino acid residues appear to fold into the \( \gamma \)-ECL domain of the *P. multocida* GshF fusion, leaving 295 amino acid residues to form the ATP-grasp domain.

Strikingly, these 295 C-terminal amino acid residues of GshF exhibit 34–36% sequence identity with a region enclosed in the bifunctional cyanophycin synthetase (CphA) (Fig. 1). The 857–901-amino acid CphA proteins, found mostly in cyanobacterial backgrounds, catalyze the ATP-dependent synthesis of the storage polymer multi-L-arginyl-poly-L-aspartic acid (cyanophycin), thereby sequentially adding an aspartic acid residue and an arginine residue to a \( \beta \)-Asp-Arg primer (38). These two ATP-dependent carboxyl-to-amine ligase reactions take place in the active sites of two distinct domains: a C-terminal domain, starting approximately just before the second half of the sequence, which falls into a superfamily of Rossman fold-containing peptide ligases including certain murein ligases and foyl poly-\( \gamma \)-glutamate ligase; and an N-terminal domain that groups within the ATP-grasp superfamily of ATP-dependent ligases (20). A multiple sequence alignment of the “founding members” of the ATP-grasp superfamily, the \( \delta \)-Ala-\( \delta \)-Ala ligase (DdlB) sequences of *E. coli* K12 and *Salmonella typhimurium* LT2 (16), together with the N-terminal portion of the CphA enzymes of *Bordetella pertussis* and *Anaabaena variabilis* and the C-terminal ATP-grasp domains of the GshF sequences of *S. agalactiae* and *P. multocida*, is presented in Fig. 3. The E. coli DdlB crystal structure revealed a three-domain organization of the ligase, of which the central (also called the lid domain) and C-terminal domains, collectively described as the ATP-grasp fold, each containing two antiparallel \( \beta \)-strands and a loop, form the active site cleft for ATPase activity (21). The ATP-grasp fold residues of the aligned sequences shown in Fig. 3 exhibit overall similarity, although two comparable and major insertions are apparent in the C-terminal domains of the aligned CphA and GshF sequences compared with the DdlB sequences; these insertions cluster the former two proteins into a phylogenetically distinct branch of ATP-grasp fold-containing proteins (25). Nonetheless, the three loops that close over the catalytic cavity in the liganded *E. coli* DdlB structure are among the most conserved regions in the alignment, and, moreover, 8 of 10 DdlB MgATP\(^2^+\)-binding residues are physicochemically conserved in both the CphA and GshF aligned sequences.

In summary, sequence analysis and secondary structure prediction demarcate two functional modules in the *P. multocida* GshF sequence. The N-terminal residues 1–462 appear to fold into a complete group 1 \( \gamma \)-ECL structure, whereas the C-terminal residues 480–757 adopt the classical two-domain ATP-grasp fold. In this scenario, the two functional modules are connected via an \( \approx \)18 amino acid linker region.

*P. multocida* Cells Acquire Glutathione Both by Biosynthesis and by Import—Because the *P. multocida* genome contains genes encoding genuine glutathione reductase and glutaredoxin, as well as the recently discovered PGdx glutathione-dependent peroxidase (39), we expected to find glutathione in crude lysates of washed *P. multocida* cells. Indeed, *P. multocida* cells grown overnight on BHI agar plates were found to contain 72 ± 12 nmol of glutathione/mg of protein, as determined by a highly specific glutathione reductase-based enzymatic recycling assay. To verify that *P. multocida* is able to synthesize rather than simply take up glutathione, as has been demonstrated for its close relative *H. influenzae* (27), cells were grown up to the early exponential phase of growth in a chemically defined liquid medium, either supplemented or not with 100 \( \mu \)M GSSG, and analyzed for intracellular glutathione. The total glutathione content of GSSG-supplemented cultures (170 ± 20 nmol of glutathione/mg of protein) was found to be 7.4-fold that of cultures grown in the absence of the oxidized tripeptide (23 ± 5 nmol of glutathione/milligram of protein). These results strongly suggest that *P. multocida* acquires glutathione by both biosynthesis and import.

Imported Glutathione Does Not Provide a Higher Level of Protection against Excessive Oxidative Stress in *P. multocida*—To rationalize the observed redundancy in glutathione acquisition, we set up disk diffusion assays by which we examined the sensitivities of *P. multocida* cells to different oxidants in relation to the availability of external glutathione. Four different oxidants were tested, each generating a specific type of oxidative stress; \( \mathrm{H}_2\mathrm{O}_2 \), \( \mathrm{t} \)-butyl hydroperoxide, diamide, and methylglyoxal generated general peroxide stress, membrane peroxidation, thiol-disulfide stress, and carbonylic stress, respectively. With the exception of methylglyoxal, the recorded growth inhibition zones for the other three oxidants were independent of available external glutathione.
FIGURE 3. Multiple amino acid sequence alignment of residues 481–757 of *P. multocida* GshF with ATP-grasp fold containing γ-Ala-γ-ligases (DdlB) and cynophycin synthetases (CphA). Ec, *Escherichia coli*; St, *Salmonella typhymurium*; Av, *Anabaena variabilis*; Bp, *Bordetella pertussis*; Sa, *Streptococcus agalactiae*; Pm, *Pasteurella multocida*. The alignment was generated using T-coffee (68) and was printed using the ESPript 2.1 software package. Conserved residues are depicted in white on a red background. Physicochemically conserved residues are depicted in red. Overall conserved regions are framed in blue. Difference regions are shown on an orange background. Top, sequence secondary structures correspond to the *E. coli* DdlB crystal structure (Protein Data Bank accession code 1I0V). Bottom, secondary structures are predicted from the GshF amino acid sequence using the PSIPRED Protein Structure Prediction Server (69). The domain organization of *E. coli* DdlB (16, 21) is depicted by color-coded bars above its sequence, with black, dark gray, and light gray representing the N-terminal domain, the central B-domain, and the C-terminal domain, respectively. The latter two domains, which characterize the ATP-grasp fold (16), form a cleft that grasps the MgATP2−/H11002 substrate with the aid of three flexible loops (framed in green), the B-loop (residues 146–152), the Ω-loop (residues 205–221), and the J-loop (residues 273–281) (framed in green), all three of which turn out to be highly conserved among the aligned ATP-grasp-like sequences. Moreover, conserved residues known to be involved in MgATP2−/H11002 binding in structurally characterized ATP-grasp enzymes are indicated in white on a purple background. Overall, 8 of 10 are also conserved in the GshF sequences. Following the Ω-loop, GshF as well as CphA sequences exhibit similarly large insertions, which cluster them into a phylogenetically distinct branch of ATP-grasp proteins (25).
thione (Fig. 4). These results suggest that glutathione acquisition by biosynthesis builds up a sufficient pool to fully protect P. multocida against oxidative stress. A recent computer simulation of the glyoxalase pathway in Leishmania infantum showed that the intracellular concentration of methylglyoxal is controlled by the rate of its formation and by the concentration of trypanothione (40), the functional counterpart of glutathione in trypanosomatids. In accordance with this model, the 7.8-fold higher levels of glutathione inside GSSG-supplemented cultures provide a higher level of protection against exogenous supranormal levels of methylglyoxal, suggesting that, in response to this type of electrophilic stress, glutathione biosynthesis inside cells grown in the absence of external glutathione is not (sufficiently) induced to produce the intracellular glutathione pools of cells that accumulate glutathione by both biosynthesis and import.

P. multocida GshF Can Complement the Glutathione Biosynthesis Deficiency Phenotype of a gshA− gshB− E. coli K12 Double Mutant—To determine whether the P. multocida GshF protein can synthesize glutathione in vivo, complementation of a glutathione-deficient gshA− gshB− E. coli K12 double mutant was assayed using a plasmid construct containing part of the P. multocida gshF gene locus (Fig. 1). As explained in the foregoing paragraph, an obvious phenotype of mutant E. coli deficient in glutathione biosynthesis is the highly increased sensitivity toward the cytotoxic effects of the electrophile methylglyoxal (41). Table 1 shows that stable introduction of the gshF subgenomic fragment into gshA− gshB− E. coli K12 fully restored the wild-type phenotype of methylglyoxal resistance, which was found to be consistent with the complementation of the double mutant to (higher than) wild-type intracellular glutathione pools. Because gshA− gshB− E. coli K12 lacks both γ-ECL and GS, the GshF enzyme of P. multocida thus catalyzes both peptide bond-forming reactions toward the synthesis of glutathione.

Expression and Purification of P. multocida GshF in E. coli—After having established the in vivo function for the GshF protein of P. multocida, we overexpressed the protein inside an E. coli background in order to conduct kinetic studies on pure protein. Therefore, the gshF gene was inserted into expression vector PET-11a, and overexpression was achieved inside E. coli BL21(DE3) cells. Recombinantly expressed GshF was highly soluble and was purified to apparent electrophoretic homogeneity using a four-step purification scheme (inset of Fig. 5). Purified recombinant GshF in 50 mM Tris-HCl (pH 7.4) containing ~100 mM NaCl could be stored at ~80 °C for up to 6 months without significant loss of activity. Purified recombinant GshF migrated as a single 85-kDa band during SDS-PAGE, which is close to the predicted molecular mass of 85,862 Da. To appreciate the quaternary structure of P. multocida GshF, purified protein was chromatographed on a Superdex 200 gel filtration column, and the molecular weight was estimated with respect to the elution positions of five standards (Fig. 5). The results indicated an apparent molecular mass of ~220 kDa, suggesting that the P. multocida GshF forms dimers in solution. This result contradicts the reported molecular weight estimation for the S. agalactiae GshF counterpart, which elutes as a monomer from a Superdex 200 gel filtration column (24). Note that the P. multocida and S. agalactiae GshF sequences share 47% sequence identity. To clarify this issue, the streptococcal GshF enzyme was expressed and purified according to a methodology identical to that described for the P. multocida enzyme and subjected to Superdex 200 gel filtration chromatography. Purified S. agalactiae eluted at a position corresponding to a molecular mass of ~191 kDa, proposing that GshF proteins in general form functional homodimers.

Characterization of the Individual γ-ECL and GS Activities of the Bifunctional GshF Protein of P. multocida—By using the steady-state coupled enzyme ATPase assay (36), which couples the rephosphorylation of the ADP product to the decomposition of NADH, the ATP-dependent synthesis of γ-EC from L-Glu and L-Cys, as well as the ATP-dependent synthesis of glutathione from γ-EC and Gly, was demonstrated to be catalyzed by the GshF bifunctional protein of P. multocida. Next, the kinetic parameters were determined for both individual activities (summarized in Table 2). For comparison, Table 2 also includes the corresponding values for the S. agalactiae GshF (24) together with those of the E. coli monofunctional counterparts (4, 42). It is interesting to note that no deviations from linearity were apparent in Eadie-Hofstee plots of the initial velocity data, indicating that both peptide bond-forming reactions are catalyzed by the bifunctional GshF without the involvement of cooperative mechanisms.

The maximal turnover rate for the γ-ECL activity catalyzed by P. multocida GshF (kcat = 25.3 s−1) was found to be consistent with the kcat values reported for monofunctional γ-ECL enzymes from E. coli to human and, notably, was almost identical to the kcat value reported for the S. agalactiae GshF catalyzed reaction (kcat = 29.8 s−1). The Km value for ATP (250 µM) is rather high when compared with the corresponding affinity constants reported for the bacterial γ-ECL activities of E. coli.

### Table 1
Characterization of Pasteurella multocida GshF

| Strain (plasmid) | Methylenylglyoxal sensitivity (zone diameter of complete inhibition)a | Intracellular glutathione concentrationb |
|------------------|---------------------------------------------------------------|---------------------------------------|
|                  | mm                             | nmol of glutathione/mg of total cellular protein |
| E. coli K12 (pGEM) | 26.0 ± 0.3                     | 21.0 ± 3.5                             |
| E. coli K12 (pGEM-gshF) | 26.2 ± 0.6                     | 42.1 ± 5.4                             |
| gshA− gshB− E. coli K12 (pGEM) | 65.6 ± 2.4                     | BLD+                                  |
| gshA− gshB− E. coli K12 (pGEM-gshF) | 26.1 ± 0.2                     | 46.2 ± 6.2                             |

a Results are obtained from three independent disk diffusion testing experiments and are presented as means ± S.E. See “Materials and Methods” for experimental details.

b Results are obtained from three independent experiments and are presented as means ± S.E. See “Materials and Methods” for experimental details.

1. Below the limit of detection.
Characterization of Pasteurella multocida GshF

TABLE 2
Steady-state kinetic constants of the individually assayed γ-ECL and GS activities of the bifunctional GshF glutathione synthetase of P. multocida (Pm)

Both activities were assayed on the basis of ADP formation as described under “Materials and Methods.” The counterpart values for the homologous GshF protein from S. agalactiae (Sa) (24) and for the monofunctional γ-ECL (4) and GS (42) ligases from E. coli strain B (Ec) are given for comparison.

| Source strain | Kinetic parameter | Variable substrates for γ-ECL activity | Variable substrates for GS activity |
|---------------|-------------------|---------------------------------------|------------------------------------|
|               | $K_m$ (μM)        | l-Glu 100 | l-Cys 100 | ATP 100 | γ-Glu-Cys 100 | Gly 100 | ATP 100 |
| Pm            |                   |           |           |         |             |         |         |
| Sa            | 5300 ± 330        | 220 ± 32 | 250 ± 24  |         |             |         |         |
| Ec            | 22000 ± 1000      | 160 ± 10 | 64 ± 11   |         |             |         |         |
| Pm            | 1900 ± 200        | 100 ± 20 | 62        |         |             |         |         |
| Sa            | 64               |           |           |         |             |         |         |
| Ec            | 104              |           |           |         |             |         |         |
| Pm            | 62               |           |           |         |             |         |         |
| Sa            | 105              |           |           |         |             |         |         |
| Ec            | 105              |           |           |         |             |         |         |

$K_m$ (62 μM) and S. agalactiae ($K_m$ = 64 μM), yet is comparable with that reported for the human enzyme ($K_m$ = 200 μM). The Michaelis constant for l-Cys (220 μM) was found to be 2-fold higher than the E. coli counterpart value (100 μM). Again, similar $K_m$ values have already been reported for eukaryotic γ-ECL activities (for example, a $K_m$ value of 310 μM for the rat liver γ-ECL ligase). The rather high $K_m$ value for l-Glu (5.3 mM) agrees well with the physiological l-Glu concentrations within Gram-negative bacteria (43).

The $k_{cat}$ value for the γ-EC-to-Gly condensation reaction catalyzed by the P. multocida GshF protein should be considered as an approximation of the real maximal turnover rate in view of the fact that substrate inhibition occurred at Gly concentrations of only 2.5-fold its $K_m$ value. Therefore, the obtained kinetic parameters shown in Table 2 are apparent values, as the pseudo first-order approximation was by no means valid during the steady-state kinetic assays. The apparent $k_{cat}$ values for γ-EC (26.9 s⁻¹) and ATP (26.5 s⁻¹), determined at ~70% Gly saturation, were found to be equal to the maximal turnover rate of the γ-ECL reaction. With respect to binding affinities, the GS activity of the P. multocida GshF protein is characterized by an apparent $K_m$ value of 81 mM for Gly, which is extremely high compared with that of monofunctional GS ligases and with respect to the reported physiological Gly concentrations of ~0.5 mM in Gram-negative bacteria (43). On the other hand, the established apparent Michaelis constant for γ-EC (90 μM) is 65-fold lower than that of the S. agalactiae GshF counterpart activity (5.9 mM), yet it is comparable with the equivalent $K_m$ values of monofunctional GS enzymes. So the question arises as to why the P. multocida GshF evolved toward a high specific activity for γ-EC, while concurrently engendering the wasteful accumulation of this intermediate as a result of highly inefficient Gly binding.

Glutathione Feedback Inhibition of the P. multocida GshF-catalyzed γ-ECL Reaction—Glutathione is a non-allosteric feedback inhibitor for all monofunctional γ-ECL enzymes studied to date. In these reports, feedback inhibition mechanisms were consistently analyzed toward l-Glu. Strikingly, the γ-ECL activity catalyzed by S. agalactiae GshF was reported to be insensitive to feedback inhibition by glutathione (24), a result that was reproduced here, as shown in Fig. 6D. To study the effect of glutathione on the γ-ECL activity catalyzed by the P. multocida GshF bifunctional enzyme, increasing glutathione concentrations were included in the assay mixtures composed to follow steady-state enzyme activities as a function of l-Glu concentration (Fig. 6). The direct plot (Fig. 6A) shows that low millimolar levels of glutathione significantly reduced activity and that increasing the concentration of l-Glu did not
overcome the inhibition. Plotting the reciprocal of velocity against the reciprocal of L-Glu concentration (1/v against 1/S, Fig. 6B) yielded all straight lines that meet in a joint intercept on the abscissa, indicating pure noncompetitive inhibition. However, a secondary plot of the ordinate intercepts against glutathione concentration is nonlinear, and therefore the true noncompetitive inhibition constant $K_i$ cannot be read directly from the x-intercept. Yet, the ordinate intercepts fit to Equation 1,

$$\text{Or} = \frac{1}{V_{\text{max}}} + \frac{[\text{GSH}]^n}{K_i V_{\text{max}}}$$

(Eq. 1)

where Or is ordinate intercept, $n$ is the Hill coefficient, $V_{\text{max}}$ is maximum velocity, and $K_i$ is true noncompetitive inhibition constant), indicating noncompetitive feedback inhibition characterized by a $K_i$ of 13.6 mM, which is sensitive to positive cooperativity characterized by a Hill coefficient of 1.65.

Enzymology of Coupled γ-ECL and GS Activities of the Bifunctional P. multocida GshF—Because of the growing evidence of the existence of tight coupling between active centers catalyzing consecutive reactions in bifunctional enzymes (44), we wanted to explore this possibility, to tackle the above mentioned paradox with respect to the established physiologically irrelevant $K_m$ value of 81 mM for Gly of the individually assayed P. multocida GshF γ-ECL reaction. Therefore, the steady-state kinetic analyses with respect to Gly were repeated, with the exception that this time the γ-EC substrate was delivered by saturating the γ-ECL reaction with the substrates L-Glu, L-Cys, and MgATP$^2^-$. Instead of the GS activity being continuously monitored via the standard ATPase assay, the rate of glutathione formation was followed via a discontinuous assay based on the direct quantification of glutathione using the GSSG reductase-based enzymatic recycling assay described under “Materials and Methods.” Fig. 7 implies that under these conditions, the P. multocida GshF-catalyzed γ-ECL to Gly condensation reaction already became saturated at about 15 mM Gly, and nonlinear fitting of the primary data yielded an apparent $K_m$ of 1.72 ± 0.15 mM. Catalysis at the γ-ECL reaction center thus appears to transmit a signal to the GS site to activate the latter by considerably increasing the affinity for Gly.

When assaying the in vitro kinetics of the individual GshF activities using the steady-state coupled enzyme ATPase assay, we were surprised to notice that under saturating concentrations of the substrates to form the γ-EC intermediate, the steady-state rate of ADP formation remained unaffected whether or not 20 mM of Gly was included in the reaction mixture. This phenomenon is also demonstrated in Fig. 8, in which the Michaelis-Menten plots of steady-state velocities against varying l-Glu concentrations are compared for assay mixtures contain-
Characterization of Pasteurella multocida GshF

FIGURE 7. Plot of the initial velocities of glutathione formation versus varying Gly concentrations of the coupled γ-ECL-GS reaction shows a physiologically relevant affinity (K_m of 1.72 mM) for the C-terminal amino acid substrate. The rates of glutathione formation were recorded via a discontinuous assay as described under "Materials and Methods." Inset, the progress curves for the γ-ECL-GS-coupled reaction saturated with its substrates L-Glu, L-Cys, and MgATP^2− versus varying concentrations of Gly. These time courses show an apparent lag period before reaching a steady state.

ing L-Glu, L-Cys, and MgATP^2− (γ-ECL reaction) and assay mixtures containing L-Glu, L-Cys, Gly, and MgATP^2− (γ-ECL + GS reactions). On the basis of this experiment, it appears as if the GS ligase reaction is increasingly inhibited by the degree of saturation of the γ-ECL activity and finally, at full saturation of the latter activity, is completely inactivated.

To explore this possibility, we needed to know the individual turnover rates of the γ-ECL and GS reactions under conditions in which the coupled reactions operate simultaneously. To obtain individual activities, the GS turnover rate was directly monitored as described above (GSSG reductase-based enzymatic recycling assay). This enabled us to calculate the γ-ECL activity simply by subtracting the rate of glutathione formation from the total ATPase rate. The rate of P. multocida GshF-catalyzed γ-ECL formation in an assay mixture containing 50 mM L-Glu, 2.5 mM L-Cys, and 5 mM MgATP^2− was found to be 24.6 s^−1. The inclusion of 20 mM Gly in an otherwise identical reaction mixture allowed the GS ligase reaction to proceed at a rate of 11.1 s^−1, while concomitantly lowering the rate of γ-ECL formation almost 2-fold to 13.5 s^−1. This result demonstrates that catalytic turnover at the γ-ECL condensation center does not transmit a signal to lower the activity at the consecutive active site. Instead, there appears to be communication in the opposite direction.

**Steady-state Transient Time Analysis Is Inconsistent with Substrate Channeling—**The presence of two consecutive enzymatic activities on GshF raises the possibility that the intermediate, γ-ECS, is channelled between the catalytic sites. Because both consecutive reactions are supposed to be irreversible (cf. irreversible monofunctional counterpart enzymes and no ADP accumulation as a result of the coupled ATPase assay to record GshF activity) and because no deviations from simple Michaelis-Menten kinetics have been observed for either reaction, the progress curves of glutathione formation shown in Fig. 7 may be described (45, 46) by

\[
[GSH]_t = v_0 t - \frac{v_0 \tau}{1 - e^{-\frac{t}{\tau}}} \tag{Eq. 2}
\]

where [GSH]_t = glutathione concentration produced at time t, v_0 is steady-state velocity, t is time, and τ is transient time. The latter parameter is actually of great importance in describing a coupled enzyme system, because it gives an indication of the speed with which the coupled enzyme system reaches a steady state. In cases wherein channeling has been proven, the channeling step is very fast (>1000 s^−1), and accordingly, no transient time, along with an immediate steady state, has been observed using steady-state kinetics (47, 48). Fitting the time course of the P. multocida GshF-coupled activity saturated with substrates (50 mM L-Glu, 2.5 mM L-Cys, 20 mM Gly, and 5 mM MgATP^2−) to Equation 2 gives a τ value of 48.4 s, indicating that substantial γ-ECS intermediate has to accumulate before the attainment of a steady state. These accumulated levels of γ-EC at the steady state are described by

\[
[\gamma - EC]_{ss} = \frac{v_0 K_m}{V_2 - V_0} \tag{Eq. 3}
\]

and were calculated to be 28 µM. When the K_m value of the GS activity of GshF for γ-EC (90 µM) is not influenced by the preceding coupled γ-ECS activity, then the steady-state γ-EC concentration may further be described (45, 46) by

\[
[\gamma - EC]_{ss} = \frac{v_0 K_m}{V_2 - V_0} \tag{Eq. 4}
\]

where [γ-EC]_{ss} is steady-state γ-EC concentration; v_0, steady-state velocity; K_m is Michaelis constant of the GS activity for γ-EC; and V_2, maximal velocity of the GS reaction center. Substituting [γ-EC]_{ss} = 28 µM, v_0 = 11.1 s^−1, and K_m = 90 µM gives a V_2 value of 46.5 s^−1, which is close to the apparent K_m value for the individual GS activity obtained in this work (Table 2). In summary, the generalized theory of the transient time for sequential noninteracting enzyme reactions obeying Michaelis-Menten kinetics, as first described by Easterby (45), is applicable to the linked consecutive peptide bond-forming reaction centers in GshF, without the need to consider metabolite channeling.

**DISCUSSION**

Gram-negative glutathione-containing cyanobacteria and proteobacteria accumulate glutathione via a *de novo* biosynthesis pathway that consists of two unrelated monofunctional ATP-dependent amino acid ligases called γ-ECL and GS. In this respect, our previous results with *H. influenzae* were surprising, as this γ-proteobacterium of the family Pasteurellaceae acquires glutathione solely via a highly specific import system (27). The motivation for the present research was to evaluate whether other Pasteurellaceae members were atypical also with respect to glutathione acquisition. By conducting this survey, novel hybrid sequences, referred to as GshF sequences, containing a typical γ-proteobacterial γ-ECL fused to an ATP-grasp-like domain, were discovered to be present in the genomes of strains of *H. somnis*, *A. pleurophomonae*, *M. succiniciproducens*, and *P. multocida*, yet no such sequences were found in the genomes of a number of *H. influenzae* and *H. ducreyi* strains. By *in vivo* complementation studies in a GshF−*E. coli* double mutant background and through *in vitro* kinetic studies with a recombinant and purified P. multocida GshF preparation, we have proven that the natural hybrid catalyzes both well known consecutive peptide bond-forming reactions toward the formation of glutathione, strongly suggesting that a large number of Pasteurellaceae strains accumulate glutathione via *de novo* synthesis, whereas others, such as *H. influenzae* and *H. ducreyi*, accumulate the tripeptide via import.

A BLASTP search with 303 microbial genomes at NCBI (37) identified 19 species, of both Gram-negative and Gram-positive signature, containing a GshF homologous sequence. Among Gram-negative bacteria, the distribution of GshF sequences appears to be confined to...
Characterization of Pasteurella multocida GshF

FIGURE 8. Plots of the steady-state initial velocities of the γ-ECL reaction of GshF in the absence (open squares) and in the presence (filled squares) of 20 mM Gly (i.e. the combined ATPase activities of the γ-ECL-GS coupled reaction) versus varying concentrations of L-Glu are indicative for domain-domain interactions according to a variant of the half-of-the-sites reactivity model. Initial velocities were recorded on the basis of ADP formation as described under “Materials and Methods.” The hyperbolic curves are simulations as explained in the Discussion. Inset, plot of the ratios of the γ-ECL-GS-coupled reaction initial velocities to the individually assayed γ-ECL reaction initial velocities (ratios of the simulations are given as a continuous curve). The simulated ratios have values of 2 and 1, respectively, as explained under “Discussion.”

members of the Pasteurellaceae family, with Desulfotalea psychrophila LSv54, a δ-proteobacterium, as the single exception. Fourteen of the 19 GshF-containing bacteria are Gram-positive species, which are classified as firmicutes. The GshF ligases of L. monocytogenes and S. agalactiae have recently been shown to be functional multidomain glutathione synthetases (24, 25) and, in case of L. monocytogenes, GshF was found to be essential for aerobic growth and virulence. Interestingly, some of the Gram-positive genera that harbor GshF, such as streptococci and enterococci, have been found to accumulate glutathione by either or both of the two presently recognized ways of glutathione acquisition, import and complete de novo synthesis (1, 24, 49).

Probing intracellular glutathione pools of cultures grown to the early exponential phase in chemically defined liquid minimal medium showed that P. multocida also accumulates glutathione by both biosynthesis and import. In this context, it would be of interest to investigate whether the uptake machineries of Gram-negative and Gram-positive GshF-containing bacteria are also related. P. multocida intracellular glutathione pools that become accumulated by biosynthesis, 23 ± 5 nmol/mg protein, are comparable with those measured for E. coli K12 (21.0 ± 3.5 nmol/mg protein (see Table I)) and, by using E. coli conversion data, these intracellular glutathione levels should correspond to about 3 mM. When the minimal growth medium contained GSSG, import accumulated intracellular glutathione more than 7-fold to 170 ± 20 nmol/mg protein or up to 20 mM. The substantial contribution of active transport in steady-state glutathione accumulation, however, does not appear to have a function in oxidative stress tolerance, as inferred from our disk diffusion assays presented under “Results” (Fig. 4). Therefore, we would like to propose that the P. multocida glutathione transport machinery serves a L-Cys/Gly salvage pathway. Indeed, L-Cys and Gly auxotrophic E. coli mutants can be rescued by adding glutathione to the minimal salts M9 growth medium, and γ-glutamyltranspeptidase, presently the only known peptidase that can cleave the glutathione γ-L-Glu to L-Cys peptide bond, is a crucial first player in the recycling pathway (50). In this respect, it is interesting to note that we have found L-Cys to be an essential amino acid for normal P. multocida growth and, additionally, despite the apparent lack of a γ-glutamyltranspeptidase homologous sequence in the sequenced genome, that we were able to demonstrate regulated γ-L-glutamyl-p-nitroanilide hydrolase activity in P. multocida crude extracts.3

All monofunctional GshA ligases studied to date, from either bacterial or animal origin (the latter consisting of a large catalytic and a small regulatory subunit), are regulated through non-allosteric feedback inhibition by glutathione. The tripeptide is believed to be a competitive inhibitor of the holoenzyme of rat kidney (Ki = 8.2 mM) and inhibits the E. coli enzyme (IC50 = ~3.0 mM), the rat kidney apoenzyme (Ki = 1.8 mM), and either human holoenzyme (Ki = 25.5 mM) or apoenzyme (Ki = 2.2 mM) in a noncompetitive manner, the latter being an example of true noncompetitive inhibition (51–54). A similar type of feedback inhibition by glutathione regulates the activity of recombinant P. multocida GshF, for which we calculated a Ki-value of 13.6 mM. In the absence of exogenous glutathione, steady-state intracellular glutathione concentrations (about 3 mM) are apparently not regulated by feedback inhibition, as the γ-GCL activity of GshF retains more than 90% of its uninhibited maximal turnover rate (Fig. 6D). It is more likely, as has been reported for rodents and human (55, 56), that the availability, in the case of P. multocida, of the essential amino acid L-Cys may be the rate-limiting factor for glutathione synthesis. Glutathione feedback inhibition then comes into play when the environment is loaded with physiologically relevant (low micromolar) levels of glutathione, because the accumulation of the tripeptide by means of active transport not only inhibits GshF but also indirectly should increase the rate of L-Cys availability. Glutathione accumulation by import can reach up to 20 mM levels, which inhibits the γ-GCL activity of P. multocida GshF more than 65%. Interestingly, glutathione has been found to marginally influence the γ-GCL activity of S. agalactiae GshF (24), a result that was reproduced in the present study (Fig. 6D). As a possible consequence, de novo GshF-catalyzed glutathione biosynthesis indeed accumulates enormous amounts of glutathione inside S. agalactiae cells (304 ± 11 nmol/mg protein) (24), meaning that under the applied assay conditions, glutathione building blocks are likely to be supplied at a nonlimiting rate.

When considering the substrates of GshF, in vitro steady-state kinetics were not indicative for cooperative mechanisms. Yet, as shown in

3 B. Vergauwen, D. De Vos, and J. J. Van Beeumen, unpublished results.
**Characterization of Pasteurella multocida GshF**

Fig. 6, glutathione appears to inhibit the γ-GCL activity of *P. multocida* GshF in a cooperative way, typified by a “Hill coefficient” of 1.65. Being close to 2, this value suggests that the glutathione binding sites within the GshF dimer communicate with each other in a positive, cooperative manner and that the glutathione binding sites are real allosteric sites. Because the *S. agalactiae* GshF ligase was reported to form monomers in solution (24), the possibility existed that only GshF dimers are sensitive toward (allosteric) glutathione feedback inhibition. Therefore, purified *P. multocida* and *S. agalactiae* GshF preparations were chromatographed on a Superdex 200 gel filtration column under identical conditions. Both proteins eluted as dimers (Fig. 5), suggesting that GshF proteins in general form dimers in solution and that feedback inhibition cannot be explained in terms of differences in quaternary structure. The molecular details that sensitize GshF proteins to glutathione feedback inhibition are currently under investigation in our laboratory.

Various metabolic pathways in prokaryotes and in eukaryotes include bifunctional enzymes that catalyze consecutive reactions, although the significance of this bifunctionality is not always clear. During the last decade, however, convincing data have accumulated showing that metabolism can benefit from bifunctionality because of three major reasons: metabolite channeling, interdomain communication, and regulatory sensitivity (44, 57). Tryptophan synthase from *S. typhimurium* (58, 59), thymidylate synthase/dihydrofolate reductase (TS-DHFR) from *Leishmania major* (48, 60), glutamate synthase from *Synechocystis* sp. (61), and imidazole glycerol-phosphate synthase from yeast (62, 63) are very well characterized examples of bifunctional enzymes that exhibit both channeling and interdomain communications. Domain-domain interactions in these enzymes become evident upon formation of substrate-enzyme complexes (or reaction intermediates) at either of the two reaction centers, which reciprocally and allosterically activate turnover at the other reaction center. Consequently, these enzymes share their two main functional features in their ability to coordinate the activity of their functional sites (i) to avoid wasteful consumption of pathway starting substrate and (ii) to avoid leakage of pathway intermediate by coupling intermediate production to the availability of final substrate. Channeling in tryptophan synthase occurs through an intramolecular tunnel (59), whereas the dihydrofolate intermediate in TS-DHFR is transferred via an “electrostatic channel” on the protein surface (48). In either case, channeling of intermediate appears to occur at a lower limit of 1000 s⁻¹ (47, 48), making this event not rate-limiting for overall catalysis. As a consequence, steady-state time courses of coupled activities proceed to generate final product with no visible lag period. Steady-state progress curves recorded for recombinant substrate saturated *P. multocida* GshF (Fig. 7) exhibit a transient time of 48.4 s, which argues against the involvement of non-leaky channeling in the reaction mechanism. Furthermore, because the progress curves can be described by the generalized free diffusion theory for coupled non-interacting enzymes (45, 46) without adjusting a single determining parameter, also leaky channeling, *i.e.* channeling by random diffusion alone as a result of the close proximity of active sites, may be considered not to be in play. This means that the γ-EC intermediate mixes entirely with the bulk phase during the transfer from the γ-ECL reaction center to the GS active site in the *P. multocida* GshF bifunctional glutathione synthetase. These predictions, however, are based on the assumption that certain kinetic constants obtained for the individually analyzed activities (such as the $K_{m}$ for γ-EC and the $k_{cat}$ of the GS activity) do not change in enzyme that simultaneously catalyzes both coupled reactions. Transient-state kinetic methods will therefore be needed to delineate the presumptions made here concerning intermediate channeling within GshF proteins.

Steady-state kinetics clearly identified the phenomenon of interdomain communication, allosterically modifying activity at both active centers of GshF. First, turnover at the γ-ECL active center stimulates activity at the GS site by drastically increasing the affinity for Gly ($k_{cat}$ values are not modified; see discussion in the previous paragraph). The coupling of the γ-ECL and GS active centers lowers the apparent $K_{m}$ of 81 mM for Gly of the individually assayed GS activity to the more physiologically relevant value of 1.72 mM. Recall from Table 2 the rather low affinities of the individually assayed GS activity of the *S. agalactiae* GshF for both γ-EC ($K_{m} = 5.9$ mM) and Gly ($K_{m} = 6.3$ mM), for which type of interdomain communication could rationalize less wasteful synchronization of active sites. An increase in activity of the second site upon catalysis at the preceding reaction center has also been reported for tryptophan synthase and TS-DHFR bifunctional enzymes. However, in these cases the activation was not the result of increased substrate affinity but was ascribed to an increase in maximal turnover rate, for example more than 8.5-fold for the DHFR reaction in case of TS-DHFR (48).

Now, why is the GS reaction virtually inactivated in the absence of L-Cys and/or L-Glu substrates (cf. under these circumstances and at the *in vivo* Gly concentration of 0.5 mM reported to be present in Gram-negative bacteria (43), by substituting the Michaelis-Menten formula, GS catalysis is calculated to proceed at only 0.6% of the maximal rate)? From a physiological viewpoint, three consequences of this mechanistic behavior may be of importance. First, the GS activity is prevented from forming glutathione from imported γ-EC substrate. Second, steady-state glutathione formation is attained more rapidly. Third, salvage of the γ-EC pool may be facilitated in times of L-Cys shortage. Alternatively, mechanistic reasons may also explain this kind of interdomain communication. As will be discussed further, the binding of Gly to the GS site may already be sufficient to influence (decrease) turnover at the γ-ECL site, which is, of course, unwanted when the rate of γ-EC production is already low because of a deficiency in either or both L-Cys and L-Glu.

The second indication for domain-domain interactions stems from our observation that catalysis at the substrate saturated γ-ECL reaction center is halved in the presence of saturating concentrations of Gly. Thus, catalysis at the GS site apparently impedes catalysis at the preceding γ-ECL active site, which is most obviously explained by a model in which Gly binding or catalysis at the GS site transmits a signal to the γ-ECL site to decrease its maximal turnover rate. This hypothesis does not make much sense from a functional and physiological point of view and also does not fit the data presented in Fig. 8, in which the pseudo-first order initial ATPase velocities of the coupled γ-ECL + GS reaction and of the individually monitored γ-ECL reaction are plotted against varying concentrations of L-Glu. The Fig. 8 inset shows that the ratio of these Michaelis-Menten plots in the limit goes from 2 at zero L-Glu to 1 at saturating L-Glu concentrations. In the case where Gly binding or catalysis at the GS site transmits a signal to the γ-ECL site to decrease its maximal turnover rate, we suspect the Gly effect will occur irrespective of the degree of L-Glu saturation of the γ-ECL site, giving identical ratios along the L-Glu saturation axis.

As catalysis at the substrate-saturated γ-ECL reaction center is approximately halved in the presence of saturating concentrations of Gly, we considered the possibility of half-of-the-sites reactivity, also known as alternating sites catalytic cooperativity. The so-called half-of-the-sites reactivity was originally proposed on the basis of biochemical analyses of certain homo-oligomeric enzymes, including glyceraldehyde-3-phosphate dehydrogenase (64) and succinyl-coenzyme A synthetase (65). The half-of-the-sites reactivity is an extreme form of negative kinetic cooperativity in which the first ligand binding induces a negative cooperativity that is so strong that the second ligand binds
Characterization of Pasteurella multocida GshF

In the absence of half-of-the-sites reactivity, lowers the fraction of GshF dimers with two fully saturated site. Because the activity of the other subunit, which in turn prevents catalysis at its GS center, the allosteric Gly effect is a function of the degree of L-Glu saturation of the GshF dimer by L-Glu irrespective of Gly availability. In this model, the allosteric Gly effect fades (ATPase activity doubles upon the addition of saturating concentrations of Gly in the limit toward zero L-Glu concentrations). This variant of the half-of-the-sites reactivity model also accurately simulates the steady-state kinetic data presented in Fig. 8. As we have discussed, reaction at the GS center is only weakly or not at all (66). In the context of the bifunctional dimeric P. multocida GshF, we put forward the following variant of the half-of-the-sites reactivity model to explain the steady-state kinetic data presented in Fig. 8. When the rate constants of both \( \gamma \)-ECL reaction centers of the GshF dimer are taken to be identical, when these rate constants are not affected by Gly availability and/or \( \gamma \)-EC accumulation, and when, at steady state, the rate of glutathione formation equals the rate of \( \gamma \)-EC reaction, the ATPase velocity is proportional to the fractional saturation (\( Y \)) of the GshF dimer by L-Glu irrespective of Gly availability. Hence, for the individually assayed \( \gamma \)-ECL reaction,

\[
Y = \frac{V_0}{V_{\text{max}}} \quad \text{(Eq. 5)}
\]

whereas for the coupled \( \gamma \)-ECL-GS reaction,

\[
Y = \frac{V_Y}{V_{\text{max}}} \quad \text{(Eq. 6)}
\]

where \( v_0 \) and \( V_{\text{max}} \) are the initial velocity and maximum velocity at infinite [L-Glu] of \( \gamma \)-ECL catalyzed ATP hydrolysis, whereas \( Y_0 \) and \( V_{\text{max}} \) are the combined initial velocity and combined maximum velocity at infinite [L-Glu] of ATP hydrolysis of the \( \gamma \)-ECL-GS-coupled reaction. In the absence of half-of-the-sites reactivity, \( V_{\text{max}} = 2V_{\text{max}} \). By theory, the \( Y \)-value for L-Glu binding to the GshF dimer is

\[
Y = \frac{[ES] + 2[ES_2]}{2[E_0]} \quad \text{(Eq. 7)}
\]

with

\[
[ES] = \frac{2[S][E_0]/K_m}{1 + (2[S]/K_m) + ([S]^2/K_mK_{m}')} \quad \text{(Eq. 8)}
\]

the concentration of GshF dimer with one \( \gamma \)-ECL site fully saturated ([L-Glu], initial L-Glu concentration; \( K_{m'} \), Michaelis constant for L-Glu binding to the free GshF dimer; \( K_{m''} \), Michaelis constant for L-Glu binding to half-occupied GshF dimer; [E\text{G}], GshF dimer concentration) and

\[
[ES_2] = \frac{[ES][S]}{2K_{m''}} \quad \text{(Eq. 9)}
\]

In the case of the individually assayed \( \gamma \)-ECL reaction, the theoretical hyperbolic \( Y \) (combining Equations 7–9), via Equation 5, gives rise to the Michaelis-Menten curve drawn to fit the \( \gamma \)-ECL-derived ATPase initial velocities in Fig. 8. Because of the presumption that \( K_{m'} \gg K_{m''} \), which states that L-Glu binding at one monomer abolishes \( \gamma \)-ECL turnover at the other, the theoretical \( Y \) (combining Equations 7–9) in the case of the coupled \( \gamma \)-ECL-GS reaction reduces to

\[
Y = \frac{[ES]}{2[E_0]} \quad \text{(Eq. 10)}
\]

which resembles the hyperbolic \( Y \) of a monomeric enzyme, the difference being that only half of all potential L-Glu binding sites are contributing. Substituting this theoretical \( Y \) in Equation 6 gives a hyperbola that fits the experimentally derived \( \gamma \)-ECL-GS-coupled reaction steady-state initial velocities depicted in Fig. 8. To the best of our knowledge, half-of-the-sites reactivity of bifunctional oligomeric enzymes, based on induced asymmetry between dissimilar domains of the subunits, has never been proposed to be involved in their reaction pathways. Half-of-the-sites reactivity, already biochemically and structurally well characterized for homo-oligomeric proteins (67), may thus be a novel functional trait/benefit of bifunctionality. The molecular basis underlying this kind of novel kinetic behavior of the bifunctional glutathione synthetase GshF of \( P. \) multocida is currently under investigation in our laboratory.

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