Structural Variation in Bacterial Glyoxalase I Enzymes

INVESTIGATION OF THE METALLOENZYME GLYOXALASE I FROM CLOSTRIDIUM ACETOBUTYLICUM*‡

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The glyoxalase system catalyzes the conversion of toxic, metabolically produced α-ketoaldehydes, such as methylglyoxal, into their corresponding nontoxic 2-hydroxycarboxylic acids, leading to detoxification of these cellular metabolites. Previous studies on the first enzyme in the glyoxalase system, glyoxalase I (GlxI), from yeast, protozoa, animals, humans, plants, and Gram-negative bacteria, have suggested two metal activation classes, Zn⁡²⁺ and non-Zn⁡²⁺ activation. Here, we report a biochemical and structural investigation of the GlxI from Clostridium acetobutylicum, which is the first GlxI enzyme from Gram-positive bacteria that has been fully characterized as to its three-dimensional structure and its detailed metal specificity. It is a Ni⁡²⁺/Co⁡²⁺-activated enzyme, in which the active site geometry forms an octahedral coordination with one metal atom, two water molecules, and four metal-binding ligands, although its inactive Zn⁡²⁺-bound form possesses a trigonal bipyramidal geometry with only one water molecule liganded to the metal center. This enzyme also possesses a unique dimeric molecular structure. Unlike other small homodimeric GlxI where two active sites are located at the dimeric interface, the C. acetobutylicum dimeric GlxI enzyme also forms two active sites but each within single subunits. Interestingly, even though this enzyme possesses a different dimeric structure from previously studied GlxI, its metal activation characteristics are consistent with properties of other GlxI. These findings indicate that metal activation profiles in this class of enzyme hold true across diverse quaternary structure arrangements.

Methylglyoxal (MG), an α-ketoaldehyde produced from a number of different enzymes and pathways, including triose-phosphate isomerase, amino acid degradation, and acetoacet-aldehyde converting monoxygenases, is found in both prokaryotic and eukaryotic organisms (1–4). MG is a cytotoxic compound that can be produced to a level as high as 0.4 mm per cell per day, leading to protein synthesis inhibition, adduct formation with proteins, DNA, and RNA and can promote advanced glycation end products (5–10).

A major contributing pathway involved in the detoxification of MG is the glyoxalase system. This two-enzyme system consists of glyoxalase I (GxlI) and glyoxalase II (GxlII) that convert α-ketoaldehydes into their corresponding 2-hydroxy-carboxylic acids (α-lactate in the case of methylglyoxal), using an intracellular thiol as a cofactor/cosubstrate (Fig. 1). The first enzyme, GxlI (S-α-lactylglutathione methylglyoxal lyase (isomerizing), EC 4.4.1.5), converts a hemithioacetal, the product of the non-enzymatic reaction between MG and a thiol, such as glutathione (GSH), to S-α-lactylglutathione. GxlI is a metalloenzyme that can be divided into two classes, Zn⁡²⁺ activation (i.e. Homo sapiens GxlI (11)) and non-Zn⁡²⁺ activation (being selectively Ni⁡²⁺/Co⁡²⁺-activated, i.e. Escherichia coli GxlI (12)).

It is likely that almost all Gram-negative prokaryotes possess Ni⁡²⁺/Co⁡²⁺-activated GxlI with the possible exception of pseudomonads (13–15). It has been reported that GxlI from Pseudomonas putida is a Zn⁡²⁺-activated enzyme (13). Interestingly, Pseudomonas aeruginosa has been reported to possess three genes coding for GxlI enzymes (gloA1, gloA2, and gloA3) (15). GloA1 and GloA2 were determined to be Ni⁡²⁺/Co⁡²⁺-activated (but Zn⁡²⁺-inactive), whereas GloA3 is a Zn⁡²⁺-activated enzyme. All Zn⁡²⁺-activated enzymes from pseudomonads have longer amino acid sequences, which are similar in length to that of H. sapiens GxlI. Metal specificity for GxlI from eukaryotes seems to vary. The protozoan Leishmania major GxlI possesses Ni⁡²⁺/Co⁡²⁺-activation properties with no activity in the presence of Zn⁡²⁺ ion (16, 17). Trypanosoma cruzi GxlI is Ni⁡²⁺/Co⁡²⁺-activated with minor but measurable activity in the presence of Zn⁡²⁺ (18). However, the malarial parasite, Plasmodium falciparum, contains a GxlI that is Zn⁡²⁺-activated (19). In addition, yeast (Saccharomyces cerevisiae), human (H. sapiens), and plant (Brassica) GxlI fall into the Zn⁡²⁺-activated class (12, 20–28).

Although several GlxI enzymes have been investigated from a number of biological sources, little is currently known concerning GlxI enzymes from Gram-positive microorganisms. Our biochemical analysis on and x-ray crystallographic structural determination of the dimeric Clostridium acetobutylicum...
glyoxalase I (CLO GlxI, AAK80149) has provided interesting insights concerning the possible structures and function of Gram-positive GlxI enzymes (PDB codes 2QH0 and 3HDP).

EXPERIMENTAL PROCEDURES

His-tagged CLO GlxI DNA Cloning and Protein Purification (High Throughput Protocols)—The gene for clo glxI (PSI Target ID NYSGXRC-11003p; Swiss-Prot: Q97H22) was amplified using PCR primers (+) 5’-AAAGTACACCATATAGGG-TATGC-3’ and (−) 5’-CTTCTTTCTAAATTTCTATCAAC-CCTA-3’ and was cloned into the pSGX4 (BC) expression vector. The pSGX4 (BC) vector containing clo glxI was transformed into Top10 competent cells, and the sequence-verified plasmid was transformed into BL21 (DE3) CodonPlus RIL cells. The protein was expressed using standard isopropyl 1-thio-β-d-galactopyranoside induction protocols. The detailed protocols of DNA cloning and protein purification are available at Protein Expression Purification and Crystallization Database website. Mass spectrometry analyses documented that the purified protein had not undergone degradation or post-translational modification before setting up crystallization experiments (data not shown). This His-tagged CLO GlxI was used for the investigation of protein stability, including consideration of the secondary and the quaternary structures of the dimeric enzyme, and crystallography with bound Zn2+ atoms.

Non-His-tagged CLO GlxI DNA Cloning and Protein Purification—All DNA manipulations and purifications were performed according to the protocols by Sambrook and Russell (29). The clo glxI was cloned into the pET-28b(+) expression vector utilizing NdeI and BamHI restriction endonuclease enzymes and PCR to generate a protein with the N-terminal His6 tag and a thrombin protease cleavage site. The forward and reverse primers were designed as follows: (+) 5’-TAATAGACTCATAATAGG-3’ and (−) 5’-GGCGAATTCGGATCC-CTACTTTTCTAAATAC-3’. The plasmid was heat shock-transformed into competent Escherichia coli DH5α cells, and its sequence was verified (Molecular Biology Core Facility, University of Waterloo, Waterloo, Ontario, Canada) before heat shock transforming into E. coli BL21 (DE3) cells for protein expression purposes.

A bacterial culture of BL21 (DE3)/pCLO-GlxI in LB media (1 liter) containing kanamycin (30 μg/ml LB) was grown at 25 °C and induced in the presence of 0.5 mM isopropyl-1-thio-β-d-galactopyranoside for 4 h. The overproduced enzyme was purified using a HisTrap™ HP Ni2+-affinity step followed by treatment with thrombin protease to remove the His6 tag. Further purification by use of HisTrap HP and HiTrap benzamidine FF affinity columns gave a purified non-His-tagged CLO GlxI, which was dialyzed against buffer containing 50 mM HEPES (pH 7.0), 200 mM KCl, 10 mM Met, and 10% glycerol. The protein concentration was determined by the Bradford assay (30) using bovine serum albumin (BSA) as a standard.

Apoenzyme preparation and metal analysis by 4-(2-pyridylazo)resorcinol assay and inductively coupled mass spectrometry was performed according to those previously described for E. coli GlxI (31–35). The subunit molecular mass was determined by positive ion mode-electrospray mass spectrometry (ESI-MS) using a Micromass Q-TOF Global Ultima mass spectrometer (Mass Spectrometry Facility, University of Waterloo, Waterloo, Ontario, Canada). The molecular mass of the native enzyme was determined by gel permeation chromatography (Superdex 75 HR 10/30 column) utilizing MOPS buffer with a flow rate of 0.5 ml/min. This non-His-tagged CLO GlxI was used for the investigations of metal activation, metal titration, pH profile, and enzyme activity of GlxI and methylmalonyl-CoA epimerase.

Protein Stability Investigation—The effect of protein concentration, buffer pH, ionic strength, additives, temperature, and metal reconstitution on the secondary structure of His-tagged CLO GlxI was investigated using circular dichroism (CD) on a Jasco J-715 spectropolarimeter (Easton, MD) and a cuvette of 1 mm path length. The CD detection was scanned between 190 and 250 nm with 0.2-nm step resolution, 100 nm/min speed, 25 accumulations, 1-s response, 1-nm bandwidth, 50 millidegree sensitivity, and 500-μm silt width. All experiments were performed under nitrogen gas.

Enzymatic Assay for Glyoxalase I Activity—An enzymatic assay was performed in 50 mM KPB (pH 6.6) (or stated otherwise), as reported previously (12, 36, 37), using a SpectraMax 190 96-well UV-visible spectrophotometer and Soft Max Pro analysis software (Molecular Devices, Sunnyvale, CA). The ini-
**TABLE 1**

X-ray data collection and statistics for Zn$^{2+}$- and Ni$^{2+}$-bound CLO

| Parameter | Zn$^{2+}$-bound | Ni$^{2+}$-bound |
|-----------|-----------------|-----------------|
| Wavelength | 0.9792 Å | 0.9792 Å |
| Space group | P4$_2$,1,2 | P4$_2$,2 |
| Resolution | 50 to 2.45 Å | 50 to 2.06 Å |
| Cell parameters a, b, and c | 71.1, 71.1, 63.8 Å | 70.3, 70.3, 66.6 Å |
| α, β, and γ | 90.0, 90.0, 90.0° | 90.0, 90.0, 90.0° |
| Redundancy$^c$ | 24.8 (20.7) | 25.5 (25.3) |
| Outermost shell | 2.54 to 2.45 Å | 2.13 to 2.06 Å |
| No. of unique reflections | 6249 | 9650 |
| $R_{merge}$ | 6.0% (14.3%) | 5% (17.2%) |
| Overall completeness$^a$ | 98.2% (97.4%) | 88.3% (99.0%) |
| ($I/σI$)$^b$ | 17.3 (3.1) | 31.6 (10.0) |

**Reefinement statistics**

| Parameter | Zn$^{2+}$-bound | Ni$^{2+}$-bound |
|-----------|-----------------|-----------------|
| Resolution range | 40.0 to 2.45 Å | 24.2 to 2.06 Å |
| Outermost shell | 2.60 to 2.45 Å | 2.19 to 2.06 Å |
| No. of reflections | 6004 | 8993 |
| $R$-factor$^c$ | 0.235 | 0.256 |
| $R$-free$^d$ | 0.299 | 0.286 |
| No. of protein atoms | 1035 | 1060 |
| No. of water molecules | 29 | 78 |
| No. of heteroatoms | 1 | 1 |

**Ramachandran plot (residues in %)**

- Core region: 94.0%
- Additionally allowed: 6.0%
- Generously allowed: 0%

**Root mean square deviations**

| Bond lengths | 0.007 Å | 0.006 Å |
| Bond angles | 1.30° | 1.20° |

$^a$ The values corresponding to the outermost shell are given in parentheses.
$^b$ $R_{merge} = \frac{\sum |I_h| - \langle I_h \rangle}{\sum I_h}$, where $\langle I_h \rangle$ is the mean intensity of symmetry-related reflections, $I_h$.
$^c$ $R$-factor = $\frac{\sum |F_o| - |F_c|}{\sum |F_o|}$, where $F_o$ and $F_c$ are the observed and calculated structure factor amplitudes, respectively.
$^d$ $R$-free is calculated for the 2% of the data that was withheld from refinement.

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**Structural Variation in Bacterial GlxI Enzymes**

Using SHARP (41) and SOLOMON (42). Automated model building with Arp/wArp yielded structural information for ~80% of the model comprising a single chain in the asymmetric unit (43), and the rest of the model was manually built using O (44). Refinement using data collected at peak wavelength was performed with CNS (45). The residue numbering 4–130 is based on Swiss-Prot data base (Swiss-Prot code Q97H22_CLOAB). The Ni$^{2+}$-bound non-His-tagged CLO GlxI structure was solved by molecular replacement with MolRep (46) using the Zn$^{2+}$-bound structure as search model.

**RESULTS**

**Protein Characterization**—His-tagged CLO GlxI was generated with a replacement of the first Met residue at the N terminus with three extra amino acids, MSL, and an additional His$_6$ tag at the C terminus (EGHHHHHHH), which corresponded to the predicted molecular mass of 15,818.1 Da and the predicted pl of 6.50 (ProtParam tool, available on line) (47). This His tag was used for purification purposes and was left uncleaved. Similar molecular weights of enzyme in buffer with and without DTT (20 mM) present were observed in gel permeation chromatography, indicating that the native His-tagged CLO GlxI is a dimer and the interaction between subunits does not involve disulfide bond formation. Similar results were observed when varying the enzyme concentrations (9.56–0.64 μM), pH (MOPS buffer pH of 6–8), ionic strength (0–500 mM KCl), and additives (0–20% (v/v) glycerol), suggesting that these factors do not affect the quaternary structure of the dimeric enzyme. However, the presence of the His tag most likely is the cause of enhanced protein aggregation for this form of the enzyme because oligomers may occur due to interaction with Ni$^{2+}$ ions in solution.

Investigation of the secondary structure using CD analysis suggested that to optimize protein stability, protein concentrations lower than 7.80 μM in Tris buffer (pH 6.0) containing 200 mM KCl in the presence of 10% (v/v) glycerol were required. The melting temperature of His-tagged CLO GlxI was ~61 °C (supplemental Fig. S1). Although protein concentration and buffer pH are the major causes of aggregation, metal reconstitution with Zn$^{2+}$ and Ni$^{2+}$ atoms exhibited no significant change in either the secondary or quaternary structure of the enzyme.

Because of its low protein stability and recovery yield, a non-His-tagged construct of CLO GlxI was engineered to investigate the active site geometry of a Ni$^{2+}$-reconstituted enzyme in comparison with its Zn$^{2+}$-bound form. This non-His-tagged CLO GlxI was prepared in its apo-form before incubating with 10 eq of NiCl$_2$. Excess metals were removed by dialyzing against metal-free buffer. Inductively coupled mass spectrometry was utilized to quantitate the amount of metal ion incorporated into the enzyme, and the ratio of 2.34 metal atoms per dimeric enzyme ratio was determined.

**Metal Specificity and Enzyme Kinetics**—These experiments were performed using both His-tagged and non-His-tagged CLO GlxI. The experimental studies indicate that the His tag does not affect the metal properties, enzymatic assay, or dimeric structural formation. Reconstitution of the apoenzyme with excess metal ions (10 eq) showed that the enzyme was
activated by Ni\textsuperscript{2+}, Co\textsuperscript{2+}, Cd\textsuperscript{2+}, and Mn\textsuperscript{2+} (Fig. 2). The activity of Co\textsuperscript{2+}-reconstituted enzyme was ~40%, although Mn\textsuperscript{2+}- and Cd\textsuperscript{2+}-activated forms were ~5% that of Ni\textsuperscript{2+}-activated enzyme. The activities with Ca\textsuperscript{2+} and Mg\textsuperscript{2+} atoms were negligible. No activity was found for the apoenzyme or for the Zn\textsuperscript{2+}-reconstituted enzyme.

Other studies on CLO GlxI, including metal titration and pH profile experiments, showed that the enzyme possessed maximum activity with ~1 mol of metal per mol of dimeric enzyme for both Ni\textsuperscript{2+} and Co\textsuperscript{2+} reconstitutions (supplemental Fig. S2A and text in supplemental material). A similar result has been observed for the E. coli GlxI, L. major, and P. falciparum GlxI (one metal per dimer for E. coli and L. major GlxI, and one metal per monomer (two active sites) for P. falciparum GlxI) (12, 16, 17, 48). By using Ni\textsuperscript{2+} activation, enzymatic activity was optimized in KPB buffer (pH 7.0) (supplemental Fig. S2B). In addition, it was found that \( V_{\text{max}}/K_m \) (4.80 ± 0.02 \( \mu \text{mol/min/mg/mM} \)) was optimal in buffer containing 0.4–0.8 mM KCl, suggesting the enzyme possesses highest activity under these buffer conditions (supplemental Fig. S2C).

In the kinetic studies, it was found that CLO GlxI (0.8 \( \mu \text{g} \)) possessed greater catalytic activity (\( k_{\text{cat}}/K_m \)) in KPB (pH 7.0) (2.6 \( \times 10^3 \text{M}^{-1} \text{s}^{-1} \)) than in pH 6.6 (2.3 \( \times 10^3 \text{M}^{-1} \text{s}^{-1} \)) (supplemental Table S1). These data matched the pH profile, as it was previously determined that the optimized pH buffer was at pH 7.0.

Crystal Structure of CLO GlxI (Inactive Zn\textsuperscript{2+}- and Active Ni\textsuperscript{2+}-bound Forms)—The CLO GlxI monomer consists of two repeating domains, the N- and C-terminal domains, with the \( \alpha/\beta \) fold (Fig. 3, A and B). The overall structure has an eight-stranded \( \beta \)-sheet generated by edge-to-edge packing of two internally duplicated \( \beta\alpha\beta\beta \) modules. The N-terminal domain consists of \( \beta1\alpha1\beta2\beta3\beta4 \) topology (residues 2–60) followed by the C-terminal domain with \( \beta5\alpha2\beta6\beta7\beta8 \) arrangement (residues 72–140). Both domains are connected by a short \( \alpha \)-helix (residues 61–71). The core of the CLO GlxI enzyme creates a large cleft and harbors the active site with the two central strands (\( \beta1 \) and \( \beta5 \)), whereas the outer strands (\( \beta4 \) and \( \beta8 \)) cover from the side. Although the two internally duplicated \( \beta\alpha\beta\beta \) modules share structural similarity (root mean square deviation (r.m.s.d.) of 1.69 Å), they show little sequence identity and similarity (12 and 18%, respectively). The Zn\textsuperscript{2+} and Ni\textsuperscript{2+} ions are bound deep in the active site cleft (supplemental Fig. S3), coordinating to the side chains of His\textsuperscript{5} (Ne2), Glu\textsuperscript{52} (Oe1), His\textsuperscript{75} (Ne2), and Glu\textsuperscript{124} (Oe1). A water molecule completes the near trigonal bipyramidal coordination in the active site of an inactive Zn\textsuperscript{2+}-bound enzyme (Fig. 3D), whereas its Ni\textsuperscript{2+}-bound form possesses octahedral geometry with two water molecules and the same four protein ligands (Fig. 3C). The metal-binding ligands come from topologically equivalent positions in the two modules as follows: His\textsuperscript{5} and Glu\textsuperscript{52} from the first and fourth \( \beta \)-strands of the N-terminal domain and His\textsuperscript{75} and Glu\textsuperscript{124} from equivalent positions in the first and fourth \( \beta \)-strands of the C-terminal domain. Despite their different metal coordinations, the overall structures of Ni\textsuperscript{2+}- and Zn\textsuperscript{2+}-bound CLO GlxI are well superimposed with r.m.s.d. of 0.434 Å for all 129 Ca pairs.

Interestingly, the dimeric structure of CLO GlxI is unique among other homodimeric GlxI. Instead of forming two active sites at the dimeric interface like other GlxI (for example, GlxI from E. coli and human), CLO GlxI forms an active site within each monomer (Fig. 4, A and B). The major difference in the structures of CLO GlxI and E. coli GlxI is a flexible loop that connects the N- and C-terminal domains. In CLO GlxI, this loop bends and curves up to form a catalytic pocket by the N- and C-terminal domains within the same subunit. Conversely, a connecting loop in E. coli GlxI (residues 63–73) extends and lies in an antiparallel fashion with a connecting loop from another subunit (Fig. 4B), thus forming a catalytic pocket by the N-terminal domain from one subunit and the C-terminal domain from another subunit. This type of dimeric formation is observed from all other investigated homodimeric GlxI. Even though the dimeric formations of CLO GlxI and E. coli GlxI are different, their metal coordinations of the active and inactive enzymes are similar. The active E. coli GlxI with bound Ni\textsuperscript{2+} in its active site possesses an octahedral geometry with four metal-binding protein residues (two from each monomer, including His\textsuperscript{5}, Glu\textsuperscript{56}, His\textsuperscript{74}, and Glu\textsuperscript{122}) and two water molecules around the metal center (PDB code 1F92) (49). Its corresponding inactive form with bound Zn\textsuperscript{2+} possesses a five-coordinated trigonal bipyramidal geometry with only one water molecule bound to the zinc ion (PDB code 1FA5) (49). Similarly, human GlxI (Zn\textsuperscript{2+}-activated enzyme) with two active sites forming at the dimeric interface also possesses an octahedral geometry in the presence of Zn\textsuperscript{2+} (PDB code 1IQN) (50).

A list of closely resembling structures in the PDB identified through a Dali search (DaliLite version 3) (51) indicated that most of these structures appear related to the family of dioxygenases, but the closest structure was MMCE from P. shermanii (PDB code 1C4) with an r.m.s.d. of 1.1 Å for 61 Ca atomic pairs (Fig. 4C). As well, based on multiple sequence alignments (supplemental Fig. S4), a phylogenetic tree suggests a closer relationship of CLO GlxI with MMCE than with GlxI that catalyzes the same enzymatic reaction (supplemental Fig. S5). Like CLO GlxI, homodimeric MMCE from P. shermanii packs back-to-back, forming an active site within one subunit (two active sites per dimer, Fig. 4C). The interactions between a dimer are composed mainly of hydrophobic as
well as hydrogen bonding interactions. Similarly to GlxI, there are four conserved metal-binding residues, including His^{12}, Gln^{65}, His^{91}, and Glu^{141}, in P. shermanii MMCE that locate at the active site (52). The active site geometry of the active MMCE also forms an octahedral coordination with four metal-binding residues and two water molecules around the metal center. The structural similarities of both enzymes lead to a common reaction mechanism for MMCE and GlxI, where the former functions as an epimerase using the thioester methylmalonyl-CoA, and the latter accomplishes an isomerization reaction upon the hemithioacetal of 2-oxoaldehydes as substrates. The activities of both enzymes also depend on the presence of divalent metals.

Because of these similarities, it was possible that CLO GlxI might exhibit bifunctional activity, glyoxalase I and methylmalonyl-CoA epimerase activity. MMCE activity of the CLO GlxI was investigated according to a previously reported HPLC assay (38, 53). However, no MMCE activity by the Co^{2+}-bound CLO...
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GlxI (1–10 μg) and its Ni\(^{2+}\)-bound form (30 μg) was observed, indicating that this enzyme does not possess epimerase activity. In addition, Zn\(^{2+}\)-activated and Ni\(^{2+}/Co\(^{2+}\)-activated homodimeric GlxI from \textit{P. aeruginosa} (GloA3 (1 μg) and GloA2 (0.048–1 μg), respectively) exhibited no detectable MMCE activity. Neither was epimerase activity observed with commercial monomeric yeast GlxI (2–9 ng), suggesting that both metal activation classes and sizes of GlxI do not exhibit epimerase activity, which is a study that has previously not been undertaken.

**DISCUSSION**

An analysis of the multiple sequence alignments of CLO GlxI with GlxI from other organisms based on conserved metal-binding residues and amino acid length indicated that this putative enzyme might be a Ni\(^{2+}/Co\(^{2+}\)-activated enzyme, which would in turn exhibit no catalytic activity with Zn\(^{2+}\) atoms (supplemental Fig. S4). This finding suggests that the initial Zn\(^{2+}\)-complex structure obtained from a high-throughput x-ray crystallographic platform was catalytically inactive. Metal activation experiments confirmed this prediction. It was found that the Gram-positive CLO GlxI is a member of the Ni\(^{2+}/Co\(^{2+}\)-activated glyoxalase I class and has no activity with Zn\(^{2+}\), which is similar to several Gram-negative bacteria such as \textit{E. coli}, \textit{Yersinia pestis} and \textit{Neisseria meningitidis} (12, 14, 31).

Kinetic studies indicate that the enzyme activity of CLO GlxI (k_{cat}/K_m of 2.6 × 10^{-3} M^{-1} s^{-1}) is lower than the activity of GlxI from yeast (k_{cat}/K_m of 3.5 × 10^{-6} M^{-1} s^{-1}), human (k_{cat}/K_m of 2.3 × 10^{-2} M^{-1} s^{-1}), and \textit{E. coli} (k_{cat}/K_m of 1.24 × 10^{-2} M^{-1} s^{-1}) (Ref. 12, and references therein). The level of activity exhibited by CLO GlxI is, however, observed in GlxI isolated from most plants (such as maize, \textit{Brassica juncea}, and \textit{Aloe vera} (22, 23, 54)) as well as nematode parasites (such as \textit{Trichostrongylus colubriformis}, \textit{Ascaris lumbricoides}, and \textit{Ascardia galli} (55)). There are several possibilities that might explain the lower activity observed in CLO GlxI. First, it is possible that more than one GlxI may exist in this Gram-positive bacterium (similar to \textit{P. aeruginosa} with three GlxI as stated previously).

Another putative GlxI from \textit{C. acetobutylicum} (NP_346890) may exist as determined by analysis of the National Center for Biotechnology Information (NCBI) protein sequence data base. The multiple sequence alignment with other GlxI shows that this putative protein possesses four conserved metal-binding residues and may behave as a Ni\(^{2+}\)-activated enzyme (with shorter amino acid sequence). In addition, putative GlxIs from other species of Clostridia also possess shorter amino acid sequences, which are more in line with the Ni\(^{2+}\)-activated enzymes (putative GlxI from \textit{Clostridium botulinum} (YP_001920932), \textit{Clostridium butyricum} (ZP_02951467), \textit{Clostridium perfringens} (YP_697773), and \textit{Clostridium tetani} (NP_782738)). Therefore, the GlxI enzymes from many Clostridia likely behave as Ni\(^{2+}\)-activated enzymes.

Moreover, the amount of GSH produced by γ-glutamylcysteine synthetase and glutathione synthetase (amino acid sequences in \textit{C. acetobutylicum} being identified by BLAST search (56)) in Clostridia (0.24 μmol/g residual dry weight in \textit{C. perfringens}) is found to be lower than that in Gram-negative bacteria (4.5 μmol/g in \textit{E. coli}), animals (16–25 μmol/g in rat liver), and plants (4.8 μmol/g in spinach leaves) (57). It is also possible that another thiol might be acting in this capacity and that our data is for glutathione, which is used by the enzyme.

As well, it is remarkable that investigations regarding pathways involved in the detoxification of MG in other Clostridia, such as \textit{C. beijerinckii} and \textit{C. difficile}, have suggested the glyceral dehydrogenases in these microorganisms as the main source of MG detoxification (58). Thus, it is possible that GlxI and GlxII, which exhibit similar biological role and use MG as a substrate, may play a lesser part in the detoxification process.

It has been determined that a protein’s amino acid length and sequence might be useful in predicting the probable metal specificity of a putative GlxI (14). GlxIs with shorter amino acid sequences (~130 amino acids in length) tend to be Ni\(^{2+}/Co\(^{2+}\)-activated (i.e. non-Zn\(^{2+}\)-activated), although longer ones (~180 amino acids in length) are likely to be Zn\(^{2+}\)-activated enzymes (14). These metal activation profiles may be explained by consideration of active site geometries. It has been proposed that only enzymes with octahedral metal environments are catalytically active, regardless of the metal activation class to which they belong (Zn\(^{2+}\)-activated or Zn\(^{2+}\)-inactive but Ni\(^{2+}/Co\(^{2+}\)-activated) (49, 50, 59). Our observations on CLO GlxI follow the same trend. The inactive Zn\(^{2+}\)-bound CLO GlxI form has a trigonal bipyramidal metal coordination, whereas its active Ni\(^{2+}\)-reconstituted form possesses an octahedral geometry in the active site. Hence, from all information on previously investigated GlxI, it appears possible to accurately predict the metal activation of a GlxI family member based on the metal coordination and amino acid sequence of the enzyme. The structural findings reported in this study indicate that metal activation profiles in this class of enzyme hold true across diverse quaternary structure arrangements. The arrangement in the active site must be critical to the chemical mechanism of GlxI, but as yet remains elusive with respect to the exact chemistry of this enzyme, although proposals have been put forth (50, 60–62).

Several proteins such as GlxI, MMCE, estradiol dioxygenase, fosfomycin resistance protein, bleomycin resistance protein, and mitomycin C resistance protein share a \(\beta\beta\beta\beta\) motif, suggesting evolutionary relatedness within this superfamily (61, 63). It was hypothesized that the dimeric members of this superfamily share the same one-module ancestor, whereupon gene duplication, gene fusion, and various mutations resulted in these proteins sharing high structural similarity but possessing low sequence homology. These enzymes may use this evolutionarily conserved structural fold as a platform but bind a diverse set of metal ions and ligands and change the active site geometry, thereby supporting diverse functions.

A proposed early event is the gene modification of a single motif ancestor to give a G2-symmetric metallo dimer that links (\(\beta\beta\beta\beta\)) and (\(\beta\beta\beta\beta\)) motifs through metal-binding ligands (model A in Fig. 5). The symmetry of the metal-binding ligands is inherent from the symmetry in the paired \(\beta\beta\beta\beta\) motifs. No known examples of a GlxI resembling model A is currently known, however. These metal-bound motifs then could evolve to form a more stable two-domain pseudosymmetric metallo-monomer as a result of gene duplication and fusion. This arrangement has indeed been detected in the metastable monomeric Zn\(^{2+}\)-activated \textit{P. putida} GlxI (model B in Fig. 5) pro-
duced from its more stable dimeric form in the presence of excess glutathione. This “monomeric” form has been found to be catalytically active, although it slowly reforms the dimer, which has substantially more catalytic activity (13). This active monomer is considered a precursor of the back-to-back dimeric structure that has been observed for MMCE and now for CLO GlxI (model C in Fig. 5), which suggests that this protein may have evolved earlier than other homodimeric proteins in this superfamily. Another dimeric arrangement may have occurred at the subunit interface and resulted in an edge-to-edge fusion of (βαββ)1 of one subunit with (βαββ)2 of another subunit, a possible result of gene modification and arrangement (model D in Fig. 5). Examples of the proteins in this model are small GlxI (i.e. GlxI from E. coli and H. sapiens), small estradiol dioxygenases, fosfomycin resistance protein, bleomycin resistance protein, and mitomycin C resistance protein. Among these proteins, MMCE and GlxI show a very close relationship. Both are metalloenzymes with four metal-binding residues, forming an octahedral geometry when bound to their activating metal ions. However, despite their structural similarity, this study has demonstrated that CLO GlxI does not exhibit MMCE activity nor do other Zn2+-activated (P. aeruginosa GloA3 and yeast GlxI) and Ni2+/Co2+-activated (P. aeruginosa GloA2) GlxI. These results indicate that GlxI is very specific to the hemithioacetal substrate of MG-GSH and that overall molecular structure alone cannot be used to unambiguously predict substrate selection.

GlxI and MMCE may have preceded the tridentate enzymes (three metal-binding ligands) such as estradiol dioxygenase and FosA that catalyze more complex bisubstrate reactions. Loss of one of the four metal-binding ligands provides further functional diversification by opening an additional coordination site to support the particular chemical reaction. Other proteins in this structural class such as the bleomycin resistance proteins and the mitomycin C resistance proteins are neither enzymes nor metalloproteins. The metal binding ability of the progenitor of these resistance proteins may have been omitted in favor of a more hydrophobic cavity to accommodate the antibiotic. Interestingly, in the case of mitomycin C resistance protein, its apo-form (PDB code 1KMZ) possesses a similar dimeric structure as CLO GlxI and MMCE, in which a binding pocket locates entirely within a single subunit (64, 65). However, its drug-bound structure exhibits two binding pockets at the dimeric interface (PDB code 1KLL) (65). The mechanism of altering the dimeric structure upon binding of the antibiotics, however, remains unknown.

These small dimeric two-subunit modules are believed to be progenitors of the larger monomeric four-domain proteins such as the large monomeric GlxI and several estradiol dioxygenases (schematically represented as model E and F in Fig. 5). Yeast and P. falciparum GlxI, for example, are large single polypeptide enzymes, wherein the N terminus resembles its C-terminal domain, and form two active sites within one polypeptide chain (48, 66). The N-terminal domain in these large GlxI likely resembles one subunit of the small GlxI, which consists of two domains (the N- and C-terminal domains). However, instead of having two polypeptide chains with identical amino acid sequences (one polypeptide with two domains) as in the small GlxI, these large GlxI contain one four-domain polypeptide. As there is no crystallographic structure available for a large GlxI, the chemical structure of its catalytic pockets remains unknown.

An attempt was made to identify other putative GlxI proteins with a similar βαββ topology to CLO GlxI in the Protein Data Bank. Among those analyzed, only two proteins, one from Bacillus halodurans (PDB code 3OA4) and the other from Listeria monocytogenes (PDB code 3E5D), were identified. One presented a dimeric structure that fits into model C in Fig. 5 (PDB code 3OA4), and the other has a molecular structure that fits into model D in Fig. 5 (PDB code 3E5D). Both might exhibit GlxI activity based on consideration of multiple sequence alignments and crystallographic structural analyses. A comparison of the structures of CLO GlxI (PDB code 2QH0) with the putative protein from B. halodurans (PDB code 3OA4) suggests that both proteins share high structural similarity with r.m.s.d. of 0.94 Å for 40 Ca pairs (supplemental Fig. S6). This protein also contains a Zn2+ atom in its active site, forming a trigonal bipyramidal geometry with four metal-binding residues and one water molecule around the metal center (supplemental material). One might hypothesize that this putative protein might indeed be a GlxI from another Gram-positive bacterium, which has a similar overall structure to CLO GlxI and is in the Ni2+/Co2+ activation class. Also, when one compares the structures of E. coli GlxI (PDB code 1FA5) with the second putative GlxI from L. monocytogenes (PDB code 3E5D), both proteins are found to possess similar overall structures with r.m.s.d. of 0.94 Å for 86 Ca pairs. This putative protein contains all four metal-binding residues. However, there is no metal observed in the active site for this reported molecular structure, thus we hypothesize that this protein might be a GlxI, but its metal activation would need to be clarified. Nevertheless, the actual
biological functions of these two proteins will require further investigation.

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REFERENCES

1. Cooper, R. A. (1984) Annu. Rev. Microbiol. 38, 49–68
2. Inoue, Y., and Kimura, A. (1995) Adv. Microbiol. Physiol. 37, 177–227
3. Richard, I. P. (1991) Biochemistry 30, 4581–4585
4. Vander Jagt, D. L., and Hunsaker, L. A. (2003) Adv. Microbiol. Physiol. 47, 105–119
5. Ariza, A., Vickers, T. J., Greig, N., Armour, K. A., Dixon, M. J., Eggleston, I. M., Fairlamb, A. H., and Bond, C. S. (2006) Biochem. Soc. Trans. 34, 1235–1240
6. Clugston, S. L., Barnard, J. F., Kinach, R., Miedema, D., Ruman, R., Daub, E., and, Honek, J. F. (1998) Biochemistry 37, 8754–8763
7. Beard, A. J., and Bello, F. (2000) Appl. Microbiol. Biotechnol. 53, 682–685
8. McCarthy, A. A., Baker, H. M., Shewry, S. C., Patchett, M. L., and Baker, E. N. (2001) Structure 9, 637–646
9. Podiliak, T., and Basch, M. E. (2004) Appl. Microbiol. Biotechnol. 63, 682–685
10. Chen, T., and Polisky, J. (2004) Phytopathology 94, 938–945
11. Brophy, P. M., Crowley, P., and Barrett, J. (1990) Int. J. Parasitol. 20, 259–261
12. Kino, K., Kuratsu, S., Noguchi, A., Kubo, M., Kozakawa, Y., Arai, T., Yagasaki, M., and Kimura, K. (2007) Biochem. Biophys. Res. Commun. 352, 351–359
13. Newton, G. L., Arnold, K., Price, M. S., Sherrill, C., Delcardayre, S. B., Aharonowitz, Y., Cohen, G., Davies, J., Fahey, R. C., and Davis, C. (1996) J. Bacteriol. 178, 1990–1995
14. Liyanage, H., Kashket, S., Young, M., and Kashket, E. R. (2001) Appl. Environ. Microbiol. 67, 682–685
15. Chen, Z. Y., Brown, R. L., Damann, K. E., and Cleveland, T. E. (2004) Phytopathology 94, 938–945
16. Brophy, P. M., Crowley, P., and Barrett, J. (1990) Int. J. Parasitol. 20, 259–261
17. Kino, K., Kuratsu, S., Noguchi, A., Kubo, M., Kozakawa, Y., Arai, T., Yagasaki, M., and Kimura, K. (2007) Biochem. Biophys. Res. Commun. 352, 351–359
18. Newton, G. L., Arnold, K., Price, M. S., Sherrill, C., Delcardayre, S. B., Aharonowitz, Y., Cohen, G., Davies, J., Fahey, R. C., and Davis, C. (1996) J. Bacteriol. 178, 1990–1995
19. Liyanage, H., Kashket, S., Young, M., and Kashket, E. R. (2001) Appl. Environ. Microbiol. 67, 682–685
20. Chen, T. Y., Brown, R. L., Damann, K. E., and Cleveland, T. E. (2004) Phytopathology 94, 938–945
21. Brophy, P. M., Crowley, P., and Barrett, J. (1990) Int. J. Parasitol. 20, 259–261
22. Kino, K., Kuratsu, S., Noguchi, A., Kubo, M., Kozakawa, Y., Arai, T., Yagasaki, M., and Kimura, K. (2007) Biochem. Biophys. Res. Commun. 352, 351–359
23. Newton, G. L., Arnold, K., Price, M. S., Sherrill, C., Delcardayre, S. B., Aharonowitz, Y., Cohen, G., Davies, J., Fahey, R. C., and Davis, C. (1996) J. Bacteriol. 178, 1990–1995
24. Liyanage, H., Kashket, S., Young, M., and Kashket, E. R. (2001) Appl. Environ. Microbiol. 67, 682–685
25. Chen, Z. Y., Brown, R. L., Damann, K. E., and Cleveland, T. E. (2004) Phytopathology 94, 938–945
26. Brophy, P. M., Crowley, P., and Barrett, J. (1990) Int. J. Parasitol. 20, 259–261
27. Kino, K., Kuratsu, S., Noguchi, A., Kubo, M., Kozakawa, Y., Arai, T., Yagasaki, M., and Kimura, K. (2007) Biochem. Biophys. Res. Commun. 352, 351–359
28. Newton, G. L., Arnold, K., Price, M. S., Sherrill, C., Delcardayre, S. B., Aharonowitz, Y., Cohen, G., Davies, J., Fahey, R. C., and Davis, C. (1996) J. Bacteriol. 178, 1990–1995
29. Liyanage, H., Kashket, S., Young, M., and Kashket, E. R. (2001) Appl. Environ. Microbiol. 67, 682–685
30. Chen, Z. Y., Brown, R. L., Damann, K. E., and Cleveland, T. E. (2004) Phytopathology 94, 938–945
31. Brophy, P. M., Crowley, P., and Barrett, J. (1990) Int. J. Parasitol. 20, 259–261
32. Kino, K., Kuratsu, S., Noguchi, A., Kubo, M., Kozakawa, Y., Arai, T., Yagasaki, M., and Kimura, K. (2007) Biochem. Biophys. Res. Commun. 352, 351–359
33. Newton, G. L., Arnold, K., Price, M. S., Sherrill, C., Delcardayre, S. B., Aharonowitz, Y., Cohen, G., Davies, J., Fahey, R. C., and Davis, C. (1996) J. Bacteriol. 178, 1990–1995
34. Liyanage, H., Kashket, S., Young, M., and Kashket, E. R. (2001) Appl. Environ. Microbiol. 67, 682–685
35. Chen, Z. Y., Brown, R. L., Damann, K. E., and Cleveland, T. E. (2004) Phytopathology 94, 938–945
36. Brophy, P. M., Crowley, P., and Barrett, J. (1990) Int. J. Parasitol. 20, 259–261
37. Kino, K., Kuratsu, S., Noguchi, A., Kubo, M., Kozakawa, Y., Arai, T., Yagasaki, M., and Kimura, K. (2007) Biochem. Biophys. Res. Commun. 352, 351–359
38. Newton, G. L., Arnold, K., Price, M. S., Sherrill, C., Delcardayre, S. B., Aharonowitz, Y., Cohen, G., Davies, J., Fahey, R. C., and Davis, C. (1996) J. Bacteriol. 178, 1990–1995
39. Liyanage, H., Kashket, S., Young, M., and Kashket, E. R. (2001) Appl. Environ. Microbiol. 67, 682–685
40. Chen, Z. Y., Brown, R. L., Damann, K. E., and Cleveland, T. E. (2004) Phytopathology 94, 938–945
41. Brophy, P. M., Crowley, P., and Barrett, J. (1990) Int. J. Parasitol. 20, 259–261