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ARTICLE *in* JOURNAL OF INORGANIC BIOCHEMISTRY · NOVEMBER 2011

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Ni^{2+}-activated glyoxalase I from *Escherichia coli*: Substrate specificity, kinetic isotope effects and evolution within the βαββββ superfamily

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ARTICLE INFO

Article history:
Received 21 July 2011
Received in revised form 11 October 2011
Accepted 11 November 2011
Available online 28 November 2011

Keywords:
Glyoxalase
Escherichia coli
Thiol cofactor
Substrate specificity
Kinetic isotope effect
Fosfomycin resistance protein

ABSTRACT

The *Escherichia coli* glyoxalase system consists of the metalloenzymes glyoxalase I and glyoxalase II. Little is known regarding Ni^{2+}-activated *E. coli* glyoxalase I substrate specificity, its thiol cofactor preference, the presence or absence of any substrate kinetic isotope effects on the enzyme mechanism, or whether glyoxalase I might catalyze additional reactions similar to those exhibited by related βαββββ structural superfamily members. The current investigation has shown that this two-enzyme system is capable of utilizing the thiol cofactors glutathionylspermidine and trypanothione, in addition to the known tripeptide glutathione, to convert substrate methylglyoxal to non-toxic D-lactate in the presence of Ni^{2+} ion. *E. coli* glyoxalase I, reconstituted with either Ni^{2+} or Cd^{2+}, was observed to efficiently process deuterated and non-deuterated phenylglyoxal utilizing glutathione as cofactor. Interestingly, a substrate kinetic isotope effect for the Ni^{2+}-substituted enzyme was not detected; however, the proton transfer step was observed to be partially rate limiting for the Cd^{2+}-substituted enzyme. This is the first non-Zn^{2+}-activated GlxI where a metal ion-dependent kinetic isotope effect using deuterium-labelled substrate has been observed. Attempts to detect a glutathione conjugation reaction with the antibiotic fosfomycin, similar to the reaction catalyzed by the related superfamily member FosA, were unsuccessful when utilizing the *E. coli* glyoxalase I E56A mutein.

1. Introduction

The intracellularly generated metabolite methylglyoxal (MG, 2-oxopropanal) acts as a potent electrophile causing irreparable cellular damage if allowed to build to cytotoxic concentrations [1–6]. The glyoxalase (Glx) system is an enzyme couple critical for the detoxification of MG. The system consists of two enzymes, glyoxalase I (GlxI, *S*-D-lactoylglutathione lyase; EC 4.4.1.5) and glyoxalase II (GlxII, *S*-2-hydroxyacylglutathione hydrolase; EC 3.1.2.6) (Fig. 1) [7]. The first enzyme, GlxI, converts the hemithioacetal, a product formed by the non-enzymatic reaction of MG and a thiol cofactor/cosubstrate such as glutathione (GSH; γ-Glu-Cys-Gly), to *S*-D-lactolyglutathione. This thioester is then hydrolyzed by the second enzyme, GlxII, to produce a non-toxic 2-hydroxycarboxylic acid in the form of D-lactate and the regenerated thiol cofactor. There is evidence that the intermediate, *S*-D-lactoylglutathione, can control intracellular pH in bacteria through interaction with the KefB potassium efflux system [8].

There are several different thiol cofactors found in various organisms. GSH is the most prominent intracellular thiol in eukaryotes and some prokaryotes [9–12]. This thiol is involved in many biological functions including aromatic metabolism, maintenance of cellular redox potentials and several detoxification mechanisms [11,13]. In *E. coli*, a novel GSH and spermidine (spd) conjugate was discovered and designated as glutathionylspermidine (GspdSH, Fig. 1). This complex glutathione and its disulfide form (glutathionylspermidine disulfide, $(GspdS)_2$), under anaerobic conditions or stationary phase, compose approximately 80% of all produced GSH in *E. coli* [14,15]. An elevated level of GspdSH production is found to be in proportion to bacterial cell density [14,15], while the amount of $(GspdS)_2$ is decreased when cellular stress conditions prevail. GspdSH is also recognized to act more efficiently toward protecting cells against radical or oxidant-induced damage than its disulfide form [14,16]. Analysis of these experimental results suggests that GspdSH could be a reasonable alternate cofactor for the Glx system under these conditions. Interestingly, the glyoxalase enzymes are also conserved in organisms that produce

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doi:10.1016/j.jinorgbio.2011.11.008

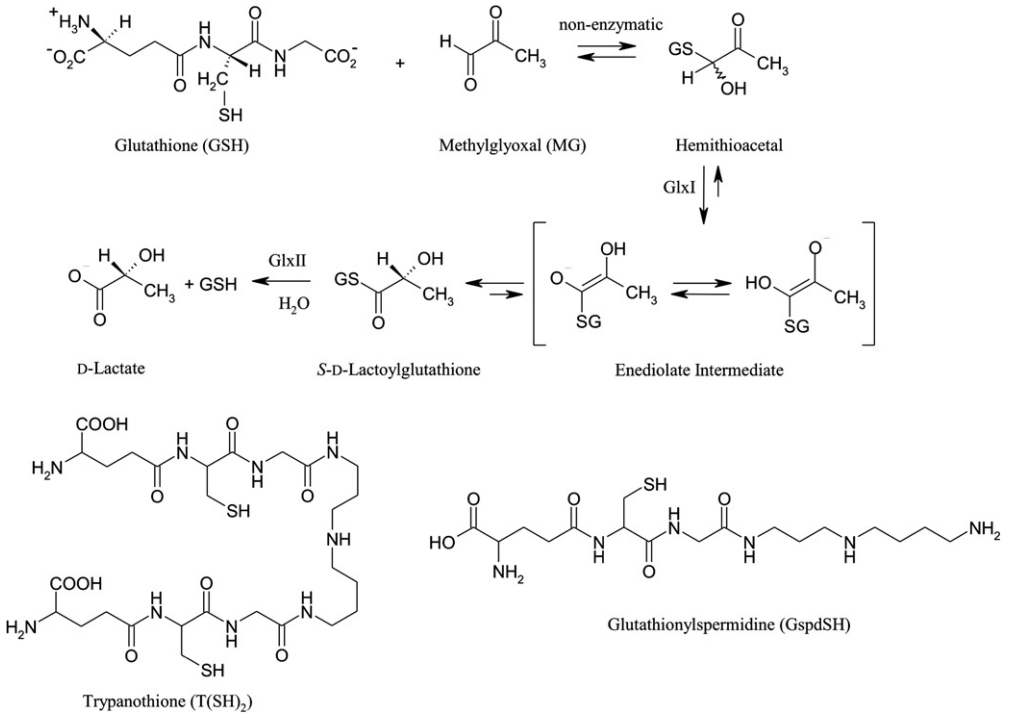

Fig. 1. The two-enzyme glyoxalase system, which consists of glyoxalase I (GlxI) and glyoxalase II (GlxII), using methylglyoxal (MG) and different thiols including glutathione (GSH), glutathionylspermidine (GspdSH) and trypanothione ($T(SH)_2$).

major intracellular thiols other than GSH. Recent studies on *Leishmania* and *Trypanosoma* have indicated that their corresponding Glx systems have evolved to use the bis-glutathionyl derivative of spermidine, trypanothione ($T(SH)_2$, N^1,N^8-bisglutathionylspermidine) (Fig. 1), the predominant thiol of these organisms, as a cofactor [17–21].

The Glx system has been investigated with respect to mechanistic, structural and metabolic aspects. GlxI enzymes are metalloenzymes that can be divided into two classes according to their metal activation profile, Zn^{2+}-activation (i.e., *H. sapiens* GlxI [22]) or Ni^{2+}/Co^{2+}-activation (non-Zn^{2+}-activated i.e., *E. coli* GlxI [23]). The active metallated forms of these enzymes require a metal environment that is octahedral in nature, with four protein residues serving as metal ligands, along with two water molecules completing the geometry [24–26]. This occurs for the Zn^{2+}-bound active human GlxI as well as the catalytically active Ni^{2+}-bound *E. coli* GlxI. However, the inactive Zn^{2+}-bound form of the *E. coli* GlxI approximates a trigonal bipyramidal geometry around the Zn^{2+} ion with only one water molecule present [26]. Mechanistically it is suggested, based on analysis of the X-ray structures of inhibitors bound to the *H. sapiens* GlxI, that the arrival of the hemithioacetal substrate at the catalytic site results in the replacement of the two active site water ligands by the two oxygens from the hemithioacetal substrate. This is likely followed by a series of deprotonation, reprotonation steps (Fig. 1), resulting in thioester product formation [24,25]. This mechanism is supported by reported solvent isotope incorporation studies as well as observations of a kinetic isotope effect using deuterated α-ketoaldehydes with yeast GlxI (Zn^{2+}-activated enzyme) [27,28]. To date, no studies have been reported that investigate the Ni^{2+}-activation class of GlxI in this manner.

GlxI is a member of the βαββββ superfamily of proteins, consisting of fosfomycin resistance protein (FosA), methylmalonyl-CoA epimerase (MMCE), extradiol dioxygenase (DIOX), mitomycin C resistance protein (MRP) and bleomycin resistance protein (BRP) [29–31]. These proteins are believed to have evolved from a common one-module ancestor, which, upon gene duplication and fusion as well as through accumulated mutagenesis, resulted in high structural similarity but with different biological functionality within this superfamily [29,30,32]. Among these structural members, FosA is mechanistically close to GlxI, as both are metalloenzymes that utilize GSH as a cofactor/cosubstrate, and both enzymes form an active site octahedral geometry around the metal center (Fig. 2) [24–26, 33, 34]. However, FosA employs three water molecules and three metal binding ligands that coordinate to the metal center via vicinal chelation [33,35], while GlxI utilizes two water molecules and four metal binding residues around the active metal [26]. FosA employs two metal binding subsites, one for the catalytically critical metal Mn^{2+} and another for a monovalent K^+ (Fig. 3) [36–38]. GlxI, on the other hand, utilizes only one metal binding site (Fig. 3) [24–26].

Fosfomycin, the substrate of FosA, is an antibiotic useful in the treatment of lower urinary tract and gastrointestinal infections due to its stability and broad spectrum of activity [39–42]. This antibiotic affects both Gram-positive and Gram-negative bacteria by interfering with MurA, the first enzyme in the bacterial cell wall biosynthetic pathway [42], a target that is lacking in humans. FosA likely evolved from "housing keeping" gene(s) within bacterial cells to resist the cytotoxicity of the natural product fosfomycin. FosA activates the epoxide moiety in fosfomycin for nucleophilic attack by glutathione, resulting in the formation of an inactive GS-fosfomycin adduct (Fig. 2). We hypothesize that FosA activity might be engineered from some proteins in the same superfamily, more specifically GlxI, due to similarities between these enzymes as stated above.

Fig. 2. The reaction catalyzed by the metalloenzyme fosfomycin (FM) resistance protein (FosA), in the presence of both divalent and monovalent metals. The enzyme catalyzes the formation of the product glutathione-fosfomycin adduct (GS-FM).

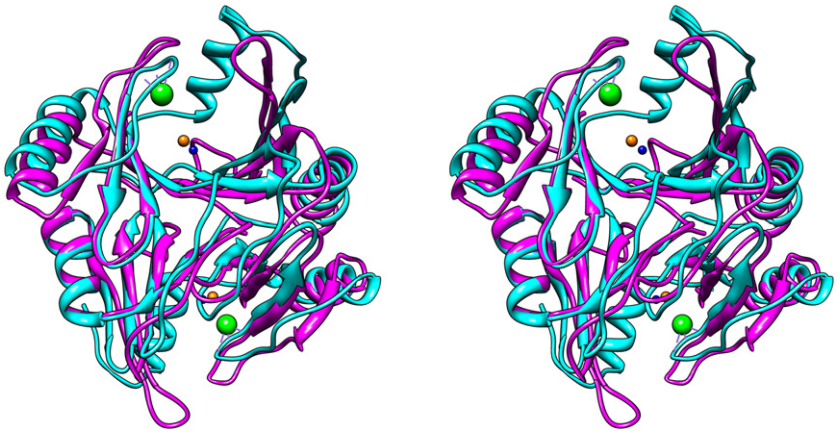

Fig. 3. A three-dimensional figure of the superimposed structures of *P. aeruginosa* FosA (cyan, PDB ID: 1LQP) and *E. coli* GlxI (magenta, PDB ID: 1F9Z) showing the active site metals including K^+ (green) and Mn^{2+} (orange) in FosA and Ni^{2+} (blue) in GlxI (wall-eyed view).

Herein, we report an investigation of the cofactor specificity of the *E. coli* Glx system using a set of thiol cofactors (GSH, GspdSH and $T(SH)_2$) to probe the extent of cofactor promiscuity by these *E. coli* enzymes and to obtain additional insight into the molecular interactions that occur between substrate and metalloprotein. As well, we report our studies on evaluating the deuterium kinetic isotope effect (KIE) on *E. coli* GlxI catalysis by utilizing α-deuteriophenylglyoxal (α-deuterioPG) or non-deuterated phenylglyoxal (PG) as substrates and GSH as the cofactor. To determine if the extent of any KIE could be metal ion dependent, both Ni^{2+}- as well as Cd^{2+}-activated GlxI were utilized in these studies. Lastly, intrigued by the mechanistic relatedness between the enzymes GlxI and FosA, we prepared the E56A mutant of *E. coli* GlxI in order to produce a GlxI mimicking the metal coordination geometry of FosA. Fosfomycin conjugation competency was evaluated for this GlxI mutein in the presence of various M^{2+} metal ions.

2. Experimental

2.1. Materials

Chemical reagents and enzymes were acquired from Sigma Chemical Company (St. Louis, MO) as follows: L- and D-lactate dehydrogenases, fosfomycin (disodium salt), bovine serum albumin (BSA), methylglyoxal (MG), phenylglyoxal (PG) and reduced glutathione (GSH). The thiols, $T(SH)_2$ and GspdSH, were purchased in their disulfide forms from Bachem Bioscience Inc. (King of Prussia, PA). Metals were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) including zinc chloride (assay, 99.3%), cobalt chloride hexahydrate (assay, 100.4%), manganese chloride tetrahydrate (assay, 98.8%) and cadmium chloride (assay, 99.4%). Magnesium chloride hexahydrate (assay, 99.2%) and calcium chloride dihydrate (assay 75.5%) were purchased from BDH Chemicals Ltd. (Poole, England). Nickel chloride hexahydrate (99.9999% pure) and cupric chloride hydrate (>99.0%) were supplied from Sigma Aldrich (St. Louis, MO). Water used in all experiments was Milli-Q water (18 MΩ-cm conductivity, Waters Associates, Milford, MA).

Protein visualization on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 8–15% acrylamide gradient gel was performed on a PhastSystem (Pharmacia, Etobicoke, ON). The enzymatic assay of GlxI was performed on a SpectraMax 190 96-well UV–visible spectrophotometer (Molecular Devices, Sunnyvale, CA) using a Soft Max Pro software (Molecular Devices). The activity assays for GlxII were performed on a Cary 3 UV–vis spectrophotometer (Varian, Mississauga, ON) using the Kinetics module of CaryWinUV version 3.0 (Varian, Mississauga, ON).

2.2. DNA cloning and manipulation

In order to mimic the active site of FosA, the multiple sequence alignment of GlxI from various organisms was performed in comparison with FosA from *Serratia marcescens* (Supplementary Information Fig. S1). There are four conserved metal binding residues in the active site of GlxI; however, only three exist in FosA. The active site residue, Glu^{56}, in *E. coli* GlxI corresponds to a smaller residue, Cys^{48}, in the *S. marcescens* FosA, which has only three protein residues ligating the metal center based on X-ray analysis. The lack of one active site metal binding residue in FosA compared to GlxI supports the two-substrate reaction of FosA, which requires more catalytic space around the metal ion than the one-substrate reaction of GlxI. Thus, Glu^{56} in *E. coli* GlxI was mutated to Ala to produce a somewhat similar ligand arrangement around the metal center as found in FosA, as well as to avoid side reactions that might occur with an oxidizable thiol group if a Cys^{56} mutation was used instead. The site-directed mutagenesis of *E. coli* GlxI-E65A was generated utilizing a QuikChange® polymerase chain reaction (PCR) protocol with an expression vector containing the *E. coli glxI* gene (pET22b-*glxI*) as previously described [23] and the following primers; (+) 5′-ACCGAAGAAGCGGTGATTGCCCT-GACCTACACCTGGGGC-3′ and (−) 5′-GCCCCAGTTGTAGGTCAGGGCAA TCACCGCTTCTTCGGT-3′. The plasmid was transformed into heat shocked competent *E. coli* DH5α cells and its sequence was verified (Molecular Biology Core Facility, University of Waterloo, Waterloo, ON) before transforming the plasmid into heat shocked *E. coli* BL21 (DE3) cells for protein expression purposes.

2.3. Protein purification

Protein purifications, apo-enzyme preparations and metal analyses including the 4-(2-pyridylazo)resorcinol (PAR) assays and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) of protein samples were performed according to those previously described [23,43,44]. The concentrations of samples of purified enzymes were calculated using the Bradford Assay with bovine serum albumin (BSA) as standard [45]. Protein molecular masses were determined by analyses of SDS-PAGE, gel permeation chromatography (Superdex75 HR 10/30 column with flow rate of 0.5 mL/min) results, and analysis of positive ion mode-electrospray ionization mass spectrometry (ESI-MS) results (Micromass Q-TOF Global Ultima mass spectrometer at the Mass Spectrometry Facility, University of Waterloo, Waterloo, ON).

2.4. GlxI activity assays

Reduction of the cofactor disulfides to their required thiol forms, $T(SH)_2$ and GspdSH, were carried out using a tris[2-carboxyethyl]

phosphine hydrochloride (TCEP) immobilized reducing gel (Thermo Fisher Scientific Inc., Rockford, IL) [17] under argon with metal-free HEPES buffer (pH 7.4) [23]. The thiol solution was extracted in degassed and argon sparged water and titrated to pH 7.0 using 0.1 mM HCl, also under inert atmosphere. The free thiol was quantitated by Ellman's reagent (5′,5′-dithiobis-(2-nitrobenzoic acid) or DTNB) using the detection at 412 nm and the ε_{412} of 14,150 M^{-1} cm^{-1} [46]. This free thiol solution was immediately frozen under argon and stored at −20 °C.

The enzyme assays were performed as previously reported [17,23,43,44] using the hemithioacetal formation between MG and thiols (GSH, GspdSH and $T(SH)_2$) in KBP (pH 7.0 or stated otherwise) with dissociation constant of 3.1 mM [47–52] on a SpectraMax 190 96-well UV–visible spectrophotometer and Soft Max Pro software (Molecular Devices, Sunnyvale, CA). The initial rate data were fitted to a Michaelis–Menten equation with least squares fit parameters using GraphPad Prism software version 5.00 for Windows (GraphPad Software, Inc., San Diego, CA, www.graphpad.com) or GraFit software version 3.01 (Erithacus Software).

2.5. GlxII activity assays

Assays to measure GlxII activities were performed as previously described [53,54]. The substrate for GlxII, *S*-D-lactoylthioester, was prepared by incubating *E. coli* GlxI with the hemithioacetal formed between MG and thiol cofactors (GSH, $T(SH)_2$ and GspdSH) for 1 h at room temperature. The enzyme was then removed by centrifugation using a Nanosep device (Millipore, Billerica, MA) with 3 kDa molecular weight cut-off. The thioester in the flow through fraction was quantitated by detection at 240 nm with ε_{240} of 2860 M^{-1} cm^{-1} [23].

L- and D-lactate dehydrogenase (LDH) assays were used to determine whether the D-stereo isomer of lactic acid was produced by the *E. coli* Glx system using *S*-lactoylGSH, *S*-lactoylTSH and *S*-lactoylGspdSH as substrates. The GlxII assays using these substrates were performed as stated above, and the completion of the reactions was monitored at 240 nm. The reaction mixture was then filtered using a Nanosep device with 10 kDa molecular weight cut-off, and the flow through fraction containing lactate was used for the LDH assays. The enzymes (2.75 units), L-LDH (EC 1.1.1.27) and D-LDH (EC 1.1.1.28), were incubated with the solution (350 μL) of isolated lactate and NAD^+ (5 mM) in 100 mM Tris buffer (pH 8.5) at 25 °C for 1 h. The assay activity was monitored at 340 nm, and the NADH formed was quantitated using the ε_{340} of 6220 M^{-1} cm^{-1} [55,56].

2.6. Assay for FosA activity

The assay for FosA activity was performed as previously reported using DTNB [33]. Fosfomycin (10 mM) was mixed with GSH (15 mM) in buffer containing 50 mM HEPES (pH 8.0) and 100 mM KCl that had been degassed under argon for 30 min prior to the reaction. The metal-reconstituted GlxI-E56A (4 μM) that was prepared by incubating the apo-enzyme with metal chloride (50 μM of $NiCl_2$, $CoCl_2$, $CuCl_2$, $ZnCl_2$, $MnCl_2$, $CdCl_2$, $MgCl_2$ or $CaCl_2$) overnight at 4 °C was added to the GSH–fosfomycin mixture (final volume of 100 μL) before incubating at room temperature under argon for 3 h. The reaction was then quenched by an addition of methanol (300 μL). The free thiol of GSH was quantitated by mixing 5 μL of the quenched reaction, 10 μL of 18 mM DTNB solution and 185 μL HEPES buffer before monitoring at 412 nm with the ε_{412} of 14,150 M^{-1} cm^{-1} [46].

An alternative qualitative assay was performed using the analysis of ESI-MS experiments. The reaction of metal-reconstituted ($NiCl_2$ and $MnCl_2$) GlxI-E56A (14.4 μM) was mixed with fosfomycin (150 mM) and GSH (150 mM) in HEPES buffer (pH 8.0) containing 100 mM KCl to a final volume of 373 μL under argon. The reaction was incubated at 30 °C for 3 h before quenching by the addition of 500 μL of methanol. The product was purified on a Sephadex G-10 26/40 column using gravity flow and fractions were monitored at 240 nm. The purified GS-fosfomycin adduct was identified by ESI-MS. This reference compound was chemically synthesized using a reported method [38] with and without microwave assistance in a Biotage Initiator system (Biotage, LLC; Charlotte, NC, USA).

2.7. Substrate kinetic isotope effect (KIE) experiments

The synthesis of α-deuteriophenylglyoxal was performed as previously described in the literature [28]. The chemical structure of the purified product was confirmed using ESI-MS and proton nuclear magnetic resonance (^1H NMR) on a Bruker 300 MHz spectrometer. This deuterated substrate was used in the GlxI assay in comparison with its non-deuterated counterpart to observe the proton transfer in the enzyme reaction as was previously undertaken for studies on Zn^{2+}-activated yeast GlxI [28]. The hemithioacetal was permitted to form (K_d of 0.6 mM [28]) between these substrates (PG and α-deuterioPG) and GSH as cofactor in 50 mM KPB (pH 7.0) at 25 °C for 10 min prior to performing the assay (1 mL). *E. coli* GlxI (16.8 nM), which had been reconstituted with either 2.5 molar equivalents of $NiCl_2$ or $CdCl_2$ was added into the reaction mixture, and the activity was monitored as the disappearance of the hemithioacetal at 263 nm, the isobestic wavelength for the free PG and the hemithioacetal [28]. The kinetic parameters were determined using the extinction coefficients for PG (ε_{240} = 5690 M^{-1} cm^{-1}) and α-deuterioPG (ε_{240} = 4740 M^{-1} cm^{-1}) [28,57].

2.8. Molecular modeling

Molecular graphics images and superimpositions of protein structures (using default settings) were produced using the UCSF Chimera package (version 1.5.3) from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIH P41 RR001081).

3. Results and discussion

3.1. Efficiency of thiol cofactor utilization by the E. coli Glx system

The discovery of a GlxI isolated from *E. coli* that was selectively activated by Ni^{2+}/Co^{2+}, and not by Zn^{2+} ion, was the first indication that two different classes of GlxI metalloenzymes existed in nature [23,26]. Previously only Zn^{2+}-activated GlxI enzymes had been reported, although these enzymes exhibited wide metal ion promiscuity, even activation by Ni^{2+} ion was observed. The ability of *E. coli* GlxI to bind, but not be activated by, Zn^{2+} ion and to be maximally activated by Ni^{2+} ion was subsequently shown to occur for certain other GlxI as well [43,44,53]. To date, the hemithioacetal formed non-enzymatically between GSH and MG has been the only substrate studied with the *E. coli* Glx metalloenzymes. However, significant amounts of GspdSH are biosynthesized by *E. coli* under increasing cell turbidity and stress, which normally occur under growth limiting conditions [14,15]. As well, other parasitic organisms such as *Trypanosoma* and *Leishmania* possess a GlxI system that utilizes alternative thiols, such as $T(SH)_2$, instead of GSH (Fig. 1) [18]. These protozoans are able to utilize the precursor of $T(SH)_2$, GspdSH, as a cofactor for their Glx reactions. The $T(SH)_2$-dependent Glx systems favor the GspdSH cofactor over $T(SH)_2$, while their enzymatic activities with GSH are very low [17,18]. Interestingly, not all protozoans prefer GspdSH and $T(SH)_2$ as cofactors for their Glx systems. The malarial parasite, *Plasmodium falciparum*, possesses a Glx system that utilizes GSH as cofactor [58]. The differences in thiol cofactor preference for various Glx systems depend on the cellular levels of specific thiols within a particular organism. *Trypanosoma* and *Leishmania* predominantly utilize trypanothione and employ a $T(SH)_2$-dependent Glx system. Interestingly, the *Leishmania major* GlxI enzyme utilizes

trypanothione as its cosubstrate and the enzyme is activated selectively by Ni^{2+} (inactive with Zn^{2+}), similar to *E. coli* GlxI [53].

Based on these considerations, it was reasonable to hypothesize that *E. coli* GlxI might utilize GspdSH as a cofactor when available in the cell. In this current investigation, GspdSH, and also $T(SH)_2$, were indeed found to be reasonable cofactors for Ni^{2+}-activated *E. coli* GlxI (Table 1). Analyses of the kinetic data indicated that the activities (k_{cat}) of Ni^{2+}-activated *E. coli* GlxI with the hemithioacetal substrates formed by reaction of MG with each of the thiol cofactors, $T(SH)_2$ and GspdSH, decreased drastically to approximately 18% in the case of GspdSH and 7% in the case of $T(SH)_2$, when compared with the natural GSH cofactor. However, the K_m for the hemithioacetal substrates formed from GspdSH and $T(SH)_2$ were found to be similar. The catalytic efficiencies (k_{cat}/K_m) determined for the hemithioacetal substrates formed from GspdSH and $T(SH)_2$ were only 5% and 2% of that of the GSH-hemithioacetal. Thus, a less sterically demanding GSH appeared to be more acceptable to the enzyme active site than the larger GspdSH and $T(SH)_2$ (Supplementary Information and Fig. S2). Although trypanothione is not present in *E. coli*, it is interesting to note the capability, albeit low, of *E. coli* GlxI to utilize this thiol.

In the second step of the Glx reaction, the thioester product is hydrolyzed by the metalloenzyme GlxII to produce D-lactate and regenerate the thiol cofactor. This enzyme in *E. coli* is a Zn^{2+} metalloenzyme [54]. In trypanosomes, thioesters of GspdSH, $T(SH)_2$ and GSH are able to act as the substrates for GlxII with the trypanothione thioester being an efficient substrate for the *Trypanosoma brucei* and *Leishmania donovani* GlxII [56,59]. Similar results were observed for *E. coli* GlxII in the present study, where the enzyme's activities for the various *S*-D-lactoyl thiol conjugates did not significantly differ from *S*-D-lactoylglutathione itself, and suggested that GlxII has lower substrate specificity than GlxI (Table 1). However, the K_m value of *S*-D-lactoyl$T(SH)_2$ as substrate with *E. coli* GlxII was increased to 226%, while that of *S*-D-lactoylGspdSH as substrate was reduced to only 24% compared to that of *S*-D-lactoylGSH as substrate. Thus, the highest efficiency of *E. coli* GlxII was observed using *S*-D-lactoylGspdSH as substrate. Analysis of this data suggested that GspdSH present in *E. coli* under various conditions could indeed be used as a cofactor to detoxify cytotoxic MG in combination with the bacterial glyoxalase system. The *E. coli* Glx system was also able to utilize $T(SH)_2$ even though this thiol is not produced within the bacterial cells. This finding is consistent with the close structural relationship held among the GlxII enzymes from different organisms (Supplementary Information and Fig. S3).

Although the Zn^{2+}-activated class of glyoxalase I enzymes are only known to produce the *S*-D-lactoylthioester as product, no study has investigated the stereospecificity of the Ni^{2+}-activated class of GlxI. Based on our experiments with Ni^{2+}-activated *E. coli* GlxI used in tandem with the Zn^{2+}-activated *E. coli* GlxII, and utilizing L-LDH as well as the D-LDH to detect the stereochemistry of the resulting lactic acid product produced from the Glx system using MG and the various thiols (GSH, GspdSH and $T(SH)_2$), only D-lactate was detected as an increase in wavelength intensity at 340 nm upon the formation of NADH. No activity was detected using either L-LDH with the same final product mixture nor using controls lacking GlxI. Analysis of these results indicated that the Ni^{2+}-activated class of GlxI has the same stereospecificity as the Zn^{2+}-activated class of GlxI, a result consistent with the stereospecificity reported for the aforementioned enzymes from the *Leishmania*, *H. sapiens*, and *Trypanosoma* organisms, among others [14, 60–62]. An exception is the Glx system from the African trypanosome, *T. brucei* [63], where GlxI appeared to be absent and L-lactate was produced as a major product in this organism.

3.2. Metal-dependent substrate deuterium isotope effect for E. coli GlxI

Primary kinetic isotope effect (KIE) experiments were undertaken in order to determine the effect of deuterium substitution on the rate of the GlxI reaction mechanism as catalyzed by a Ni^{2+}-activated GlxI class enzyme. The enzyme from *E. coli* was chosen as the prototype of this class as the apo-enzyme can be readily prepared and easily reconstituted with activating metals such as Ni^{2+}, Co^{2+}, Mn^{2+} and Cd^{2}, with Ni^{2+} substitution producing the most active enzyme ($k_{cat} = 338\ s^{-1}$; $K_m = 27 \pm 0.4\ \mu M$; $k_{cat}/K_m = 12.4 \times 10^6\ M^{-1}\ s^{-1}$) and Cd^{2+} producing the least active holoenzyme ($k_{cat} = 21\ s^{-1}$; $K_m = 9 \pm 0.4\ \mu M$; $k_{cat}/K_m = 2.4 \times 10^6\ M^{-1}\ s^{-1}$) using MG and GSH [23,64]. Metal removal from the Ni^{2+}/Co^{2+}-activated enzyme (i.e., *E. coli* GlxI) can be readily accomplished using ethylenediaminetetraacetic acid (EDTA) or isoelectric focusing; the apo-enzyme being catalytically inactive [23]. On the other hand, the Zn^{2+}-activated class of enzyme (i.e., *H. sapiens* GlxI) requires stronger conditions for metal removal such as use of dipicolinic acid (DPA) to remove bound metals. Metal reconstitution of the apo-enzyme from *Pseudomonas aeruginosa* GloA3 (a Zn^{2+}-activated GlxI enzyme) for example, only recovers approximately 80% activity in the presence of Zn^{2+} compared to the pre-DPA treated enzyme [44]. Thus, *E. coli* GlxI is a more suitable model for probing potential metal-ion dependent characteristics of the catalyzed reaction than a Zn^{2+}-activated class enzyme. As well, a potential metal-dependent effect on the KIE of a GlxI of any class has, to our knowledge, never been reported. We therefore chose to investigate substrate-based isotope effects on both the Ni^{2+}- as well as the Cd^{2+}-activated *E. coli* GlxI. For various technical reasons and for comparison with the literature when available, phenylglyoxal as well as its α-deuterioPG analog were used as substrates in these KIE investigations. Additionally, the X-ray structures of both the Ni^{2+}- and the Cd^{2+}-substituted have been determined (Supplementary Information Fig. S4) and have reported highly similar metal ligand arrangements around the metal centers; however, several metal coordination distances differed between the two metallated forms of the enzyme (Supplementary Information Table S1).

In our experiments, the enzyme kinetics of either Ni^{2+}- or Cd^{2+}-reconstituted GlxI were recorded for varying concentrations of the hemothioacetal substrates formed from MG, PG or α-deuterioPG

Table 1
Kinetics of *E. coli* GlxI and GlxII using different thiols (GSH, GspdSH and $T(SH)_2$) in the presence of methylglyoxal.

Cofactor[a]	GlxI[b]				GlxII[c]			
	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\ s^{-1}$)	Relative k_{cat}/K_m	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\ s^{-1}$)	Relative k_{cat}/K_m
GSH	344 ± 8	61 ± 4	5.6×10^6	100	61 ± 0	184 ± 22	0.3×10^6	23
GspdSH	61 ± 3	204 ± 39	0.3×10^6	5	58 ± 1	45 ± 0.1	1.3×10^6	100
$T(SH)_2$	24 ± 0	199 ± 25	0.1×10^6	2	70 ± 1	415 ± 11	0.2×10^6	15

[a] GSH is commercially available as the reduced-form; however, the disulfide forms of GspdSH and $T(SH)_2$ require reduction by immobilized TCEP prior to performing the kinetic assays (see text).
[b] The GlxI assays (200 μL) were performed using Ni^{2+}-reconstituted GlxI (3 nM, 48 h incubation at 4 °C with 2.5 equivalents of metal) and 0.04–0.7 mM hemithioacetal substrate in 100 mM KPB (pH 7.0) at room temperature.
[c] The GlxII assays (1 mL) were performed using Zn^{2+}-bound GlxII (70 nM) and 0.02–0.1 mM thioester substrate in buffer containing 100 mM KPB (pH 8.0) and 3.5 mM DTT.

and GSH as the cofactor (Table 2). For the Ni^{2+}-activated *E. coli* GlxI, the enzyme efficiencies using non-deuterated and deuterated PG-GSH substrates were determined to be similar, suggesting that there was no substrate deuterium kinetic isotope effect on the k_{cat} nor on the K_m for this metallated form of the *E. coli* enzyme. These data were in contrast to the effect of the α-hydrogen substitution for the same substrates with the yeast GlxI enzyme. The yeast GlxI enzyme was observed to exhibit substrate deuterium isotope effects of 3.3 and 3.2 on K_m and k_{cat}, respectively, which suggested that proton-transfer from substrate is a major contributor to the rate-determining step for this particular enzyme [28]. Assuming the mechanism of Ni^{2+}-substituted *E. coli* GlxI is the same as for the Zn^{2+}-activated GlxI class enzymes, the substrate isotope effect could have been obscured by another step in the catalytic mechanism. This step would not have been detectable by the experiments typically used to evaluate the contribution of the proton transfer to substrate conversion.

The activities of the Cd^{2+}-substituted *E. coli* GlxI using PG-GSH and α-deuterioPG-GSH substrates contrasted markedly with the results observed for the Ni^{2+}-holoenzyme. A drastic decrease in catalytic activity of the Cd^{2+}-substituted enzyme was observed using the deuterated aldehyde. This significant reduction in substrate conversion rate for the Cd^{2+}-substituted enzyme indicated the presence of a kinetic isotope effect for this form of the enzyme when handling PG-GSH as substrate. The K_m was also affected modestly by isotopic replacement in the aldehyde, while analysis of the observed $k_{cat(H)}/k_{cat(D)}$ value of 2.3 indicated that the α-proton transfer was partially rate-limiting in the enzymatic reaction. The effect of deuterium substitution on the ratio of k_{cat}/K_m was observed to be low (a value of 1.7) and did not indicate that the enzyme–substrate complex off-rate was influencing the value of the intrinsic isotope effect. The result was consistent with the interpretation that the proton abstraction was partially rate determining in the enzyme mechanism, and that other steps contributed to product formation as well.

Analysis of the kinetic data obtained for the Ni^{2+}- and the Cd^{2+}-activated forms of the *E. coli* GlxI enzyme with the hemithioacetal substrates MG-GSH and PG-GSH indicated that both metal activation classes were comparable in efficiently processing the bulkier PG aldehyde co-substrate. The activities of the Ni^{2+}-reconstituted enzyme with the hemithioacetals, MG-GSH and PG-GSH, were found to be similar; however, the catalytic efficiency was approximately 4.8-fold lower for the reaction using PG-GSH as the substrate. Interestingly, the Cd^{2+}-reconstituted enzyme exhibited lower activity with the hemithioacetal of MG-GSH than with PG-GSH, which suggested that the substrate specificity of *E. coli* GlxI may be dependent upon the specific metal ion activating the enzyme. However, both metal-reconstituted forms exhibited a lower K_m for MG-GSH than for PG-GSH, indicating that the larger PG aldehyde did not interact as well with the active site of the enzyme compared to the less bulky MG molecule.

The differences in the kinetic results between Ni^{2+} and Cd^{2+} for the *E. coli* GlxI could be due to the difference in the extent of polarization that may occur with the bound substrate for each metal ion, Cd^{2+} being less capable of lowering the energy barrier to proton abstraction compared to Ni^{2+}, causing a lower catalytic rate to be observed for the Cd^{2+}-GlxI with MG and PG but also allowing for a KIE to be detected for Cd^{2+}-GlxI upon use of deuterated PG as substrate. An additional factor that may contribute in some fashion to the catalytic differences observed between the Ni^{2+} and the Cd^{2+} forms of GlxI is that of the difference in ligand substitution rates for each metal. Specifically, the rate of water–ligand exchange from Cd^{2+} centers is faster (~10^4×) than the exchange rates observed for water–ligands associated with Ni^{2+} centers [65]. The impact of water–ligand exchange rates on the overall rate of the GlxI-catalyzed reaction can be considered under the assumption that substrate binding occurs in the active site with bound metal via vicinal oxygen chelation with concomitant displacement of the resting-state water ligands. This has been suggested as a likely stage in the chemical mechanism of GlxI. Hydroxamate analogs, which are believed to mimic the enediolate (Fig. 1) intermediate(s) have been studied with respect to the Zn^{2+} center in *H. sapiens* GlxI as well as with the Ni^{2+} center in *E. coli* GlxI [25,66]. In both cases displacement of the aqua ligands around the metal center were observed, although it should be stated that hydroxamates may be poor choices for representatives of the reaction intermediates of GlxI due to their strong metal binding properties which might nullify their application as reaction intermediate analogs for GlxI. If the rate constant for association of the substrate hemithioacetal with the active site metal is low relative to the rate constant for the chemical deprotonation/reprotonation step, the KIE should be suppressed. The active site glutamate residue which serves as one of the metal ligands has been implicated as a potential catalytic base in the enzyme reaction (Glu^{122} in *E. coli* GlxI) [66,67]. It would be interesting to determine whether such an inhibitor has a different rate of association with a Cd^{2+} center versus a Ni^{2+} center in *E. coli* GlxI. The most notable observation from these kinetic experiments is that different metal ions in the active site of *E. coli* GlxI exhibit different kinetic isotope effects when an α-deuterio-substituted substrate is evaluated.

Table 2
Kinetics of metal reconstitutions (Ni^{2+} and Cd^{2+}) of *E. coli* GlxI using the different hemithioacetal substrates, including MG-GSH, PG-GSH and α-deuterioPG-GSH. The assays were performed using 0.02–0.5 mM hemithioacetal substrate in KPB (pH 7.0) at room temperature.

Metal	Substrates	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	$k_{cat(H)}/k_{cat(D)}$	$K_{m(H)}/K_{m(D)}$	$(k_{cat(H)}/K_{m(H)})/(k_{cat(D)}/K_{m(D)})$
Ni	MG-GSH[a]	338	27 ± 0.4	12×10^6	–	–	–
	PG-GSH	322	127 ± 23	2.5×10^6	0.85	0.76	1.1
	α-deuterioPG-GSH	381	168 ± 25	2.3×10^6			
Cd	MG-GSH[b]	21	9 ± 0.4	2.4×10^6	–	–	–
	PG-GSH	182	100 ± 25	1.8×10^6	2.28	1.37	1.64
	α-deuterioPG-GSH	80	73 ± 9	1.1×10^6			

V_{max} (μmol/min/mg): Ni^{2+}-GlxI (MG-GSH (676 ± 17), PG-GSH (648 ± 54), α-deuterioPG-GSH (766 ± 69)).
Cd^{2+}-GlxI (MG-GSH (43 ± 5), PG-GSH (366 ± 59), α-deuterioPG-GSH (161 ± 5)).
[a] Data obtained from Clugston et al. [23].
[b] Data obtained from Clugston et al. [64].

3.3. Structural relatedness between GlxI and FosA

The gene coding for FosA has been detected in the *P. aeruginosa* genome and shares 60% sequence identity with the plasmid encoded protein from transposon Tn2921 [68]. From this information, it is possible that genomically encoded FosA might have evolved from a protein having a similar fold that already existed in an ancestral organism and likely acted as a "housekeeping" enzyme. GlxI with its high structural similarity to FosA (Fig. 3) could be a possible candidate as a progenitor to FosA such that a gene duplication event followed by multiple rounds of mutations, deletions and insertions could have permitted the evolution of a GlxI molecular framework that developed fosfomycin inactivating properties. In an attempt to investigate the mechanistic relatedness between these two proteins, the deletion of one of the metal binding ligands in GlxI was performed. Based on a multiple sequence alignment, the *E. coli* GlxI-E56A was chosen to produce a protein containing a similar metal ligand environment around the active site metal ion to what is found in the active sites of several FosA enzymes (1× Glu, 2× His and 3 aqua ligands surrounding the metal center) (Fig. 4; Supplemental Information and Fig. S5). The single point mutation on *E. coli glxI* was generated (E56A), and the existence of the purified mutein was confirmed by analysis of its electrospray mass spectrum, which exhibited the molecular weight of a single

subunit at 14861.5 Da (calculated MW is 14861.8 Da, data not shown). The mutein's molecular structure was shown to remain unchanged by a variety of biophysical experiments (Supplementary Information Fig. S6 and S7).

The enzymatic activity of the mutein was then studied to evaluate its glyoxalase I as well as its possible FosA-like activities. The Ni^{2+}-reconstituted *E. coli* GlxI-E56A protein possessed a similar metal activation profile for its glyoxalase I activity as does wild-type GlxI, where enzyme activity was detected for the Ni^{2+}- and Co^{2+}-reconstituted forms of the enzyme (Supplementary Information Fig. S8A). The maximum glyoxalase I activity for the E56A mutein was achieved with Ni^{2+}-reconstitution (k_{cat} of 11.5 s^{-1} and K_m of 0.68 ± 0.08 mM) and approximately 60% activity was observed with Co^{2+}-reconstitution (k_{cat} of 7.3 s^{-1} and K_m of 0.93 ± 0.17 mM). However, in comparison to wild-type *E. coli* GlxI with a reported k_{cat} of 338 s^{-1} [23], the activity of the mutein was reduced to only <4%. The enzyme reconstitution with other divalent metals such as Mn^{2+} and Cd^{2+} resulted in only trace GlxI activities. Analyses of metal titration profiles indicated that the enzyme required at least 10 equivalents of metal ion to attain maximum catalytic activity (Supplementary Information Fig. S8B). This mutein therefore has reduced metal ion affinity as a result of the mutation of one of the four metal binding residues. These results are consistent with studies undertaken on the *H. sapiens* (E99N) and *Pseudomonas putida* (E93D) GlxI wherein these corresponding mutations reduced enzymatic activities to 1.5% and <0.02% of corresponding wild type activities, respectively [67,69]. Metal analysis of the human GlxI E99N mutant indicated that the mutein only contained 0.3 mol zinc per subunit enzyme [67]. Thus, low metal content in the enzyme may explain the decrease in glyoxalase I activity for the *E. coli* GlxI-E56A mutein observed in the current study; however, saturating amounts of metal ions produced no more than <4% of the wild type activity.

The fosfomycin conjugating activity of the *E. coli* Glx-E56A mutein, however, was undetectable by use of an established DTNB assay, which previously had been successfully utilized to measure FosA activity [33]. The GlxI-E56A mutein was incubated in the presence of GSH, fosfomycin and various concentrations of a variety of metal ions for various lengths of time. The reaction was subsequently quenched by the addition of methanol. The unreacted GSH in the solution was then monitored by DTNB detection, thus the enzyme kinetics was examined in terms of the decrease in substrate thiol levels. However, the absorbance of the quenched reaction mixture were similar to background detection, suggesting that there was either little or no formation of the target GS-fosfomycin adduct by the GlxI-E56A mutein under the studied conditions. Variation of the divalent metals utilized ($NiCl_2$, $CoCl_2$, $CuCl_2$, $ZnCl_2$, $MnCl_2$, $CdCl_2$, $MgCl_2$ and $CaCl_2$) in the incubation did not result in any detectable fosfomycin-conjugating activity. As high background levels of detectable GSH were produced in this reported assay, an alternative method to detect the GS-fosfomycin adduct was also undertaken. This approach utilized a series of ESI-MS experiments. The products of the various enzyme incubation reactions were isolated utilizing Sephadex G-10 gel permeation chromatography to reduce background contamination. Chemically synthesized GS-fosfomycin adduct was utilized to determine product retention characteristics on the Sephadex G-10 column. The purified synthetic product-containing fractions were confirmed by ESI-MS analysis, for which the presence of a compound of the expected mass of 444 Da indicated the likely presence of the target GS-fosfomycin adduct. Analysis of the ESI-MS spectra indicated unresolved purification however, where GSH was found to co-elute with the compound whose mass corresponded to the expected GS-fosfomycin adduct. Detection of the adduct in the ESI-MS of the control experimental fractions (without addition of the E56A mutein) as well as the enzymatic reaction mixture were found to be similar, suggesting that small amounts of the GS-fosfomycin adduct could form without addition of the mutein. Analysis of the results suggested that the mutein had little or no detectable fosfomycin conjugating activity. The presence of varying amounts of divalent metals (Ni^{2+} and Mn^{2+}) did not alter the negative outcome of the experiments.

Even though the active site of *E. coli* GlxI was mutated in order to mimic the metal liganding environment present in FosA, other functional parts in the active site structure were different. Both share overall structural similarity; however, the differences can be clearly observed in the connecting loops (Fig. 3). As well, limited catalytic space in GlxI might not support the binding of the two substrates, fosfomycin and GSH. Several critical residues present in FosA but absent in the *E. coli* GlxI-E56A mutein likely further explain the absence of detectable fosfomycin-conjugating activity by GlxI-E56A (Supplementary Information Fig. S5).

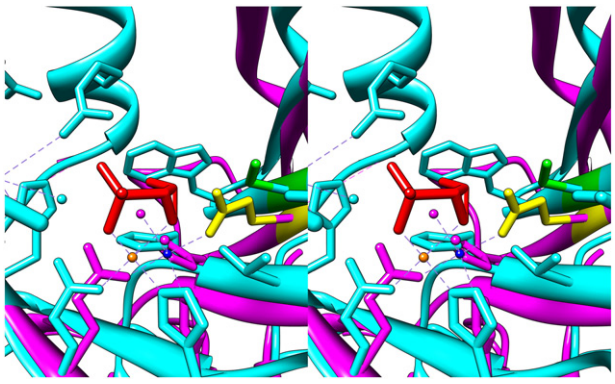

Fig. 4. A three-dimensional figure of the detailed active sites for the superimposed structures of *P. aeruginosa* FosA (cyan, PDB ID: 1LQP) and *E. coli* GlxI (magenta, PDB ID: 1F9Z) showing the active site metals Mn^{2+} (orange) in FosA and Ni^{2+} (blue) in GlxI. The oxygen atoms of the two water molecules bound to Ni^{2+} in the GlxI active site are magenta spheres. The mutated Glu56 in the GlxI is colored yellow and the corresponding residue in FosA, Cys48, is colored green. A fosfomycin molecule (red) bound to FosA in the X-ray structure is also shown to elucidate the fosfomycin binding area (wall-eyed view).

4. Conclusions

The current investigation has extended our understanding of the Ni^{2+}-activated GlxI class of metalloenzyme. The *E. coli* Glx system, composed of GlxI and GlxII, has been shown to utilize a variety of thiol cofactors. GSH, GspdSH and $T(SH)_2$ cofactors can be utilized to stereospecifically convert MG to D-lactate by the *E. coli* Glx system. As well, the metal ion-dependent presence of a deuterium isotope effect in *E. coli* GlxI is the first non Zn^{2+}-dependent GlxI enzyme characterized by such experimental approaches. An observable deuterium kinetic isotope effect has been detected for the Cd^{2+}-substituted *E. coli* GlxI indicating that α-proton transfer is partially rate-limiting in the enzymatic reaction.

Due to the high structural similarity between GlxI and FosA, it is possible that GlxI directed evolution experiments could produce FosA enzymatic activity in a GlxI enzyme. Although the current investigation was unable to detect FosA activity by mutation of a key metal liganding residue in the active site of *E. coli* GlxI, additional studies on the evolution of antibiotic (FosA) and toxic metabolite (Glx) resistance proteins from ancestral protoresistance proteins is a subject of exciting current interest [70] and worthy of further investigation.

Abbreviations

ESI-MS	electrospray ionization mass spectrometry
FosA	fosfomycin resistance protein A
Glx	glyoxalase
GSH	glutathione
GspdSH	glutathionylspermidine
$(GspdD)_2$	glutathionylspermidine disulfide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

ICP-MS	inductively coupled plasma mass spectrometry
KIE	kinetic isotope effect
D-LDH	D-lactate dehydrogenase
L-LDH	L-lactate dehydrogenase
MG	methylglyoxal
PAR	4-(2-pyridylazo)resorcinol)
PCR	polymerase chain reaction
PDB	protein data bank
PG	phenylglyoxal
Spd	spermidine
TCEP	Tris[2-carboxyethyl]phosphine hydrochloride
$T(SH)_2$	trypanothione (reduced state)

Acknowledgments

The authors would like to thank Dr. Richard Smith for electrospray ionization mass spectrometry analysis of the samples and Dr. Zhengding Su for sharing his knowledge and suggestions. This research was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) (JFH), University of Waterloo (Ontario, Canada), an NSERC graduate scholarship (to NS), the Government of Thailand (graduate scholarship to US) and the Government of China (visiting scientist award to YR). The funding sources had no involvement in any of the aspects of the experimental research, the interpretation of results or the preparation of this article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jinorgbio.2011.11.008.

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