

# Distinct classes of glyoxalase I: metal specificity of the *Yersinia pestis*, *Pseudomonas aeruginosa* and *Neisseria meningitidis* enzymes<sup>1</sup>

Nicole SUKDEO, Susan L. CLUGSTON<sup>2</sup>, Elisabeth DAUB and John F. HONEK<sup>3</sup>

Department of Chemistry, University of Waterloo, 200 University Avenue, Waterloo, Ontario, Canada N2L 3G1

The metalloisomerase glyoxalase I (GlxI) catalyses the conversion of methylglyoxal-glutathione hemithioacetal and related derivatives into the corresponding thioesters. In contrast with the previously characterized GlxI enzymes of *Homo sapiens*, *Pseudomonas putida* and *Saccharomyces cerevisiae*, we recently determined that *Escherichia coli* GlxI surprisingly did not display Zn<sup>2+</sup>-activation, but instead exhibited maximal activity with Ni<sup>2+</sup>. To investigate whether non-Zn<sup>2+</sup> activation defines a distinct, previously undocumented class of GlxI enzymes, or whether the *E. coli* GlxI is an exception to the previously established Zn<sup>2+</sup>-activated GlxI, we have cloned and characterized the bacterial GlxI from *Yersinia pestis*, *Pseudomonas aeruginosa* and *Neisseria meningitidis*. The metal-activation profiles for these additional GlxIs firmly establish the existence of a non-Zn<sup>2+</sup>-dependent

grouping within the general category of GlxI enzymes. This second, established class of metal activation was formerly unidentified for this metalloenzyme. Amino acid sequence comparisons indicate a more extended peptide chain in the Zn<sup>2+</sup>-dependent forms of GlxI (*H. sapiens*, *P. putida* and *S. cerevisiae*), compared with the GlxI enzymes of *E. coli*, *Y. pestis*, *P. aeruginosa* and *N. meningitidis*. The longer sequence is due in part to the presence of additional regions situated fairly close to the metal ligands in the Zn<sup>2+</sup>-dependent forms of the lyase. With respect to sequence alignments, these inserts may potentially contribute to defining the metal specificity of GlxI at a structural level.

**Key words:** glyoxalase, lyase, metal activation, metalloenzyme, nickel, zinc.

## INTRODUCTION

The glyoxalase system has been recognized for over 90 years as a critical enzymic detoxification route that is widely distributed across major taxonomic divisions [1–9]. This two-enzyme system accepts  $\alpha$ -oxoaldehydes ( $\alpha$ -ketoaldehydes) as substrates for conversion into corresponding  $\alpha$ -hydroxyacids [10]. GlxI [glyoxalase I; S-D-lactoylglutathione methylglyoxal lyase (isomerizing), EC 4.4.1.5] is a metalloisomerase that converts the non-enzymically formed MG (methylglyoxal)–GSH hemithioacetal adduct into the corresponding thioester (Scheme 1). This product is subsequently hydrolysed by glyoxalase II (S-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6) generating D-lactate and regenerating GSH [11].

GlxI is considered to be a member of the  $\beta\alpha\beta\beta$  and VOC (vicinal oxygen chelate) superfamilies [12–16]. The former superfamily is distinguished by proteins that possess tandem arrangements of  $\beta\alpha\beta\beta$  that form a cleft, which, in the case of GlxI, comprises the metal-binding active site. The VOC superfamily refers to a grouping of enzymes defined by the mechanistic feature of co-ordination by vicinal substrate oxygen atoms to the catalytic metal centre. The defining attributes of these two superfamilies as they manifest in GlxI imply the importance of metal-ion binding for the function of this lyase.

The GlxI enzyme has been isolated from several sources, mostly of eukaryotic origin. Characterization of GlxI enzymes, particularly those from *Homo sapiens* and *Saccharomyces cerevisiae*, has revealed selectivity for Zn<sup>2+</sup> activation [17,18]. In fact, the classification of GlxI as a Zn<sup>2+</sup>-dependent lyase has persisted. However, initial enzymological investigation of *E. coli* GlxI revealed that this metalloisomerase was not activated by

Zn<sup>2+</sup> ions, but instead was optimally activated in the presence of Ni<sup>2+</sup> ions [19].

The question arises as to whether Ni<sup>2+</sup> (or rather non-Zn<sup>2+</sup>) activation as observed in the *E. coli* enzyme is mirrored by GlxI enzymes from other bacterial sources, potentially indicating a second, previously unrecognized class of this metalloenzyme. A putative division of metal selectivity for GlxI enzymes on the basis of biological origin (eukaryotic compared with bacterial) could provide an interesting structure–function target for developing antibacterial agents, if this difference is proven and is biochemically exploitable, given the continuing interest in the glyoxalase system from a medicinal perspective [20–27]. However, the *Pseudomonas putida* (prokaryotic) GlxI has been shown to be a Zn<sup>2+</sup> enzyme, implying that a division in metal activation along evolutionary lines may not be quite so clearly defined [28]. To investigate this possibility, we have cloned and overexpressed several putative bacterial GlxI-encoding sequences from the micro-organisms *Yersinia pestis*, *P. aeruginosa* and *Neisseria meningitidis* [29].

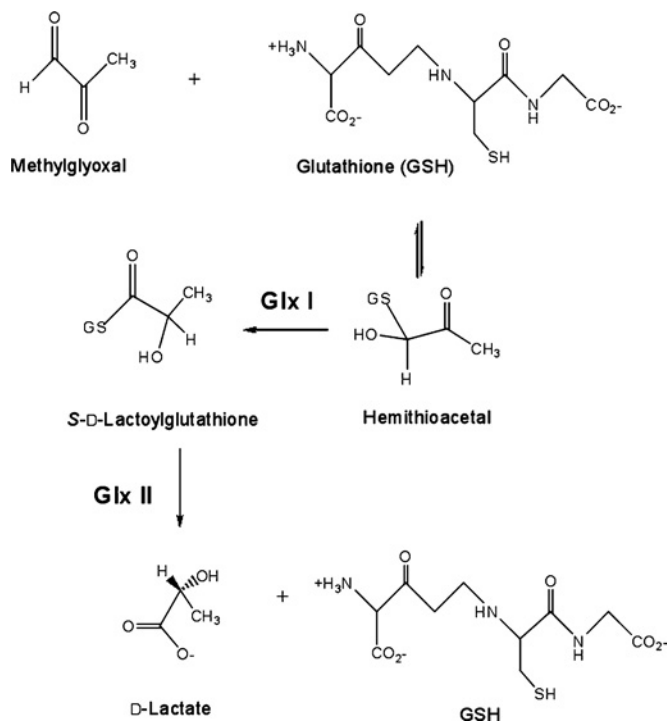
The results of our biochemical characterization of these purified enzymes indicate that the *Y. pestis*, *P. aeruginosa* and *N. meningitidis* putative GlxI sequences do indeed code for MG–GSH-isomerizing GlxI enzymes. Of seminal importance, our results show that these prokaryotic GlxI enzymes are maximally active in the presence of Ni<sup>2+</sup> and do not exhibit activity in the presence of Zn<sup>2+</sup>. This assembly of metal activation data establishes the existence of a previously undetected class of GlxI enzymes, specifically a class of non-Zn<sup>2+</sup>-dependent metalloisomerases compared with a Zn<sup>2+</sup>-dependent class. A biochemical study of GlxI in this respect exemplifies the limits of assigning enzymic characteristics from bioinformatic methods. Although amino acid

Abbreviations used: DTT, dithiothreitol; GlxI, glyoxalase I; MG, methylglyoxal; VOC, vicinal oxygen chelate.

<sup>1</sup> This paper is dedicated to Professor C. T. Walsh (Harvard Medical School, Boston, MA, U.S.A.) in celebration of his 60th birthday.

<sup>2</sup> Present address: Discovery Technology Center, Pfizer, Cambridge, MA 02139, U.S.A.

<sup>3</sup> To whom correspondence should be addressed (email jhonek@uwaterloo.ca).



**Scheme 1** Reactions of the glyoxalase system

sequence comparison was sufficient to assign putative GlxI functions to these proteins, metal activation could not be inferred except by investigation of the isolated enzyme. The characterization of these bacterial enzymes therefore represents a critical contribution to the identification of the scope of differential metal activation in a metalloenzyme class formerly considered to be exclusively  $Zn^{2+}$ -dependent.

## EXPERIMENTAL

### Materials

Nickel (II) chloride hexahydrate (99.9999% pure) was from Aldrich (Oakville, Ontario, Canada), cobalt chloride hexahydrate (assay, 100.4%; specification, 98–102%), cadmium chloride (assay, 99.4%), manganous chloride tetrahydrate (assay, 98.8%) and zinc chloride (assay, 99.3%) were obtained from J. T. Baker (Toronto, Ontario, Canada), and magnesium chloride hexahydrate (assay, 99.2%) and calcium chloride dihydrate (assay, 75.5%) was obtained from BDH (Toronto, Ontario, Canada).

Chelex 100 resin was obtained from Bio-Rad (Mississauga, Ontario, Canada). Q-Sepharose Fast Flow anion exchange resin was obtained from Amersham Biosciences (Uppsala, Sweden). For the isoelectric focusing stage of protein purification, a Bio-Rad Rotofor Isoelectric Focusing Unit was employed. This apparatus was used with Bio-Rad Rotolytes (pH 3.9–5.6), with a Mes/Gly-Gly ratio of 1:1. In purifying *N. meningitidis* GlxI, 200 mM Mopso [3-(*N*-morpholino)-2-hydroxypropanesulphonic acid; Sigma-Aldrich, St. Louis, MO, U.S.A.] and 200 mM  $\beta$ -alanine solutions (Sigma-Aldrich) were mixed at a 7:3 ratio for use as the isoelectric focusing solution as suggested by Bio-Rad [30]. Water used in all experiments was purified using a Milli-Q RG Ultrapure water system (18 M $\Omega$ -cm; Waters Associates, Milford, MA, U.S.A.).

## Methods

Protein samples were concentrated using an Amicon Ultra-filtration cell with a YM10 or PM10 membrane, or by using a Centricon 10 (Amicon, Beverly, MA, U.S.A.). Protein quantification was performed using the method of Bradford, with BSA as the standard as described previously [31].

### Bacterial strains and sources of template DNA

*E. coli* strains BL21( $\lambda$ DE3) and DH5 $\alpha$  were used as GlxI overexpression and plasmid propagation hosts respectively. *E. coli* strain MG1655 was used as an overexpression host for the variant *E. coli* GlxI enzymes. Genomic template DNA used for cloning of the *Y. pestis* GlxI gene was from the strain CO92. A sample of this DNA was obtained from Dr K. Isherwood at the Biomedical Sciences Department of Chemical and Biological Defence (CBD) establishment at Porton Down (Wiltshire, U.K.). Genomic DNA was isolated from wild-type *P. aeruginosa* strain PA01. This strain was obtained as a gift from Dr J. Lam of the University of Guelph (Ontario, Canada). Genomic DNA from a *N. meningitidis* clinical isolate was obtained from Dr D.A.A. Ala'Aldeen, from the Division of Microbiology at the University of Nottingham (Nottingham, U.K.) [32]. Although identification of a GlxI from *N. meningitidis* has been reported, detailed biochemical characterization of the enzyme was not carried out [32].

### Oligonucleotides and DNA sequencing

DNA primers for isolating GlxI-encoding genes of *Y. pestis*, *P. aeruginosa* and *N. meningitidis* were obtained from MOBIX (McMaster University, Hamilton, Ontario, Canada) or Sigma Genosys (Oakville, Ontario, Canada). Sequencing of the plasmid constructs for overexpression of *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI-encoding genes was performed by MOBIX.

### DNA manipulation

All manipulation of DNA and plasmid isolation was performed according to the protocols of Sambrook et al. [33].

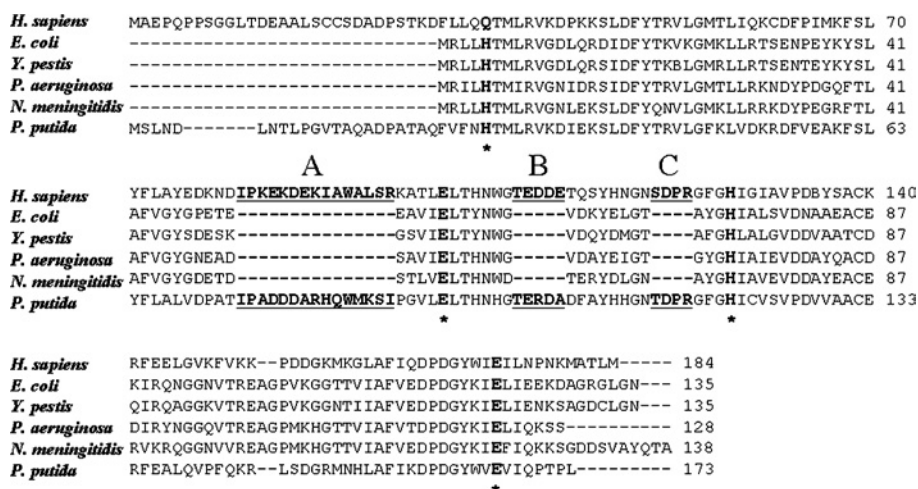
### Cloning of *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI-encoding genes

The putative GlxI DNA sequences used to design oligonucleotide primers were those determined from previous database searches [29]. The *Y. pestis* GlxI gene was amplified from the aforementioned template DNA by PCR, using the following primer pair: (+) 5'-CCAGAATTCCATATGCGCTTACTCCATACC-ATG-3', (−) 5'-CCAAAGCTTGGATCCTCAGTTTCCGAGGCA-GTCACC-3'.

The *P. aeruginosa* GlxI gene was amplified from the aforementioned template DNA by PCR, using the following primer pair: (+) 5'-CCAGAATTCCATATGCGCATTCTCCATACCA-TG-3', (−) 5'-CCAAAGCTTGGATCCTCAGGAAGACTTCT-GGATCAG-3'.

The *N. meningitidis* GlxI gene was amplified from the aforementioned template DNA by PCR, using the following primer pair: (+) 5'-CCACTGCAGCATATGCGCTTACTCCATACTA-TGC-3', (−) 5'-CCAGGATCCTCAGGCAGTTTGATAGGCAA-CC-3'.

All three amplification products and the pET22b expression vector (amp<sup>R</sup>, T7 *lac* promoter, florigin of replication; Novagen, Madison, WI, U.S.A.) were subjected to restriction endonuclease digestion with *Nde*I and *Bam*HI. The digestion products were



**Figure 1** Amino acid sequence alignment for *H. sapiens*, *E. coli*, *Y. pestis*, *P. aeruginosa*, *N. meningitidis* and *P. putida* GlxI enzymes

Conserved metal ligands are indicated in bold lettering with the position marked by an asterisk. The additional regions of the *H. sapiens* and *P. putida* GlxI enzymes are indicated in bold with underlining. The configuration of sequences in this alignment was obtained from a previous comparative study [29].

subsequently ligated to generate the overexpression constructs designated pYPG1, pPAG1 and pNMG1 respectively. These plasmids denote constructs carrying the cloned *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI-encoding genes respectively. All three plasmid constructs were transformed into *E. coli* BL21( $\lambda$ DE3) cells, yielding the final overexpression systems for these GlxI enzymes.

### Growth and induction of GlxI-overexpressing cells

The protocol for induction of *Y. pestis* [using cell line BL21( $\lambda$ DE3)/pYPG1] and *P. aeruginosa* [BL21( $\lambda$ DE3)/pPAG1] GlxI was similar to that used for induction of the *E. coli* GlxI-overexpression system [19], except that no NiCl<sub>2</sub> was added to the growth medium. Also, cells were induced with IPTG (isopropyl  $\beta$ -D-thiogalactoside) for only 4 h.

Growth and induction of BL21( $\lambda$ DE3)/pNMG1 for overexpression of *N. meningitidis* GlxI was also similar to the protocol outlined in [19], including the aforementioned modifications for BL21( $\lambda$ DE3)/pYPG1 and BL21( $\lambda$ DE3)/pPAG1. TB (terrific broth) was employed as the growth medium for BL21( $\lambda$ DE3)/pNMG1 as opposed to LB (Luria-Bertani) medium, and again the medium was not supplemented with NiCl<sub>2</sub>. Cells were harvested and stored as indicated in [19].

### Purification of *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI

Purification of GlxI from BL21( $\lambda$ DE3)/pYPG1, BL21( $\lambda$ DE3)/pPAG1 and BL21( $\lambda$ DE3)/pNMG1 cells was performed using the protocol optimized for *E. coli* GlxI [19], with the following modifications indicated in this section. For *Y. pestis* GlxI purification, the Q-Sepharose Fast Flow (Amersham Biosciences) elution buffers [no-salt: 20 mM Tris/HCl, 30% (v/v) glycerol, pH 7.0; high-salt: no-salt + 1 M KCl] and the storage buffer (50 mM Chelex-treated Mops, pH 7.0) contained DTT (dithiothreitol) at a final concentration of 1 mM. This reducing agent was employed since initial attempts at purifying *Y. pestis* yielded GlxI as a tetramer (confirmed by electrospray ionization MS and native PAGE), probably attributable to an intermolecular disulphide bond between dimers as a consequence of a second cysteine residue in the amino acid sequence at position 132 (Figure 1). This residue is likely to be an exposed cysteine, based on com-

parison with the *E. coli* GlxI X-ray crystal structure determined previously [34]. DTT concentrations were decreased by dilution of the GlxI sample with storage buffer (50 mM Chelex-treated Mops, pH 7) not containing the thiol reducing agent, such that DTT concentrations during enzymic assays did not inhibit the isomerization reaction (i.e. GSH concentration was at least 120-fold in molar excess of DTT during assay). Initial chromatographic purification of *N. meningitidis* GlxI was performed on a Q-Sepharose Fast Flow column with the no-salt and high-salt elution buffers. The GlxI-containing fractions were pooled and dialysed against 20 mM Tris, 10% (v/v) glycerol, pH 7.5, and then applied to a Mono-Q (Amersham Biosciences) column. The elution buffers used with this column were identical with those used in the initial chromatographic step, except that the glycerol content was 10% (v/v). The GlxI-containing fractions were pooled and dialysed into 10% (v/v) glycerol before being subjected to the isoelectric focusing step, with the modifications as indicated above. Purified apo-GlxI was concentrated and stored in 50 mM Chelex-treated Mops, pH 7.0, in acid-treated plasticware at 4 °C as described in [19].

### Molecular-mass determination

Electrospray ionization MS was used to confirm the molecular mass of the overproduced bacterial GlxI enzymes in crude lysate and in purified samples. Analysis was performed on protein samples using a Micromass Q-TOF Ultima Global (Manchester, U.K.) mass spectrometer at the Waterloo Chemical Analysis Facility, University of Waterloo. Samples were introduced using eluants consisting of 1:1 water/acetonitrile plus 0.2% methanoic (formic) acid. Electrospray ionization was performed in positive ion mode. The peaks of higher molecular mass following the major GlxI peaks in the obtained spectra were attributed to sodium adducts of the purified enzymes (see Figure 2).

SDS/PAGE was employed to screen for the presence of GlxI enzyme during purification. Separation (using precast homogeneous 20% gels) and visualization (Coomassie Brilliant Blue staining) of protein samples by SDS/PAGE was performed using a PhastSystem™ (Amersham Biosciences).

Gel-filtration chromatography was employed to assess whether the *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI enzymes were dimeric in native form, as is the case with the *E. coli*

enzyme. The *Y. pestis* and *P. aeruginosa* GlxI samples were purified for this analysis using Q-Sepharose Fast Flow and Mono-Q columns. The *N. meningitidis* GlxI analysed was prepared as an apoenzyme, as described above. A Superdex 75 10/30 column was employed for molecular-mass determination, using 20 mM Tris/HCl, pH 7, and 100 mM KCl as the eluent. The column was calibrated using commercially obtained protein standards.

### GlxI enzymic assay

The assays and assay conditions used for quantification of GlxI activity were identical with those described in [19]. Initial rate data was fitted by non-linear regression analysis using the software GraFit version 3.01 (Erithacus Software).

### Metal titration of apo-GlxI

Metal-titration experiments were conducted as an initial assessment of metal activation for the GlxI enzymes under study. The standard GlxI activity assay was used to survey activation when enzyme was pre-incubated with differing concentrations of metal chloride. A sample containing a particular GlxI enzyme was diluted in 50 mM Mops and metal chloride was added to an approximate range of 0–7 molar equivalents of metal to dimeric enzyme (see Figure 4). Assays were performed using a MG–GSH concentration of 0.5 mM, with 0.7–2.5  $\mu\text{g}$  of protein per assay (see Figure 4). The purified *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI enzymes were confirmed to be active as homodimers by gel-filtration chromatography (results not shown), as observed with *E. coli* GlxI (gel-filtration chromatography, X-ray diffraction methods) [19,34]. Activity assays were performed in triplicate at a substrate concentration of 0.5 mM, with samples assayed in order of increasing metal concentration. For the apoenzyme control assays, cuvettes were prepared by soaking in concentrated nitric acid for 10 min, followed by soaking in 1 mM EDTA for 10 min, followed by liberal rinsing with Chelex-treated Milli-Q water before assaying the first replicate. This pre-treatment of the cuvettes was administered to minimize recorded enzyme activity for the apo-control due to the tendency for metal abstraction from the cuvette itself [19,35]. Before the second and third replicates of the apo-control sample, cuvettes were soaked in 1 mM EDTA, followed by liberal rinsing with Chelex-treated Milli-Q water. No further cuvette treatment was employed for the remaining samples assayed during the titration.

### Metal-activation studies

Profiles for metal activation for each of the purified bacterial GlxI enzymes with various bivalent metal chlorides were obtained using the methods described previously [19,35]. Samples of a particular apo-GlxI were diluted in 50 mM Chelex-treated Mops, pH 7, and pre-incubated with either 10 molar equivalents of metal chloride or activated with a 0.5 mM final concentration of metal chloride in the enzyme dilution. Enzyme activity was assayed at a 0.5 mM substrate concentration in triplicate. Amounts of GlxI used per assay ranged from 0.2 to 0.9  $\mu\text{g}$ . Activation was assessed using the following metal chlorides:  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$ . It must be noted that assays for  $\text{Mn}^{2+}$  activation of *Y. pestis* GlxI were performed in Mes buffer. Also, the maximal activity values for  $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ - and  $\text{Zn}^{2+}$ -reconstituted *Y. pestis* GlxI were obtained from metal-titration data (see Figure 3).

### Determination of kinetic parameters

The Michaelis–Menten constants ( $K_m$ ) and maximal enzyme velocities ( $V_{\text{max}}$ ) for *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI

enzymes were determined experimentally by measurement of initial reaction rate [19]. Rate data was collected at substrate concentration ranges between 0.025 and 1 mM. Stock enzyme was diluted with 50 mM Chelex-treated Mops pH 7.0 and pre-incubated with  $\text{NiCl}_2$  or  $\text{CoCl}_2$  at a final concentration of 0.5 mM. Enzyme activity at each concentration was measured in triplicate. The entire set of triplicates was measured twice, and the values from the duplicate experiments were averaged to obtain the final parameter values.

## RESULTS

### Overexpression and purification of *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI enzymes

Robust expression of GlxI enzymes was observed with induction of all three overexpressing clones [BL21( $\lambda$ DE3)/pYPG1, BL21( $\lambda$ DE3)/pPAG1 and BL21( $\lambda$ DE3)/pNMG1]. Sequencing of all three plasmids confirmed that these GlxI-encoding genes were isolated in entirety with correct amplification of the template gene (results not shown). Metal activation for all three GlxI enzymes was determined on a preliminary basis using the crude lysate from small-scale induced cultures (25 or 50 ml). The lysates were assayed for GlxI activity in the presence of  $\text{NiCl}_2$  and  $\text{ZnCl}_2$  at final concentrations of 0.5 mM metal chloride. The results of this screening indicated that *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI sequences do indeed encode for GlxI enzymes capable of isomerizing a MG–GSH hemithioacetal substrate. Furthermore, the MG–GSH hemithioacetal isomerizing activity associated with all three lysates was enhanced in the presence of  $\text{NiCl}_2$ . This result also indicates that some of the *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI enzyme was produced in the apo form. Enhancement of enzyme activity was not observed during the assay of lysate in the presence of  $\text{ZnCl}_2$ . Observed relative increases in GlxI activity were based on comparison with lysate activity with no metal chlorides added to the substrate.

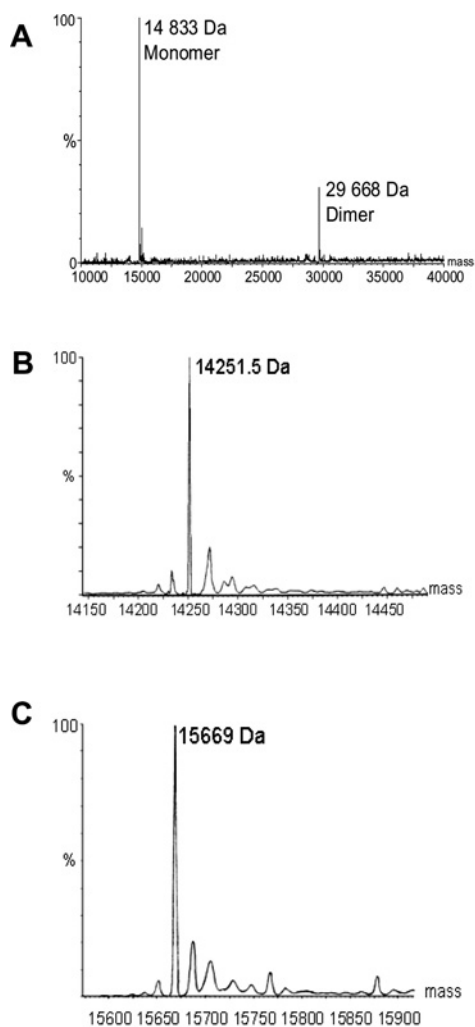
The protocol employed for purification of *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI enzymes was similar to that optimized for isolation of *E. coli* GlxI. The GlxI purification protocol as applied to *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI enzymes, yielded substantial quantities of each protein as purified isolates characterized by electrospray ionization MS.

The molecular mass of the *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI monomers were confirmed by electrospray ionization MS to be 14833 Da, 14251 Da and 15669 Da respectively, in agreement with their predicted values (14834 Da, 14251 Da and 15669 Da respectively; Figure 2). Although each of these enzymes was overproduced in an *E. coli* host strain, the electrospray ionization mass spectra did not indicate the presence of any *E. coli* GlxI (predicted mass, 14919 Da) in any of the purified enzyme preparations. Furthermore, endogenous levels of *E. coli* GlxI are extremely low [19,35].

### Relative metal activation of *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI enzymes

The results of these experiments are summarized in Figure 3. The propensity toward  $\text{Ni}^{2+}$ -activation of all three GlxI enzymes is readily apparent from these profiles. The series of activating metals ( $\text{Ni}^{2+} > \text{Co}^{2+} > \text{Mn}^{2+} > \text{Cd}^{2+}$ ) for each of these newly characterized bacterial GlxI enzymes is similar to that documented for the *E. coli* lyase. Comparison of these four bacterial GlxI enzymes together reveals some slight differences in relative activation conferred by the aforementioned metal ions.

The activation profiles for *Y. pestis* and *P. aeruginosa* GlxI enzymes show a similar trend towards activation with  $\text{Ni}^{2+}$  over

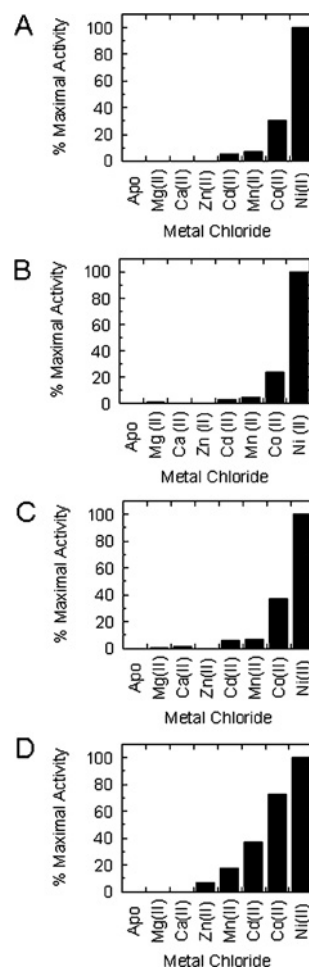


**Figure 2** Electrospray ionization mass spectra of (A) *Y. pestis* (reconstructed spectrum) (B) *P. aeruginosa* GlxI and (C) *N. meningitidis* GlxI enzymes

The expected molecular masses of the monomeric enzymes are 14834 Da, 14251 Da and 15669 Da respectively.

$\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Cd}^{2+}$ , as observed with *E. coli* GlxI (see Figure 3). Notable features of *N. meningitidis* GlxI are the higher levels of activation, specifically with  $\text{Co}^{2+}$ , but also with  $\text{Mn}^{2+}$  and  $\text{Cd}^{2+}$  relative to the maximal activity obtained by reconstitution with  $\text{Ni}^{2+}$  (Figure 3). Although it appears in this data set that the activity level in the presence of  $\text{Zn}^{2+}$  is elevated, no increase in activity relative to the apo-control was observed during titration of *N. meningitidis* GlxI with this metal (results not shown).

The second defining attribute of these characterized bacterial GlxI enzymes, which is of critical importance, is the non-activation of these apoenzymes in the presence of  $\text{Zn}^{2+}$ . This differing metal-dependence observed with *E. coli* GlxI in contrast with previously characterized GlxI enzymes (*H. sapiens*, *P. putida* and *S. cerevisiae*) is also exhibited by the *Y. pestis*, *P. aeruginosa* and *N. meningitidis* enzymes investigated. Titrations with  $\text{ZnCl}_2$  were also performed on *Y. pestis*, *P. aeruginosa* and *N. meningitidis* apo-GlxI, confirming that this bivalent ion is ineffectual as a co-factor, as no increase in activity was observed with increasing amounts of  $\text{Zn}^{2+}$  (results not shown).



**Figure 3** Profiles of apoenzyme reactivation with various bivalent ions for (A) *E. coli* [19] (B) *Y. pestis* (C) *P. aeruginosa* and (D) *N. meningitidis* GlxI enzymes

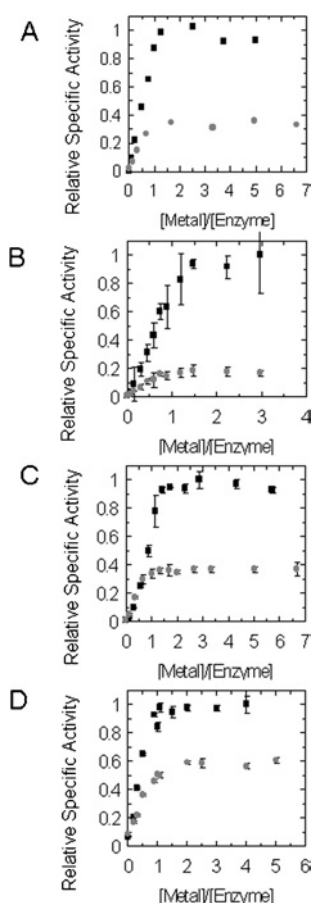
To obtain the relative maximal activity values for these graphs, baseline apo-control assays were subtracted from all activities so that relative maximal activity was calculated using the specific activity – apo-specific activity values.

### $\text{Ni}^{2+}$ and $\text{Co}^{2+}$ titration of *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI enzymes

These titrations in comparison with similar reconstitutions of *E. coli* apo-GlxI reveal the overall similarity in selectivity of  $\text{Ni}^{2+}$  over  $\text{Co}^{2+}$  for activation (in roughly similar proportions) (Figure 4). The higher relative  $\text{Co}^{2+}$  reconstitution of *N. meningitidis* GlxI observed in the previous activation profile is supported by the ratio of  $\text{Ni}^{2+}$  to  $\text{Co}^{2+}$  activation observed in the titration experiments. Furthermore, titrations for *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI in comparison with the *E. coli* enzyme reveal that all four enzymes are strong recruiters of metal ions, based on the low stoichiometric equivalents of  $\text{Ni}^{2+}$  or  $\text{Co}^{2+}$  relative to amount of enzyme, that confer maximal GlxI activation (Figure 4).

### Kinetic parameters of *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI enzymes

Initial comparison of the kinetic data obtained for the *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI enzymes reveals overall similarities in metal selectivity to *E. coli* GlxI (Table 1). Greater



**Figure 4** Titration of GlxI apoenzymes with various NiCl<sub>2</sub> (black squares) and CoCl<sub>2</sub> (grey circles) ratios: (A) *E. coli* [19], (B) *Y. pestis*, (C) *P. aeruginosa* and (D) *N. meningitidis* GlxI enzymes

Data points and error bars in (B), (C) and (D) represent the specific activity  $\pm$  S.D. for triplicate readings. Relative specific activity values were calculated as the proportion of activity relative to the highest specific activity values obtained during the NiCl<sub>2</sub> titration.

**Table 1** Kinetic parameters for *E. coli*, *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI enzymes with MG-GSH substrate

*E. coli* GlxI kinetic data with Ni<sup>2+</sup> was obtained from [19] and with Co<sup>2+</sup> was obtained from [35]. Results are means  $\pm$  S.D.

GlxI source	Metal chloride	$K_m$ ( $\mu$ M)	$V_{max}$ ( $\mu$ mol/min per mg of protein)	$k_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_m$ (M <sup>-1</sup> · s <sup>-1</sup> )
<i>E. coli</i>	Ni <sup>2+</sup>	27.0 $\pm$ 0.4	676 $\pm$ 17	338	1.2 $\times$ 10 <sup>7</sup>
	Co <sup>2+</sup>	12 $\pm$ 2	213 $\pm$ 33	106	8.8 $\times$ 10 <sup>6</sup>
<i>Y. pestis</i>	Ni <sup>2+</sup>	56.0 $\pm$ 0.6	618 $\pm$ 48	306	5.5 $\times$ 10 <sup>6</sup>
	Co <sup>2+</sup>	29 $\pm$ 5	140 $\pm$ 6	69	2.4 $\times$ 10 <sup>6</sup>
<i>P. aeruginosa</i>	Ni <sup>2+</sup>	32 $\pm$ 2	571 $\pm$ 28	271	8.5 $\times$ 10 <sup>6</sup>
	Co <sup>2+</sup>	16 $\pm$ 3	180 $\pm$ 7	86	5.4 $\times$ 10 <sup>6</sup>
<i>N. meningitidis</i>	Ni <sup>2+</sup>	45 $\pm$ 5	390 $\pm$ 5	204	4.5 $\times$ 10 <sup>6</sup>
	Co <sup>2+</sup>	28.0 $\pm$ 0.5	279 $\pm$ 24	146	5.2 $\times$ 10 <sup>6</sup>

activation with Ni<sup>2+</sup> over Co<sup>2+</sup> is characteristic of all four enzymes. In contrast, the selectivity between Ni<sup>2+</sup> and Co<sup>2+</sup> activation is narrowed for the *N. meningitidis* enzyme. The deviations in measured kinetic parameters ( $K_m$  and  $V_{max}$ ) of *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI are slight, not exceeding an order of magnitude in comparison with the *E. coli* lyase. The catalytic efficiency values ( $k_{cat}/K_m$ ) obtained for the *Y. pestis*,

*P. aeruginosa* and *N. meningitidis* GlxI enzymes indicate another similar attribute to that of *E. coli* GlxI, namely catalytic function at a close to diffusion-controlled rate (Table 1).

## DISCUSSION

We have previously investigated the *E. coli* GlxI, including enzymological aspects and three-dimensional structure, and our results revealed a novel metal-activation profile in contrast with previously studied GlxI enzymes [19,35–38]. However, further biochemical study of other prokaryotic GlxI enzymes was imperative to categorize the metal activation of *E. coli* GlxI, either as an exception to the previously established Zn<sup>2+</sup>-selectivity of this protein or as a member of a novel, but previously unrecognized, non-Zn<sup>2+</sup>-activated class. The characterization of three bacterial GlxI enzymes in addition to the *E. coli* enzyme has now established that non-Zn<sup>2+</sup>-activation of this lyase occurs within the prokaryotic domain of life. The results of this biochemical investigation indicate the existence of two classes of GlxI.

A structural basis for differential metal selectivity of GlxI enzymes might be implied from amino acid sequence analysis. Comparing the four metal ligands of *E. coli* (His<sup>5</sup>, Glu<sup>56</sup>, His<sup>74</sup> and Glu<sup>122</sup>) and *H. sapiens* GlxI (Gln<sup>34</sup>, Glu<sup>100</sup>, His<sup>127</sup> and Glu<sup>173</sup>), all positions are conserved with the exception of the His<sup>5</sup>/Gln<sup>34</sup> variation. The presence of a histidine residue at a position analogous to Gln<sup>34</sup> of the human enzyme can be observed in a number of bacterial GlxI sequences including those examined in the present study. This ligand variation does not represent an unambiguous correlation with metal activation however, since *P. putida* GlxI has a histidine residue at the 'Gln<sup>34</sup>' position and is Zn<sup>2+</sup>-dependent (Figure 1). Furthermore, enzymological studies of the *E. coli* H5Q (His<sup>5</sup>  $\rightarrow$  Gln) GlxI variant has shown that the 'humanized' active site promotes a minor shift towards Zn<sup>2+</sup> activation, yet confers a severely compromised affinity for all activating metal ions [35]. So in spite of the influence of this ligand on GlxI metal selectivity, it is clearly not a dominant contributor to promoting shifts in metal activation while maintaining the catalytic capacity of the enzyme.

The initial study of *E. coli* GlxI revealed marked sequence conservation to *H. sapiens* GlxI (36% identity) [19]. However, the *H. sapiens* and *P. putida* GlxI amino acid sequences are significantly longer than those for *E. coli*, *Y. pestis*, *P. aeruginosa* and *N. meningitidis* GlxI enzymes (Figure 1). Alignment of these bacterial GlxI sequences makes apparent the additional regions of the *H. sapiens* and *P. putida* lyases that are systematically absent in the non-Zn<sup>2+</sup>-dependent counterparts investigated here. The *P. putida* GlxI primary sequence more closely resembles the *H. sapiens* enzyme in this respect, in contrast with other bacterial GlxI sequences. The striking architectural similarity between the *E. coli* and *H. sapiens* GlxI active sites, however, does not provide any evidence by visual inspection of any obvious secondary or tertiary dissimilarities that would influence metal selectivity on a differential basis [39,34].

These findings emphasize that caution must be exercised in attempts at inferring enzymic attributes, in this case metal selectivity, from primary structure data. The consequences of assuming that zinc activation is a widespread feature of GlxI enzymes can be potentially deleterious in attempts for identifying this enzyme in other biological sources. Specifically, attempts at identifying GlxI activity in cell/tissue lysates using metal reconstitution would be best performed using a variety of bivalent metals during screening, which would take into account the potential for novel metal activation in newly identified forms of this metalloisomerase. This caveat would also extend to more systematic assessments of metal utilization of other metalloenzymes whose properties may or may

not resemble characterized counterparts from which its putative function was deduced.

In conclusion, our characterization of several bacterial GlxI enzymes has now firmly established the existence of two classes of this lyase that differ in metal activation. This difference in metal selectivity cannot yet be associated with any specific structural feature of GlxI enzymes, but this class division is a real occurrence amongst these lyases, only recently discovered after 91 years of investigation of the glyoxalase system. Further investigation of GlxI enzymes is essential from a structure–function perspective, to elucidate the fundamental structural parameters that have given rise to these two metal activation classes. Intense current interest is focused on the additional inserts found in the Zn<sup>2+</sup>-activated GlxI enzymes as potential mediators of metal specificity.

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