Overproduction and Characterization of a Dimeric Non-Zinc Glyoxalase I from *Escherichia coli*: Evidence for Optimal Activation by Nickel Ions[†,‡]

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ABSTRACT: The ubiquitous glyoxalase system converts toxic α-keto aldehydes into their corresponding nontoxic 2-hydroxycarboxylic acids, utilizing glutathione (GSH) as a cofactor. The first enzyme in this system, glyoxalase I (GlxI), catalyzes the isomerization of the hemithioacetal formed nonenzymatically between GSH and cytotoxic α-keto aldehydes. To study the *Escherichia coli* GlxI enzyme, the DNA encoding this protein, *gloA*, was isolated and incorporated into the plasmid pTTQ18. Nucleotide sequencing of the *gloA* gene predicted a polypeptide of 135 amino acids and M_r of 14 919. The *gloA* gene has been overexpressed in *E. coli* and shown to encode for GlxI. An effective two-step purification protocol was developed, yielding 150−200 mg of homogeneous protein per liter of culture. Electrospray mass spectrometry confirmed the monomeric weight of the purified protein, while gel filtration analysis indicated GlxI to be a homodimer of 30 kDa. Zinc, the natural metal ion found in the *Homo sapiens* and *Saccharomyces cerevisiae* GlxI, had no effect on the activity of *E. coli* GlxI. In contrast, the addition of $NiCl_2$ to the growth medium or to purified *E. coli* apo-GlxI greatly enhanced the enzymatic activity. Inductively coupled plasma and atomic absorption analyses indicated binding of only one nickel ion per dimeric enzyme, suggesting only one functional active site in this homodimeric enzyme. In addition, the apoprotein regained maximal activity with one molar equivalence of nickel chloride, indicative of tight metal binding. The effects of pH on the kinetics of the nickel-activated enzyme were also studied. This is the first example of a non-zinc activated GlxI whose maximal activation is seen with Ni^{2+}.

The ubiquitous glyoxalase system is composed of two enzymes, glyoxalase I [GlxI; *S*-D-lactoylglutathione methylglyoxal lyase (isomerizing), EC 4.4.1.5] and glyoxalase II (GlxII; *S*-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6) (*1*). The first enzyme of this system, GlxI,[1] is typically a zinc metalloprotein which catalyzes the isomerization of the hemithioacetals formed nonenzymatically between glutathione (GSH) and cytotoxic α-keto aldehydes, such as methylglyoxal (MG) (Figure 1). Recent work has shown that GlxI from various sources accepts both isomers of the hemithioacetal substrate (*2*−*5*). Studies have indicated that this reaction proceeds through an enediol(ate) intermediate

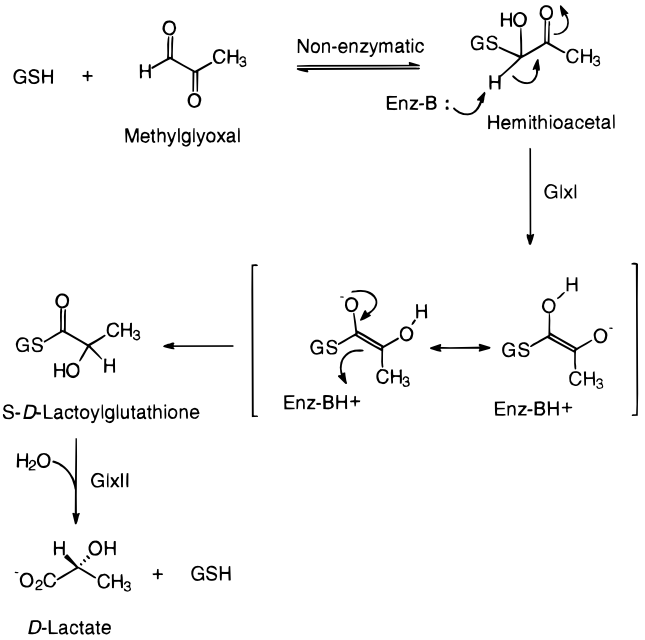

FIGURE 1: The glyoxalase system.

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[1] Abbreviations: AA, atomic absorption; Amp, ampicillin; Carb, carbenicillin; DHAP, dihydroxyacetone phosphate; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; EPR, electron paramagnetic resonance; ESMS, electrospray mass spectrometry; *gloA*, DNA encoding *E. coli* glyoxalase I; GlxI, glyoxalase I; GlxII, glyoxalase II; GSH, glutathione; ICP, inductively coupled plasma; IPTG, isopropyl 1-thio-β-D-galactopyranoside; LB, Luria−Bertani; MES, 2-(*N*-morpholino)ethanesulfonic acid; MG, methylglyoxal; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; OD, optical density; PMSF, phenylmethylsulfonyl fluoride; PVDF, poly(vinylidene difluoride); ^{R}, resistance/resistant; SD, Shine−Dalgarno sequence; SDS−PAGE, sodium dodecyl sulfate−polyacrylaminde gel electrophoresis; TBE, Tris-borate-EDTA buffer; TIM, triosephosphate isomerase; Tris, tris(hydroxymethyl)aminomethane.

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(*6*), and that the substrate interacts directly with a metal-bound water molecule (*7*, *8*). GlxII is responsible for the hydrolysis of the GSH-thioester product of the GlxI reaction, producing *D*-lactate and GSH. GlxI is a catalytically efficient enzyme, with k_{cat} and k_{cat}/K_m values of 1500 s^{-1} and 2.3×10^7 sec^{-1} M^{-1}, respectively, for the recombinant human enzyme (*9*).

Methylglyoxal can be produced from dihydroxyacetone phosphate (DHAP) by methylglyoxal synthase (*10*) and by the deamination of aminoacetone by monoamine oxidase (*11*). It has also been reported that MG is a minor side-product of the interconversion of DHAP and glyceraldehyde-3-phosphate catalyzed by triosephosphate isomerase (TIM) (*12*). Given the concentrations of triose phosphates and TIM in cells, 4×10^{-5} M and 1×10^{-5} M, respectively, it has been predicted that 0.4 mM MG/cell/day would be produced (*13*). MG has been found to produce covalent DNA adducts and to react with arginine and lysine residues in proteins (*14*−*16*). It has been noted that cells having high glycolytic rates, such as certain tumor cell lines and the parasite *Plasmodium falciparum*, have increased levels of GlxI, which may serve a protective effect against high levels of MG produced by the side reaction of TIM (*17*, *18*). Although other pathways are available for the detoxification of MG (including MG reductase, MG dehydrogenase, and aldose reductase) (*19*), it appears that in most cases the glyoxalase system is the major physiological pathway for the removal of cytotoxic MG.

Interruption of the glyoxalase system by selective inhibitors could cause cellular toxicity and may be of possible chemotherapeutic value (*20*). Inhibitors of GlxI have been found to exhibit antiproliferative effects on cancerous cells as well as on parasitic infections, a result possibly due to the inability of the blocked glyoxalase system to detoxify cellular MG (*16*, *21*). In humans, this system also appears to be linked to complications involved in diabetes mellitus as a result of increased levels of MG, and affected patients have higher than normal levels of GlxI (*22*, *23*).

Although the glyoxalase system has been known for 85 years (*24*, *25*), the mechanistic details of the enzymatic reaction are still unclear. The recently determined crystal structure of the human glyoxalase I (*26*) has revealed the overall topography of the enzyme and identified the zinc binding site, but the roles of the metal ion and neighboring residues are unknown. Little additional insight has been provided from the extensive number of sequences of known and predicted glyoxalase I enzymes (for a review, see refs *26* and *27*). To more fully understand the structural and mechanistic enzymology of glyoxalase I, we undertook a study of the bacterial enzyme from *Escherichia coli*. In addition any structural differences between the *E. coli* and human enzymes might be advantageously used in the development of novel antibacterial agents.

In our pursuit of the isolation and characterization of glyoxalase I from *E. coli* we find that contrary to initial predictions (*28*) this bacterial enzyme is dimeric in nature and, unlike other glyoxalase I enzymes, is maximally activated by Ni^{2+} and shows no activity with Zn^{2+}. Investigations on this *E. coli* enzyme should therefore provide important information in understanding not only the chemical mechanism of this enzyme but also should improve our knowledge of metalloenzyme metal selectivity.

MATERIALS AND METHODS

Materials. Reagents and protocols for the isolation and sequencing of the *E. coli* GlxI are as described elsewhere unless noted (*27*). Ampicillin (Amp) and carbenicillin (Carb) were used at a concentration of 100 μg/mL in Luria−Bertani (LB) plates for selection or 30−50 μg/mL in LB broth. MG (*27*) was used in freshly poured LB agar (1 day old maximum) at concentrations of 1−5 mM.

Yeast glyoxalase I (Grade IV) and bovine liver glyoxalase II were obtained from Sigma. Nickel(II) chloride (hexahydrate; 99.9999%) was obtained from Aldrich. Other metal chlorides were of reagent grade quality. Chelex 100 resin was obtained from BioRad. Q-Sepharose Fast Flow and Superdex 200 columns were obtained from Pharmacia. Isoelectric focusing utilized a BioRad Rotofor Preparative Isoelectric Focusing Unit, using RotoLytes (pH 3.9−5.6) with a MES:Gly-Gly ratio of 1:1. The water used in all experiments was purified with the use of a Milli-Q RG Ultrapure water system (18 MΩ-cm; Waters Associates).

Protein samples were concentrated using either a Centricon 10 or a Diaflo Ultrafiltration Membrane in a 50 mL Amicon stirred cell utilizing a PM10 membrane (Amicon, Beverly, MA). Protein concentrations were determined by the Bradford method (*29*), utilizing bovine albumin as the protein standard. In addition, the accuracy of this method was confirmed by utilizing the theoretical extinction coefficient of the denatured protein (6.0 M guanidine hydrochloride) predicted for the sequence in Figure 2 ($\epsilon = 15\,930$ M^{-1} cm^{-1} for a single subunit), calculated by PC/Gene 6.85 software (IntelliGenetics Inc., Mountain View, CA), based on the method of Gill and von Hippel (*30*). Because of the presence of a single Cys residue in each subunit of the homodimeric protein, quantitation of the Cys in the denatured GlxI protein by 5,5′dithio-bis(2-nitrobenzoic acid) based on the method of Riddles et al. (*31*) was performed and found to be in complete agreement with the determination of the protein concentration of the previous two methods.

Bacterial Strains. *E. coli* strains MG1655 (prototrophic; laboratory collection) and DH5α (F⁻Φ*lacZ*Δ*M15*Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r^-m^+) *supE44* λ^-*thi1 gyrA relA*; laboratory collection) were used for transformation of plasmid DNA. Plasmid-bearing MG1655 strains were used for partial purification of the glyoxalase I activity. Strain MG1655 was used as a source of *E. coli* chromosomal DNA.

DNA Methods. Plasmid isolation and all standard DNA manipulations were performed according to Sambrook et al. (*32*). A *Sau*3AI-partial library of *E. coli* (MG1655) DNA was constructed in pBR322 cut with *Bam*HI. The library was transformed into competent DH5α cells ($CaCl_2$ treatment), selecting for ampicillin resistance (Amp^R). The Amp^R colonies were pooled and used for direct selection.

Small-scale plasmid DNA preparations were prepared using a boiling lysis method and were analyzed on 0.8% or 1.5% agarose in TBE after restriction enzyme digestion. DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (*33*), following the T7 Sequencing Kit directions (Pharmacia).

Isolation of Methylglyoxal-Resistant (MG^R) Colonies. The *E. coli* library consisted of four independent pools of 2000−4000 colonies per pool in DH5α. The Amp^R colonies were pooled and plated on LB_{Amp} plates with 3 mM MG to select

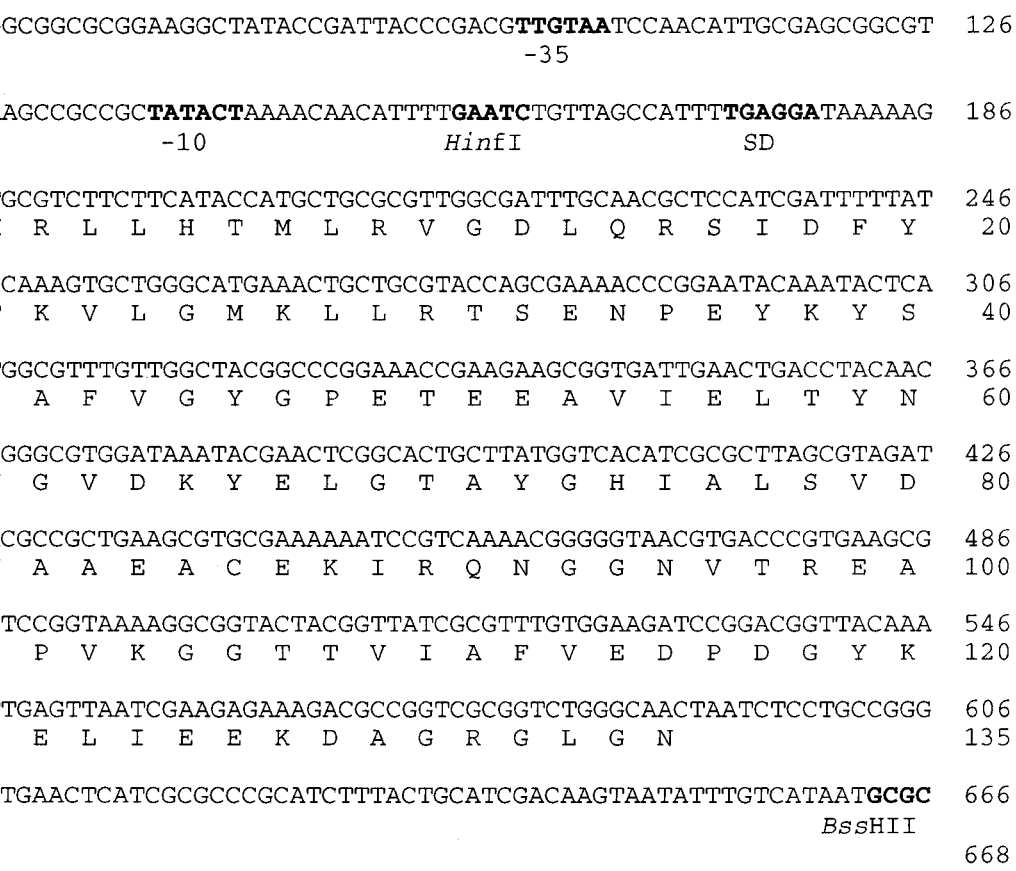

FIGURE 2: The nucleotide sequence of the *Escherichia coli gloA* gene. (Accession Number U57363). Possible promoter regions (the −35 and −10 regions) and a potential Shine−Dalgarno sequence (SD) are labeled and are in bold type. The *Bss*HII site used for generating pDM7 and *Hin*fI used to generate pGL10 are indicated.

for MG^R colonies as previously described for *Salmonella typhimurium* (*27*). To further characterize the plasmids that conferred MG^R on DH5α, plasmid DNA was isolated from the candidates and retransformed into both naive DH5α and MG1655, and retested for the MG^R phenotype on LB_{Amp} with 3 mM MG (*27*). Plasmid DNA from the MG^R colonies was purified by CsCl centrifugation and digested with numerous restriction enzymes to produce a restriction map.

Sequence Comparisons. Protein sequence comparisons and alignments were produced by the paired and clustal alignment programs in the PC/Gene 6.85 software, based on the methods of Myers and Miller (*34*) and Higgins and Sharp (*35*), respectively.

Partial Enzyme Purification and Activity Assays. The DH5α cells containing the plasmids to be tested for *E. coli* glyoxalase I, MG reductase, and MG oxidase activities were grown and used for partial enzyme purification using the techniques described previously (*27*).

Protein Blotting for N-Terminal Sequencing. Protein samples were separated by SDS−PAGE over a 15% gel. Blotting of protein bands present in SDS−PAGE onto poly(vinylidene difluoride) (PVDF) membranes was conducted by electrophoretic transfer (*36*), and the bands were stained with 0.1% Coomassie Blue R-250. The protein band of interest was excised from the PVDF membrane and N-terminal protein sequencing was performed at the Hospital for Sick Children-Pharmacia Biotechnology Centre, Toronto. Protein sequencing was performed with on-line phenylthiohydantoin analysis with a Porton gas-phase Micro-sequencer, model 2090.

Construction of Plasmid pGL10. Plasmid pDM1 was shown to confer the MG^R phenotype on *E. coli* (MG1655 and DH5α) and was used for the construction of the expression plasmid, pGL10. The 2252 bp *Eco*RI-*Bam*HI fragment from pDM1 was isolated and ligated with pUC18 restricted with *Eco*RI and *Bam*HI, to make pUC-*gloA*. This plasmid was further modified by digestion with *Bss*HII and *Bam*HI, treatment with DNA polymerase to form blunt ends, and ligation to form pDM7 (this regenerates the *Bam*HI site). The *Hin*fI-*Bam*HI fragment from pDM7 (containing the *gloA* gene) was isolated and ligated with pTTQ18 (*37*) which had been digested with *Eco*RI and *Bam*HI (the *Hin*fI and *Eco*RI sites were made blunt with the Klenow fragment of DNA polymerase). In this construction, 29 bp upstream of the ATG for *gloA* and 73 bp downstream of the termination codon remain in the *E. coli* DNA. The final plasmid, pGL10, contains the *gloA* gene from *E. coli* under the control of the *tac* promoter and the native *gloA* ribosome binding site. The construct, pGL10, was transformed into *E. coli* MG1655 cells.

Production and Purification of E. coli Glyoxalase I. E. coli MG1655/pGL10 was grown at 37 °C in LB medium, supplemented with 50 μg/mL carbenicillin (LB_{carb}) and 1 mM filter sterilized $NiCl_2$, unless otherwise indicated. The culture ($OD_{600} \approx 0.5$) was induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for 6 h (as determined by an induction time course for optimal enzyme production). The cells (3.5 g/L) were then collected (15 300 g, 15 min), washed with 20 mM Tris pH 7.0, frozen in liquid N_2, and stored at −80 °C.

E. coli MG1655/pGL10 cells in 20 mM Tris pH 7.0 (10 mL/g) were disrupted by sonication, followed by the addition of phenylmethylsulfonyl fluoride (PMSF) to 1 mM, and glycerol to 30% (v/v). The cell debris was removed by centrifugation (48 300 g, 15 min).

The crude extract was applied to a Q-Sepharose Fast Flow column (HR 10/30), equilibrated with 20 mM Tris, 30% glycerol, pH 7.0, and eluted with a linear gradient to 1 M KCl, in the same buffer. The fractions containing GlxI activity (30−50% KCl) were pooled and dialyzed overnight (10% glycerol/H_2O, 4 °C) with frequent additions of PMSF.

The dialyzed protein sample was further purified by preparative isoelectric focusing (Rotofor containing RotoLytes, final concentration of 100 mM each MES and Gly-Gly; pH gradient of 4.5−5.0). Active fractions were pooled and concentrated by ultrafiltration (10 kDa cutoff) with a buffer change to 50 mM MOPS, 10% glycerol, pH 7.0. The protein under these conditions was stable to storage at 4 °C for several months.

Molecular Weight Determination. Subunit molecular mass was determined by SDS−PAGE based on the method by Laemmli (*38*). The quaternary structure of the GlxI protein was determined by gel filtration using a Superdex 200 HR 10/30 column (50 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl, 2.5% glycerol), employing the appropriate protein standards. *E. coli* GlxI, both before the isoelectric focusing step and after isoelectric focusing (apoenzyme), was analyzed.

Mass Spectrometry. Electrospray mass spectrometry (ESMS) was performed on a Micromass Quattro II triple-stage quadrupole mass spectrometer equipped with an electrospray ionization source. Protein sample was introduced in an eluant of 1:1 H_2O/CH_3CN (containing 0.1% acetic acid). Data analyses were performed with the Masslynx software (version 2.0), and the multiply charged distribution profile was subjected to the MaxEnt algorithm to produce true molecular mass spectra with associated errors.

Glyoxalase I Enzymatic Assay. The glyoxalase I enzymatic assay was performed in 50 mM potassium phosphate buffer, pH 6.6, maintaining the GSH concentration at 0.1 mM to avoid enzyme inhibition (*39*). The concentration of the hemithioacetal substrate, formed from the equilibrium of MG and GSH, was calculated using a K_{diss} of 3.1 mM (*40*). The change in absorbance was monitored at 240 nm, and the initial rate data ($\epsilon = 2860\ M^{-1}\ cm^{-1}$; refs *41*, *42*) were fit by nonlinear regression analysis.

Metal Analysis. Growth studies to determine the effect of metals on enzyme production and activity were performed with 0.1 mM $ZnCl_2$ or 1 mM $NiCl_2$ added to the growth media. These levels were not detrimental to *E. coli* MG1655/pGL10 cell growth.

For metal analysis, the protein solution from the isoelectric focusing step was changed to Chelex-treated buffer (50 mM MOPS, 10% glycerol, pH 7.0) by ultrafiltration, and stored in plasticware which had been presoaked with nitric acid. For increased accuracy metal chlorides were weighed in an argon atmosphere. Prior to the metal activation assays, the quartz cuvettes were treated with 1:1 nitric acid:sulfuric acid and then 1 mM EDTA each for 15 min, and rinsed with Chelex-treated Milli-Q H_2O. This minimized the activation of the enzyme by metals leaching from the quartz cuvettes. This was performed prior to the apoenzyme control assays for each replicate of every metal. Following the control measurement, the other metal activation assays were performed in increasing concentrations of metal.

For atomic absorption (AA) and inductively coupled plasma (ICP) analyses of the *E. coli* GlxI protein, the purified protein was prepared in metal free solutions. Samples supplied for metal analyses were the following: (1) enzyme isolated from *E. coli* grown in the presence of $NiCl_2$ (1 mM) and purified according to the outlined procedure, including the isoelectric focusing step, with no subsequent metal addition; (2) purified GlxI with $NiCl_2$ added to the protein after isoelectric focusing; (3) GlxI isolated from cells grown without $NiCl_2$ in the growth media, purified using the Q-Sepharose Fast Flow column followed by dialysis; (4) protein from cells grown with $NiCl_2$ (1 mM) or $ZnCl_2$ (0.1 mM) but purified as in sample 3. For sample 2, $NiCl_2$ (2.5 molar equivalence) was added, and the protein was dialyzed overnight against 1 L of Chelex-treated 50 mM MOPS, 10% glycerol, pH 7.0, with a few grams of Chelex 100 resin in the buffer. In each case samples and controls were passed through Chelex 100 resin to remove loosely bound metals, and acidified to below pH 2 with high purity HCl (J. T. Baker, reagent grade for trace metal analysis). To ensure that the Chelex was not removing bound metal from the enzyme or affecting the activity in any other manner, the activity of the sample was tested before and after passage through the Chelex resin. Metal analyses were performed at the Water Quality Laboratory (Department of Earth Sciences, University of Waterloo). Nickel and zinc levels were measured utilizing a Varian Model 1475 Atomic Absorption Spectrophotometer, with instrument conditions set according to the manufacturer's specifications. Inductively coupled plasma analyses were performed using a Thermo Instruments IRIS Plasma Spectrometer (ICP). The following metals were tested for: Al, As, B, Ba, Ca, Cd, Co, Cr, Cu, Fe, Hg, Mg, Mn, Mo, Ni, Pb, Se, Sr, Ti, V, and Zn. Commercial standards were prepared in house in duplicate.

Metal Activation Studies. Incubation of the apoenzyme with different concentrations of each of the metal chlorides permitted an assessment of the binding ratios and affinities of the metal ions to the enzyme, in addition to the level of enzyme activation. In each case, the metal chloride was incubated with the purified apoenzyme produced by isoelectric focusing for a minimum of 10 min prior to the first activity assay, to compensate for any time dependence in binding at various metal concentrations. The following metal chlorides were tested: Mg^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+}.

Kinetic Analysis. Determination of the Michaelis constant, K_m, and the maximal enzyme velocity, V_{max}, for *E. coli* GlxI involved measurement of the initial reaction rate utilizing 10 substrate concentrations ranging between 0.025 and 1.0 mM. The enzyme stock was diluted with 50 mM MOPS, 10% glycerol, pH 7.0, containing 2.5 molar equivalence of nickel to dimeric enzyme. The activity at each substrate concentration was measured in triplicate and the entire set of measurements was performed in duplicate.

To ensure that nickel chloride did not affect the stability of the *S*-D-lactoylglutathione product or the hemithioacetal substrate, each was incubated separately with $NiCl_2$ in the assay buffer, in the absence of GlxI, and the absorbance at

240 nm was monitored over time. To determine if there was any time dependence in the binding of the Ni^{2+} to the enzyme, the activity of the enzyme was monitored at 1 min after $NiCl_2$ addition and at intervals over a 30 min period, using 0.5 mM substrate and 2.5 molar equivalents of $NiCl_2$.

A profile of the effect of pH on the enzyme activity was performed by altering the pH between 5.0 and 8.2. This is within the range that the dissociation constant for the substrate formation is valid (3.1 mM; refs *40*, *41*). Potassium acetate buffer was utilized for pH 5.0 and 5.5, potassium phosphate between pH 5.85 and 8.0, and *N*-methyldiethanolamine for pH 8.2.

Circular Dichroism. The circular dichroism data were collected between 250 and 180 nm, with a Jasco J-715 spectropolarimeter, in a 0.1 cm quartz cell, with a bandwidth of 1.0 nm, 20 nm/min scan rate, resolution of 0.5 nm, 8 s response, and smoothing factor of 2. Apoenzyme (10 μM in 20 mM potassium phosphate buffer, pH 6.0) and nickel-activated enzyme (2.5 molar equivalents) were scanned.

RESULTS

Isolation of MG^R Colonies. Wild-type *E. coli* DH5α was shown to be sensitive to concentrations of MG as low as 1 mM in LB agar (this work). Since glyoxalase I detoxifies MG by converting it into *D*-lactate (in conjunction with glyoxalase II), it was likely that overproduction of glyoxalase I would confer MG resistance to the cells. This was the strategy employed in the successful isolation of the glyoxalase I gene in *Pseudomonas putida*, and our previously reported *S. typhimurium* isolation (*27*, *43*). The overexpression of the gene could be achieved by the presence of a multicopy plasmid containing the glyoxalase I gene. A plasmid library of *E. coli* DNA (constructed in the vector pBR322) was transformed into *E. coli* strain DH5α, selecting for Amp^R and MG^R. The strain DH5α/pDM1 had significantly elevated glyoxalase I activity (151 times over wild-type), whereas the activities of MG reductase and MG dehydrogenase were not notably elevated as compared to DH5α/pBR322 (wild-type).

N-Terminal Protein Sequence. *E. coli* GlxI was purified by ion exchange chromatography to 90% homogeneity and was subjected to electrophoresis and electroblotting onto a PVDF membrane to isolate homogeneous protein for N-terminal sequencing. The first eleven amino acids were determined: ^{1}Met-Arg-Leu-Leu-His-Thr-Met-Leu-Arg-Val-Gly^{11}.

DNA Sequence Determination. The DNA sequence of the region of insert DNA in pDM1 from the *Bam*HI site in pBR322 (bp 1) to bp 1575 of the insert was determined. Using the protein N-terminal sequence as a guide, an open reading frame of 408 bp (135 amino acids) was found (bp 187−595), and the gene isolated was named *gloA*.

The relevant portion of the nucleotide sequence determined from pDM1 (bp 67−668), as well as the deduced amino acid sequence for the *gloA* gene product, GlxI, is reported in Figure 2. In the upstream region, bp 101−106 is a potential −35 region, and bp 138−143 is a potential −10 region for RNA polymerase binding (*44*). The region corresponding to bp 174−179 is a possible ribosome binding site consensus sequence (*45*). The *E. coli* DNA sequence reported here is 82% identical at the DNA level and 91% identical in amino acid sequence to the *S. typhimurium gloA* sequence we previously reported (*27*).

The 5′ and 3′ ends of the pDM1 insert sequence were found to overlap the upstream region of the *rnt* gene (coding for RNase T; known to map to minute 36; refs *46*, *47*), as well as the C-terminal and downstream region of the *nemA* gene (coding for *N*-ethylmaleimide reductase; ref *48*). The position of this gene was also mapped by P1 transduction with known markers; the *gloA* gene is 6% linked to *ksgB1* in FS173 (*49*) and about 30% to *zdg-299*::Tn*10* in GI42 (data not shown; refs *46*, *50*). Confirmation of the *gloA* DNA sequence was made after our deposition into the NCBI database as a result of the *E. coli* genome mapping project (*51*). However, in the current report we have also unambiguously determined that the *gloA* gene is indeed responsible for the MG^R phenotype and codes for *E. coli* glyoxalase I.

Overproduction of Glyoxalase I. Initial isolation of the *gloA* gene onto pDM1 allowed for the sequencing of the open reading frame associated with *gloA* and low level protein production (microgram quantities per liter of growth). In an effort to place the *gloA* gene downstream of the strong *tac* promoter and under control of the $lacI^Q$ gene product, a 510 bp fragment, containing the complete *gloA* gene was isolated onto the plasmid pTTQ18 (*37*). The resultant plasmid, pGL10, was used to transform *E. coli* MG1655 cells, allowing for the production of sufficient quantities of GlxI for biochemical studies.

Summary of Purification. The glyoxalase I enzyme has been successfully purified by the two-step purification procedure outlined, consistently producing between 150 and 200 mg/L and representing >50% of the total soluble protein. The purified GlxI protein appeared homogeneous by SDS−PAGE with Coomassie staining (Figure 3A). A molecular mass of 14 919 was determined from ESMS analysis of the purified GlxI protein (Figure 3B), and is in complete agreement with the mass predicted from the deduced amino acid sequence of the protein. It is interesting to note that the utilization of ESMS was helpful during the development of the purification process as it allowed for the detection of a fully active, but C-terminal truncated protein of lower molecular mass, 14 578. This corresponded to *E. coli* GlxI cleaved between Arg^{131}-Gly^{132}, presumably produced by an endogenous *E. coli* endoprotease. An increase in quantities of PMSF added to the dialysis solution was found to completely suppress this cleavage process. In addition, during early purification attempts utilizing SDS−PAGE separation and PVDF blotting, ESMS identified a protein of a higher molecular mass. The mass of this species, 14 990 (+71 mass units), is consistent with the formation of an adduct between the protein (most probably the single cysteine in the denatured protein) and unpolymerized acrylamide. Similar modifications during electrophoresis have been observed previously with other proteins (*52*, *53*).

Characterization of E. coli Glyoxalase I. *E. coli* GlxI from both before and after isoelectric focusing exhibit homodimeric structures of approximately 30 kDa, based on Superdex 200 gel filtration. No evidence for a native GlxI monomer has been observed. The isoelectric point of the enzyme, determined from isoelectric focusing, was found to be between 4.6 and 4.9. This pI is consistent with the calculated value of 4.7 (PC/Gene 6.85 software). Although precipitation did occur during the isoelectric focusing of large

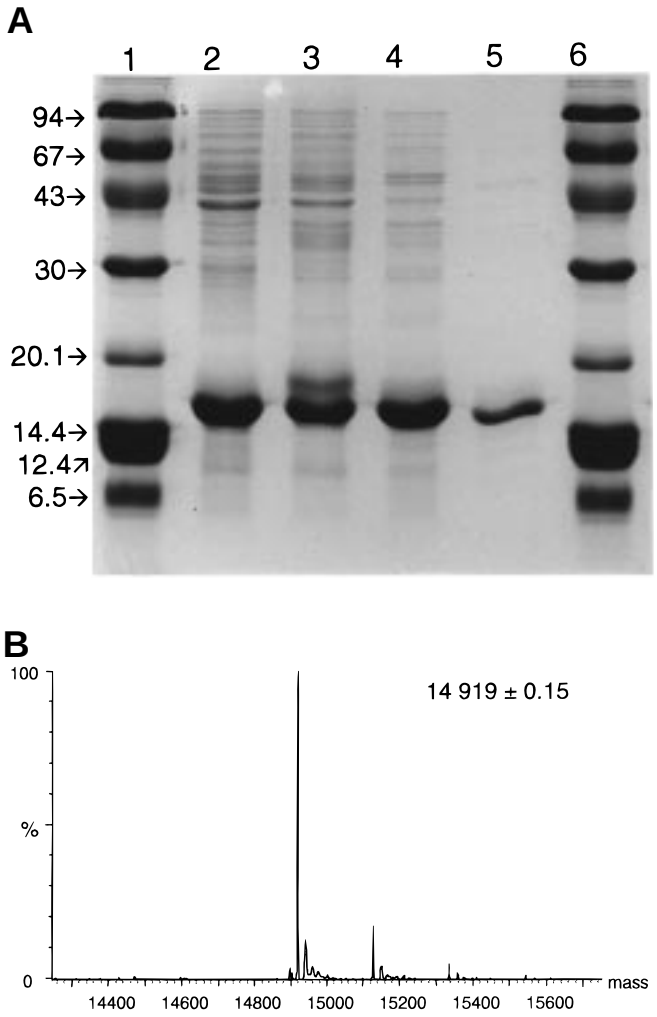

FIGURE 3: (A) Fifteen percent (Coomassie stained) SDS−PAGE illustrating the stages of the purification of *E. coli* glyoxalase I, produced in *E. coli* MG1655/pGL10 cells. Lanes 1 and 6 contain low molecular mass standards (in kilodaltons). Lane 2 contains cell break; lane 3, post Q-Sepharose Fast Flow column; lane 4, post dialysis; lane 5, post isoelectric focusing. (B) Reconstructed electrospray mass spectrum of purified *E. coli* glyoxalase I, indicating the observed subunit molecular mass in Daltons (Calculated = 14 919). Other small peaks corresponding to noncovalent adducts with sodium, potassium, and MOPS buffer are evident. No other peaks were observed in the spectra over a mass range of 1000−100000 Da.

quantities of protein, this precipitate was soluble in the standard MOPS, pH 7.0 storage buffer and the protein could be reactivated by the addition of nickel chloride.

Metal Effects. Preliminary work with partially purified *E. coli* GlxI from early constructs, such as DH5α/pDM1, produced an enzyme with a specific activity of <100 μmol min^{-1} mg^{-1}. Addition of 1 mM EDTA or EGTA reduced enzyme activity by >95%. However, addition of metals to this partially purified enzyme, which had not been treated with a metal chelator, revealed interesting enzymatic activation. Unlike zinc, magnesium, calcium, sodium, and potassium, which did not activate the protein, manganese, cadmium, cobalt, and notably nickel enhanced the enzymatic activity substantially. These studies were extended with GlxI protein purified from MG1655/pGL10.

Based on a number of experiments, including variation of induction time (2−6 h) and the metal added to the growth media, the following observations were made. When $NiCl_2$ was added to the growth media, Ni^{2+} was incorporated into the produced protein as determined by ICP and AA analyses. In addition, the activity of the overproduced enzyme directly correlated with the amount of Ni^{2+} incorporated into the protein during cell growth (based on ICP and kinetic analyses). However, incorporation of Zn^{2+} (available from the growth media with and without supplementation with $ZnCl_2$) into the overproduced enzyme resulted in production of inactive holoenzyme. The amount of enzyme produced was not affected by the presence of Ni^{2+} or Zn^{2+} in the growth media. Because of the high efficiency of protein production from our construct (150−200 mg/L with a 6 h induction time), substantial amounts of apoenzyme (50−70%) were always produced. The remaining amount of apoenzyme could be converted to active holoenzyme upon addition of nickel to the protein solution. Addition of $ZnCl_2$ did not activate the apoenzyme.

In the protein purification protocol, only the isoelectric focusing step was found to remove metal from the protein. Metal analyses by ICP and AA indicated only trace levels of various metals, including copper, zinc, nickel, and cadmium, following isoelectric focusing. In addition the activity of the protein after isoelectric focusing was only ~6 μmol min^{-1} mg^{-1}, but was >600 μmol min^{-1} mg^{-1} upon addition of $NiCl_2$ to the purified enzyme. Therefore the apoenzyme produced by the isoelectric focusing was used for metal activation studies.

A thirty minute time course on the activation of the apoenzyme indicated that there was no evident time dependence for binding of nickel to the enzyme over the time period examined (1−30 min). Addition of millimolar concentrations of nickel chloride produced no observable changes in hemithioacetal or *S*-lactoylglutathione stability. To eliminate the possibility that the nickel-activated enzyme catalyzed the oxidation of reduced GSH to its disulfide and not to *S*-lactoylglutathione, the enzymatic reaction was carried out under fully anaerobic conditions (argon). These conditions did not alter the course of the enzymatic reaction. Once the enzymatic reaction was completed, excess GlxII was added and was found to decrease the absorption at 240 nm, indicating that *S*-lactoylglutathione was indeed the product of the nickel-activated enzymatic reaction. Hence, any change in observable enzyme activity was due solely to activation of the enzyme by Ni^{2+}.

The results of the activation of various metals with the *E. coli* GlxI apoenzyme are summarized in Figure 4A. The titration of cadmium, manganese, cobalt, and nickel into the protein (Figure 4B) indicated that maximal enzyme activity was reached at approximately 1 molar equivalence of metal to dimeric enzyme. In the case of nickel activation, this is in agreement with the results of the AA and ICP analyses, which indicated that fully active enzyme contained only one atom of nickel bound per dimeric enzyme.

During the activity assays on the apoenzyme, an increasing activity over time was seen under the assay conditions. This was a source of error in the accurate measurement of the apoenzyme activity, which varied between 2 and 12 μmol min^{-1} mg^{-1}. Treatment of the cuvettes with 1:1 nitric acid:sulfuric acid and EDTA reduced this effect, suggesting that the enzyme was binding trace metals from the quartz cuvette.

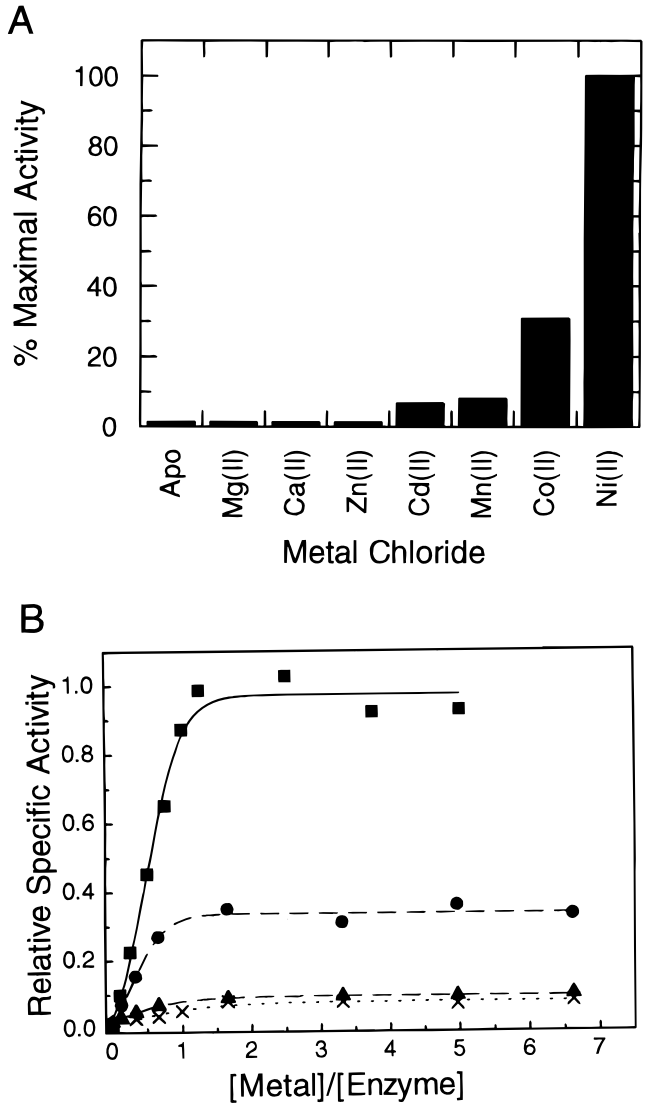

FIGURE 4: (A) Activation of the apo form of *E. coli* GlxI by various metal ions (10× molar equivalence). (B) Titration of GlxI with the four activating metals: $NiCl_2$ (■) in triplicate, and $CoCl_2$ (●), $MnCl_2$ (▲), and $CdCl_2$ (×) in duplicate. A 0.5 mM hemithioacetal substrate concentration was utilized for experiments in both (A) and (B), and homogeneous apoenzyme from the isoelectric focusing step was used. Activities are relative to the nickel-activated enzyme.

Based on these results, a standard assay for *E. coli* glyoxalase I was developed. For maximal activity and to ensure sufficient nickel was available, the enzyme was incubated with 2.5 molar equivalence of nickel chloride prior to activity measurements.

Kinetic Analysis. Under the described conditions (Materials and Methods) the kinetic parameters for the MG-GSH hemithioacetal substrate were measured. The K_m was found to be 27.2 ± 0.4 μM, and the V_{max} 676 ± 17 μmol min^{-1} mg^{-1}. From these results, the k_{cat} is calculated to be 338 s^{-1}, and the corresponding k_{cat}/K_m is 1.24×10^7 M^{-1} sec^{-1}. In comparison, *Saccharomyces cerevisiae* glyoxalase I has a k_{cat} of 1120 s^{-1} (*54*), and the recombinant *Homo sapiens* GlxI, 1500 s^{-1} (*9*). However, the k_{cat}/K_m values from these sources are similar to that of the *E. coli* GlxI, being 0.35×10^7 M^{-1} sec^{-1} and 2.3×10^7 M^{-1} sec^{-1} from *S. cerevisiae* and *H. sapiens*, respectively (*9*, *54*). The k_{cat}/K_m values indicate that the *E. coli* glyoxalase I, containing nickel, is very efficient, functioning at close to a diffusion-controlled rate. Recent studies on the kinetic properties of the yeast GlxI enzyme utilizing various viscosometric methods supports the suggestion that GlxI functions at a diffusion-controlled rate, under physiological conditions (*55*).

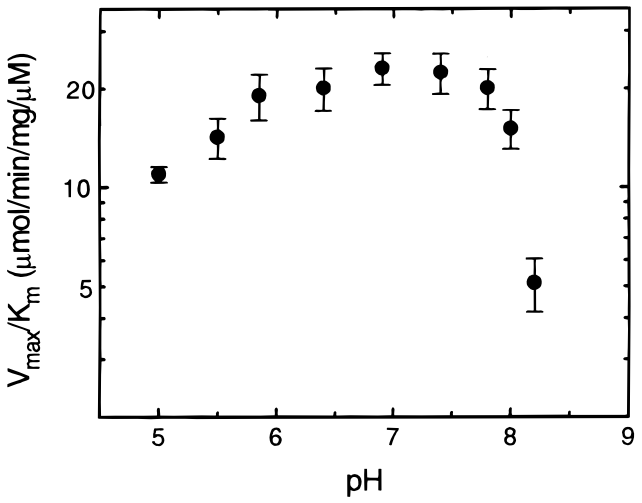

FIGURE 5: pH dependence of V_{max}/K_m for Ni^{2+}-activated *E. coli* glyoxalase I. Buffer ionic strength was maintained at 0.2 M, half contributed by buffer and half from KCl.

pH Activity Profile. A preliminary study of the effect of pH of the assay buffer on the kinetic parameters of the enzyme was undertaken (Figure 5). The maximal velocity (V_{max}) remained relatively constant throughout the pH range examined, while V_{max}/K_m exhibited a 2-fold increase from pH 5 to 7 and then rapidly decreased above pH 8.0. The activity of the enzyme was too low for accurate determination of the kinetic parameters at pH 8.4 (diethanolamine buffer). Preincubation of the enzyme in buffers of varying pH (pH 5−9) followed by assays at pH 6.6 indicated that the enzyme was stable in this region. Alteration in the ionization state of glutathione in this pH region has been considered to be unlikely from studies with yeast GlxI by Vander Jagt and Han (*54*). This sudden change in the binding of the substrate suggests that there are critical residues within the active site involved in substrate binding that are affected by the change in pH.

As expected, incubation of the protein in buffers that are known to chelate metal, such as imidazole, histidine, bicine, and glycine, reduced the level of enzymatic activity (*56*). The presence of glycylglycine as one of the ampholytes used in the isoelectric focusing purification step may account for the removal of metal ions from the focused protein.

Circular Dichroism. There was no significant change in the circular dichroism spectrum of *E. coli* glyoxalase I produced by isoelectric focusing, in the absence or presence of $NiCl_2$. Therefore it appears that no significant structural change occurs upon binding Ni^{2+}.

DISCUSSION

To pursue fundamental investigations on the structural and mechanistic aspects of GlxI, especially as it may be a member of a previously unrecognized superfamily of divalent metal-ion-dependent enzymes (*57*), an overexpression system to obtain sufficient quantities of this bacterial protein was required. Although endogenous *E. coli* glyoxalase I activity

has been noted previously, a partial purification produced an unstable enzyme and was never further characterized (*58*). As outlined in Results, we developed a two-step protocol for the purification of large quantities of homogeneous apoenzyme from MG1655/pGL10.

An investigation of the metal activation of the *E. coli* apoenzyme indicated a requirement for Ni^{2+}, with no evidence of an active Zn^{2+}-containing enzyme. This is in contrast to the active forms of *P. putida*, *S. cerevisiae*, and *H. sapiens* GlxI which have been shown to contain Zn^{2+} (*1*, *59*, *60*). The *H. sapiens* GlxI apoenzyme has been shown to be fully reactivated by the addition of Zn^{2+} and Mg^{2+}, and to a lesser extent, Mn^{2+}, and Co^{2+} (*7*, *61*). Addition of Cu^{2+} and Cd^{2+} did not recover enzyme activity (*62*, *63*). The *S. cerevisiae* enzyme was found to be partially reactivated by the addition of Mg^{2+}, Ca^{2+}, and Mn^{2+}, but not by Fe^{2+}, Co^{2+}, or Ni^{2+} (*64*). Unlike the *S. cerevisiae* enzyme, the *E. coli* GlxI apoenzyme could be fully reactivated. Interestingly, activation was found with Co^{2+}, Mn^{2+}, and Cd^{2+}, but maximal activation was found with the addition of Ni^{2+} (Figure 4A). Analysis of the nickel-activated enzyme indicated that although the V_{max} is independent of pH (between pH 5.0−8.2), the K_m is optimal between 6 and 8.

Higher enzymatic activity was found following cell break when *E. coli* MG1655/pGL10 was grown in the presence of $NiCl_2$. This is supported by the results of the ICP and AA analyses which indicated that higher levels of Ni^{2+} were incorporated into the produced protein when cells were grown in the presence of $NiCl_2$. This indicates that Ni^{2+} is transported into the cells, most likely through use of the *nik* transport system (*65*), where it is taken up by the glyoxalase I protein. Due to the efficiency of the protein production from this construct, an increase in enzymatic activity could be obtained by the addition of $NiCl_2$ after cell disruption. This is a result of the conversion of the remaining apoenzyme produced during cell growth, to holoenzyme. Although nickel was found to activate the enzyme, varying amounts of zinc could also be detected in the enzyme preparations prior to isoelectric focusing. Complete removal of all metals from the protein could be accomplished by isoelectric focusing which produced a homogeneous apoenzyme, allowing for the detailed investigation of metal activation and stoichiometry.

Based on determination of the subunit molecular mass by SDS−PAGE and ESMS, and the native molecular mass by gel permeation chromatography, the *E. coli* GlxI protein is dimeric. The quaternary structure appears not to be controlled by the presence of Ni^{2+}. Based on circular dichroism studies, no major conformational change appears to occur on binding Ni^{2+} to the apo form of the enzyme. This suggests that the metal binding site is most likely intact in the apoenzyme.

The *H. sapiens* GlxI enzyme has been recently shown by crystallographic studies to be a homodimeric protein with two Zn^{2+} atoms per active enzyme molecule. The two active sites are located at the interface between subunits (*26*). The *E. coli* GlxI protein has 36% identity with the *H. sapiens* GlxI protein sequence (alignment not shown). Included in this homology are three of the four known metal ligands based on the reported crystal structure of the *H. sapiens* GlxI (*26*), wherein residues Gln-34 and Glu-100 from one subunit and His-127 and Glu-173 from the second subunit act as zinc ligands. These amino acids correspond to His-5, Glu-56, His-74, and Glu-122, in the *E. coli* GlxI sequence. Despite the presence of these potential ligands, examination of the activation of the *E. coli* apoenzyme by all four activating metals indicated that maximum activity is reached at a molar ratio of approximately 1 metal:1 intact (dimeric) protein molecule, with nickel producing highest activity (Figure 4B). These studies indicate that metal binding to this enzyme is very tight and that only one metal binding site is required for full activity. In the case of nickel, metal analyses of the holoenzyme by ICP and AA indicated that indeed only one metal ion is bound per intact enzyme. This is an unexpected result as we have shown the *E. coli* GlxI to be a homodimeric protein, with the assumption that both active sites might be arranged similarly to the *H. sapiens* enzyme.

It is also of interest to note that only the eukaryotic sequences from *H. sapiens* (*66*, *67*), *Lycopersicon esculentum* (*68*), and *Brassica juncea* (*69*) contain the Gln metal ligand. In the remaining known and postulated GlxI sequences including *E. coli*, there is a histidine at this position. It is not known at this point what effects this change may have on metal selectivity. For example, a His→Asn mutation in the zinc binding site of carbonic anhydrase II has been found to alter metal coordination geometry around the metal center, as shown by X-ray crystallography (*70*). Although the *S. cerevisiae* GlxI enzyme contains a His rather than a Gln ligand, as does the *E. coli*, the active form of the *S. cerevisiae* enzyme has been shown to contain zinc (*60*). However, the absence of X-ray data does not allow the unambiguous assignment of the metal ligands nor the metal coordination geometry. Therefore this very unusual preference for nickel by *E. coli* GlxI cannot be simply explained by Gln→His alone, but must also depend on the structural context of the particular protein.

As seen in the work on astacin (*71*), a zinc-endopeptidase, the positions of the metal ligands affect metal selectivity, and in concert with the orientation of metal coordinated water molecules, affect enzyme activity (*72*, *73*). The active site zinc of this endopeptidase is coordinated by five ligands in a trigonal bipyramidal fashion. Replacement of zinc with either cobalt or copper produced an active enzyme, with the same metal ligand arrangement. However, the protein exhibited no catalytic activity when zinc was replaced with either nickel or mercury. A definitive X-ray crystallographic study indicated a close relationship between the catalytic properties and ground-state coordination of the metal in this protein. Differences in the orientation of metal-bound water molecules appeared critical for catalytic activity (*71*). Information based on the *H. sapiens* glyoxalase I crystal structure (*26*) and on EPR and NMR experiments indicate the importance of at least one or two water molecules interacting with the metal and the substrate (*7*, *8*). The critical orientation of metal-coordinated water molecules in *E. coli* GlxI may, in like fashion, be quintessential for enzyme activity. It is possible that nickel provides an optimal orientation of metal-coordinated water molecules in the *E. coli* GlxI active site compared with other metals. As well, due to the variation in nickel-bound water pK_a's, which is dependent upon coordination number and actual protein−metal geometry [10.6 for the aquo−nickel complex (*74*); 7.4 for nickel-substituted phophotriesterase (*75*)], the ionization

state of this metal-bound water molecule could contribute to the pH profile seen in Figure 5, at pH values greater than 8.

Unlike the *H. sapiens* GlxI enzyme, which is homodimeric with two Zn^{2+} atoms per active enzyme molecule (*26*), the monomeric *S. cerevisiae* GlxI contains one Zn^{2+} atom per active enzyme (*60*). The sequence of the *S. cerevisiae* enzyme has been suggested to be a product of a gene duplication event resulting in a fused dimer gene product (*26*, *27*, *76*). Cameron and co-workers have suggested that the presence of this gene fusion in the yeast system may prevent one of the two active sites from reaching an optimal configuration (*26*). This may explain the presence of only one metal ion per intact enzyme, despite conservation of the zinc ligands in both halves of the *S. cerevisiae* sequence. This suggests that the yeast enzyme contains only one functional active site. The *E. coli* GlxI enzyme, however, is not a product of a gene fusion event. The exact structural reasons for a single metal binding site in the homodimeric *E. coli* enzyme is currently unknown. However, other examples of ligand-induced molecular asymmetry have been observed (*77*−*79*), and based on the circular dichroism studies in the absence and presence of nickel, these changes, if they occur, are subtle in the *E. coli* GlxI case. Availability of sufficient quantities of apo and metal-substituted forms of the *E. coli* glyoxalase I protein will be useful in future investigations on the role of metal ions in the enzymatic mechanism of this enzyme and in elucidating the factors involved in controlling metal ion selectivity in this protein.

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