Investigation of metal binding and activation of *Escherichia coli* glyoxalase I: kinetic, thermodynamic and mutagenesis studies

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GlxI (glyoxalase I) isomerizes the hemithioacetal formed between glutathione and methylglyoxal. Unlike other GlxI enzymes, *Escherichia coli* GlxI exhibits no activity with Zn^{2+} but maximal activation with Ni^{2+}. To elucidate further the metal site in *E. coli* GlxI, several approaches were undertaken. Kinetic studies indicate that the catalytic metal ion affects the k_{cat} without significantly affecting the K_m for the substrate. Inductively coupled plasma analysis and isothermal titration calorimetry confirmed one metal ion bound to the enzyme, including Zn^{2+}, which produces an inactive enzyme. Isothermal titration calorimetry was utilized to determine the relative binding affinity of GlxI for various bivalent metals. Each metal ion examined bound very tightly to GlxI with an association constant (K_a) $>10^7$ M^{-1}, with the exception of Mn^{2+} (K_a of the order of 10^6 M^{-1}). One of the ligands to the catalytic metal, His^5, was altered to glutamine, a side chain found in the Zn^{2+}-active *Homo sapiens* GlxI. The affinity of the mutant protein for all bivalent metals was drastically decreased. However, low levels of activity were now observed for Zn^{2+}-bound GlxI. Although this residue has a marked effect on metal binding and activation, it is not the sole factor determining the differential metal activation between the human and *E. coli* GlxI enzymes.

Key words: glyoxalase I, isothermal titration calorimetry, kinetics, mutagenesis, nickel.

INTRODUCTION

Metal ions play critical roles in the structure and function of countless enzymes [1–3]. However, the exact role(s) that the metal ion plays in enzymic catalysis and the molecular basis of metal specificity by metalloenzymes are currently subjects of intense interest [4–8]. Variations of the protein ligands to bound metal ions and the environment surrounding the metal centre itself have been shown to affect metal affinity and activation of the protein [7,9–12]. However, an improved understanding of the factors contributing to metal selectivity and affinity is required and it necessitates additional studies on metal centres. An analysis of the metal binding and activation of GlxI [glyoxalase I; *S*-lactoylglutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5] from *Escherichia coli* has been undertaken to improve our understanding of this enzyme and the role that the metal centre plays in the enzymic steps.

GlxI is the first of the two enzymes in the two-component Glx system, which functions to remove cellular cytotoxic α-ketoaldehydes. The metalloenzyme GlxI catalyses the isomerization of the non-enzymically formed hemithioacetal of methylglyoxal and glutathione (GSH) to *S*-D-lactoylglutathione (Figure 1). GlxII [*S*-(2-hydroxyacyl)glutathione hydrolase (EC 3.1.2.6)] hydrolyses the product of the GlxI reaction to D-lactate [13].

The recent solution of the X-ray structure of the *E. coli* and *Homo sapiens* GlxI enzymes [14,15] have not only provided substantial information regarding the protein's structure in general, placing GlxI in a novel structural superfamily composed of $\beta\alpha\beta\beta\beta$-motifs [16], and mechanistically into the vicinal oxygen chelate super-family [17], but has also provided valuable knowledge about the catalytic metal site. Examination of the region surrounding the metal in the active site of the *H. sapiens* and *E. coli* GlxI enzymes has revealed that there are no suitable residues close enough to function as a catalytic base. This has led to the assumption that the metal-bound water molecules may be important, as suggested by previous studies, or that one of the ligands to the metal has a dual role [18,19]. Further studies with the *H. sapiens* enzyme have led to the suggestion that the metal ligand Glu^{173}, in *H. sapiens* GlxI, is directly involved in the catalytic mechanism of the GlxI reaction, perhaps serving as the catalytic base for removal of the C-1 proton from the substrate [20,21]. Recent spectroscopic results suggest that this is also plausible for *E. coli* GlxI, wherein Glu^{122} may act similarly [22]. The extent of the contributions of such factors for general acid–general base catalysis and electrostatic stabilization of the mechanism also needs to be delineated [23,24].

Our previous studies have demonstrated that *E. coli* Glx I is a metalloenzyme that functions as a homodimer [25]. The addition of metal neither appears to affect the overall structure of the enzyme, nor influences the dimeric nature of the protein [14,25,26]. One of the unique characteristics of *E. coli* GlxI compared with GlxI from other sources is its activation with Ni^{2+} rather than Zn^{2+} [25]. This is in marked contrast with GlxI from *H. sapiens*, *Saccharomyces cerevisiae* and *Pseudomonas putida* that are all activated with Zn^{2+} [27–29]. This is especially puzzling given the relatively high amino acid sequence similarity between the *E. coli* and *H. sapiens* GlxI [15,25].

Glx I, and the comparison of metal selectivity of *E. coli* and *H. sapiens* in this enzyme, provides an ideal system to probe the underlying factors for metal selectivity at an atomic level and explore the factors producing this altered metal activation. The present investigations were focused specifically on the metal activation and metal-binding properties of *E. coli* GlxI. Kinetic parameters of the enzyme activated with several metal ions have been examined. ITC (isothermal titration calorimetry) was performed to determine the ratio of metal bound to the apoenzyme and the relative binding affinities of *E. coli* GlxI for the various

Abbreviations used: ESMS, electrospray mass spectrometry; GlxI, glyoxalase I; ICP, inductively coupled plasma; ITC, isothermal titration calorimetry.

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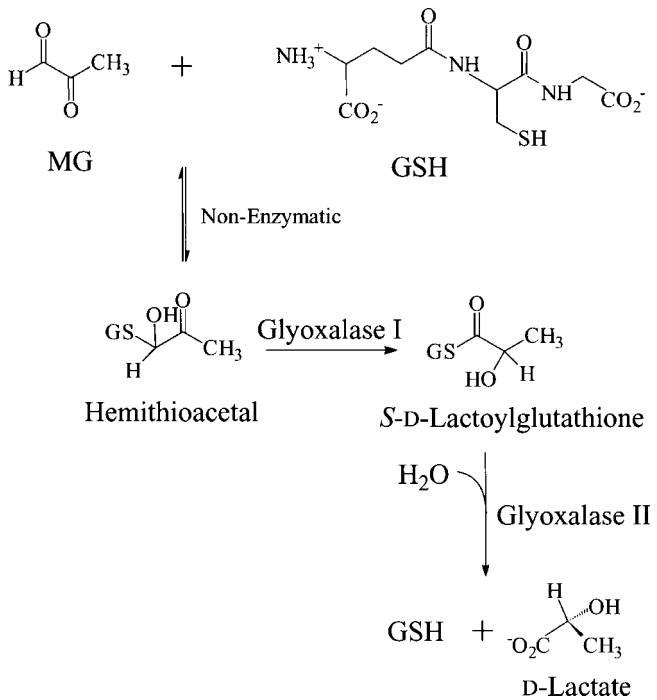

Figure 1 The reactions catalysed by the two component Glx system

bivalent metal ions. As one metal ligand in *H. sapiens* GlxI differs from that found in *E. coli* GlxI, His^5 in *E. coli* versus Gln^{34} in *H. sapiens* GlxI, the metal activation profile of the H5Q ($His^5 \rightarrow$ Gln) mutation was investigated in *E. coli* GlxI to determine the effect this alteration has on metal binding and activation profiles.

EXPERIMENTAL

Materials

The bacterial cell lines employed, all reagents utilized in the cell growth and protein purification and the enzymic assay have been described previously [25,30].

Preparation of metal-free buffers and plasticware

To remove extraneous metals from buffers and water, solutions were passed through Chelex 100 resin (Na^+ form; Bio-Rad Laboratories, Mississauga, ON, Canada) before use (approx. 5 ml of resin/l of buffer or less) using a peristaltic pump. To 'Chelex-treat' a protein sample, <5 ml of metal-substituted protein solution was passed over approx. 3 ml of resin. All Chelex-treated solutions and apoenzyme samples were stored in plasticware presoaked in 10% (v/v) nitric acid to minimize leaching of metals into the solution. To minimize the level of metal leaching from the quartz cuvettes during the kinetic measurements of apoGlxI, the cuvettes were soaked in 1:1 nitric acid/sulphuric acid for 15 min followed by exhaustive washing with Chelex-treated water before use.

Primer synthesis and DNA sequencing

The DNA primers employed in the mutagenesis and plasmid sequencing were obtained from MOBIX (McMaster University, Hamilton, ON, Canada). Sequencing of the mutated plasmid, pGL11, was also performed by MOBIX.

Mutagenesis

The H5Q mutant *E. coli* GlxI was constructed using the one-step mutagenesis protocol based on the QuikChange™ site-directed mutagenesis protocol from Stratagene (La Jolla, CA, U.S.A.). The plasmid containing the DNA for wild-type *E. coli* GlxI, pGL10 [25] was utilized as the template and Pwo DNA polymerase enzyme (Boehringer Mannheim/Roche Diagnostics, Uppsala, Sweden) for DNA amplification. Primers homologous with the DNA encoding GlxI and the DNA upstream, within the *gloA* insert, were designed with a single base change to mutate H5Q (CAT → CAA). Their sequences from 5′ to 3′, with the mutated codon in bold italics, were as follows: GGATAAAA-AGATGCGTCTTCTT***CAA***ACCATGCTGCGCG and CGCGC-AGCATGGT***TTG***AAGAAGACGCATCTTTTTATCC. Six reaction conditions were prepared, varying the $MgSO_4$ concentrations between 0.2 and 2.5 mM. PCR amplification was performed in the following manner. A 'hot start' or 'jump start' was performed in which the tubes were heated to 95 °C for 1 min before addition of the Pwo DNA polymerase enzyme. This was followed by 12 cycles of 95 °C 30 s, 55 °C 60 s, 68 °C 10 min, ending with a 15 min incubation at 68 °C to ensure all extensions were completed. PCR product mixtures were incubated with *Dpn*I at 37 °C for 1 h to digest the methylated wild-type template DNA. The plasmid containing the mutated *gloA* gene, pGL11, was first transformed into *E. coli* DH5α. Methylated pGL11 was then isolated from DH5α and transformed into *E. coli* MG1655 for effective protein expression. The pGL11 DNA sequence was confirmed by sequencing and confirmed the desired mutation (CAT → CAA) and that the remainder of the gene sequence was intact.

Expression and protein purification

Wild-type *E. coli* GlxI was expressed and purified from *E. coli* MG1655/pGL10 as described previously [25]. Protein concentrations were determined by the Bradford method [31]. Using alternative techniques, cysteine titration and molar absorption coefficient, we have previously demonstrated this to be a valid measurement of GlxI protein concentration [25], with approx. 15% variance between the methods. *E. coli* MG1655/pGL11 was grown and the mutant protein expressed as described for the wild-type enzyme [25], except that 50 μg/ml ampicillin was utilized rather than carbenicillin, with no effect on cell growth or protein expression. The purification method utilized for the wild-type GlxI enzyme was also employed to purify the H5Q mutant GlxI with the following exception. After the isoelectric focusing stage of the purification, the protein was subjected to separation on a Superdex 75™ HR 10/30 (Amersham Biosciences, Uppsala, Sweden) gel-filtration column, equilibrated with 50 mM Mops, pH 7.0 (Chelex-treated). Samples of <500 μl were applied and the protein was eluted with buffer at 0.5 ml/min for 60 min.

ESMS (electrospray mass spectrometry) analysis

The integrity of each purified protein sample was monitored by ESMS, provided by the Biological Mass Spectrometry Laboratory (University of Waterloo, ON, Canada) under conditions described previously [25].

Kinetic analyses

Determination of the Michaelis constant, K_m, and the maximal enzyme velocity, V_{max}, for wild-type *E. coli* GlxI activated with various metal chlorides involved measurement of the initial reaction rate utilizing ten substrate concentrations ranging from

0.005 to 2.0 mM. The enzyme stock solution was diluted with 50 mM Mops (pH 7.0) and 2.5 mol equivalents of metal to dimeric enzyme were added unless otherwise noted. The data were fited by non-linear regression analysis using GraFit 3.01 (Erithacus Software).

As iron (II) chloride readily oxidizes in air and in oxygenated aqueous solutions, care was taken to prevent exposure to air. The metal powder and prepared solutions were handled under a flow of argon. All buffers and solutions were degassed and sparged with argon before use.

To ensure that the metal chlorides did not affect the stability of the *S*-D-lactoylglutathione product or the hemithioacetal substrate, solutions of the substrate or the product were incubated separately with the individual metal salts in the assay buffer, in the absence of GlxI and the absorbance at 240 nm was then monitored over time. Possible spectral interference by metal ions was determined by performing a scan from 800 to 200 nm of 0.2 mM *S*-lactoylglutathione (reaction product) in water with and without the addition of the metal chloride, in the absence of GlxI.

Kinetic analyses of the H5Q mutant GlxI were performed in 50 mM potassium phosphate buffer (pH 6.6), the standard conditions for GlxI analysis [32], as described previously for the wild-type enzyme [25] or in 50 mM Mes (pH 6.6). For each metal tested, the desired metal chloride concentration was added directly to the substrate solution immediately before performing the assay. In addition, the enzyme was preincubated for a minimum of 10 min with the same metal ion concentration to ensure that there were no differential concentrations or time dependence of metal binding. As low levels of substrate breakdown were detected with the higher levels of $ZnCl_2$ used in the analysis of the H5Q GlxI enzyme, these values (slopes over the time period of the enzyme-catalysed assay) were subtracted from the enzyme-catalysed reaction values.

ICP (inductively coupled plasma) analysis

For ICP emission spectroscopy analyses of the *E. coli* GlxI protein, the purified protein was prepared in metal-free solutions. For the various metal-substituted forms of the enzyme analysed, apoGlxI was reconstituted with 2.5 mol equivalents of metal chloride ($ZnCl_2$, $CoCl_2$, $CdCl_2$, $MnCl_2$, $CuCl_2$, $MgCl_2$ and $CaCl_2$) and the solution dialysed overnight versus 2 × 500 ml of 50 mM Mops (pH 7.0; Chelex-treated) to remove any unbound metal ions, as described previously for GlxI reconstituted with $NiCl_2$ [25]. Initially, samples and controls were passed over Chelex 100 resin to remove loosely bound metals, and acidified to below pH 2 with high-purity nitric acid. As this was not found to be necessary, subsequent samples were not passed over Chelex resin before acidification. To ensure that the Chelex treatment was not removing bound, activating 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 as well as before and after the dialysis.

Metal analyses by ICP were performed at the Solutions Analytical Laboratory (Department of Earth Sciences, University of Waterloo, ON, Canada) as described previously [25].

ITC

ITC titrations were performed in a Calorimetry Sciences Corporation (CSC; Spanish Fork, UT, U.S.A.) 4200 Isothermal Titration Calorimeter, with Hastelloy stainless-steel cells, at a constant temperature of 25 °C and at a stirring rate of 297 rev./min. Titrations were performed with purified apoGlxI and several metal chlorides ($NiCl_2$, $ZnCl_2$, $CoCl_2$, $CdCl_2$, $MnCl_2$, $MgCl_2$ and $CaCl_2$).

Table 1 Kinetic analyses of wild-type *E. coli* GlxI with various activating metals

Ten substrate concentrations between 0.005 and 2.0 mM were measured in triplicate, and each set of kinetic measurements were performed 2–3 times (minimum) for each metal. Kinetic measurements with $MnCl_2$ were performed in Mes buffer, whereas all other measurements with the wild-type enzyme were performed in the standard assay buffer, 50 mM potassium phosphate (pH 6.6).

Metal chloride	V_{max} (μmol · min^{-1} · mg^{-1})	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} · s^{-1})
Ni^{2+}*	676 ± 17	27.0 ± 0.4	338	12×10^6
Co^{2+}	213 ± 33	12 ± 2	106	8.8×10^6
Mn^{2+}	121 ± 10	10 ± 2	60.2	6.0×10^6
Fe^{2+}	112 ± 20	10 ± 3	55.7	5.6×10^6
Cd^{2+}	43 ± 5	8.9 ± 0.4	21.4	2.4×10^6

* From [25].

The enzyme concentration in each titration was between 0.06 and 0.14 mM dimeric apoGlxI in 50 mM Mops (pH 7.0) (Chelex-treated), or 3.6–8.4 mg of protein/run to fill the 1.260 ml of cells plus the associated access portals. Metal concentrations were between 1 and 2 mM in matching buffer. Before each titration, the cells were rinsed with Chelex-treated Mops buffer, a buffer that matched the protein solution buffer. The reference cell was filled with degassed Milli-Q water. Titration into apoGlxI with each metal was performed a minimum of three times, with the exception of Mg^{2+} and Ca^{2+} which were only measured twice.

Titration results were collected with the 'shell' software provided with the instrument. The area of the peaks in the binding isotherm, corresponding to the heat absorbed or released when the ligand interacted with the protein, was analysed with ITC Data Works™ (version 1.0), and the thermodynamic parameters determined utilizing BindWorks™ (version 1.0). The heat of dilution was calculated by determination of the area of each titration peak at the end of the titration subsequent to saturation. This value was added or subtracted to each value during the titration. A sample titration of each metal into buffer was first performed to ensure that the heat of dilution was not concentration-dependent.

Before analysis of protein–metal interactions, the ITC was calibrated utilizing the internal electrical heater to ensure that the heat measured by the thermoelectric device was properly converted into μW. In addition, a chemical calibration was performed [33] titrating barium chloride (Aldrich, Milwaukee, WI, U.S.A.) into 18-crown-6 (Aldrich) and the exact cell volume was found to be 1260 μl. Test titrations were also performed with 2′-CMP and RNase A (Sigma). The thermodynamic parameters determined were within the acceptable limits of the published values [34], confirming that the instrument was functioning properly and the techniques utilized for titration set-up were suitable for the GlxI experiments.

RESULTS

Kinetic analyses of wild-type GlxI in the presence of various metal ions

The kinetic parameters determined for *E. coli* GlxI activated with various bivalent metal chlorides are listed in Table 1. There is only a slight alteration in the Michaelis constant, K_m, for the MG-GSH hemithioacetal adduct with the various metal ions, suggesting that the alteration of the active-site metal is not contributing to substrate binding in a substantive way. For the five metal-activated forms of *E. coli* GlxI, the primary alteration in their kinetics is seen in the V_{max} or k_{cat} values. There is approx. a 15-fold difference in the activity range of these five metallated forms of the enzyme.

As described previously [25], the Zn^{2+}-active forms of GlxI from *S. cerevisiae*, *H. sapiens* and *P. putida* have higher k_{cat} values, but their k_{cat}/K_m values are in the same range as those observed for *E. coli* GlxI activated with various metal ions. The k_{cat}/K_m values for *S. cerevisiae*, *H. sapiens* and *P. putida* were found to be 3.5×10^6, 23×10^6 and 1.3×10^6 $M^{-1} \cdot s^{-1}$ respectively [28,35,36].

During these experiments, several difficulties were encountered. Exposure of the Fe^{2+} solutions to air during analysis was minimized to avoid oxidation, as described in the Experimental section. However, initial attempts to introduce the Fe^{2+}-activated GlxI into the cuvette with a gas-tight Hamilton syringe proved problematic. The enzyme appeared to be activated by contact with the syringe needle. As these needles are made of stainless steel, which contains nickel, it is probable that this is the source of the enzyme activation. As a result, the Fe^{2+}-activated enzyme solution had to be introduced with a standard pipettor. The enzymic assay is monitored for only a few minutes and the substrate solution was sparged with argon to minimize possible oxidation of the metal Fe^{2+} to Fe^{3+}. Using this technique it was possible to determine the V_{max} and K_m values for Fe^{2+}-activated GlxI.

An additional problem was encountered in the analysis of Mn^{2+}–GlxI. After addition of the metal-activated enzyme to the substrate solution, the activity appeared to decrease slowly during the course of the reaction. It was found that addition of excess metal to the substrate solution lowered the rate of this decrease, suggesting that the metal was being lost in the reaction mixture. Analysis of the enzyme activity using Mes as the assay buffer rather than phosphate eliminated this problem. The pH was maintained at 6.6 in order not to alter the equilibrium for the MG-GSH substrate. This loss of metal was also observed, and to a much greater extent, for the H5Q mutant form of GlxI discussed below. The kinetic parameters for wild-type Ni^{2+}–GlxI were measured using Mes for the assay buffer and no significant differences on the enzyme activity were observed. Hence, all subsequent Mn^{2+}–GlxI activity assays were performed in Mes. This result can be rationalized by the metal stability constants for the two buffers. Although phosphate does have a relatively low affinity for metals [40], it may actually be higher than the affinity of the protein for the Mn^{2+} ion. As a result, the buffer may be chelating the metal making it inaccessible to the protein. However, Mes has negligible metal affinity [40] and does not appear to compete with the protein for metal ions. The ITC experiments, discussed below, demonstrate that the wild-type enzyme has an extremely high metal affinity, and hence the low metal affinity of the phosphate buffer is most probably insignificant for the assays with these metal ions.

Metal-binding ratio

ICP analysis was used to determine if each of the activating metals bind in the same ratio as seen for nickel and to determine if the metals that do not activate the enzyme, such as Zn^{2+} and Mg^{2+}, are bound by the enzyme, but in an inactive form. Table 2 summarizes the results of these analyses. For each of the activating metals, Ni^{2+}, Co^{2+}, Cd^{2+} and Mn^{2+}, one metal ion was detected per enzyme dimer. To confirm that our results were accurate, ITC was performed in conjunction with the metal analyses. This analytical technique confirmed the ratio of bound metal ions to the apoenzyme in each case (Table 2). These results clearly indicate that 1 mol of metal is bound per mol of dimeric enzyme, for every metal analysed.

Interestingly, Zn^{2+} and Cu^{2+} do in fact bind to the enzyme in the same ratio, but produce an inactive holoenzyme. However, Mg^{2+} and Ca^{2+} do not appear to bind to *E. coli* GlxI, explaining the lack of activation. This is in contrast with the *H. sapiens* GlxI enzyme, which is equally active with Zn^{2+} or Mg^{2+} [37]. *S. cerevisiae* GlxI has been found to contain Zn^{2+} as its natural metal but partial activity is recovered by addition of Mg^{2+} or Ca^{2+} to the apoenzyme [38].

Table 2 Results of the ITC and ICP analyses of *E. coli* GlxI with various metal ions

For the ITC measurements, metal chloride (1–2 mM) was titrated into apoGlxI (0.06–0.14 mM) at 25 °C and a stirring rate of 297 rev./min. The results were analysed using the ITC Data Works and BindWorks software. ICP measurements were performed on GlxI reconstituted with 2.5 mol equivalents of metal chloride to dimeric apoenzyme, followed by dialysis to remove unbound metal.

Metal chloride	*n* (binding sites)	ΔH (kJ/mol)	K_a (M^{-1})	Metal/dimeric enzyme by ICP (mol)
Nickel	1.04 ± 0.11	-16.4 ± 0.9	$> 10^7$	1.2
Zinc	0.93 ± 0.05	-10.8 ± 0.4	$> 10^8$	0.80
Cobalt	0.92 ± 0.02	-10.9 ± 0.2	$> 10^7$	0.80
Manganese	0.90 ± 0.06	-14.0 ± 0.5	$3.9 \pm 1.0 \times 10^6$	0.84
Cadmium	1.00 ± 0.03	-31.8 ± 0.6	$> 10^8$	0.95
Magnesium	0			0
Calcium	0			0
Copper	n.d.*			1.2

* Not determined.

Affinity of *E. coli* GlxI for various metal ions

ITC also provided information regarding the thermodynamic parameters of the interaction between the protein and metal ions. For each metal tested, the data were best fit by a single site-binding model. As seen in Table 2, the equilibrium binding constant or association constant (K_a) is very high for most of the metals analysed. Unfortunately, the affinity is too high for precise determination by ITC in most instances. This has also been observed for metal binding to other enzymes, such as carbonic anhydrase [39]. Only a general range could be determined, e.g. Ni^{2+} binding to *E. coli* GlxI appears to have a K_a value $> 10^7$ M^{-1}, which corresponds to a dissociation constant (K_d) of 10^{-8} or in the nanomolar range. Figure 2 illustrates a sample titration of $NiCl_2$ into apoGlxI and a plot of the integrated heat measured. The relative affinities, as listed in Table 2, can be determined from these K_a values for each of the metals. Ni^{2+} and Co^{2+} have affinities ($> 10^7$ M^{-1}) intermediate between Mn^{2+} and the tightly bound Cd^{2+} and Zn^{2+} ($> 10^8$ M^{-1}). The enzyme appears to have a lower affinity for Mn^{2+} allowing for more accurate determination of the K_a (3.9×10^6 M^{-1}). The enthalpy change (ΔH) for the interaction of each metal with apoGlxI is in the same range, although ΔH for Cd^{2+} is somewhat lower. Without a precise K_a value, other thermodynamic parameters of the interactions (e.g. Gibbs free energy change and entropy change) could not be determined. Preliminary differential scanning calorimetry studies have indicated that the denaturation temperature (T_m) for each of the various metal-substituted forms of *E. coli* GlxI follows the same trend as observed for their K_a values determined by ITC. Mn^{2+}–GlxI was found to have the lowest T_m, followed by Co^{2+}–, Ni^{2+}– and Cd^{2+}–GlxI with intermediate T_m values, and Zn^{2+}–GlxI was observed to have the highest T_m observed under the conditions studied (S. L. Clugston, H. Frey and J. F. Honek, unpublished work).

A complication was encountered during the analysis of the metal binding by ITC. The calorimeter cells in the ITC are made of Hastelloy stainless steel, which contains approx. 60 %

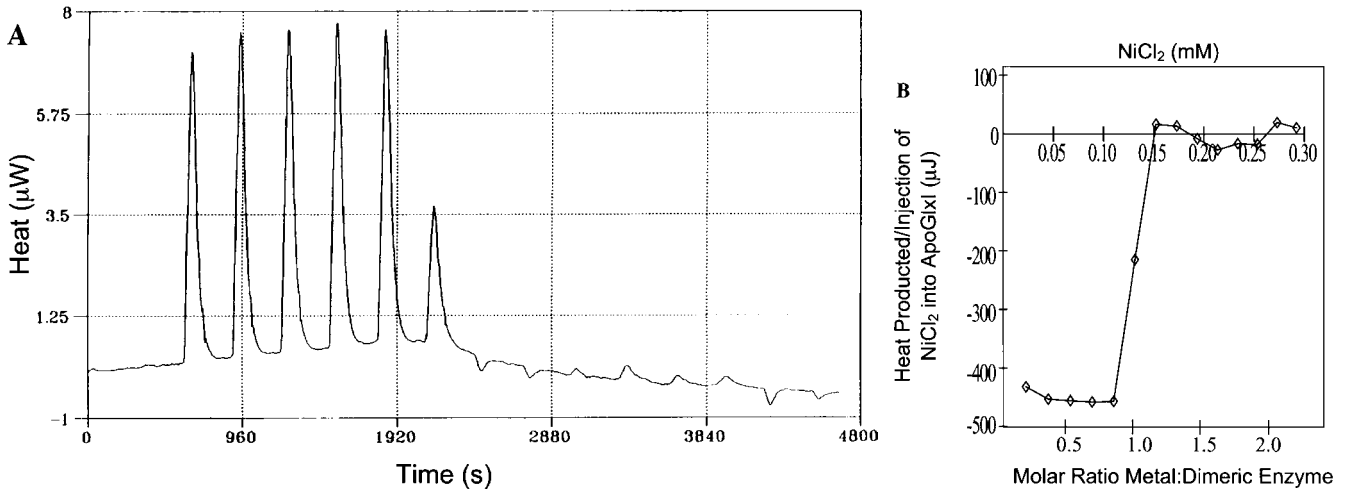

Figure 2 Titration of $NiCl_2$ into apo *E. coli* Glx I

(**A**) Binding isotherm illustrating the heat flow versus time measured for the incremental titration of $NiCl_2$ (15 μl; 1.89 mM stock) into the apoenzyme (0.13 mM) at 25 °C and 297 rev./min stirring. The upward deflection of the peaks is the convention of the CSC software for this exothermic reaction. (**B**) Integrated peak areas for the titration. Heat (μJ) measured at each injection versus total amount of metal ligand present is illustrated. The numbers on the lower axis indicate the molar ratio of metal to dimeric enzyme, with the heat returning to baseline levels after one equivalent of metal was added.

nickel (Alfa Aesar 1999–2000 Research Chemicals, Metals and Materials Catalogue). As *E. coli* GlxI readily binds nickel, there were problems with baseline stability during several of the titrations. Whereas the apoenzyme was present in the ITC cell for approx. 1 h equilibration before the start of the titration, a small steady production of heat was observed. Even after the start of the titration, the baseline often had a slight positive slope. When approximately 1 equivalent of metal chloride was titrated into the protein solution, the baseline rapidly decreased and levelled out, suggesting that the increase was due to binding metal in the system. A sample of protein was removed from the ITC after approx. 90 min in the stainless-steel cell without any metal having been titrated into the solution. The relative activity was approx. 10 % when compared with that seen with fully active Ni^{2+}–GlxI, which may explain why in some instances the number of binding sites (n) determined by ITC was slightly below 1.

H5Q GlxI mutagenesis, protein production and purification

There was no apparent effect on the level of protein production with or without 1 mM $NiCl_2$ in the growth media. The expression of the H5Q mutant protein was significantly less than that seen with the wild-type expression system [25], 30 mg/l versus 150–200 mg/l.

H5Q GlxI protein characterization and metal effects

ICP metal analysis was performed on the purified H5Q GlxI, which confirmed that it was an apoenzyme, with only trace levels of metals detected (results not shown), as reported previously for the purified wild-type apoGlxI enzyme [25]. ESMS analysis confirmed the expected protein molecular mass (14.910 kDa monomer predicted based on sequence; Figure 3). H5Q GlxI is also a dimer as determined by gel filtration (results not shown).

Preliminary experiments indicated that the H5Q mutant GlxI was active with the addition of both $NiCl_2$ and $ZnCl_2$. This was an interesting result, and led to a detailed analysis of the metal activation in the H5Q GlxI protein.

Addition of increasing amounts of metal to the H5Q mutant enzyme stock solution did not result in a significant increase in enzymic activity. Addition of the H5Q enzyme, which had been preincubated with high levels of the specific metal (1–2 mM), into the assay solution produced substantially higher initial enzymic activities (for first 10 s). This suggested that the enzyme initially could bind metal, but on dilution into the assay buffer, dissociation of the metal from the enzyme, possibly followed by buffer competition with protein for metal, resulted in decreased catalytic activity over time. If these high levels of metal were also added to the substrate assay solution, a substantial and sustainable increased activity was observed. These observations indicate that the metal affinity of the mutant protein is very low compared with wild-type enzyme. For this reason, assays were performed with an equal concentration of the desired metal in the assay solution and in the preincubation mixture containing the mutant enzyme. Therefore there would be no concentration difference of metal between the preincubation mixture and the assay mixture and no time delays would occur during the kinetic studies owing to the rate of initial metal binding to the enzyme.

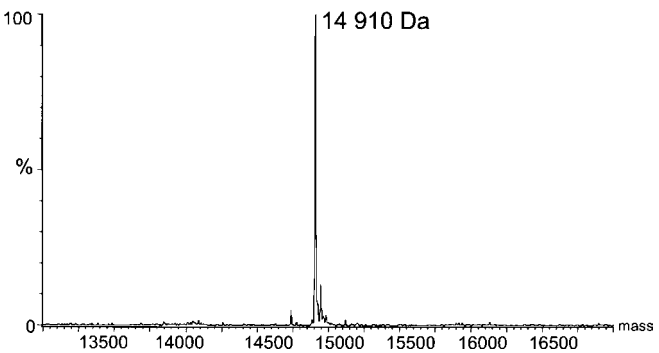

Figure 3 Reconstructed electrospray mass spectrum of the purified mutant *E. coli* H5Q GlxI

This spectrum demonstrates the protein is of the correct mass, unmodified, and that no contaminating wild-type *E. coli* GlxI enzyme is detected under these conditions.

To determine the maximal enzymic activity for *E. coli* H5Q GlxI and the required amount of metal to reach this activity, a metal activity titration was performed in which the enzymic

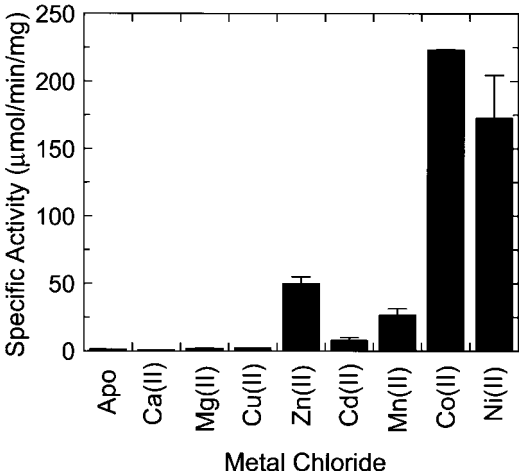

Figure 4 Activation of H5Q GlxI with various metal chlorides

Assays were performed in triplicate in Mes buffer, (pH 6.6) with 0.5 mM substrate, approx. 2.5 μg of enzyme and the entire set of measurements duplicated. Three concentrations of each metal were tested and the maximal activity observed is displayed. Ca^{2+}, Mg^{2+} and Mn^{2+} were tested at 0.25, 1.0 and 2.0 mM; Cu^{2+} and Cd^{2+} at 0.05, 0.1 and 0.25 mM; Co^{2+}, Zn^{2+} and Ni^{2+} data taken from titration curves performed over a metal concentration range of up to 2 mM. Precipitation of Cu^{2+} and Cd^{2+} precluded measurements above 0.25 mM.

activity was monitored with increasing concentrations of metal. Similar experiments with wild-type *E. coli* GlxI previously indicated that maximal enzymic activity was reached with 1 mol of metal/mol of dimeric enzyme [25]. As we have demonstrated with the Mn^{2+}-activated wild-type enzyme that phosphate can interfere with metal binding, the assays on the H5Q mutant enzyme were performed in Mes buffer (pH 6.6). At a $NiCl_2$ concentration of 2 mM the specific activity of the H5Q mutant enzyme was 173 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} (Figure 4). It was noted that very high levels of $NiCl_2$ in the assay solution and protein stock (>2 mM) inhibited the wild-type GlxI activity. At 2 mM $NiCl_2$, the activity of the wild-type GlxI enzyme was 90% of that observed with low levels of metal and decreased further to 75% at 10 mM. The H5Q GlxI activity was only moderately increased at 5 mM and decreased at 10 mM $NiCl_2$. For this reason, it was concluded that the enzymic activity should not be measured above 2 mM metal chloride. However, in any event, these activating Ni^{2+} levels would not be physiologically significant.

A similar activity titration was performed for $CoCl_2$ and $ZnCl_2$. For $ZnCl_2$, the maximal enzymic activity (50 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1}) for the mutant GlxI enzyme was observed at 0.25 mM metal, and a decrease in activity was noted above 0.5 mM. As the activity for the H5Q mutant GlxI enzyme did not reach saturation, it was not feasible to extract the affinity of the mutant protein for the metal from these results. However, the requirement of excessive amounts of metal to detect enzymic activity strongly implies a very low affinity for the metals. Although it was not possible to determine a meaningful V_{max} value for the H5Q mutant enzyme under these conditions, the maximal activities were determined for this mutant GlxI with each of the metals tested (Figure 4).

DISCUSSION

The potential for the Glx system to be an effective drug target, taking advantage of the toxic effects of methylglyoxal, is receiving renewed interest, particularly in the area of anticancer agents [41]. Alterations between eukaryotic GlxI enzymes and those from bacterial sources such as *E. coli* might also be targeted for

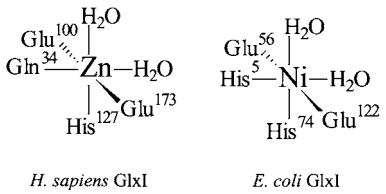

Figure 5 Active-site structure in *H. sapiens* GlxI, compared with the active form of *E. coli* GlxI

novel antibacterial agents [42–46]. In addition, the enzyme has structural and possibly mechanistic characteristics related to the vicinal oxygen chelate superfamily of enzymes [47]. Therefore a detailed study of GlxI provides an additional perspective on this superfamily, as well as unprecedented information on the subtle molecular interactions that relate to metal selectivity and metal activation of metalloproteins. A unique characteristic of *E. coli* GlxI, compared with other GlxI enzymes, is its metal activation, and the goal of the present study was to probe the factors influencing this metal activation.

The detailed kinetic studies on *E. coli* GlxI in the presence of various metals indicate the enzyme is maximally active with Ni^{2+} and exhibits lower levels of activity with Co^{2+}, Mn^{2+}, Fe^{2+} and Cd^{2+} (Table 1). No activity was observed in the presence of Zn^{2+}, Cu^{2+}, Mg^{2+} or Ca^{2+}. ICP metal analysis on the enzyme reconstituted with each metal ion indicated that each of the activating metals was bound to the enzyme with a ratio of one metal per dimeric enzyme (Table 2), as reported for Ni^{2+}–GlxI [25]. Furthermore, although producing an inactive enzyme, Zn^{2+} and Cu^{2+} were found to bind to *E. coli* GlxI in the same ratio. Ca^{2+} and Mg^{2+} did not bind to *E. coli* GlxI.

Our X-ray crystallographic studies have provided a possible explanation for why *E. coli* Zn^{2+}–GlxI is an inactive enzyme [14]. Although the same four metal ligands are found in the active site of Zn^{2+}–GlxI [Protein Database (PDB) accession no. 1FA5] and each of the active metallated forms of GlxI (Ni^{2+}, PDB accession no. 1F9Z; Co^{2+}, 1FA6; Cd^{2+}, 1FA7; and Mn^{2+}), there is only one water molecule acting as a metal ligand in the inactive Zn^{2+}–GlxI, compared with two molecules found in the active forms. This results in a trigonal bipyramidal co-ordination, as opposed to the octahedral co-ordination observed for the active forms of *E. coli* GlxI [14]. Why an octahedral co-ordination cannot be achieved in the *E. coli* enzyme with bound Zn^{2+} is unclear when the active form of the *H. sapiens* GlxI contains an octahedral Zn^{2+}. Metal ion sites in metalloproteins are designed for selective uptake and catalytic activity. The protein scaffold probably contributes not only to the proper spatial positioning of the side chains important in metal binding but also their orientation to the metal. The result is the proper positioning of what appears to be the essential two water molecules bound to the metal ion. In the *H. sapiens* GlxI enzyme, the protein's molecular structure allows for the formation of an octahedral geometry for the zinc ion, with two water molecule ligands available (Figure 5). For *E. coli* Glx I, the protein environment also provides this for Ni^{2+}, Co^{2+}, Mn^{2+}, Fe^{2+} and Cd^{2+}, but not for Zn^{2+}. All the activating metals have octahedral geometry with two positioned water molecules as ligands. The *E. coli* Zn^{2+} complex differs from the *H. sapiens* enzyme zinc environment in that the ligand geometry is now trigonal bipyramidal. Unlike Ni^{2+} (d^8), Zn^{2+} (a d^{10} co-ordination metal) has no strong preference for an octahedral over a trigonal bipyramidal geometry from considerations based on energetics or ligand-field theory [48,49]. Hence, the *H. sapiens* protein scaffold probably plays a decisive role in determining the octahedral

co-ordination surrounding zinc. For the *E. coli* enzyme, obviously, the protein scaffold is unable to provide this decisive role for zinc. Furthermore, the ligand differences (histidine in *E. coli* and glutamine in *H. sapiens*) definitely contribute to this variation. In addition, the type of co-ordination, the nitrogen imines from the histidine in *E. coli* versus the oxygen from the glutamine in the human enzyme, may contribute in some way to preferential formation of a trigonal bipyramidal geometry for Zn^{2+}. The requirement for the precise positioning of the two water molecules as ligands is therefore prevented.

In addition to providing information regarding the metal binding ratio, ITC was utilized to provide information regarding the thermodynamics of the interaction between apoGlxI and each metal. Analyses of the ITC results indicate that the metal ions are tightly bound to the active site with binding affinities (K_a) at the limits of detection ($> 10^7$–10^8 M^{-1}). The exception is Mn^{2+}, which has a lower affinity, 3.9×10^6 M^{-1} (Table 2). Interestingly, although Zn^{2+} produces an inactive enzyme, the K_a for this metal is quite high.

These thermodynamic results, metal analyses and kinetic studies all indicate that the enzyme is maximally active with one metal ion bound per dimeric enzyme. As *E. coli* GlxI is homodimeric with high homology to the *H. sapiens* enzyme, it is possible, and expected, that a second putative metal-binding site exists in *E. coli* GlxI. One metal ion is indeed observed in each active site at essentially equivalent positions in the crystal structure of the enzyme with Ni^{2+}, Cd^{2+}, Co^{2+} or Zn^{2+} bound [14]. Furthermore, the enzyme does have two active sites capable of binding GSH-based inhibitors, as determined by analysis of non-covalent complexes by MS [26]. However, these contradictory findings suggest that the two active sites may not be completely equivalent. One possibility could be that the affinity of the enzyme for a second metal ion is significantly lower when compared with that for the first metal bound and does not affect the level of enzyme activity. Addition of a very large excess of $NiCl_2$ to the wild-type enzyme (approx. 1000 equivalent) did not increase the enzymic activity above that observed when only one equivalent of $NiCl_2$ was added. Any binding to a second site does not appear to affect the enzyme activity. ITC experiments using an excess of metal (20-fold) gave no evidence for a second binding event.

The concentration of enzyme (12–37 mg/ml or 0.4–1.2 mM dimeric enzyme), and hence metal, required in the protein crystallization conditions are significantly higher when compared with that employed in standard kinetic experiments. For kinetic analyses, the enzyme is generally stored at a concentration of approx. 5 mg/ml and diluted before analysis, in which ng to μg of enzyme are used. Metal analyses were performed at enzyme concentrations of 0.5–1.0 mg/ml. This concentration factor may contribute to the ability of the enzyme to bind the second metal under the crystallization conditions. Alternatively, it is probable that the protein crystal is formed only if both sites are symmetric and hence occupied by a metal ion. Further investigation into this aspect of the bacterial GlxI is actively being pursued.

Although three of the four protein ligands to the metal are identical in the *E. coli* and *H. sapiens* GlxI enzymes, His^5 in the *E. coli* enzyme is replaced by a glutamine (Gln^{34}) in the *H. sapiens* enzyme (Figure 5). Analysis of the *E. coli* H5Q mutant revealed that the affinity of the protein for metal ions was greatly decreased. Furthermore, this mutation resulted in an enzyme that now has some measurable enzyme activity in the presence of Zn^{2+}, although the activity is extremely low and is much decreased from the activity seen with either Ni^{2+} or Co^{2+}. This is a very intriguing observation, as a change of this one ligand was not expected to produce such a drastic effect, owing to the observation that the *S. cerevisiae* and *P. putida* GlxI are active with Zn^{2+} and yet both contain a histidine at this position, as in the *E. coli* enzyme [28,29]. Most of the known and postulated GlxI enzymes contain a histidine at this position [42].

Mutation of the ligands to the metal in the *H. sapiens* GlxI enzyme has also been shown to result in a decreased metal affinity in some instances [20]. Mutation of E100Q (equivalent to Glu^{56} in *E. coli* GlxI; Figure 5) resulted in a protein with almost no metal bound. Further mutations of E173Q, the postulated catalytic residue (Glu^{122} in *E. coli*), and the double mutant Q34E/E100Q were observed to contain a reduced amount of bound catalytic zinc and minimal enzymic activity [20]. However, the metal content was determined after standard purification of the enzyme. Additional metal salts were not added to the protein after purification or during the enzymic assays. As a result, these proteins may have had some residual metal-binding properties and activity, but it was not detected under the conditions reported. Recent mutagenesis studies on the *S. cerevisiae* GlxI enzyme demonstrated that mutation of the postulated catalytic ligand in each active site, Glu^{163} or E318Q (corresponding to Glu^{122} in *E. coli* GlxI and Glu^{173} in *H. sapiens*), affects the activity of the enzyme and the type of metal ion bound, but does not significantly affect the total metal content [50]. These studies suggest that the two active sites appear to have different metal-binding preferences, binding one Fe^{2+} ion and one Zn^{2+} or Mn^{2+} ion.

One possible problem to note with the expression system used in our *E. coli* mutagenesis studies is that the H5Q GlxI mutant was not expressed in a GlxI-deficient cell line. *E. coli* MG1655 utilized to express the wild-type enzyme was also employed in the present study. As such, there could be contaminating low levels of chromosomally encoded wild-type GlxI from this strain of *E. coli*. This is not believed to be a factor for several reasons. There was no detectable wild-type enzyme in the ESMS (Figure 3) when analysed under the same conditions used for wild-type GlxI analysis. In addition, the activities measured and the observed affinity of the enzyme for various metals are drastically different from those seen for the wild-type enzyme. Even low levels of wild-type GlxI would be activated by trace amounts of metal and this activity would be detected. The fact that no activity is evident without a large excess of metal supports the fact that we are studying the mutant enzyme and that the activity measured is truly from the H5Q GlxI and not simply residual wild-type activity.

The observation that the enzyme (as the H5Q mutant protein) is now active with $ZnCl_2$, although at low levels, is intriguing. Unfortunately, the metal ion affinities of this mutant protein are very low. The observation that *P. putida* and *S. cerevisiae* GlxI have the same four ligands as the *E. coli* enzyme, yet tightly bind and are active with Zn^{2+}, indicates that the histidine versus glutamine metal ligand difference is not the critical factor determining the metal selectivity. Results shown in the present study clearly indicate that, although affecting the metal activation profile, the H5Q mutation is not the sole or even the major contributing factor involved in the differential metal activation observed between the *E. coli* and *H. sapiens* enzymes. Far more subtle changes in the protein play a role in metal specificity, which are difficult to predict from primary sequence or even three-dimensional structure comparisons.

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