# MICROBIAL GLYOXALASE ENZYMES: METALLOENZYMES CONTROLLING CELLULAR LEVELS OF METHYLGLYOXAL

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**SUMMARY**

The glyoxalase system consists of two enzymes, glyoxalase I and glyoxalase II. This system is important in the detoxification of methylglyoxal. Detailed studies have determined that the glyoxalase I from *Escherichia coli*, *Neisseria meningitidis* and *Yersinia pestis* are maximally activated by $Ni^{2+}$ and $Co^{2+}$, and are inactive with $Zn^{2+}$, a situation quite different from the human glyoxalase I enzyme, which is activated by $Zn^{2+}$. Recent studies on the *Pseudomonas aeruginosa* genome have led to the characterization of three different glyoxalase I enzymes, two of which follow a $Ni^{2+}/Co^{2+}$ activation profile and the third exhibits a human-like preference for $Zn^{2+}$.

**KEY WORDS**

glyoxalase, *Escherichia coli*, *Pseudomonas aeruginosa*, nickel

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## 1. INTRODUCTION

The seemingly ubiquitous nature of the cellular formation of methylglyoxal (MG), produced by a number of different pathways, has resulted in the development of major detoxification systems for this reactive compound /1-7/. One such system consists of the enzymes glyoxalase I and glyoxalase II (GlxI and GlxII) that catalyze the conversion of MG to *S*-D-lactoylglutathione (GlxI) and subsequently, to D-lactate and glutathione (GlxII) /8/. This is shown in Figure 1 with the possible intermediates that might be found in this reaction. Although the glyoxalase system was first reported in 1913, it has only been relatively recently that the molecular structures of these enzymes have been elucidated /9/.

Methylglyoxal has been shown to be cytotoxic and its intracellular concentration can reach cytotoxic levels in a short time period (please see various articles in this volume for elaboration of MG biosynthesis and chemical mechanisms of its toxicity) /5,10,11/. The glyoxalase system is one manner by which MG levels can be lowered by conversion of this molecule into D-lactate. However, in the Enterobacteriaceae, the enzyme methylglyoxal synthase has been identified which catalyzes the formation of MG from dihydroxyacetone phosphate /6,12/. It has been proposed that dihydroxyacetone phosphate conversion to MG by methylglyoxal synthase and subsequently to D-lactate by the glyoxalase system occurs. The D-lactate can be converted to pyruvate through the action of lactate dehydrogenase. The overall process then becomes a bypass to the glycolytic pathway /13/. Inorganic phosphate has been shown to inhibit methylglyoxal synthase which suggests that this bypass is activated under low phosphate conditions for these cells in order to maintain critical inorganic phosphate levels /14/. However, it is generally considered that a major role for glyoxalase I and II is to control the cytotoxic levels of MG (and perhaps other α-ketoaldehydes) within the cell. Nevertheless, recent reports have implied that MG and the glyoxalase enzymes may have important direct/indirect roles in controlling various cellular functions /15-25/. For example, the product of GlxI, *S*-D-lactoylglutathione, may play a role in the control of potassium efflux pumps in *Escherichia coli* /26/.

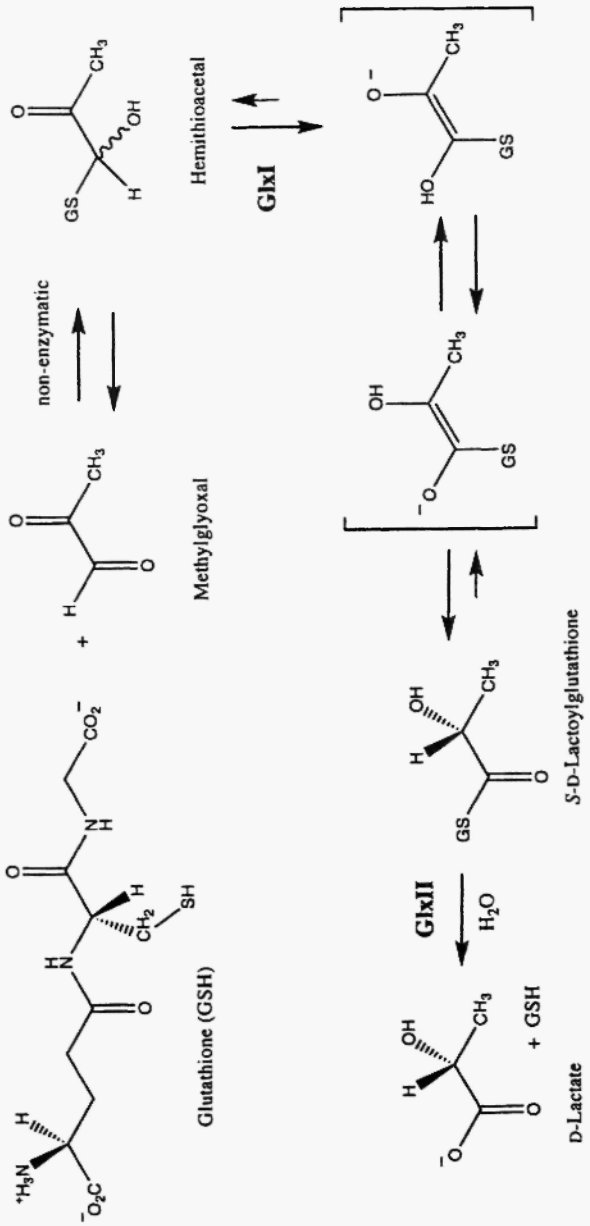


**Fig. 1:** Reactions of the glyoxalase system that is composed of glyoxalase I (GlxI) and glyoxalase II (GlxII).

## 2. THE GLYOXALASE ENZYME SYSTEM

### 2.1. Glyoxalase I

The glyoxalase system is widespread in Nature with examples of both prokaryotic as well as eukaryotic organisms utilizing these enzymes /1,8,27,28/. Calf liver Glxl was the first Glxl studied that was shown to be dependent upon metal ions for activity /29/. Since that time a range of Glxl enzymes have been shown to be metal-dependent, notably the metal ion $Zn^{2+}$ being an essential component of glyoxalase I enzymes, although $Mg^{2+}$ was at first considered to be the activating metal *in vivo* /30-32/. Detailed studies on a number of Glxls have appeared with studies on *Homo sapiens*, *Saccharomyces cerevisiae* and *Pseudomonas putida* Glxl providing critical insight into the mechanism of this enzyme and their metal specificities /33,34/.

In studying metalloenzymes, a key aspect is the determination of the metal specificity of the enzyme. However, the exact nature of the metal utilized *in vivo* can sometimes be difficult to determine experimentally, as metal ion availability present in various growth media or in the environment may vary and therefore metal reconstitution experiments on the apo (non-metallated) form of the enzyme are usually undertaken /35/. The apo forms of Glxl from *H. sapiens* and *P. putida* can be reactivated to varying extents by various metal ions including $Ni^{2+}$, $Co^{2+}$ and $Mg^{2+}$ as well as $Zn^{2+}$ /33,36/. However, $Zn^{2+}$ is considered to be the *in vivo* activating metal for a large number of Glxl enzymes. Nevertheless, in studies on the *Escherichia coli* Glxl, evidence had been reported that the apoenzyme, unlike other Glxls known at the time, is not activated by $Zn^{2+}$, although this metal ion has been shown by isothermal titration calorimetry and X-ray crystallography to bind to the active site of the *E. coli* Glxl /37,38/. Reconstitution experiments of the apoGlxI from *E. coli* with a variety of metals indicated that metals such as $Ni^{2+}$, $Co^{2+}$, $Cd^{2+}$, $Mn^{2+}$ and $Fe^{2+}$ are activating, with $Ni^{2+}$ activation producing the highest activity /37/. The kinetic constants were determined to be as follows: $Ni^{2+}$ ($K_m$ = 27 μM, $V_{max}$ = 676 μmol/min/mg, $k_{cat}$ = 338 $s^{-1}$); $Co^{2+}$ ($K_m$ = 12 μM, $V_{max}$ = 213 μmol/min/mg, $k_{cat}$ = 106 $s^{-1}$); $Cd^{2+}$ ($K_m$ = 8.9 μM, $V_{max}$ = 43 μmol/min/mg, $k_{cat}$ = 21 $s^{-1}$); $Mn^{2+}$ ($K_m$ = 10 μM, $V_{max}$ = 121 μmol/min/mg, $k_{cat}$ = 60 $s^{-1}$); $Fe^{2+}$ ($K_m$ = 10 μM, $V_{max}$ = 112 μmol/min/mg, $k_{cat}$ = 56 $s^{-1}$) /37,38/. $Zn^{2+}$ can bind to the enzyme but

the resulting enzyme is inactive. $Mg^{2+}$ does not bind to the apoGlxI as determined by isothermal titration calorimetry /38/.

Extended X-ray absorption fine structure (EXAFS) and X-ray absorption near-edge spectroscopy (XANES) studies of the inactive $Zn^{2+}$ form and the active $Ni^{2+}$ form of *E. coli* Glxl indicated that the $Zn^{2+}$ ion was most likely in a trigonal bipyramidal five-coordinate geometry whereas the $Ni^{2+}$ ion was in an octahedral six-coordinate geometry /39,40/. These biophysical results were confirmed by the determination of the X-ray structure of the *E. coli* enzyme reconstituted with a variety of metal ions /41/. The *E. coli* Glxl enzyme is dimeric in nature, which is similar to the structure of the human enzyme /42/ (Fig. 2). A comparison of the X-ray structures of the *E. coli* Glxl reconstituted with $Zn^{2+}$ (inactive) and with the activating metals $Co^{2+}$, $Cd^{2+}$ and $Ni^{2+}$ revealed that all the activating metals have an octahedral environment, but the $Zn^{2+}$-bound form of the enzyme resulted in a trigonal bipyramidal five-coordinate environment around the metal. Figure 3 shows the active site structure of the $Zn^{2+}$-bound and the $Ni^{2+}$-bound forms of *E. coli* Glxl. A key difference observed in these structures was that *E. coli* Glxl containing activating metals all have two water molecules bound to the active site metal along with four protein side chains (two side chains from each of the two subunits [A and B] making up the homodimer of the enzyme: His5A, Glu56A, His74B and Glu122B). The inactive $Zn^{2+}$-bound enzyme has the same four protein side chains bound to the metal but only one water molecule was coordinated to the $Zn^{2+}$. Recombinant overproduction of the *E. coli* Glxl in an *E. coli* host in the presence of $Ni^{2+}$ ion in the growth medium results in the formation of active enzyme when isolated directly from the cytosolic fraction. However, overproduction of the *E. coli* Glxl in the presence of $Zn^{2+}$ ion results in the formation of the inactive $Zn^{2+}$-bound enzyme /37/. Removal of the metal using isoelectric focusing produces the apoform of the enzyme, which could be readily reconstituted with a variety of metal ions. It is interesting to note that the affinity of the *E. coli* apoenzyme for $Ni^{2+}$ was confirmed by the observation that a slow but measurable exothermic reaction in the absence of added metal was seen during isothermal titration calorimetry experiments. Upon further investigation it was discovered that the apoenzyme was able to remove nickel ions from the Hastelloy stainless steel calorimetry cell (which is composed of approximately 60% nickel) /38/.

**Fig. 2:** *E. coli* glyoxalase I dimer shown in **A**) cartoon form (each subunit shown in different shading and one active site is shown with a bound $Ni^{2+}$ ion [sphere]) and **B**) solvent accessible surface area emphasizing the position of the two active sites. Figures were generated using Accelrys DS Visualizer version 1.7 (Accelrys Inc). Based on coordinates from PDB 1fgz.

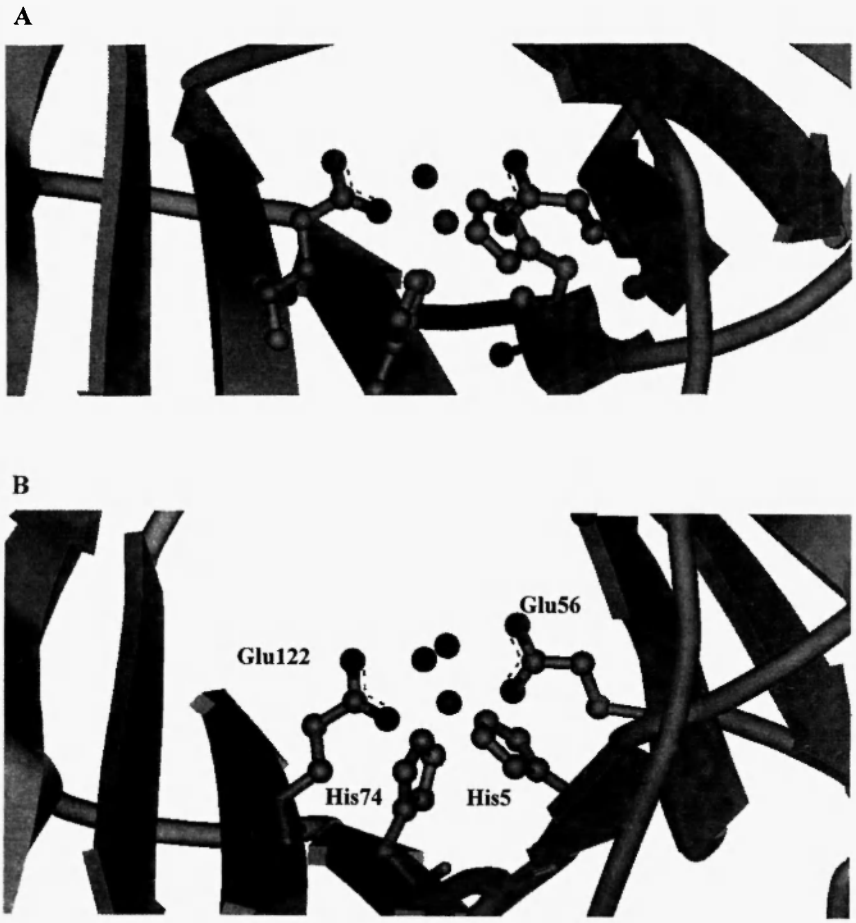


**Fig. 3:** Active site metal coordination of the *E. coli* GlxI bound to **A**) $Zn^{2+}$ (inactive) and **B**) $Ni^{2+}$ (active). The water spheres represent ligation of oxygen atoms from either $H_2O$ or hydroxide. Based on coordinates from A) PDB 1fa5 and B) PDB 1f9z.

The above discovery of a GlxI that was not activated by $Zn^{2+}$ (nor $Mg^{2+}$ as was also observed) but was maximally activated by $Ni^{2+}$ appeared to indicate that two separate classes of Glxl might exist. In order to determine whether the *E. coli* enzyme was a paradigm for a new class of Glxl, a bioinformatics analysis of the genome databases was undertaken to detect additional putative bacterial Glxl /27,43/.

This led to the investigation of the metal specificity of several additional bacterial Glxl enzymes, namely those from *Yersinia pestis*, *Neisseria meningitidis* and *Pseudomonas aeruginosa* /44/. Although the *N. meningitidis* enzyme had been isolated previously, no information was available on its metal activation profile /45/. Isolation and characterization of the proteins overproduced from these genes confirmed the glyoxalase I activity of these putative Glxls and allowed for detailed analysis of their metal activation profiles. It was determined that these Glxls were also inactive with $Zn^{2+}$ but were activated by $Ni^{2+}$. These findings provide additional support for a second class of Glxl enzymes.

Although Glxl enzymes have been studied from organisms that contain glutathione as their major intracellular thiol, other organisms utilize different intracellular thiols. For example, flagellates of the genera *Leishmania* and *Trypanosoma* are eukaryotic parasites and their intracellular biochemistry makes use of two major thiols, glutathione and the more complex trypanothione [*N*1,*N*8-bis(glutathionyl) spermidine] (Fig. 4) /46-48/. Infection by these protozoa produces clinically and agriculturally important diseases, such as African sleeping sickness (*Trypanosoma brucei*) and Chagas disease (*T. cruzi*) /49/. Infections by *Leishmania*, such as *Leishmania tropica*, *L. donovani*, and *L. braziliensis*, are believed to infect millions of people worldwide /49/. Fairlamb and coworkers have reported the isolation, characterization and X-ray structure of the *L. major* GlxI. The

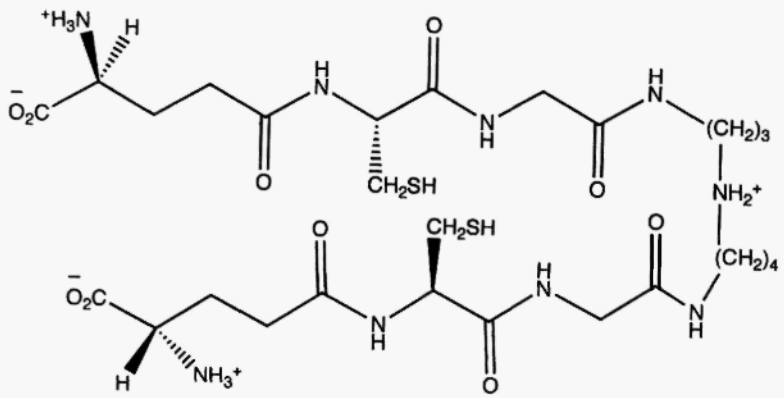


**Fig. 4:** Chemical structure of trypanothione.

molecular structure has strong resemblance to the *E. coli* Glxl structure and the *L. major* enzyme is also a $Ni^{2+}$-activated enzyme (inactive with $Zn^{2+}$) /50,51/. The *L. major* Glxl preferentially utilizes the hemithioacetal formed between MG and trypanothione as the substrate. The length of the amino acid sequence of the *L. major* enzyme is also similar to the *E. coli* and other $Ni^{2+}$-activated enzymes. These enzymes are shorter in sequence than the $Zn^{2+}$-activated enzymes. Glyoxalase activity has been detected in *L. braziliensis* and reports have recently appeared on the preliminary characterizations of the Glxl from *L. donovani* ($Ni^{2+}$-activated) and *T. cruzi* (major activation by $Ni^{2+}$ and $Co^{2+}$ but interestingly also exhibits some measurable activation by $Zn^{2+}$) which will lead to further investigations into the glyoxalase systems of these medically important parasites /52-55/. Reports have already appeared that focus on the medicinal aspects of these glyoxalase systems and the critical nature of MG concentrations in these parasites /55-58/.

Interestingly the quaternary structures of Glxl can also be different. For example, Glxl from several plants, yeast and the malaria parasite *Plasmodium falciparum* are approximately twice the length of the homodimeric Glxl enzyme from humans and *E. coli* /59-61/. The *P. falciparum* enzyme is composed of a single polypeptide chain containing two active sites. $Zn^{2+}$ activates this enzyme /61/. Recently it has been shown that there is allosteric coupling between the two active sites in *P. falciparum* Glxl /62/. Since malaria is a major pathogen affecting the world's population with 350-500 million cases occurring worldwide, with an estimated one million deaths occurring per year (http://www.cdc.gov/malaria/facts.htm), further investigation on the critical nature of Glxl in this organism is certainly warranted. Efforts along these lines are being reported /63-66/.

Another structural aspect to the Glxl enzymes is that they are members of the βαβββ superfamily of proteins /67/. The three-dimensional structure of Glxl enzymes is composed of a βαβββ secondary structural motif which is found in a number of other proteins. Proteins in this category, which have different functions, are: 2,3-dihydroxy-biphenyl 1,2-dioxygenase /68/, mitomycin resistance protein /69/, fosfomycin resistance protein (FosA) /70/, methylmalonyl-CoA epimerase /71/, and bleomycin resistance protein /72/. Connected βαβββ motifs are found in the monomeric subunits as well as proximal to each other in three-dimensional space from neighboring

subunits /36,67,73/. This arrangement forms a bowl-shaped region whose functional significance is that it is the binding site for a ligand (bleomycin and mitomycin resistance proteins) or the active site for catalysis (for Glxl, extradiol dioxygenases and methylmalonyl-CoA epimerase).

Although there now appears to be no doubt about the existence of two classes of Glxl based on metal activation profiles, would it be possible to predict the metal specificity a new Glxl might have based on its sequence analysis? It does appear that shorter sequences, comparable to the *E. coli* enzyme, exhibit $Ni^{2+}$-activation but little or no $Zn^{2+}$-activation /44,50,74/. Can we extend this prediction to the type of organism under study? This does not appear to be the case since the *P. putida* Glxl does utilize and is isolated as a $Zn^{2+}$-bound enzyme and yet a recent report has shown that a Glxl from another Pseudomonad, *P. aeruginosa*, contains a $Ni^{2+}$-activated enzyme (inactive with $Zn^{2+}$) /44/. The *P. putida* enzyme has a longer amino acid sequence as does the *H. sapiens* enzyme, which is also $Zn^{2+}$-activated. Surprisingly, a further analysis of the *P. aeruginosa* PAO1 genome, however, recently revealed two additional putative Glxl enzymes, GloA2 and GloA3, one of which (GloA2) was similar in size to the *E. coli* Glxl and the previously reported *P. aeruginosa* Glxl (GloA1) and the other, GloA3, was longer than the *E. coli* enzyme but similar to the *H. sapiens* enzyme in length /75/. Isolation and over-production of these two putative Glxl enzymes from *P. aeruginosa* PAO1 using *E. coli* expression systems allowed for examination of their potential catalytic activity as well as for detailed analysis of their metal activation profiles. Both gene products were found to exhibit high Glxl enzyme activity. The GloA2 enzyme exhibited $Ni^{2+}$-activation and no $Zn^{2+}$-activation. However, the GloA3 enzyme exhibited what we are defining as Class I activity, which is $Zn^{2+}$-activation. In fact, as isolated, the GloA3 enzyme has $Zn^{2+}$ tightly bound to the enzyme, so much so that removal of the $Zn^{2+}$ required more forceful conditions than metal removal from the Class II or non-$Zn^{2+}$ ($Ni^{2+}$-activated) enzymes. The kinetic properties of the three *P. aeruginosa* Glxl are as follows: GloA1 (with $Ni^{2+}$: $K_m$ = 32 μM, $V_{max}$ = 571 μmol/min/mg, $k_{cat}$ = 271 $s^{-1}$; $k_{cat}/K_m$ = 8.5 x $10^6$ $M^{-1}s^{-1}$); GloA2 (with $Ni^{2+}$: $K_m$ = 21 μM, $V_{max}$ = 497 μmol/min/mg, $k_{cat}$ = 247 $s^{-1}$; $k_{cat}/K_m$ = 1.2 x $10^7$ $M^{-1}s^{-1}$) and GloA3 (with $Zn^{2+}$: $K_m$ = 287 μM, $V_{max}$ = 1176 μmol/min/mg, $k_{cat}$ = 787 $s^{-1}$; $k_{cat}/K_m$ = 2.8 x $10^6$ $M^{-1}s^{-1}$). Their

amino acid sequences are presented in Figure 5 along with several other Glxl enzymes discussed in this article. The *P. aeruginosa* PAO1 study is the first report of a eubacterial species containing several Glxl encoding genes, and also an organism which possesses Glxl enzymes from both metal activation classes. A detailed scan of the National Center for Biotechnology Information (NCBI) repository of protein and nucleic acid sequence databases did not reveal any additional organisms that could contain more than one Glxl gene. Other Pseudomonads whose genomes have been completely sequenced, such as *P. syringae*, *P. putida* and *P. fluorescens*, appear to possess a single Glxl gene with significant similarity to the *P. aeruginosa* GloA3 protein, the $Zn^{2+}$-activated Glxl. It will be interesting to determine what factors (such as metal availability) might control the expression of each of these separate genes in *P. aeruginosa*. The metal activation profiles for several Glxls studied in the literature are presented in Figure 6 /30, 37,38,44,50,54,75-79/. The figure qualitatively indicates the different metal profiles between the class of Glxl that are maximally activated by $Ni^{2+}$ and show little to no activity with $Zn^{2+}$ and the class of Glxl that are activated by $Zn^{2+}$, $Mg^{2+}$ and other metals (including $Ni^{2+}$ in some cases). This class has rather broad metal activation characteristics, as can be seen from the Figure.

### 2.2. Glyoxalase II

The second enzyme of the glyoxalase system, glyoxalase II (GlxII), is involved in the hydrolysis of the *S*-D-lactoylglutathione product formed by the action of Glxl on the hemithioacetal-glutathione adduct (Fig. 1). GlxII from *Homo sapiens* and *Arabidopsis thaliana* are two of the best characterized enzymes /80-86/. GlxII has been found to be a binuclear metalloenzyme with $Zn^{2+}$ as the probable active site metal ion. The cytosolic and the mitochondrial GlxII from *A. thaliana*, however, have been reported to contain varying ratios of $Zn^{2+}$, $Fe^{2+}$ and $Mn^{2+}$ and exhibit broad metal activation /87,88/. The X-ray structures of GlxII from *H. sapiens* and *A. thaliana* have been published /82,84/ and, recently, the unpublished structure of the GlxII from *Salmonella typhimurium* LT2 has been released to the RCSB protein structure databank (PDB 2obw) /89/. As of this writing, the unpublished three-dimensional structure of the *Leishmania infantum* GlxII enzyme has been deposited but not released in the RCSB protein structure databank (PDB 2p18) /90/. The molecular structures of the *A.*

**Fig. 5:** Sequence alignment of Glxl amino acid sequences from *E. coli* (accession number AAC27133), *Y. pestis*, *L. major* (AAZ14524), *N. meningitidis* (CAA74673), *P. aeruginosa* (where GloA1, GloA2 and GloA3 sequences are indexed using the accession numbers AAG06912, AAG04099 and AAG08496, respectively), *P. putida* (NP_745896) and *H. sapiens* (NP_006699). All accession numbers correspond to entries in the NCBI protein database. Asterisks indicate conserved metal binding residues of the active site in the Figure. This alignment was created using CLC Free Workbench Version 4.0.1 (CLC bio A/S, Aarhus C, Denmark).

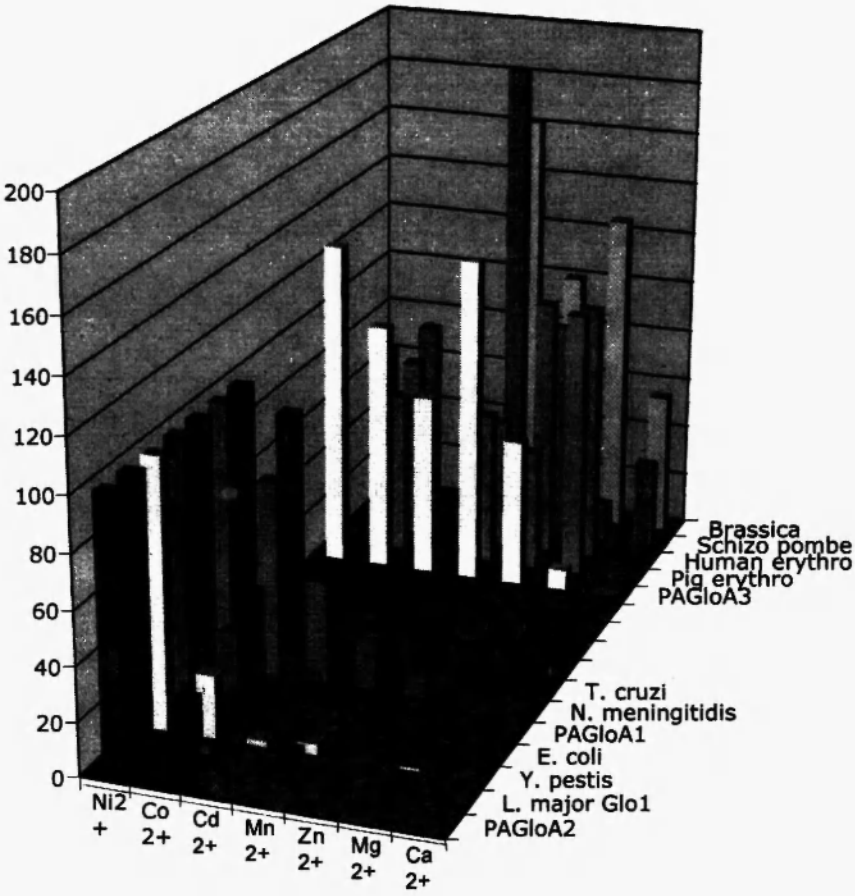


**Fig. 6:** Relative metal activation profiles for several glyoxalase I enzymes. Data taken from references as stated in the text. PAGloA1, PAGloA2 and PAGloA3 refer to the three *Pseudomonas aeruginosa* glyoxalases. Human erythro and pig erythro refer to the corresponding erythrocyte enzymes. Schizo pombe refers to the fission yeast *Schizosaccharomyces pombe*. Brassica refers to the *Brassica juncea* plant enzyme.

*thaliana* and the *H. sapiens* GlxII share the same overall fold as the $Zn^{2+}$-dependent metallo-β-lactamases, which are themselves members of the larger $Zn^{2+}$-metallohydrolase structural family /91/. The three-dimensional structure of the *A. thaliana* GlxII is shown in Figure 7. The GlxIIs from *H. sapiens*, *A. thaliana* and *Candida albicans* are monomeric in nature /80,86,92/.

Due to the discovery of the existence of two classes of GlxI based on their metal activation profiles, it was of interest to determine whether the GlxII enzyme in an organism that has a Class II ($Zn^{2+}$-inactive but $Ni^{2+}$ activated) Glxl enzyme would also have a non-$Zn^{2+}$ but $Ni^{2+}$-activated enzyme. A recent report has presented evidence that the *E. coli* GlxII enzyme is in fact isolated with approximately two

**Fig. 7:** Diagram of the *A. thaliana* GlxII structure showing key active site residues in ball-and-stick form. Coordinates taken from PDB 1xm8.

moles of $Zn^{2+}$ bound per mole of GlxII and that the enzyme is active in this form /93/. This was determined by spectrophotometric as well as inductively coupled plasma mass spectrometric techniques and is consistent with previous studies on other GlxIIs. Conditions were reported that allowed for the preparation of high levels of the apoenzyme, which could then be used to evaluate whether enzyme activity could be regained upon incubation with various metal ions. Activity was regained by the addition of either $Mn^{2+}$ or $Co^{2+}$, but no activity was seen with the addition of $Ni^{2+}$. Interestingly re-addition of $Zn^{2+}$ resulted in a further inhibition of the residual enzyme activity of the incompletely demetallated apoGlxII. Although somewhat surprising, there is literature evidence for $Zn^{2+}$-inhibition of apoenzymes for which $Zn^{2+}$ is the activating metal ion /94,95/. In the case of the *E. coli* GlxII, the presence of six cysteine residues in its amino acid sequence may result in $Zn^{2+}$-thiolate interactions that could be detrimental to reconstituted enzyme activity. Several studies have appeared that focus on GlxII as a target for the development of new drugs and this area will certainly be extended with the availability of new structural information on GlxII from a number of sources /55,57,63,96,97/.

## 3. GLYOXALASE III

Evidence has appeared for the existence of an *E. coli* protein able to convert MG into D-lactate without the need for glutathione /98/. Termed GlxIII, this enzyme has been determined to be a homodimer of ~82 kDa. No sequence or further characterization has been published for this enzyme, although a recent report has indicated the possible role of rpoS in the regulation of GlxIII in *E. coli* /99/.

## 4. CONCLUDING REMARKS

As is evident from the articles in this Special Issue, the area of methylglyoxal biochemistry and toxicology continues to receive intense investigation. In the area of enzymatic control of methylglyoxal levels, much new knowledge has been gained concerning the enzymology of the glyoxalase system. Not only has this new information expanded our fundamental understanding of this important set of enzymes, but it has also provided information on

several new potential drug targets that might be the focus of future investigations in the control of human disease. Professor Szent-Györgyi would be pleased to know that his legacy continues with the new discoveries that are being made on the biochemistry of this 'simple' three carbon aldehyde.

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