Glyoxalase II from *A. thaliana* requires Zn(II) for catalytic activity

Michael W. Crowder*, Mrinal K. Maiti, Linda Banovic, Christopher A. Makaroff

Department of Chemistry, Miami University, Hughes Hall, Oxford, OH 45056, USA

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Abstract **Cytosolic glyoxalase II from *Arabidopsis thaliana*, GLX2-2, was overexpressed and purified to homogeneity using Q-sepharose chromatography. MALDI-TOF mass spectrometry studies indicated a molecular weight of 28 767 Da. Using steady-state kinetics studies, the purified enzyme exhibited a K_m of 660 ± 100 μM and a k_{cat} of 484 ± 92 s^{-1} at 37°C. Metal analyses demonstrated that the enzyme binds 2.1 ± 0.5 moles of Zn(II) per monomer; the binding of Zn(II) is essential for enzyme viability and activity. Sequence comparison of glyoxalase II enzymes from human, *A. thaliana*, and yeast and the metallo-β-lactamases reveal that all metal binding ligands of the metallo-β-lactamases are conserved in glyoxalase II enzymes, suggesting that all glyoxalase II enzymes are Zn(II) metalloenzymes. These results and their implications are discussed in light of previous studies on glyoxalase II, and an active site for the glyoxalase II enzymes is proposed.**

Key words: Glyoxalase II; Zn(II); *Arabidoposis thaliana*

1. Introduction

The glyoxalase system catalyzes the conversion of 2-oxoaldehydes into hydroxyacids using two consecutive enzymatic reactions, the first of which requires the presence of glutathione [1]. Glyoxalase I is a Zn(II)-containing metalloenzyme that catalyzes the isomerization of a hemimercaptal species, formed spontaneously from 2-oxoaldehydes and glutathione, to form *S*-(2-hydroxyacyl)glutathione [2]. Methylglyoxal is thought to be the principle biological substrate for glyoxalase I; it is produced primarily from dihydroxyacetone and glyceraldehyde 3-phosphate in glycolysis or from acetone or threonine catabolites [3–5]. The product when glyoxalase I uses methylglyoxal as a substrate is *S*-D-lactoylglutathione [2]. Glyoxalase II catalyzes the hydrolysis of *S*-D-lactoylglutathione into D-lactate and glutathione [6,7]. The glyoxalase system appears to be ubiquitous in nature, appearing in the cytosol and mitochondria of animals and plants, and in bacterial cells in high levels [1,3]. The exact physiological role of the glyoxalase system is unknown; however, there is some evidence that the system is used to detoxify methylglyoxal and other 2-oxoaldehydes, which are cytotoxic and mutagenic by-products of lipid and glucose metabolism. There is compelling evidence emerging that inhibitors of the glyoxalase system may be anti-tumor and anti-malarial agents and may also offer treatment to complications associated with diabetes [8–12].

Glyoxalase II has been isolated from numerous mammalian tissues and from higher plants, and has been partially characterized using biochemical and kinetics studies [6,7,13–21]. Chemical modification studies identified evidence for active site amine, arginine, and histidine residues [6]. The latter residue has been implicated as a nucleophile which is thought to attack the putative activated thiol ester to form an acyl intermediate. The role of an essential histidine is supported by sequence comparisons of all glyoxalase II enzymes, which revealed a strictly conserved T-H-X-H-X-D-H motif and two other conserved histidines [6,20,21]. Makaroff and coworkers have recently noted that the former motif is also present in metallo-β-lactamases [19], which have recently been shown to bind 2 moles Zn(II) per mole of enzyme [22,23]. Based on this observation, Maiti et al. suggested that glyoxalase II enzymes may bind at least one Zn(II) ion [19].

Previous biochemical studies had been used to conclude that the glyoxalase II enzymes do not bind metal ions [6,20,21,24]. Surprisingly though, there is no report, to our knowledge, of metal analyses performed on any glyoxalase II enzyme. Given the biological roles of the glyoxalase system and its involvement in disease, glyoxalase II is an excellent target for the generation of rationally designed drugs. However, detailed structural knowledge of the enzyme is lacking.

In an effort to directly address whether glyoxalase II is a metalloenzyme, the cloned glyoxalase II from *A. thaliana* was purified, kinetically characterized, and analyzed for metal ions. We show here that *A. thaliana* glyoxalase II binds 2 moles of Zn(II) per mole of enzyme and that glyoxalase II may utilize Zn(II) binding motifs similar to those used by the metallo-β-lactamases. Sequence comparisons of glyoxalase II from *A. thaliana* with other glyoxalases suggest that all glyoxalase II enzymes, like the glyoxalase I enzymes, are Zn(II) metalloenzymes.

2. Materials and methods

2.1. General

S-D-lactoylglutathione was purchased from Sigma. A bicinchonicic acid (BCA[1]) kit was purchased from Pierce Chemical company. All chromatographic steps were performed on a Pharmacia FPLC operating at 4°C. Metal standards were purchased from Fisher and were diluted with Nanopure purified H_2O. All other chemicals used in this study were purchased commercially and were of highest quality available.

2.2. Overexpression and purification of glyoxalase II from A. thaliana

The construction of the overexpression plasmid, pT7-7/GLX2-2, has been described previously [19], and this plasmid was used to transform *E. coli* BL21(DE3)pLysE for overproduction of enzyme. Cultures of these cells were grown at room temperature in ZY media containing 200 μg AMP/ml until the cell density measured an

*Corresponding author. Fax: +1 (513) 529-5715.
E-mail: crowdemw@muohio.edu

Abbreviations: AMP, ampicillin; BCA, bicinchonicic acid; e, extinction coefficient; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; FPLC, fast performance liquid chromatography; ICP, inductively coupled plasma; IPTG, isopropyl thio-β-D-galactoside; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MOPS, 3-(*N*-morpholino)propane sulfonic acid; OD, optical density; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SLG, *S*-D-lactoylglutathione

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OD_{600nm} of 0.4. Protein production was induced by making the culture 0.2 mM in IPTG, and the cultures were allowed to shake at room temperature for 8 h. The cells were collected by centrifugation, washed with multiple additions of ddH_2O to remove salt, and lysed by a passage through a french press at 16000 psi. Cell debris was removed by centrifugation, and the cleared supernatant was loaded directly onto a Q-sepharose column, preequilibrated with 10 mM MOPS, pH 7.2. Protein was eluted from the column using a linear, 0–500 mM NaCl gradient, and glyoxalase II eluted at ca. 50 mM NaCl. Enzyme purity was assessed by SDS-PAGE and judged to be >90% pure. Glyoxalase II was quantitated using a BCA kit according to manufacturer's instructions and by amino acid analysis (Commonwealth Biotechnologies, Inc.). Six preparations of the enzyme yielded an extinction coefficient of $\varepsilon_{280}=2.8\pm0.4$ ml/mg·cm. This value was used to quantitate enzyme using its absorbance at 280 nm.

Mass spectra were acquired on a Bruker Reflex II time-of-flight (TOF) mass spectrometer operating in the linear mode. Ions were produced by matrix-assisted laser desorption ionization (MALDI) employing the 355 nm line of a New Wave Research MiniLase-10 Nd: YAG laser. The matrix solution for MALDI was saturated α-cyano-4-hydroxycinnamic acid in a solvent system of acetonitrile/water (70/30 v/v) with 0.01% trifluoroacetic acid. The enzyme sample solution (11 μM) was mixed with the matrix solution at a ratio of about 2:5. In order to increase the mass accuracy, bovine serum albumin was added to sample solutions as an m/z-calibrate at levels of ca. 10^{-5} M. The $[M+2H]^{2+}$ and $[M+3H]^{3+}$ of the calibrant (m/z 33216 and 22144, respectively) bracketed the enzyme sample's measured m/z of 28767.

2.3. Metal analyses

Purified glyoxalase II was dialyzed versus 3×1 l of freshly prepared, chelexed 30 mM MOPS, pH 7.2 at 4°C to remove any loosely bound metal ions. Metal analyses were performed on a Varian Inductively Coupled Plasma Emission spectrometer (ICP). Calibration curves with 5 standards and correlation coefficients of better than 0.9999 were used. The final dialysis buffer was used as a blank. Three trials of each sample were taken and averaged. The following absorbances for the indicated metal ions were used to ensure the lowest detection limit possible: Zn, 213.856 nm; Cu, 324.754 nm; Ni, 321.604 nm; Co, 238.892 nm; Fe, 259.940 nm; Mn, 257.610 nm; and Cd, 228.802 nm.

2.4. Steady-state kinetics studies

The activity of glyoxalase II from *A. thaliana* was assayed by measuring the initial rate of hydrolysis of *S*-D-lactoylglutathione to yield reduced glutathione and D-lactic acid in 30 mM MOPS, pH 7.2 at 20°C and 37°C using a HP5483 Diode Array UV-Vis Spectrophotometer or a Cary 1E UV-Vis Spectrophotometer, respectively. The rate of hydrolysis was followed by measuring the absorbance at 240 nm and by using the molar absorptivity of the substrate ($\varepsilon_{240}=-3100$ l/mol·cm). The observed hydrolysis rates were corrected for any background hydrolysis (in the absence of the enzyme) at each substrate concentration tested. Substrate concentrations were varied between 15 and 600 μM, but concentrations higher than 600 μM were not used due to an observed substrate inhibition.

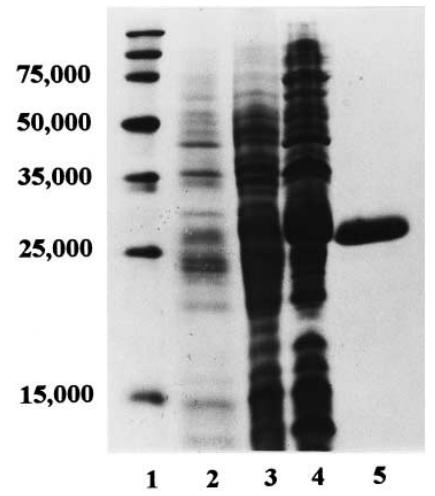

Fig. 1. SDS-PAGE of GLX2-2 purification. Lane 1: Novagen perfect protein molecular weight markers, lane 2: boiled cell fraction of BL21(DE3) containing pT7-7/GLX2-2 before induction, lane 3: boiled cell fraction of BL21(DE3) containing pT7-7/GLX2-2 after 2 h induction with 0.2 mM IPTG, lane 4: crude protein after french press, and lane 5: purified GLX2-2.

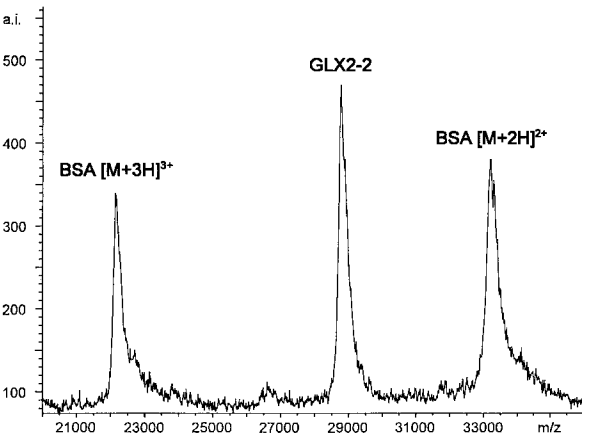

Fig. 2. MALDI-TOF mass spectrogram of purified GLX2-2. The masses of the peaks are: GLX2-2 ($[M+H^+]^+$), 28767 m/z; and bovine serum albumin ($[M+2H^+]^{2+}$, 33216 m/z) and ($[M+3H^+]^{3+}$, 22144 m/z. The sample was prepared as described in Section 2.

3. Results

3.1. Purification and characterization of glyoxalase II from A. thaliana

Makaroff and coworkers have previously cloned and overexpressed a cytosolic isozyme of glyoxalase II from *A. thaliana* (GLX2-2); test cultures demonstrated that large quantities of both soluble and insoluble glyoxalase II are produced by this overexpression system [19]. Since plant glyoxalase II enzymes have pI values less than 7 (the cytosolic *A. thaliana* enzyme has a predicted pI of 6.2), an attempt to purify the soluble, overexpressed enzyme with Q-sepharose chromatography was undertaken. As shown in Fig. 1, glyoxalase II, purified with Q-sepharose chromatography, was shown to be >90% pure by SDS-PAGE. This purification protocol yielded >15 mg of pure, active glyoxalase II from 1 l of culture. The N-terminal analysis of the purified enzyme revealed the predicted sequence of M-K-I-F [19]. Steady-state kinetics studies were performed on three different samples of purified GLX2-2 using *S*-D-lactoylglutatione as the substrate, and the k_{cat} and K_m values were 484 ± 92 s^{-1} and 660 ± 100 μM, respectively.

MALDI-TOF mass spectrometry was used to determine the molecular weight of the purified, recombinant glyoxalase II from *A. thaliana* (Fig. 2). The peak at 28767 m/z is assigned as the $[M+H]^+$ peak for GLX2-2. This value represents an error of 0.25% in the mass predicted from the DNA sequence (predicted value of 28838 Da). Including the mass of two Zn(II) atoms in the predicted mass increases the error to 0.70%, suggesting that the Zn(II) ions may not remain bound during the MALDI process.

3.2. Metal analyses on recombinant glyoxalase II from A. thaliana

In order to address directly whether glyoxalase II is a met-

Table 1
Metal content of glyoxalase II from *A. thaliana*

Metal ion	Moles metal/mole enzyme
Zn	2.1 ± 0.5
Cu	< 0.005
Co	< 0.005
Mn	0.063 ± 0.016
Fe	0.41 ± 0.24
Ni	0.45 ± 0.31

alloenzyme, metal analyses on the purified enzyme were conducted. After elution from the Q-sepharose column, the enzyme was concentrated using an Amicon concentrator with a YM-10 membrane and dialyzed versus 3×1 l of freshly prepared, chelexed 30 mM MOPS, pH 7.2 buffer. As shown in Table 1, the only metal ion detectable in significant amounts is Zn(II), and the metal to enzyme stoichiometry is 2.1 ± 0.5. Six additional preparations of the enzyme yielded similar values for the metal content of glyoxalase II, even if the dialysis steps were left out. This metal content was also unchanged if Zn(II) was included in the chromatography buffers at a concentration of 200 μM.

Prolonged dialysis of the purified enzyme (2 days at 4°C) against 10 mM EDTA in 10 mM MOPS, pH 7.2 resulted in precipitation of 90% of the enzyme. The enzyme that did not precipitate after EDTA dialysis had no observable activity and did not bind any metal ions. We were unable to reconstitute enzymatic activity via addition of or dialysis against Zn(II). This result suggests that the enzyme that remained in solution was irreversibly inactivated, in agreement with previous metal-reconstitution studies on apo-glyoxalase II [6]. Further support for a role of Zn(II) in glyoxalase II comes from experiments when GLX2-2 is incubated at pH 7.0 versus 5 mM EDTA for 2 min before a kinetics assay; the k_{cat} of the enzyme dropped by 80%. These experiments clearly show that GLX2-2 from *A. thaliana* tightly binds 2 moles of Zn(II) per mole of enzyme and that the presence of Zn(II) is required for catalytic activity. Further experiments are necessary to address whether the Zn(II) serves a catalytic or a structural role in these enzymes.

4. Discussion

The observation that glyoxalase II from *A. thaliana* contains the -H-X-H-X-D- motif which is conserved in the metallo-β-lactamases, lead Makaroff and coworkers to propose that glyoxalase II may also bind at least one Zn(II) ion [19]. A sequence comparison of three glyoxalase II isozymes from *A. thaliana* (GLX2-1, GLX2-2, and GLX2-3), human glyoxalase II, two yeast glyoxalase II enzymes (yeast GLO1 and GLO2) with the metal binding regions of the sequenced metallo-β-lactamases reveals a striking homology among these enzymes (Fig. 3). All of the ligands to the first Zn(II) site in the metallo-β-lactamases (His135, His137, and His201) are strictly conserved in the glyoxalase II enzymes. The second Zn(II) site in the metallo-β-lactamases contains a His (His272), a Cys (Cys225 in all metallo-β-lactamases except the one from *X. maltophilia* which has an aspartic acid [25]), and an Asp (Asp139). Glyoxalase II enzymes contain all of these ligands. In addition, the relative spacing between the ligands is also conserved (Fig. 3). These observations along with our data showing that *A. thaliana* GLX2-2 binds two moles of Zn(II) per mole of enzyme lead us to predict that a dinuclear Zn(II) binding site is present in the active site of the glyoxalase II enzymes.

This conclusion contradicts the long held belief that glyoxalase II enzymes are not metallohydrolases. As recently as this year, it was concluded that the cytosolic isozyme of *A. thaliana* glyoxalase II does not depend on metal ions for activity [21]. The conclusion that glyoxalase II is not a metalloenzyme is based on early biochemical studies on glyoxalase II enzymes from various sources. The enzymes from human liver and from rat erythrocytes were reported to be inactivated by long term exposure to or dialysis against chelating agents, such as EDTA, EGTA, 1,10-phenanthroline, and 8-hydroxyquinoline; however, the addition of divalent metal ions to the dialyzed apoenzyme failed to restore activity [6,24,26]. This result was used to conclude that glyoxalase II is not a metalloenzyme; however, an equally plausible explanation of these early results is that many of the glyoxalase II enzymes are irreversibly denatured upon dialysis [7,26] (Crowder, unpublished). In our hands, the addition of Zn(II) to apoGLX2-2 from *A. thaliana* also does not yield an active enzyme; our inability to restore activity was due to protein precipitation during dialysis. The inability to observe reversible metal binding in a metalloenzyme is not unprecedented; the apo-metallo-β-lactamase from *B. fragilis* cannot be reconstituted by addition of Zn(II) [23]. These earlier studies also reported that short incubations of glyoxalase II with EDTA at pHs greater than 7.0 did not affect the activities of the enzymes [6,24,26]. This result is not surprising because at these slightly basic pHs, the metal binding ligands would not be significantly protonated, and the lability of the metal ion would be expected to be low. When *A. thaliana* GLX2-2 is incubated

	131		198		222		270
A.t. GLX2-1	ILNTHHHDDHIGG	(47)	TPGHTQGH	(11)	FTGDLI	(33)	CGRENT
A.t. GLX2-2	VLTTHHHWDHAGG	(46)	TPCHTKGH	(15)	FTGDTL	(34)	CGHEYT
A.t. GLX2-3	AMNTHVHADHVTG	(44)	TPGHTAGC	(17)	FTGDAV	(36)	PAHDYK
HUMAN GLX2	VLTTHHHWDHAGG	(44)	TPCHTSGH	(16)	FTGDTL	(34)	CGHEYT
YEAST GLX2-1	IVNTHHHYDHSGG	(51)	TPCHTKDS	(15)	FTGDTL	(39)	CGHEYT
YEAST GLX2-2	IVNTHHHYDHADG	(51)	TPCHTRDS	(15)	FTGDTL	(39)	PGHEYT
R.b. ORF	ILLTHHHDDHIQA	(46)	VPGHTRGH	(11)	FTGDSL	(33)	SGHDYL
X.m. β-LACT	ILLSHAHADHAGP	(64)	MAGHTPGS	(17)	ATADSL	(37)	TPHPGA
B.c. β-LACT	VIITHAHADRIGG	(51)	GKGHTEDN	(11)	AGGCLV	(37)	TPHPGA
B.f. β-LACT	FIPNHWHGDCIGG	(51)	GGGHATDN	(11)	FGGCML	(37)	PGHPDY
S.m. β-LACT	SISSHFHSDSTGG	(50)	GPGHTPDN	(11)	FGGCFI	(34)	PSHSEV
A.h. β-LACT	VINTHYHTDRAGG	(67)	GPAHTPDG	(11)	YGNCIL	(33)	GGHSDP

Fig. 3. Sequence comparison of glyoxalase II enzymes and metallo-β-lactamases in the regions of the metal binding ligands of the metallo-β-lactamases. The numbering is based on the sequence of *A. thaliana* GLX2-1. The numbers in parentheses reflect the number of amino acids between the motifs. The abbreviations of the organisms are: *A.t.*, *Arabidopsis thaliana*; *R.b.*, *blastica*; *B.c.*, *Bacillus cereus*; *B.f.*, *Bacteroides fragilis*; *S.m.*, *Serratia marcescens*; *A.h.*, *Aeromonas hydrophila*.

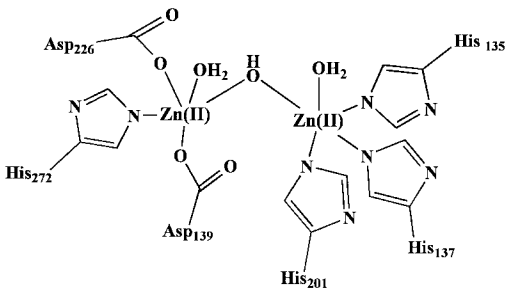

Fig. 4. Proposed active site of GLX2-2.

for short times with EDTA at pH 7.0, the activity of the enzyme drops by 80%. At this lower pH, the lability of the metal ion is expected to be greater.

Based on (1) our data that demonstrate that *A. thaliana* GLX2-2 binds 2 moles of Zn(II) per mole of enzyme and that metal binding is necessary for catalysis, (2) the observation that all of the ligands associated with the dinuclear Zn(II) center of β-lactamases, as well as their relative positions, are conserved in glyoxalase II proteins, and (3) previous biochemical data that identified potential active site residues [6], we propose a potential active site model for glyoxalase II. A representation of this proposed metallo active site, which is based in part on the crystal structure of metallo-β-lactamase from *B. fragilis* [22] is shown in Fig. 4. The proposed dinuclear site has three histidine ligands and a bridging hydroxide (or water) binding to the first Zn(II) ion and 1 histidine, 2 aspartic acids, a bridging hydroxide (or water) and a terminal water bound to the second Zn(II) ion. The crystal structure of metallo-β-lactamase from *B. fragilis* identified an asparagine and a lysine that are thought to be involved in substrate activation and binding, respectively [22]. Interestingly, previous chemical modification studies identified catalytically important amine and arginine residues [6]. Arginine, lysine, and asparagine residues are conserved in most or all of the glyoxalase II enzymes identified to date (data not shown). This suggests that the metallo-β-lactamases and glyoxalase II enzymes utilize similar reaction mechanisms to perform their catalytic functions.

The use of glyoxalase I inhibitors as anti-tumor and antimalarial agents and as a possible intervention in complications of diabetes has been proposed [3,12]. The inhibition of glyoxalase I could result in high cellular levels of methylglyoxal, which has been shown to be toxic to cells by inhibiting cell division, DNA synthesis, and microtubule assembly [8,12,27,28]. Glyoxalase I inhibitors demonstrate IC_{50} concentrations of the inhibitor in the micromolar range on cancer cell lines [8]. The inability to observe IC_{50}s of sub-micromolar concentrations was attributed to the poor penetration of inhibitors into the cells [8]. However, the partial removal of methylglyoxal from these cells by other means such as diffusion or reaction with aldose reductase cannot be totally discounted [11,29].

Glyoxalase II may be a better target for anti-tumor compounds. The putative physiological substrate for glyoxalase II is *S*-D-lactoylglutathione (SLG), which has been shown to be toxic and selective for rapidly proliferating cells, it is involved in the inhibition of DNA synthesis [12]. Several thiocarbonate and thiocarbonate-carbamate derivatives of glutathione have been shown to inhibit glyoxalase II in vitro, and diester analogs of these inhibitors have been shown to be effective on cancer cell lines, with IC_{50} values in the micromolar range [9,10,30]. The data presented here along with specific structural information on the glyoxalase II active site should allow us to take a more rational approach to the design of glyoxalase II inhibitors. Detailed studies on the structure of the metal binding site in GLX2-2 and the role of this site in catalysis are currently in progress.

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