OVER-EXPRESSION AND CHARACTERIZATION OF A MITOCHONDRIAL GLYOXALASE II FROM ARABIDOPSIS THALIANA

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In an effort to characterize mitochondrial glyoxalase II from *A. thaliana*, GLX 2-5, was cloned and over-expressed in *Escherichia coli*. This is the first time a plant mitochondrial glyoxalase II has been heterologously over-expressed in *E.coli*. GLX 2-5 was overexpressed in rich media supplemented with iron, zinc, both iron and zinc, and no added metal. Although, metal analysis showed differences in metal ion composition among the enzymes produced under different conditions, no significant difference in catalytic activity was observed. EPR spectroscopy indicated the presence of two dinuclear metal centers; Fe(III)-Zn(II) and Fe(III)-Fe(II). $^1$H NMR spectroscopy indicated the binding of Fe(II) to two histidine and an aspartate residues. Preliminary X-ray crystallographic data confirmed that GLX 2-5 is a FeZn protein and contains five histidines, two aspartate, and a bridging water as ligands in the metal binding site. A model for the active site of *A. thaliana* GLX 2-5 is proposed based on this study.
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Chapter 1
Introduction

The Glyoxalase System

The glyoxalase system is comprised of two enzymes, glyoxalase I (lactoylglutathione lyase, EC 4.4.1.5) and glyoxalase II (hydroxyacylglutathione hydrolase, EC 3.1.2.6). It converts acyclic alpha-oxoaldehydes to their corresponding alpha-hydroxyacids using reduced glutathione (GSH) as a cofactor. The hemithioacetal formed non-enzymatically from an alpha-oxoaldehyde and GSH is isomerized to a S-2-hydroxyacylglutathione derivative by glyoxalase I. Glyoxalase II catalyses the hydrolysis of this thiolester into the corresponding alpha-hydroxy acid, regenerating free GSH. The primary physiological substrate of the glyoxalase system is thought to be methylglyoxal. This system has been reported from many organisms including mammals, plants, bacteria, yeast and protozoa. In contrast, in Escherichia coli, a single enzyme, glyoxalase III, converts methylglyoxal into D-lactate without using GSH. Interestingly, protozoan glyoxalase II from African trypanosomes uses trypanothione-thioesters as substrates.

Although the glyoxalase system seems to be ubiquitous, variation in the expression of glyoxalase I and glyoxalase II has been observed. Rapidly dividing cancer cells, and plant shoot and root tips showed increased levels of glyoxalase activity. Human colon carcinoma cells exhibited increased glyoxalase I gene expression. The expression of glyoxalase I in Brassica juncea was up regulated in response to salt, water and heavy metal stresses. Also, glyoxalase I was induced in response to drought and cold stresses in Arabidopsis. It has been observed that the ratio of glyoxalase I to glyoxalase II is initially very high in Bufo bufo embryonic cells and the ratio gradually decreases during development. Glyoxalase I activity was high in proliferating, embryonic tissues whereas glyoxalase II activity was high in differentiated, adult tissues. The rat spermatogenesis-associated protein RSP29 was shown to be an homolog of human glyoxalase II. RSP29 was highly expressed in testis and showed stimulatory effects on Sertoli cells suggesting that it may play an important role in the regulation of spermatogenesis.
Figure 1.1. The glyoxalase system. The conversion of methylglyoxal (the main physiological substrate of the glyoxalase system) into D-lactic acid regenerating reduced glutathione by the glyoxalase system. The pathway involves three steps: a spontaneous reaction and glyoxalase I- and glyoxalase II-catalyzed reactions.
Methylglyoxal (MG) is considered to be the main physiological substrate of this system (19). MG is produced during carbohydrate and lipid metabolism (20, 21). MG is also produced during threonine catabolism, the metabolism of ketone bodies, the fragmentation of glycated proteins and photosynthesis. Triosephosphate isomerase and methylglyoxal synthase are involved in enzymatic reactions to give MG from dihydroxyacetone phosphate (1, 22).

Methylglyoxal is a cytotoxic and mutagenic metabolite. MG reacts with arginine and lysine residues to give glycosylamine derivatives and with cysteine residues to give hemithioacetals (22, 23). It modifies guanylate residues in DNA and RNA and induces apoptosis (22, 24). It produces interstrand crosslinks in AT-rich regions in duplex DNA (22) and single-strand breaks in DNA (25). It is clear that MG removal is important for the viability of a living organism. Thus the high ratio of glyoxalase I to glyoxalase II that has been observed in embryonic development and is a characteristic of the proliferative stage, assures a good scavenging action against the potentially cytotoxic effect of methylglyoxal (17).

S-D-lactoylglutathione (SLG) is the typical substrate of glyoxalase II and is formed by the enzymatic reaction of glyoxalase I in the cytosol. SLG is a cytotoxic compound that can induce growth arrest in human leukemia cells in vitro through inhibition of de novo pyrimidine synthesis (26, 27). SLG does not transport across plasma membranes (28). However, γ-glutamyltranspeptidase converts SLG into N-D-lactoylcysteinylglycine which can cross cell membranes (29).

Diseases Associated with the Glyoxalase System

Increased concentrations of glyoxalase metabolites (methylglyoxal, S-D-lactoylglutathione and D-lactate) have been reported from diabetic patients (30). Alpha-oxoaldehydes undergo non-enzymatic reactions with proteins, nucleotides and basic phospholipids (31). The resulting advanced glycation end products (AGEs) cause protein denaturation, enzyme inactivation, mutagenesis, apoptosis, and membrane lipid bilayer disruption (31). In diabetes mellitus, accumulation of AGEs may give rise to biochemical dysfunctions; such as neuropathy, nephropathy and retinopathy (32). Glyoxalase I contributes an enzymatic defense against glycation by detoxifying α-oxoaldehydes. Thus,
removal of α-oxoaldehydes by the action of glyoxalase I can be employed in the disease therapy (1).

Increased glyoxalase activity has been observed in human colon carcinoma and *Plasmodium falciparum* (14, 33). Thus elevating the levels of methylglyoxal can be expected to cause cytotoxicity in tumor cells and the malarial parasite. This is an ideal strategy to help alleviate cancer and malarial conditions. The development of cell-permeable, structure- and mechanism-based inhibitors of glyoxalase I would help achieve this goal.

**Inhibitors of the Glyoxalase System**

Bivalent transition-state analogues that simultaneously bind the active site on each subunit of the homodimeric human glyoxalase I and bromoacetyl esters of GSH that function as active-site-directed irreversible inhibitors are strong inhibitors of glyoxalase I. However, to date, the strongest competitive inhibitor for human glyoxalase I is a transition state analog inhibitor, [CHG(β-ala)*n*]_2_ suberate diamide where the number of β-alanine residues (*n*) can vary from 1 to 7; it is a homodimeric compound (34). Furthermore, reactive sulphoxide esters are one of the innovative prodrugs that deliver GSH-based inhibitors into tumor cells. They are converted to the inhibitors and polymethacrylamide esters of the inhibitors by acyl exchange with endogenous GSH. Polymethacrylamide esters of the inhibitors were reported to be tumor selective (35).

*S*-p-Bromobenzylglutathione diethyl ester induced toxicity in the malarial parasite *P. falciparum* in infected human red blood cells *in vitro* (36). The ester showed inhibitory effects for incorporation of [³H]hypoxanthine in nucleotide synthesis and for incorporation of [¹⁴C]isoleucine into protein. The prospective mechanism implied that the de-esterified metabolite, *S*-p-bromobenzylglutathione, inhibited glyoxalase I activity (36). By a similar mechanism, *S*-p-bromobenzylglutathione cyclopentyl diester inhibited tumor growth by inducing the accumulation of methylglyoxal in tumor cells, and the induction of apoptosis of human leukemia 60 (HL60) cells *in vitro* (37). However, *S*-glutathione derivatives were not effective inhibitors *in vivo*, possibly because cell membranes were impermeable to them (29).

Partially purified glyoxalase I of Ehrlich ascites carcinoma (EAC) cells have been tested with physiologically important aldehydes by Biswas *et al.* (38). The results showed
that $D$, and L-lactaldehyde are strong non-competitive inhibitors of glyoxalase I and that the effect with the $D$-isomer is more pronounced. In contrast, both $D$- and $L$-glyceraldehyde and acetaldehyde are moderately inhibitory and the nature of inhibition is strictly competitive. (38).

Methylglyoxal inhibits cell proliferation. Ayoub et al. demonstrated that proliferation of human leukemia 60 cells (HL60 cells) is inhibited by methylglyoxal in vitro (39). Research on the antiproliferative action of methylglyoxal has been done using rat enterocytes and colonocytes and suggested that the antiproliferative action may occur through the inhibition of macromolecular synthesis of protein, DNA and RNA (40). $S$-$\delta$-hydroxyacylglutathione derivatives were found to induce growth arrest and toxicity in human leukemia 60 cells in culture (41). $S$-$D$-lactoylglutathione was the most effective with IC$_{50}$ of 82 µM. Monoethyl ester derivatives of the $S$-$\delta$-hydroxyacylglutathiones also induced growth arrest and toxicity but were less effective than the corresponding unesterified compounds. $S$-$\delta$-hydroxyacylglutathione derivatives also inhibited the incorporation of [$^3$H] thymidine into DNA early in the development of toxicity (41). In addition, $S$-$D$-lactoylglutathione exhibited anti-proliferative activity of HL60 cells in vitro that was mediated by inhibition of uridylate synthesis (42).

In the absence of the glyoxalase II hydrolysis reaction, $\gamma$-glutamyl transferase and dipeptidase convert SLG into N-$D$-lactoylcysteinylglycine and N-$D$-lactoylcysteine. N-$D$-lactoylcysteine showed inhibition of human leukemia 60 cell growth through the inhibition of de novo pyrimidine synthesis (43).

Glyoxalase II catalyzes the rate-limiting step in the conversion of methylglyoxal to $D$-lactate. Therefore glyoxalase II should be the most preferred therapeutic target in anti-tumor drug design. It has been demonstrated that some nucleotides, such as guanosine and adenosine triphosphates and their analogs, inhibit rat liver glyoxalase II activity in vitro (44). The authors proposed that inhibition of glyoxalase II by nucleotides suggest a mechanism whereby SLG levels can be modulated in vivo (44).

Inhibition studies on corn glyoxalase II showed that glutathione derivatives containing a thioether bond are weaker inhibitors than those containing a thioester bond or a carbonyl group (45). For example, $p$-nitrobenzyl-S-glutathione, $p$-chlorophenacyl-S-derivative and carbobenzoxy-S-derivatives exhibit increasing inhibitory activities (45). Also
glyoxalase II from *C. albicans* was inhibited strongly by S-carbobenzoxyglutathione and poorly by *p*-chlorophenacylglutathione, whereas thioether derivatives were totally ineffective (46). S-FMOC, S,N-dicarbobenzoxy- and S-carbobenzoxy-glutathione were effective competitive inhibitors of glyoxalase II from *Aloe vera* with both S-\(D\)-lactoylglutathione and S-acetylglutathione as the substrate (6). End product inhibition studies with cytosolic glyoxalase II from *A. thaliana* revealed that glutathione is a mixed inhibitor whereas \(D\)-lactate is a weak competitive inhibitor (47). Also significant differences between the \(K_I\) of glutathione (0.4 mM) and \(D\)-lactate (122 mM) suggested that product release was in the order of \(D\)-lactate to glutathione (47).

A kinetics study on cytosolic human glyoxalase II and bovine liver mitochondrial glyoxalase II in the presence of liposomes made of different phospholipids showed that neutral phospholipids did not affect the enzymatic activity (48). However, negatively charged phospholipids exerted noncompetitive inhibition on cytosolic glyoxalase II only. This study suggested a possible role of negatively charged phospholipids in the regulation of lactoylglutathione in the cell (48).

Inhibitors having high specificity toward mammalian glyoxalase II, S-fluorenylmethoxycarbonylglutathione (FMOC-G) and N,S-bis-fluorenylmethoxycarbonylglutathione (DiFMOC-G) were found to be competitive inhibitors. However, since these compounds were not efficiently transported across the plasma membranes, diesters (dimethyl, diethyl, and diisopropyl) of FMOC-G and DiFMOC-G have been synthesized, as proinhibitors, to improve transport into mammalian tumor cells in culture. The diesters were inhibitory to cell growth and viability (49, 50). A series of N- and S-blocked glutathione compounds containing 9-fluorenylmethoxycarbonyl (FMOC) and phenylmethoxycarbonyl (Cbz) protecting groups has been tested for the inhibition of glyoxalase II from *A. thaliana*. This study showed that di-FMOC and di-Cbz compounds were the best inhibitors of GLX2-2. It was postulated that tight binding of inhibitors to the enzyme is not through the interactions of protecting groups but is due to entropy effects (51).

It is clear from these inhibition studies that the use of glyoxalase II as a therapeutic intervention requires a detailed structure of the active site and reaction mechanism of the enzyme to design effective inhibitors or clinical drugs. Also, though the glyoxalase system is ubiquitous in nature, its exact physiological role is not known. It may
have additional physiological roles, in addition to detoxification of methylglyoxal. Thus, it is important to study the glyoxalase system to determine its significance on life, in addition to its involvement in tumors, malaria and diabetes.

**Glyoxalase I**

Glyoxalase I belongs to the metalloglutathione transferase superfamily. Its activity has been detected only in the cytosol. It converts diastereomers of thiohemiacetal into S-D-lactoylglutathione via a 1,2-hydride shift. Human glyoxalase I is a homodimeric Zn(II) metalloisomerase with one active site per monomer (52) whereas glyoxalase I from *Escherichia coli* is a Ni(II) metalloenzyme with two active sites per monomer (53). X-ray crystallography and site-directed mutagenesis studies showed that the mechanism of glyoxalase I is a base-mediated, proton-transfer in which the bound diastereomeric substrates undergo catalytic interconversion before the 1S-diastereomer goes to product via a Zn(II) coordinated, cis-enediolate intermediate (54). The carboxyl groups of Glu172 and Glu99 of human glyoxalase I were found to be involved in this mechanism (54).

**Glyoxalase II**

Glyoxalase II belongs to the metallo-β-lactamase superfamily of proteins, possessing the characteristic dinuclear active site (55). Generally, glyoxalase II is a monomeric protein with a molecular mass varying in the range of 18-30 kDa (56). In general, plant and yeast glyoxalase II possess acidic pI values whereas animal glyoxalase II is basic (6, 46, 57). Biochemical and molecular research demonstrated that glyoxalase II exists as multiple isozymes in many organisms, including plants, mammals and yeast (2, 58, 59) whereas glyoxalase I is a single isozyme. For example; five glyoxalase II isozymes have been identified in *A. thaliana*, two in yeast, four in spinach (2, 59, 60). Glyoxalase II activity was found in both the cytosol and mitochondria (59, 61, 62). In most cases, separate genes encode cytosolic and mitochondrial glyoxalase II isozymes. However, in humans a single gene produces both forms of glyoxalase II (63). The single gene gives rise to two distinct mRNAs transcribed from either 9 or 10 exons. The mRNA with 9 exons can give rise to both the mitochondrial and cytosolic forms of glyoxalase II whereas mRNA with 10 exons produces only the cytosolic form (63). Interestingly, rat liver mitochondrial glyoxalase II
isoforms (pI 6.6-8.1) were localized in both the mitochondrial matrix (five forms) and intermembrane space (two forms) (58, 62). Isoelectric focusing and electrophoretic studies on rat liver glyoxalase II showed that two of the mitochondrial forms have counterparts in the cytosol (62). Immunoblotting experiments further proved that the cytosolic and intermembrane space enzymes are similar by the observation of positive cross-reaction of the antibody raised for cytosolic enzyme with intermembrane space enzymes but not with matrix enzymes (58). Bovine liver mitochondria resolved into five isoforms (pI 6.3 to 7.9). They do not have a counterpart for the single cytosolic form (pI 7.5)(64). In spinach, there were three cytosolic forms (pI 5.3, 5.8, and 6.2) and one mitochondrial form (pI 4.8) of glyoxalase II (60).

Many different thioesters of glutathione (S-D-lactoylglutathione (SLG), S-D-mandeloylglutathione, S-D-acetylglutathione, S-D-acetoacetylglutathione, S-D-formylglutathione, S-D-glycolylglutathione and S-D-lactonylglutathione) have been shown to be substrates of glyoxalase II. However, SLG is the preferred substrate for glyoxalase II characterized from many sources including human, yeast, spinach, Zea mays, Aloe vera, and Candida albicans (2, 6, 45, 46, 56, 60). While thioesters of glutathione are substrates for most characterized glyoxalases II, interestingly, African trypanosomes prefer thioesters of trypanothione as substrates (9). The catalytic efficiencies (k<sub>cat</sub>/K<sub>M</sub>) were very high with mono-(lactoyl)trypanothione (5.3x10<sup>5</sup> M<sup>-1</sup>s<sup>-1</sup>) and bis-(lactoyl)trypanothione (6.9x10<sup>5</sup> M<sup>-1</sup>s<sup>-1</sup>) compared to that of S-lactoylglutathione, the typical substrate of glyoxalase II (0.015 M<sup>-1</sup>s<sup>-1</sup>). The authors suggested that this difference in substrate preference may be due to lack of some basic residues at the enzyme active site that accommodate the glycine carboxylate moiety of glutathione in the mammalian enzyme (9).

Glyoxalase II is a metalloenzyme that shows different metal binding preferences. Although there was a question of whether glyoxalase II is a metalloenzyme, Crowder et al. demonstrated that A. thaliana glyoxalase II binds Zn (II) in its active site and that Zn is required for its catalytic activity (65). The zinc-binding domain (THXHXDH) is highly conserved in all glyoxalase II enzymes reported so far and is similar to that of metallo-β-lactamases and arylsulfatases (9, 47, 66, 67). The prediction that glyoxalase II can bind two metal ions, which was based on similarity to the metal binding site of the metallo-β-lactamases was proven by the crystal structure of human glyoxalase II. The crystal structure
demonstrated that human glyoxalase II has a dinuclear zinc center that is similar to the metal binding site of metallo-β-lactamases from Stenotrophomonas maltophilia (3). A site-directed mutagenesis study on cytosolic glyoxalase II from A. thaliana demonstrated that it has iron and zinc in its binuclear metal center that is required for substrate binding and catalysis (47). Based on similarity to the metallo-β-lactamases, it has been proposed that cytosolic glyoxalase II from A. thaliana utilizes five histidines, two aspartates and a bridging water molecule at its dinuclear active site (47). Chemical modification studies on glyoxalase II from human brain suggested that arginine and histidine residues were involved in catalysis whereas serine or tryptophan residues were not (68). Furthermore, glyoxalase II from rat erythrocytes utilized an active site arginine and histidine for substrate binding and catalysis, respectively. Also, serine or cysteine residues were not found at the active site of this enzyme (69).

Kinetic properties of glyoxalase II vary depending on their source. Principarto et al. showed that human brain glyoxalase II (pI 8.5) prefers S-D-mandeloylglutathione over S-D-lactoyl- and S-D-glycolylglutathione as the substrate (68). Binding of S-D-lactoylglutathione exhibited effective catalysis (68). Glyoxalase II from both the intermembrane space and the matrix of the rat liver hydrolyzed S-D-lactoylglutathione most efficiently. In contrast, significant differences in substrate specificities and $V_{\text{max}}$ values have been observed between the two enzymes with S-acetoacetylglutathione, S-acetylglutathione, S-propionylglutathione and S-succinylglutathione as substrates (58). Two isozymes of glyoxalase II from the cytosol showed a similar trend in substrate specificity and $V_{\text{max}}$ values (62). Kinetic studies performed on glyoxalase II from bovine liver mitochondria demonstrated that efficiency of hydrolysis decreased in the order of S-lactoylglutathione, S-acetoacetylglutathione, S-succinylglutathione, and S-acetylglutathione (64). The best substrate for glyoxalase II from C. albicans was S-D-lactoylglutathione; S-acetoacetylglutathione and S-succinylglutathione were next in decreasing order as shown by catalytic efficiency (46). The kinetic parameters of yeast cytoplasmic and mitochondrial glyoxalase II were dependent on pH for the substrate SLG (2). Pronounced activity of the cytoplasmic enzyme was between pH 7-8 whereas the mitochondrial protein was active over broad pH range (pH 6.5-9.0). The $K_M$ of the former showed a five-fold increase with
increasing pH from pH 5.5 to 9.0 while the $K_M$ of the later remained nearly constant. Both showed low preference for S-D-mandeloylglutathione (2).

While glyoxalase I is a single isozyme, the presence of multiple forms of glyoxalase II is interesting. More specifically, the presence of mitochondrial glyoxalase II is intriguing because the glyoxalase I reaction and hence its product SLG have been observed only in the cytosol (21). Yet, mitochondrial glyoxalase II from yeast, rat and bovine liver, appear to utilize SLG as the preferred substrate (2, 58, 64). Thus, the physiological substrate/s and the role of mitochondrial glyoxalase II are not clear and further research is required in order to obtain detailed information on the mitochondrial enzyme.

**Glyoxalase II of A. thaliana**

In *A. thaliana*, five genes have been identified to encode putative glyoxalase II isozymes. They are localized in mitochondria (GLX 2-1, GLX 2-4, and GLX 2-5) and cytosol (GLX 2-2 and GLX 2-3). Northern analyses have demonstrated that transcripts for the mitochondrial and cytosolic isozymes were highest in roots and flower buds respectively (59, 70). Recombinant forms of these isozymes have been generated and their characterization is underway.

GLX 2-2 is the most well characterized glyoxalase II isozyme of *A. thaliana*. The complete open reading frame (ORF) has been used for expression of GLX 2-2. GLX 2-2 shares 39% amino acid identity and 60% similarity with the mitochondrial forms (59). GLX 2-2 is also the most similar to mammalian cytoplasmic glyoxalase II (55% identity and 71% similarity) (59) (Figure 1.2). The catalytic activity of GLX 2-2 (28 kD) is dependent on the binding of metal in the active site of the enzyme (65). The most recent study on GLX 2-2 demonstrated that it binds a mixture of iron, zinc and manganese (55). The ratio of metal content was dependent on the availability of metal/s in the preparation media. These enzyme preparations yielded $k_{cat}/K_M$ values in the range 1.5-1.9 s$^{-1}$µM$^{-1}$ with the substrate SLG (55, 71).

Recombinant GLX 2-3 is distantly related to the other isozymes and does not utilize SLG as a substrate. When compared with human glyoxalase II, GLX 2-3 was found to lack of several residues that are involved in SLG binding (70). At the amino acid level, GLX 2-3 is only 17% identical to mitochondrial and cytosolic glyoxalase II (59).
Figure 1.2. Alignment of amino acid sequences of glyoxalase II of *Arabidopsis thaliana*, *Homo sapiens*, and *Rhodopseudomonas blastica*. Sequences were aligned using the Clustal method. Shaded boxes show conserved amino acids residues. Gaps introduced by the alignment were shown in dashes.
As part of studies to biochemically characterize GLX 2-1, a serine residue at position 76 of the ORF has been changed to methionine for the expression of GLX 2-1. This was chosen based on the amino terminus of purple bacterium, *Rhodopseudomonas blastica* (Figure 1.2), given that the mature amino terminus of GLX 2-1 protein was not known and that GLX 2-1 resembles the *R. blastica* protein (43% identity and 63% amino acid similarity) (59). The shortening of the N-terminus has also been reported for recombinant yeast mitochondrial glyoxalase II in order to obtain active expressed protein in *E. coli* (61). Later, MALDI-TOF MS analysis revealed that the predicted N-terminal sequence (MSLK) used in the construction of the GLX 2-1 overexpression plasmid may not be the mature amino terminus of the protein (72).

Detailed characterization of mitochondrial glyoxalase II will help elucidate the structure, function and mechanism of these enzymes. This will contribute to the ultimate goal of glyoxalase research in search of inhibitors that can be used as potential drugs in the treatment of glyoxalase system-related diseases. Towards this target, this thesis presents for the first time, the cloning, molecular, biochemical and spectroscopic characterization of a mitochondrial glyoxalase II (GLX 2-5) from *A. thaliana*. 
References


Chapter 2
Over-expression and Characterization of Recombinant Glyoxalase 2-5
from Arabidopsis thaliana

1. Introduction

In addition to its main role in detoxification, the glyoxalase system has also been reported to regulate spermatogenesis (1), control cell differentiation and proliferation (2), and increase tolerance to stress conditions (3). Glyoxalase I and glyoxalase II have been characterized from many different sources, including mammals (4), plants (5), bacteria (6), and yeast (7). Glyoxalase I is localized in the cytosol (8), whereas glyoxalase II shows variation in its localization. Glyoxalase II has been purified from the cytosol and mitochondria of spinach (9), humans (4), and yeast (10) and appears to exist as multiple isozymes in many organisms (11, 12). To date there is no known significance for the existence of the different isozymes. In particular the role of the mitochondrial isozymes is unknown.

In Arabidopsis thaliana, five different putative isozymes of glyoxalase II have been identified. Three of them (GLX 2-1, GLX 2-4, GLX 2-5) are mitochondrial in origin and the other two (GLX 2-2, GLX 2-3) are localized in the cytosol. They show somewhat different expression patterns in plant tissues. For example, the levels of the mitochondrial and cytosolic isozymes are found to be high in roots and flower buds, respectively (13). This observation suggests that the different isozymes may perform different functions.

Human cytosolic glyoxalase II showed 51% identity and 68% similarity with mitochondrial GLX 2-1 (14) and 55% identity and 71% similarity with the cytosolic GLX 2-2 (13) from A. thaliana. GLX 2-2 of A. thaliana showed 39% identity and 60% similarity to the mitochondrial isozymes whereas they both showed 17% identity to GLX 2-3 (13) (see Chapter 1, Figure 1.2). When compared to the cytosolic enzymes, the mitochondrial forms possess amino-terminal extensions that are required for the mitochondrial localization. Analysis of the predicted sequences for the three mitochondrial enzymes indicate that, like all glyoxalase II enzymes, they contain a conserved metal binding domain THXHXDH, which is also conserved in the metal binding domain of metallo-β-lactamases (13). Based on the crystal structure of metallo-β-lactamase L1 from Stenotrophomonas maltophilia (15), the
proposed active site of glyoxalase II (GLX 2-2) contains five histidines, two aspartates, and a bridging water molecule in its binuclear metal center (16). Furthermore, the conserved metal binding ligands in different classes of metallo-β-lactamases are found to bind zinc but no other metal in their native state (17). Though metallo-β-lactamases bind only zinc, proteins that contain the β-lactamase fold have been reported to bind zinc, iron, and manganese (18, 19). Thus, the presence of this metal binding domain strongly suggests that glyoxalase II from A. thaliana is a metalloenzyme (13).

The cytoplasmic glyoxalase II from A. thaliana, GLX 2-2, has been well characterized (13, 16, 20-23). However, the mitochondrial forms have not yet been characterized. GLX 2-2 has been cloned and over-expressed in Escherichia coli by two independent groups (13, 20). It is a 29 kD protein with a slightly acidic isoelectric point of 6.86. Steady-state kinetic studies revealed that the enzyme prefers S-D-mandeloylglutathione to S-D-lactoylglutathione but that the catalytic efficiency with both substrates are similar in magnitude (20).

Crystallographic data on human glyoxalase II suggested that its N-terminal domain is structurally similar to the metallo-β-lactamases. Also, the active site and metal binding site resembles that of metallo-β-lactamase from S. maltophilia (15, 24). A mutational analysis of GLX 2-2 indicated that the relative stoichiometry of metals bound to glyoxalase II is dependent on the environment of the metal binding site (21). For example, the R248W and C140A mutants of GLX 2-2 showed a higher preference for zinc than iron, whereas the wild-type enzyme binds more iron (21). Furthermore, alteration in the metal-binding ligands resulted in a significant reduction in enzyme activity. The proposed mechanism for the hydrolysis reaction of GLX 2-2 involves nucleophilic attack of the bridging hydroxide to lactoyl carbonyl group yielding a tetrahedral transition state (21). In fact, this mechanism showed that both metal ions participate in substrate binding, transition state stabilization, and the hydrolysis reaction (21).

As stated earlier, glyoxalase II can accommodate different metal ions. GLX 2-2 produced in metal-supplemented rich media and Hepes-minimal media are found to bind a mixture of iron, zinc, and manganese (18, 22, 23). Even though the metal content was variable and enriched with the metal ion/s added in the culture media, these enzyme variants showed similar catalytic efficiencies (22, 23). EXAFS revealed that the metal-metal distance
of the dinuclear active site is $3.18 \pm 0.06$ Å. The enzyme utilizes $2.5 \pm 0.5$ histidine and $2.5 \pm 0.5$ oxygen ligands in 5-6 fold coordination for metal binding (18). EPR studies showed evidence for the presence of various dimetal sites, including spin-coupled Fe(III) Fe(II), Fe(III) Zn(II), and Mn(II) Mn(II) centers (18). EPR and EXAFS spectroscopy data confirm the presence of a dinuclear metal center, which shows flexible preference and positive cooperativity in metal binding. However, the site-specific binding of the metal ions was not determined (18, 23).

As a first step in the characterization of mitochondrial GLX 2-1 from *A. thaliana*, it was cloned into pT7-7 and over-expressed by Maiti *et al.* (13). However, its insolubility and low activity restricted further characterization. The authors proposed that this may have been due to incorrect folding of the protein that arose from expressing the protein with an incorrect N-terminus (13).

As a further attempt to characterize mitochondrial forms of glyoxalase II, we have characterized GLX 2-5 by over-expression, purification, and detailed kinetics and spectroscopic studies. GLX 2-5 was cloned into pT7-7 and sequenced. Differentially metal-supplemented GLX 2-5 was over-expressed and purified using a Q-Sepharose anion exchange column. Purified enzyme was used to determine the molar extinction coefficient, substrate preference, metal content, and kinetic parameters of the enzyme. Circular dichroism and fluorescence studies have provided information on secondary structural elements and the tertiary conformation of the protein, respectively. The proposed metal binding sites have been probed with EPR and $^1$H-NMR spectroscopies to gather evidence for the types of metal centers as well as the number and identity of metal-binding ligands for GLX 2-5. Most importantly, we sent a sample to solve the crystal structure of GLX 2-5. This vital information will contribute to the ultimate goal of glyoxalase research to design structure- and/or mechanism-based inhibitors for malaria, cancer, and diabetes treatments.

2. Materials and Methods

PCR reagents, Deep Vent DNA polymerase, restriction enzymes (*NdeI* and *XhoI*), and S-∗D-lactoylglutathione were purchased from Sigma (St. Louis, MO) and New England Biolabs (Beverly, MA). Oligonucleotide primers were synthesized by Integrated DNA
Technologies (Coralville, IA). All chromatographic steps were carried out on a Pharmacia Biotech Fast Protein Liquid Chromatography (FPLC) system operating at 4 °C. Columns and resins for FPLC were purchased from the same company. All protein and DNA quantitations were performed on an Agilent 8453 UV-Vis spectrophotometer.

2.1 Construction of Over-expression Plasmid

The cDNA encoding *A. thaliana* GLX 2-5 was isolated, and the cDNA sequence was reported previously by Maiti *et al.* (13) (Figure 2.1). The original over-expression plasmid GLX 2-5/pET15b was constructed by Zang (25). In the current study, a truncated GLX 2-5 was subcloned into pT7-7 as shown in Figure 2.2. PCR was conducted with the GLX 2-5/pET15b plasmid as template using a 5’ primer (Glx 2-5 NdeI primer) designed to create a NdeI restriction enzyme site at the 5’ end and a 3’ primer (Oligo dT adaptor primer) to create a XhoI site. The PCR program consisted of: 1 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 42 °C, 75 s at 72 °C; and 8 min at 72 °C. Deep Vent DNA polymerase was used, and PCR amplification was carried out using a Peltier Thermal Cycler-200 DNA Engine (MJ Research). The products were analyzed by electrophoresis on a 0.7% (w/v) agarose gel in TAE buffer using λ DNA/HindIII fragments as DNA markers to determine size. The expected band (975 bp) was gel-isolated and purified using a GeneClean II kit. The gel purified DNA fragment was digested with NdeI and XhoI restriction enzymes sequentially and ligated with T4 ligase into the pT7-7 vector (2473 bp) as an NdeI and XhoI fragment. The ligation produced the GLX 2-5/pT7-7 plasmid, which was then transformed into competent DH10B *E. coli* cells using a standard heat shock method (26). Positive constructs were verified by PCR using gene-specific primers, Glx 2-1/4/5, 5’ primer, and Glx 2-5 3’ primer. The PCR program consisted of: 1 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 52 °C, 1 min at 72 °C; and 5 min at 72 °C.

Cells from positive clones were further analyzed by DNA sequencing. DH10B cells were grown in 5 mL of LB media containing 150 µg/ mL ampicillin at 37 °C overnight. Plasmid DNA was isolated using a GenElute Plasmid Miniprep kit (Sigma). Cycle sequencing PCR on this plasmid was carried out using the T7 promoter primer (forward reaction) and T7 terminator primer (reverse reaction) in separate reaction mixtures, and DNA
Figure 2.1. The cDNA sequence and deduced amino acid sequence of GLX 2-5. Solid arrows show the locations of the primers used in the construction of GLX 2-5/pT7-7. Bold letters on sequences shows location of start codon generated by Glx 2-5 NdeI primer and □ and ○ shows tyrosine and tryptophan residues, respectively.
Figure 2.2. GLX 2-5/ pT7-7 plasmid construction. The GLX 2-5 gene was cloned into the pT7-7 vector plasmid as a Ndel and Xhol fragment. The pT7-7 vector is a derivative of the pET system and contains ampicillin resistance and a T7 promoter.
sequencing was conducted by Sanger’s dideoxy chain terminator method using a DYEnamic ET Terminator Cycle Sequencing Kit. The alignment of the forward and reverse sequences with the cDNA sequence using DNASTAR software verified the sub-cloning. The GLX 2-5/pT7-7 plasmid was then transformed into *E. coli* over-expression cells (BL21 (DE3)-Rosetta) with the heat shock method. The oligonucleotide primers used in this study are given in Table 2.1.

### 2.2 Over-expression and Purification

In order to ascertain the effect of different growth conditions on the over-expression of GLX 2-5, the above transformed cells were grown in 5 mL overnight cultures of either LB or ZY media containing 150 µg/mL ampicillin, with or without adding desired metals and at different induction temperatures (15 °C, 28 °C or 37 °C) for different time periods (8 h, 12 h, 24 h).

Cells containing the construct GLX 2-5/pT7-7 were grown at 37 °C in LB media containing 150 µg/mL ampicillin overnight as pre-cultures. LB media was composed of 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter with the pH adjusted to 7.5. The cell pellet from the pre-culture was introduced into one-liter of LB media containing 150 µg/mL ampicillin and 8 mL/L glycerol, and the culture was grown with shaking at 37 °C to an optical density of 0.6-0.8 at 600 nm. Protein production was induced making the culture a final concentration of 0.2 mM IPTG (isopropyl-1-thio-β-D-galactopyranoside), and the induction temperature was brought to 15 °C for 24 h. To produce metal-supplemented enzyme variants, 250 µM of the desired metal ions [Fe(NH$_4$)$_2$(SO$_4$)$_2$, Zn(SO$_4$)$_2$] were introduced to the media at induction. After induction for 24 h, the cells were harvested by centrifugation at 7000 rpm for 8 min and washed three times with cold, sterile ddH$_2$O to remove salts. Harvested cell pellets were stored at -80 °C until further use.

The cell pellet was resuspended in 30 mL of 10 mM MOPS [3-(N-morpholino)propanesulfonic acid] buffer at pH 7.2 containing 0.1 mM PMSF (phenylmethylsulfonyl fluoride). The cells were lysed by passage twice through a French Press at 16000 psi, and the cell debris was removed by centrifugation at 12,500 rpm for 45 min. The soluble GLX 2-5 protein was released into the supernatant. The cleared supernatant was purified using Fast Protein Liquid Chromatography (FPLC) with a Q-Sepharose column as described.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glx 2-5 Ndel</td>
<td>CTCCCATATGCAAATTGAACTGGTGCCCTT</td>
<td>Cloning</td>
</tr>
<tr>
<td>Oligo dT adaptor</td>
<td>CGAGGATCCTCGGTCGACGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT</td>
<td></td>
</tr>
<tr>
<td>Glx 2-1/4/5, 5’</td>
<td>CATCATCATTATGATCACATTTGG</td>
<td>Glx 2-5</td>
</tr>
<tr>
<td>Glx 2-5 3’</td>
<td>CAACATGAGCTGCATAAGAC</td>
<td>gene-specific</td>
</tr>
<tr>
<td>T7 promoter</td>
<td>AATACGACTCACTATAGGG</td>
<td>DNA</td>
</tr>
<tr>
<td>T7 terminator</td>
<td>GCTAGTTATTGCTCAGCGG</td>
<td>sequencing</td>
</tr>
</tbody>
</table>

Table 2.1. Oligonucleotide primers used in the construction of GLX 2-5/ pT7-7 plasmid.
previously (16). In brief, the supernatant was loaded onto a column that was pre-equilibrated with 10 mM MOPS, pH 7.2, and the protein was eluted with a 0 to 500 mM linear NaCl gradient in the same buffer. GLX 2-5 eluted at approximately 125 mM NaCl. The purity of the enzyme was assessed by 12% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis), and fractions containing pure enzyme were pooled and concentrated using an Amicon concentrator with an YM-10 membrane. The enzyme was quantitated by measuring the absorbance at 280 nm and using a molar extinction coefficient for the enzyme of 37,753 M\(^{-1}\)cm\(^{-1}\).

2.3 Determination of molar extinction coefficient

The molar extinction coefficient was determined by the Edelhoch method described by Gill & Von Hippel (27) and by PIXE experiments. Recombinant GLX 2-5-FeZn sample was purified as described earlier, using 10 mM TRIS (tris(hydroxymethyl)aminomethane) buffer at pH 7.2. PIXE requires the absence of sulfur and chloride ions. Therefore, TRIS buffer was used instead of MOPS buffer, and the pH of TRIS buffer was adjusted using acetic acid instead of HCl. The sample was sent to Dr. W. Meyer-Klaucke at EMBL Outstation, Hamburg, Germany for PIXE analysis as described (28).

2.4 Substrate specificity studies

A series of thiolesters of glutathione were used for the preliminary investigation of substrate preferences of the GLX 2-5-wt. The substrates used were S-D-lactoylglutathione (SLG, \(\varepsilon_{240} 3,100 \text{ M}^{-1}\text{cm}^{-1}\)), S-D-mandeloylglutathione (\(\varepsilon_{263} 4,200 \text{ M}^{-1}\text{cm}^{-1}\)), S-D-acetylglutathione (\(\varepsilon_{240} 2,980 \text{ M}^{-1}\text{cm}^{-1}\)), S-D-acetoacetylglutathione (\(\varepsilon_{240} 3,400 \text{ M}^{-1}\text{cm}^{-1}\)), S-D-formylglutathione (\(\varepsilon_{240} 3,300 \text{ M}^{-1}\text{cm}^{-1}\)), S-D-glycolylglutathione (\(\varepsilon_{240} 3,260 \text{ M}^{-1}\text{cm}^{-1}\)), and S-D-lactonylglutathione (\(\varepsilon_{240} 3,310 \text{ M}^{-1}\text{cm}^{-1}\)) and were synthesized by Dr. Kewu Yang of the Crowder Group, Miami University, as described elsewhere (29), except commercially purchased SLG. The initial hydrolysis of thiolesters by GLX 2-5-wt was monitored at 240 nm (except at 263 nm for S-D-mandeloylglutathione) over 30 s at 25 °C using a UV-Vis spectrophotometer. The concentrations of substrate and enzyme were 200 µM and 10 µM, respectively.
2.5 Producing polyclonal antibody to GLX 2-5

GLX 2-5 was over-expressed and purified as described above and the purity was verified by 12% SDS-PAGE. Antibodies to GLX 2-5 were raised using a standard protocol (30). The enzyme (20 µL, 165 µM) in 0.7 mL of PBS (phosphate-buffered saline) buffer was mixed with 0.3 mL of Freund’s complete adjuvant (Sigma) and was injected into a rabbit. The rabbit was boosted four times with purified enzyme and 0.3 mL Freund’s incomplete adjuvant (Sigma) at six-week intervals. Blood was collected 10 days after each injection except the first injection and was kept at 4 °C overnight. It was centrifuged twice at 7,000 rpm and 13,000 rpm, and the cleared supernatant was collected. The rabbit anti-GLX 2-5 antibody was aliquoted and stored at -80 °C.

The antibody was tested using a western blot. Samples of GLX 2-2, GLX 2-3, and GLX 2-5 were resolved on a 12% SDS-PAGE. The protein bands were transferred electrophoretically onto a nitrocellulose membrane. The membrane was incubated with primary rabbit anti-GLX 2-5 antibody and secondary antibody, goat anti-rabbit IgG conjugated with alkaline phosphatase. The presence of GLX 2-5 was visualized by the reaction with a substrate solution containing NBT (nitro blue tetrazolium) and BCIP (5-bromo-4-chloro-3-indolyl phosphate) followed by quenching the reaction with 20 mM EDTA.

2.6 Metal analysis

Metal analyses were performed on a Varian-Liberty 150 inductively coupled plasma spectrometer with atomic emission spectroscopy detection (ICP-AES) as described elsewhere (16). The concentration of GLX 2-5 was 10 µM in 10 mM MOPS buffer, pH 7.2. A calibration curve with 5 standards and a correlation coefficient of greater than 0.998 was generated using Zn, Mn, Fe, and Cu reference solutions. The following emission wavelengths were chosen to ensure the lowest detection limits possible: Zn, 213.856 nm; Mn, 257.610 nm; Fe, 259.940 nm; and Cu, 324.754 nm. Metal concentrations were obtained and averaged from at least three preparations of enzyme.
2.7 Steady-state kinetic studies

The steady-state kinetic parameters of the GLX 2-5-catalyzed hydrolysis of S-D-lactoylglutathione were determined at 25 °C in 10 mM MOPS, pH 7.2, using a HP5483 Diode Array UV-Vis spectrophotometer. The rate of hydrolysis was monitored by measuring the absorbance at 240 nm for 30 µM to 600 µM of S-D-lactoylglutathione ($\varepsilon_{240} = -3100 \text{ M}^{-1} \text{cm}^{-1}$) over a 30 s reaction period (16). Using the experimental data on Michaelis-Menten mode of the Igor Pro program, the kinetic parameters were obtained.

2.8 Fluorescence Emission Spectroscopy

The intrinsic fluorescence emission spectra of GLX 2-5 samples and their H$_2$O$_2$ treated samples were monitored using a Perkin-Elmer LS55 Luminescence Spectrometer tuned to an excitation wavelength of 295 nm and fluorescence emission was monitored in the wavelength range of 300-500 nm with a slit width of 5 nm. A 4-mm quartz cuvette was used to load samples. The concentration of the samples was 5 µM in 10 mM MOPS buffer, and 10 mM MOPS buffer was used as a blank.

2.9 Circular Dichroism Spectroscopy

Samples were prepared by dialyzing GLX 2-5 samples versus 2 x 2 L of 5 mM phosphate, pH 7.0, for 4 h. The samples were diluted with the same buffer to a final concentration of 75 µg / mL. A JASCO J-810 CD spectrometer operating at 25 °C and a 0.1 mm quartz cuvette were used to collect CD spectra. The CD spectra were then analyzed for secondary structural components using the CDSSTR simulation programs at the DICHROWEB internet site, http://www.cryst.bbk.ac.uk/cdweb/html/home.html. The standard NRMSD goodness of fit parameter was used to determine which program best fits the data (31-33).

2.10 EPR Spectroscopy

Electron paramagnetic resonance (EPR) spectra were recorded on a Bruker ESP-300E spectrometer equipped with an Oxford Instruments ESR-900 helium flow cryostat operating at 4.7 K with 2 mW microwave power, 9.48 GHz frequency, 10 G modulation amplitude,
100 kHz modulation frequency, sweep time of 41.9 s, receiver gain of $10^5$, 41 ms conversion time, 82 ms time constant, center field of 3350 G, and sweep width of 6400 G. EPR samples were made by pipetting 400 µL of 345 µM GLX 2-5 enzyme in 10 mM MOPS, pH 7.2, into a 4 mm o.d. quartz EPR tube, and then the samples were frozen by slow immersion in a liquid nitrogen bath.

2.11 $^1H$ - Paramagnetic NMR Spectroscopy

A GLX 2-5-FeZn sample was analyzed by NMR spectroscopy. The sample contained approximately 10% D$_2$O for locking, and the concentration was 1.8 mM. The sample in D$_2$O was made by performing three or more dilution/concentration cycles in a Centricon-10 to a final concentration of 1.6-1.8 mM. A GLX 2-5-FeZn sample (1.8 mM) in 10% D$_2$O was oxidized using one equivalent of H$_2$O$_2$. The samples were then loaded into Wilmad 5-mm tubes for NMR. NMR spectra were collected on a Bruker Avance 500 spectrometer operating at 500.13 MHz, 298 K, and a magnetic field of 11.7 T, recycle delay (AQ), 41 ms; sweep width, 400 ppm. Protein chemical shifts were calibrated by assigning the H$_2$O signal the value of 4.70 ppm. A modified presaturation pulse sequence (zgpr) was used to suppress the proton signals originating from water molecules (34, 35).

2.12 X-ray Crystallography

GLX 2-5-FeZn was prepared and purified as described in the over-expression and purification section. Enzyme purity was ascertained by SDS-PAGE to be >95%, and the concentration was 12 mg/mL (0.4 mM). The sample (2 mL) was drop frozen in liquid nitrogen and shipped on dry ice to Dr. Craig Bingman at the Center for Eukaryotic Structural Genomics, University of Wisconsin, Madison. A slightly different procedure was followed for the over-expression and purification of GLX 2-2-FeZn (16) and the X-ray crystallographic sample was prepared as described above.
3. Results

3.1 Construction of Over-Expression Plasmid

The construction of GLX 2-5/ pT7-7 was successful. GLX 2-5 contains a long N-terminal extension (69 amino acids) compared to the cytosolic forms of *A. thaliana* and human (see Chapter 1, Figure 1.2). Since the N-terminal sequence of native mature protein was not known, the start codon was generated at bp 297 (based upon the numbering of the cDNA sequence) using the Glx 2-5-*NdeI* primer. This removed the N-terminal extension and gave an N-terminal sequence of MQIELVP (Figure 2.1). This construct maintains a conserved Q as the second amino acid. The PCR fragment was cloned into the pT7-7 vector, and the plasmid was transformed into DH10B competent cells. PCR verified the size of the insert as well as that it was a positive clone. DNA sequencing further confirmed the construct and indicated that it did not contain any mutations. The plasmid was then transformed to *E. coli* BL21(DE3) Rosetta cells for protein production.

3.2 Over-Expression and Purification

The GLX 2-5/pT7-7 construct showed over-expression in preliminary assays. It was then important to optimize the over-expression conditions, as this was the first time that the GLX 2-5/pT7-7 construct was used. *E. coli* Rosetta cells containing GLX 2-5/pT7-7 were grown in LB and ZY culture media containing ampicillin in the presence of bivalent metals. Protein production was induced by IPTG, and cultures were grown at different temperatures for different time periods. Protein production was verified by 12% SDS-PAGE. Strong over-expression of GLX 2-5 was observed in LB Amp media, with or without added metal ions for 24 h induction at 15 °C.

Recombinant GLX 2-5 was then over-expressed in *E. coli* and purified by FPLC as described in Materials & Methods. For characterization purposes, four different types of the enzyme were prepared in media supplemented with iron (GLX 2-5-Fe), zinc (GLX 2-5-Zn), both iron and zinc (GLX 2-5-FeZn), and without any metal (GLX 2-5-wt). All showed similar, high levels of soluble protein production.

Q-Sepharose anion exchange chromatography was then employed to purify the enzyme. The protein eluted at pH 7.2 and approximately 125 mM NaCl. This single-step, typical purification lead to approximately 50 mg of protein from a one-liter culture and was
>95% pure as shown by SDS-PAGE. A typical SDS-PAGE of FPLC column fractions of GLX 2-5 protein purification is shown in Figure 2.3. Generally two or three pure fractions were combined, concentrated, and used for subsequent characterization.

### 3.3 Determination of Molar Extinction Coefficient

Initially, GLX 2-5 was quantitated using its absorbance at 280 nm and the molar extinction coefficient of GLX 2-2 (\( \varepsilon = 69,040 \text{ M}^{-1}\text{cm}^{-1} \)) \(^{16} \). To determine the molar extinction coefficient for GLX 2-5, the Edelhoch method was used \(^{27} \). It involves measuring the absorbance of equally concentrated samples of denatured and native enzyme at 280 nm. The extinction coefficient of the denatured protein is calculated using amino acid sequence data and the following equation,

\[
\varepsilon_{\text{denatured}} = a \cdot \varepsilon_{\text{(Tyrosine)}} + b \cdot \varepsilon_{\text{(Tryptophan)}} + c \cdot \varepsilon_{\text{(Cysteine)}}
\]

where, \( a, b, \) and \( c \) are number of residues of tyrosine, tryptophan, and cysteine, respectively. The concentration is then calculated using Beer’s Law, \( A = \varepsilon c l \), where, \( A, c, \) and \( l \) are absorbance, concentration, and path length of the cell, respectively. Finally, as both denatured and native sample were equal in concentration, the extinction coefficient of native GLX 2-5 can be calculated using Beer’s Law. The \( \varepsilon \) for GLX 2-5 using this method was determined to be 18,220 \( \text{M}^{-1}\text{cm}^{-1} \).

PIXE (Particle-induced X-ray emission) was also used to determine the molar extinction coefficient of recombinant GLX 2-5. PIXE measures the total sulfur content of protein, which can be used to determine protein content of the sample using its amino acid sequence data. Then using the absorbance of the sample at 280 nm, a molar extinction coefficient can be calculated as described previously. This method yielded an extinction coefficient of 37,753 \( \text{M}^{-1}\text{cm}^{-1} \).

Molar extinction coefficient for GLX 2-5 was chosen as 37,753 \( \text{M}^{-1}\text{cm}^{-1} \) throughout this study because it was obtained by an experimental method with fewer variables.

### 3.4 Substrate Specificity Studies

Since most glyoxalase II enzymes show a preference for S-\( D \)-lactoylglutathione as the substrate, seven related thioesters of glutathione were used to determine the substrate
Figure 2.3. A SDS-PAGE gel of typical purification profile of recombinant GLX 2-5 from *A. thaliana*. Lanes were loaded with 10 µL of FPLC column fractions, and the gel was stained with Coomassie Brilliant Blue. The protein eluted at pH 7.2 and approximately 125 mM NaCl.
preference of GLX 2-5. The substrates used were S-D-lactoylglutathione (SLG), S-D-mandeloylglutathione, S-D-acetylglutathione, S-D-acetoacetylglutathione, S-D-formylglutathione, S-D-glycolylglutathione, and S-D-lactonylglutathione. Initial rate assays on these substrates using GLX 2-5-wt showed that SLG was the preferred substrate (Table 2.2). S-D-acetoacetylglutathione and S-D-glycolylglutathione were next and exhibited less than half the initial rates of SLG. GLX 2-5 showed very low rates of hydrolysis of S-D-mandeloylglutathione, S-D-acetylglutathione, and S-D-lactonylglutathione, whereas S-D-formylglutathione was not hydrolyzed.

3.5 Polyclonal antibodies to GLX 2-5

Antibodies to GLX 2-5 were raised in rabbits for localization studies on GLX 2-5 in plants. Purified GLX 2-5 was used as an antigen to raise polyclonal antibodies in rabbits. Western blot analysis was carried out to check the purity of antibody (Figure 2.4). As shown in Figure 2.4, GLX 2-5 antibodies cross-reacted with GLX 2-5 and GLX 2-2 but did not react with GLX 2-3. This reaction is expected from a polyclonal antibody. This observation further confirms that antibody to GLX 2-5 has been generated.

3.6 Metal content

Since glyoxalase II is a metalloenzyme, metal analyses of GLX 2-5 enzyme variants were performed using ICP-AES. Also, the metal preference of the dinuclear metal center was studied by over-expressing GLX 2-5 in the presence of added metal ions. The metal content of the metal-enriched and the wild-type enzyme preparations are given in Table 2.3. The data suggested that the metal content of the enzyme clearly depends on the growth condition, i.e. the metal present during enzyme production. All enzyme variants were found to have approximately two equivalents of metals except the wild-type, GLX 2-5-wt, which had approximately one equivalent of metal. The data on GLX 2-5-wt indicate that the enzyme can incorporate both Fe and Zn with approximately similar affinity. While the enzyme could be enriched in either Fe or Zn, the samples always contained a mixture of both. This result along with the fact that both GLX 2-5-FeZn and GLX 2-5-wt contained nearly equal ratios of Fe to Zn indicate that GLX 2-5 likely contains a Fe/Zn site. However, in the
Table 2.2. Substrate preference studies of GLX 2-5-wt monitored by initial rate of hydrolysis. The hydrolysis was carried out in 10 mM MOPS, pH 7.2, 25 °C, over 30 s. The concentrations of substrate and enzyme were 200 µM and 10 µM, respectively.
Figure 2.4. Western blot on glyoxalase II isozymes from *A. thaliana* immunoblotted using GLX 2-5 antibody. Lane 1: GLX 2-2; Lane 2: GLX 2-3; and Lane 3: GLX 2-5. Anti-GLX 2-5 antibody showed cross-reaction with GLX 2-2 and GLX 2-5 proteins.
Table 2.3. Metal contents of GLX 2-5 samples. The metal content of the GLX 2-5 samples was measured with a Varian Inductively Coupled Plasma spectrometer with atomic emission spectroscopy detection (ICP-AES). Each metal content is shown as equivalent of metal per enzyme.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Iron</th>
<th>Zinc</th>
<th>Manganese</th>
<th>Copper</th>
<th>Total metal</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLX 2-5-FeZn</td>
<td>1.04 ± 0.15</td>
<td>1.31 ± 0.05</td>
<td>0.016 ± 0.02</td>
<td>0.001 ± 0.0</td>
<td>2.37 ± 0.11</td>
</tr>
<tr>
<td>GLX 2-5-Zn</td>
<td>0.40 ± 0.12</td>
<td>1.20 ± 0.27</td>
<td>0.012 ± 0.03</td>
<td>0.003 ± 0.001</td>
<td>1.61 ± 0.21</td>
</tr>
<tr>
<td>GLX 2-5-Fe</td>
<td>1.43 ± 0.19</td>
<td>0.53 ± 0.14</td>
<td>0.011 ± 0.01</td>
<td>0.0 ± 0.0</td>
<td>1.96 ± 0.17</td>
</tr>
<tr>
<td>GLX 2-5-wt</td>
<td>0.61 ± 0.07</td>
<td>0.58 ± 0.15</td>
<td>0.010 ± 0.01</td>
<td>0.0 ± 0.0</td>
<td>1.19 ± 0.10</td>
</tr>
</tbody>
</table>
presence of high concentrations of one metal ion in the growth media, it picks up more of that metal. This confirms that GLX 2-5 is a metalloenzyme and has dinuclear metal center. GLX 2-5 does not bind significant amounts of either Mn or Cu as detected by ICP, whereas Arabidopsis GLX 2-2 can bind Mn (22). The metal analysis data clearly indicate the high affinity of GLX 2-5 for Fe and Zn ions.

3.7 Steady-state Kinetics

The steady-state kinetic constants of GLX 2-5 were evaluated to determine the effect of metal content on substrate binding and catalysis. The kinetic parameters, Michaelis constant, $K_M$ and catalytic constant, $k_{cat}$, for GLX 2-5 variants are given in Table 2.4. GLX 2-5-wt shows the highest $k_{cat}$ value when the ratio of Fe to Zn is 1:1. The second highest $k_{cat}$ value is displayed by GLX 2-5-FeZn with a similar ratio (Fe to Zn ratio of 1:1.3). The $k_{cat}$ of GLX 2-2-wt ($430 \pm 71 \text{ s}^{-1}$) is considerably higher than all of the GLX 2-5 variants analyzed (18). When compared with GLX 2-2-wt, GLX 2-5-FeZn, GLX 2-5-Zn, and GLX 2-5-Fe exhibit 81%, 60%, and 37% relative activities, respectively. Comparison of the GLX 2-5-Fe and GLX 2-5-Zn reveals that the Zn analog is almost 50% more efficient than the Fe analog.

With the exception of GLX 2-5-FeZn, the $K_M$ values of all enzyme variants of GLX 2-5 are very similar and within experimental error (Table 2.4). The lowest $K_M$ value is observed when the total metal content is almost one and the ratio of Fe to Zn is 1:1. Even though the $K_M$ value is found to increase with increasing total metal content, the increase is moderate when the total metal content is below two equivalents. The total metal content of GLX 2-5-FeZn is 2.35 equivalents, and there is nearly a 75% increase in $K_M$ compared to that of GLX 2-5-wt, indicating decreased substrate binding affinity with increased total metal content. The catalytic efficiency of GLX 2-5-wt is maximal and was two-fold higher than other GLX 2-5 enzyme variants. This is primarily due to the lower $K_M$ value.

3.8 Circular Dichroism Spectroscopy

CD spectra were collected to identify possible secondary structural changes caused by the different metal compositions in the GLX 2-5 samples. The far-UV (240-180 nm) CD spectra of proteins are due to peptide bond absorption, and analysis of the spectra gives an approximate estimation of the content of different secondary structural elements of a protein.
Table 2.4. Steady-state kinetic constants for SLG hydrolysis by GLX 2-5 samples.

Steady-state kinetic study was carried out in 10 mM MOPS, pH 7.2, 25 °C, and $K_M$ and $k_{cat}$ values were obtained using the Michaelis-Menten equation on IgorPro Software.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (s$^{-1}$µM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLX 2-5-FeZn</td>
<td>391 ± 48</td>
<td>129 ± 10</td>
<td>0.33</td>
</tr>
<tr>
<td>GLX 2-5-Zn</td>
<td>248 ± 67</td>
<td>93 ± 9</td>
<td>0.37</td>
</tr>
<tr>
<td>GLX 2-5-Fe</td>
<td>243 ± 27</td>
<td>58 ± 2</td>
<td>0.24</td>
</tr>
<tr>
<td>GLX 2-5-wt</td>
<td>213 ± 20</td>
<td>157 ± 6</td>
<td>0.73</td>
</tr>
</tbody>
</table>
Figure 2.5 shows CD spectra of GLX 2-5 containing different metal ion compositions. All spectra suggest similar structural features, negative bands at 208 nm and 220 and a positive band approximately at 195 nm although the mean residue ellipticity of GLX 2-5-Zn exhibited a slight deviation. The secondary structural analysis of the CD spectra by the CDSSTR algorithm is given in Table 2.5 (31). However, it should be noted that the CDSSTR method only gives an approximate estimation of secondary structural elements. The secondary structure composition of GLX 2-5-wt is 37% α-helix, 16% β-sheet, 18% turns, and 30% unordered. These values closely match those of GLX 2-5-FeZn and GLX 2-5-Fe. The CDSSTR fit for GLX 2-5-Zn indicates lower amounts of unordered structure than in the other enzymes, as seen in Table 2.5.

3.9 Fluorescence Emission Spectroscopy

The effect of metal binding on the fluorescence emission of the GLX 2-5 variants was investigated in order to determine whether the different metal content of the GLX 2-5 variants induced conformational perturbations. The fluorescence emission spectra of the GLX 2-5 variants prepared in different metal-supplemented media are shown in Figure 2.6. Although GLX 2-5 contains a single tryptophan residue, intense fluorescence emission can be observed.

When excited at 295 nm, the fluorescence emission spectra of GLX 2-5 samples with varying Fe and Zn contents display differences in both fluorescence intensity and red-shifted peak maxima. The fluorescence emission spectra of GLX 2-5-FeZn and GLX 2-5-wt display almost identical fluorescence intensity. The emission maxima of both spectra were at ~ 345 nm, which is typically observed for partially solvated tryptophan emission (38). The emission spectra of GLX 2-5-Zn and GLX 2-5-Fe display a nearly 40–60% decrease in fluorescence intensity compared to the GLX 2-5-FeZn and GLX 2-5-wt preparations. The second notable difference in the spectra is the clear red-shift in both GLX 2-5-Zn and GLX 2-5-Fe compared to that of GLX 2-5-FeZn and GLX 2-5-wt. The emission maxima of GLX 2-5-Zn and GLX 2-5-Fe were shifted to the 350-450 nm range.

In order to monitor the effect of an oxidant, H₂O₂, on the fluorescence emission spectra, all of the samples were treated with H₂O₂. It was anticipated that if iron is present in the Fe(II) state, it would be oxidized to Fe(III), which in turn may have some impact on the
Figure 2.5. CD spectra of GLX 2-5 samples. Samples were 75 µg/mL in 5 mM phosphate, pH 7.0. CD spectra were recorded with a JASCO J-810 CD spectrometer operating at 25 °C.
<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>% α-Helix</th>
<th>% β-Sheet</th>
<th>% Turns</th>
<th>% Unordered</th>
</tr>
</thead>
<tbody>
<tr>
<td>GLX 2-5-FeZn</td>
<td>39</td>
<td>17</td>
<td>16</td>
<td>26</td>
</tr>
<tr>
<td>GLX 2-5-Zn</td>
<td>41</td>
<td>24</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>GLX 2-5-Fe</td>
<td>35</td>
<td>21</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>GLX 2-5-wt</td>
<td>37</td>
<td>16</td>
<td>18</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 2.5. Comparison of CD spectral data of GLX 2-5 samples analyzed with CDSSTR (31).
Figure 2.6. Fluorescence emission spectra of GLX 2-5 samples. Sample concentration was 5.0 µM in 10 mM MOPS, pH 7.2, at 25 °C, and the excitation wavelength was 295 nm. Spectra were recorded on a Perkin-Elmer LS55 Luminescence Spectrometer.
Figure 2.7. Fluorescence emission spectra of GLX 2-5 samples in the absence and presence of H$_2$O$_2$. GLX 2-5 sample concentrations were 5.0 µM in 10 mM MOPS, pH 7.2, at 25 °C, and the excitation wavelength was 295 nm. Oxidized samples were prepared by adding > 1 molar equivalent of H$_2$O$_2$. 
the fluorescence emission spectra. However, there was no change in the fluorescence emission intensity after addition of one molar equivalent of H_2O_2. But addition of excess (> 5 molar equivalents) showed a slight increase in the fluorescence emission in all the spectra except GLX 2-5-FeZn (Figure 2.7). However, it is uncertain if the difference is due to the effect of oxidation or denaturation of protein.

### 3.10 EPR Spectroscopy

The interaction of a paramagnetic metal center with an applied magnetic field gives information such as the oxidation states of metal ions, especially Fe(II) and Fe(III) and possible spin coupled dinuclear centers (39). The EPR spectra of GLX 2-5-Zn, GLX 2-5-Fe, and GLX 2-5-FeZn are shown in Figure 2.8. All sample concentrations were 345 µM. Because the EPR spectra of GLX 2-5-Zn and GLX 2-5-Fe display relatively low intensity features (Figure 2.8), the spectra of GLX 2-5-Zn and GLX 2-5-Fe were blown up in scale to show their EPR features and are shown in Figure 2.9.

The metal analyses of GLX 2-5-Zn, GLX 2-5-Fe, and GLX 2-5-FeZn revealed the presence of Fe (Table 2.3) in all the samples, as was observed in GLX 2-2 (18). This prompted us to investigate all the samples using EPR spectroscopy. The EPR spectrum of GLX 2-5-FeZn showed four distinct sets of signals (1) g = 8.4, (2) g = 5.25, (3) g = 2, and (4) g = 1.71. The EPR spectra of GLX 2-5-Zn and GLX 2-5-Fe showed all the above four set of signals as more or less intense features (Figure 2.8). A six line hyperfine pattern at g = 2 is characteristic of Mn(II) and has been previously observed in GLX 2-2 (18). The features at g = 5.25 and g = 8.4 are indicative of bound Fe(III) (40, 41) (Figure 2.9).

### 3.11 ^1H-Paramagnetic NMR Spectroscopy

The ^1H-NMR spectrum of GLX 2-5-FeZn is shown in Figure 2.10. There are nine well-resolved peaks between 70 ppm and -30 ppm. In addition, there are a few distinguishable peaks in the 10 – 15 ppm region; however, assignment of these peaks to specific protons requires additional experiments. Among the nine peaks, four of them (Table 2.6, Peaks A, B, C, D) are well shifted into the downfield region of protons where the protons of metal bound ligands usually appear (42).
**Figure 2.8. EPR spectra of GLX 2-5 samples.** The enzyme concentrations were 345 µM, in 10 mM MOPS, pH 7.2. The EPR parameters were: 4.7 K, 1.997 mW microwave power, 9.46 GHz frequency, 10 G modulation amplitude, 100 kHz modulation frequency, sweep time of 83.88 seconds, receiver gain of $10^5$, 81.9 ms conversion time, 82 ms time constant.
Figure 2.9. EPR spectra of GLX 2-5 samples. These spectra are the expanded versions of the EPR spectra shown in Figure 2.8. The enzyme concentrations were 345 µM, in 10 mM MOPS, pH 7.2. The EPR parameters were: 4.7 K, 1.997 mW microwave power, 9.46 GHz frequency, 10 G modulation amplitude, 100 kHz modulation frequency, sweep time of 83.88 seconds, receiver gain of $10^5$, 81.9 ms conversion time, 82 ms time constant.
In the spectrum of a 90% D$_2$O treated GLX 2-5-FeZn sample, two peaks (A and D) disappeared. Deuterium exchangeable protons are marked with asterisks (‘*’) in the spectrum and Table 2.6. The $^1$H-NMR spectrum of GLX 2-5-FeZn treated with one molar equivalent of H$_2$O$_2$ is shown in Figure 2.11. The spectrum of H$_2$O$_2$ treated GLX 2-5-FeZn displays essentially similar features observed in the GLX 2-5 / H$_2$O spectrum except the disappearance of peak E.

3.12. X-ray Crystallography

While this thesis was being written, Professor Craig Bingman solved the crystal structure of GLX 2-5-FeZn. He provided the preliminary data on the metal centers and the metal binding ligands. GLX 2-5-FeZn clearly contains a dimetal center with Fe and Zn. The crystal structure demonstrated that Fe is bound to two histidines and two aspartates, and Zn is bound to three histidines (Table 2.7).
**Figure 2.10.** $^1$H - NMR spectrum of GLX 2-5-FeZn. The sample concentration was 1.8 mM and was in 10 mM MOPS, pH 7.2, in 10 % D$_2$O. Solvent exchangeable peaks are labeled with an asterisk. The spectrum was collected at a temperature of 25 °C on a 500 MHz Bruker Avance NMR spectrometer.
Figure 2.11. $^1$H - NMR spectrum of GLX 2-5-FeZn treated with $\text{H}_2\text{O}_2$. GLX 2-5-FeZn (1.8 mM) in 10 mM MOPS, pH 7.2, containing 10 % $\text{D}_2\text{O}$ was treated with one molar equivalent of $\text{H}_2\text{O}_2$. The spectrum was collected at a temperature of 298 K on a 500 MHz Bruker Avance NMR spectrometer.
Table 2.6. $^1$H- NMR chemical shift assignments of GLX 2-5-FeZn spectra. Solvent exchangeable proton signals are marked with an asterisk (‘*’).
<table>
<thead>
<tr>
<th>Metal Center</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe</td>
<td>His59 Ne2</td>
</tr>
<tr>
<td></td>
<td>His169 Ne2</td>
</tr>
<tr>
<td></td>
<td>Asp58</td>
</tr>
<tr>
<td></td>
<td>Asp131</td>
</tr>
<tr>
<td>Zn</td>
<td>His54 Ne2</td>
</tr>
<tr>
<td></td>
<td>His56 Nδ1</td>
</tr>
<tr>
<td></td>
<td>His112 Ne2</td>
</tr>
<tr>
<td>Between Fe and Zn</td>
<td>1 H$_2$O Bridge</td>
</tr>
</tbody>
</table>

Table 2.7. The metal binding ligands of GLX 2-5-FeZn based on X-ray crystallographic data.
4. Discussion

The *Arabidopsis* mitochondrial isozyme, GLX 2-5, has been successfully cloned and heterogeneously over-expressed in *E. coli*. Previously, GLX 2-5 had been cloned into pET15b using the W-9/Nco primer generating an N-terminal sequence of MEIELV(25). It was over-expressed in *E. coli* RIL Codon Plus BL21 (DE3) pLysS cells. However, the protein solubility was only approximately 30% and under the experimental conditions used, the above plasmid did not show good over-expression of GLX 2-5 (25). Therefore in order to isolate more soluble protein, an attempt was made to re-subclone GLX 2-5.

In the construct GLX 2-5/pT7-7, the GLX 2-5 gene is under the IPTG-inducible control of the T7 promoter, and thus it was possible to induce protein production using IPTG. The over-expression and soluble protein production of GLX 2-5 in the NdeI/pT7-7 construct was high compared to the earlier GLX 2-5-Nco/pET15b construct. It is unclear if the differences in expression of soluble protein is due to the different expression vectors used in these two constructs or a change in the second amino acid residue E to Q. It is also known that Rosetta cells enhance expression of proteins that have codons rarely used in *E.coli*. It supplies tRNAs for six rare codons AUA, AGG, AGA, CUA, CCC, and GGA. Therefore, the increased expression could also be due to the Rosetta cells. This is the first report that a plant mitochondrial glyoxalase II has been over-expressed in *E. coli* in good yield; the yeast mitochondrial glyoxalase II has been over-expressed and purified by Bito *et al.* (43). FPLC purification using Q-Sepharose as an anion exchange resin facilitated the binding efficiency of the acidic enzyme (pI = 5.67). The single-step purification gave similar purity and appeared more efficient than the two-step purification used by Zang (25).

The molar extinction coefficient of GLX 2-2 has been determined through amino acid analyses on cytoplasmic glyoxalase II (16). It was necessary to determine the molar extinction coefficient for GLX 2-5, because GLX 2-5 is a mitochondrial protein and also extinction coefficients are specific for a given protein. We have determined the molar extinction coefficient of GLX 2-5 using two different methods. The Edelhoch method is a calculation-based method (27), whereas PIXE is an experimental method. An extinction coefficient of 37,753 M\(^{-1}\)cm\(^{-1}\) used to determine protein concentration resulted in protein that had approximately two mole equivalents of metal, which is consistent with the crystal structure. Protein concentration based on the Edelhoch method resulted in protein predicted
to contain only one mole equivalent of metal. Thus it was reasonable to use the PIXE-determined molar extinction coefficient of 37,753 M$^{-1}$cm$^{-1}$ for GLX 2-5 throughout this study.

The glyoxalase II activity is expected to take place in the cytosol for two reasons: (1) SLG, the substrate for glyoxalase II is produced in the cytosol by the catalytic reaction of glyoxalase I, and (2) there is no evidence to support SLG transport through cellular membranes. Surprisingly, mitochondrial glyoxalase II from many species utilizes SLG as their substrate (5, 43, 44). In the current study, SLG was the preferred substrate for GLX 2-5 among all the substrates tested. It has been reported that $S$-$D$-acetoacetylglutathione, $S$-$D$-glycolylglutathione, $S$-$D$-acetylglutathione, and $S$-$D$-mandeloylglutathione were acceptable substrates for glyoxalase II (14, 20, 45, 46). GLX 2-5 was found to utilize SLG, but showed little to no activity towards $S$-$D$-formylglutathione.

The crystal structure of human glyoxalase II revealed the identities of several residues that are involved in glutathione substrate binding. These residues were Arg249, Asn179, Lys252, Lys143, Cys141, Tyr175, and Tyr145 (24). All of these residues except Tyr145 are conserved in GLX 2-5. Tyr145 was also not conserved in Arabidopsis GLX 2-2 (see Figure 2.12), which also has a preference for SLG. Arg249 and Lys252 are within hydrogen bonding distance of the carboxylate of glutathionyl glycine residue, whereas Tyr145 and Tyr175 were reported to be distantly interacting (24). Therefore, the presence of substrate binding residues is consistent with our finding that GLX 2-5 can utilize glutathione analogs as substrates.

Anti-GLX 2-5 antibodies were raised in rabbits. In order to ascertain the mitochondrial localization of glyoxalase II, a closely-related, readily available, mitochondria-rich Brassica oleracea plant tissue had been previously used in our lab. The tissue extract from B. oleracea was fractionated into cytoplasmic, mitochondrial, and chloroplast fractions, and a western blot experiment on each fraction using GLX 2-5 antibodies showed mitochondrial localization of glyoxalase II (47).

The observation that GLX 2-5 can bind both iron and zinc is consistent with the results on cytoplasmic glyoxalase II from A. thaliana (21) and human (24). Furthermore in all three enzymes; GLX 2-5, GLX 2-2, and human glyoxalase II, the metal binding ligands; five histidines and two aspartate residues are conserved (Figure 2.12) (18, 24). The fact that
GLX 2-5 binds nearly equal ratios of Fe:Zn (1:1 to 1:1.3) in the wild-type (GLX 2-5-wt) and GLX 2-5-FeZn enzymes, respectively, suggested that the enzyme is a binuclear iron/zinc protein. Furthermore, when the over-expression media was supplemented with a specific metal ion (Fe or Zn), the metal content of the enzyme shifts towards the added metal ion. The $k_{\text{cat}}$ values of the GLX 2-5 variants display a trend with the metal contents. The observation that GLX 2-5-wt and GLX 2-5-FeZn showed highest activity indicates that the enzyme prefers a Fe: Zn ratio of approximately 1:1 for maximum catalytic activity. Furthermore a 50% reduction in the activity of GLX 2-5-Fe compared to GLX 2-5-Zn suggests that Zn may be important for catalysis. On the other hand, though the content of zinc is almost identical in GLX 2-5-Fe and GLX 2-5-wt, the GLX 2-5-Fe enzyme exhibited the lowest activity. Therefore it is possible that presence of high levels of Fe leads to partial inactivation of the enzyme. GLX 2-5-wt exhibited a relatively higher binding affinity for S-$D$-lactoylglutathione than the other glutathione analogs; the $K_M$ value was comparable to that of GLX 2-2 (22) (Table 2.8). Surprisingly, GLX 2-5-wt showed the lowest $K_M$ when the total metal content was one equivalent. Therefore, binding of the extra metal ions to the protein may be inducing minor structural changes causing a reduced substrate binding affinity as seen in the other enzyme variants (Table 2.4). In general, wild-type glyoxalase II enzymes from *A. thaliana* demonstrated lower substrate binding affinity for S-$D$-lactoylglutathione when compared with its human and yeast analogs (Table 2.8).

The slight deviation observed in the CD spectrum of GLX 2-5-Zn compared to the other GLX 2-5 variants may be due to subtle differences in the secondary structure or possibly in the concentration of the enzyme. Comparison of the values of CD curve-fitting indicate that the secondary structures of GLX 2-5 variants are within 5% of each other. Thus, in general it can be concluded that the composition of secondary structural elements do not vary significantly in the GLX 2-5 variants. The differences in CD spectra could be due to difference in concentration that depends on different molar extinction coefficient for enzyme variants. Though CD spectroscopy gives an estimation of the components of secondary structures of a protein, it is also possible to distinguish the tertiary structural class of a protein by certain spectral features (48). The main spectral features are two negative bands at 208 and 220 nm, and a positive band around 190-195 nm, which are common to the $\alpha/\beta$ class and $\alpha+\beta$ class of proteins (48). These features are in agreement with CD spectral data of wild-
Figure 2.12. Alignment of amino acid sequences of *A. thaliana* glyoxalase II (GLX 2-5 from mitochondria and GLX 2-2 from cytosol) with human glyoxalase II (cytosolic). The leader peptide of GLX 2-5 is not shown. Sequences are aligned using the Clustal method. Shaded box shows conserved amino acids residues. Gaps introduced by the alignment are shown in dashes. Metal binding ligands are shown by * and substrate binding ligand are shown by #.
<table>
<thead>
<tr>
<th>Source</th>
<th>$K_M$ ($\mu$M)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M \times 10^6$ (M$^{-1}$s$^{-1}$)</th>
<th>Note</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human (Cytosolic)</td>
<td>187</td>
<td>780</td>
<td>4.17</td>
<td>$37^\circ$C</td>
<td>(14)</td>
</tr>
<tr>
<td>A. thaliana (Cytosolic)</td>
<td>245 ± 65</td>
<td>430 ± 71</td>
<td>1.76</td>
<td>$25^\circ$C</td>
<td>(22)</td>
</tr>
<tr>
<td>A. thaliana (Mitochondrial)</td>
<td>213 ± 20</td>
<td>157 ± 6</td>
<td>0.73</td>
<td>$25^\circ$C</td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae (Mitochondrial)</td>
<td>72</td>
<td>723</td>
<td>10</td>
<td>$37^\circ$C</td>
<td>(43)</td>
</tr>
</tbody>
</table>

Table 2.8. Comparison of kinetic constants of recombinant glyoxalase II enzymes on the hydrolysis of SLG.
type human glyoxalase II (38). According to Manavalans’ classification, GLX 2-5 is in the \(\alpha/\beta\) class of proteins, and it contains a \(\beta\)-lactamase type of fold (48). The \(\alpha/\beta\) class of proteins possess alternating \(\alpha\)-helix and \(\beta\)-sheet structures, and the distinguishing feature of the \(\alpha/\beta\) class of protein is an overlap of the 208 and 220 nm bands to give a single broad minimum that is skewed towards 220 nm (48).

The single tryptophan residue has served as an excellent probe to monitor the fluorescence emission changes in all forms of GLX 2-5 in our study. This intrinsic fluorescence emission can serve as an indicator of structural changes that could lead to a change in the microenvironment of tryptophan (49, 50). The notable feature of all the spectra shown in Figure 2.6 is the overall shape. None of the spectra were symmetric as observed in other proteins such as metallo-\(\beta\)-lactamases L1 and tyrosine hydroxylases (38, 51, 52). The extent of asymmetry is much higher in the GLX 2-5-Zn and GLX 2-5-Fe samples. Typically asymmetric spectral peaks occur due to overlapping of peaks. Therefore, the asymmetric feature observed in the fluorescence spectra of GLX 2-5 samples can be explained assuming that these spectra were composed of more than one emission spectra, \(i.e\). more than one fluorescence-active species in these enzyme preparations. Furthermore, the composition of different fluorescence-active species in each enzyme preparation varied.

A plausible explanation for the fluorescence data can be provided based on the varying metal composition in the GLX 2-5 preparations. It is well known that the fluorescence emission can be quenched by the presence of paramagnetic divalent metal ions such as Fe(II), Mn(II), and Co(II) (52). EPR studies on GLX 2-2 demonstrated the presence of dimetal centers (18). Our EPR study on GLX 2-5 (see below) indicates the presence of Fe(II)-Fe(III), Fe(III), and Mn(II) centers. Furthermore, the metal analyses indicate the presence of zinc. Therefore, the difference in fluorescence emission spectra of GLX 2-5-FeZn, GLX 2-5-Fe, GLX 2-5-Zn, and GLX 2-5-wt can be explained by assuming that they contain varying compositions of GLX 2-5 loaded with Fe(II)-Fe(III), Fe(III)-Zn(II), and Zn(II)-Mn(II) dimetal centers.

In human tyrosine hydroxylase, which binds iron and zinc, it has been shown that reconstitution of apo protein with paramagnetic Fe(II) quenches fluorescence emission (52). Further it has been shown in metallo-\(\beta\)-lactamase L1 that the addition of Zn(II) enhances fluorescence emission (51). The counteracting fluorescence properties of Fe(II) and Zn(II)
lead to a conclusion that the GLX 2-5-FeZn and GLX 2-5-wt may predominantly have a GLX 2-5 form loaded with a Fe(III)-Zn(II) dimetal center resulting in enhanced fluorescence. Whereas GLX 2-5-Fe and GLX 2-5-Zn may predominantly have Fe(II)-Zn(II) and Fe(II)-Fe(III) loaded forms of GLX 2-5 leading to relatively quenched fluorescence emission. However we were unable to estimate the relative amounts of these different dimetal center containing GLX 2-5 enzymes. Another contribution to the lowered fluorescence emission could be due to slightly different protein folding (53).

Typically a red-shift is observed in fluorescence emission in an unfolded protein compared to the folded protein (38, 51). The red-shift observed in our study may be due to folding differences between the different dimetal forms of GLX 2-5. It is also possible that the samples contain different amounts of denatured protein. Since we lack structural information on the position of the single tryptophan residue relative to the metal binding sites, it is difficult to determine whether a metal-binding, a folding difference or both contributed to the red-shift.

Electron paramagnetic resonance (EPR) is widely used to probe the environment around paramagnetic metal ions and give information about metal ligands and coordination geometries (46). Application of EPR spectroscopy to study high spin Fe(II) and Fe(III) centers in enzymes is well known (40, 54). The interpretation of the EPR spectra of iron-containing systems is usually complicated by the presence of mixed oxidation states. Model compounds or computer simulations are usually used in the interpretation of experimental EPR (55). However, model compounds do not exactly represent a metal ion center in an enzyme to provide information about the geometry and electronic structure. Computer simulation of experimental line shape to obtain EPR parameters is also becoming popular (56). Simulation can provide information about the geometry, types of ligand, and electronic symmetry around the metal ion (57). GLX enzymes have been probed using EPR previously (18, 22). The interpretation of EPR spectra of glyoxalase enzymes are complicated by the presence of mixed metal valencies of Fe and other metal ions such as Mn(II).

EPR spectra of GLX 2-5-FeZn, GLX 2-5-Fe, and GLX 2-5-Zn, all exhibit features indicating the presence of multiple metal centers. GLX 2-2 enzymes are known to contain varying compositions of multiple metal centers (18). The GLX 2-5-FeZn spectrum shows three distinct features that identify different metal centers. The presence of signals at $g = 8.4$
and 5.25, which according to experimental and theoretical studies indicate that these signals arise from a single protein-bound, high spin Fe(III) (S = 5/2) species (40, 41, 54, 58). GLX 2-5-FeZn exhibits approximately one equivalent of Zn(II), therefore, it is very likely that Fe(III) center may exist as a Fe(III)–Zn(II) dinuclear center. In GLX 2-2 it has been proposed that this Fe(III) center exists primarily in dinuclear centers (23).

The second feature in the GLX 2-5-FeZn spectrum is a prominent signal at g = 4.2, which is typically identified as adventitiously bound Fe(III) (generally referred to as “junk Fe”). This Fe(III) could be bound to the surface of the GLX 2-5 enzyme. The third feature, centered around g = 2, is more complicated. It is obvious from the multiple line pattern (6 lines) that a Mn(II) center is present. The presence of Mn(II) is further confirmed by the g value being 2 (18). Typically, a clear 6-line pattern is observed for Mn(II), but here it is obscured by the overlap of a Fe(II)–Fe(III) spin system in the same region. The g-values of 1.71, 1.8, and 1.98 are typical for a Fe(II)–Fe(III) mixed valence system (18, 40). As proposed earlier, it is possible that this Mn(II) could exist as a Mn(II)–Zn(II) center considering the relatively small amount of Mn observed in the metal analysis.

These three features; (1) protein bound Fe(III), (2) adventitious Fe(III), and (3) the presence of signals for Mn(II) and Fe(II)–Fe(III) centers, are also present in the GLX 2-5-Fe, and GLX 2-5-Zn samples as well. The intensities of these signals in the GLX 2-5-Fe and GLX 2-5-Zn spectra are very much reduced. In the GLX 2-5-Fe sample, the protein-bound Fe(III) is less prominent than in GLX 2-5-FeZn but more pronounced than that of GLX 2-5-Zn (see Figure 2.8). The metal analyses shows a ratio of Fe : Zn in GLX 2-5-FeZn as 1 : 1.3 and in GLX 2-5-Fe as 1.4 : 0.5. Therefore the low intensity of the protein-bound Fe(III) in the GLX 2-5-Fe spectrum may be due to the formation of lower amounts of the Fe(III)–Zn(II) dinuclear center in GLX 2-5-Fe. Since we did not spin quantitate the EPR active species, we were unable to determine quantitatively how Fe is distributed among these three Fe-containing centers. In addition to these three features, a spike around g = 2 is observed in the GLX 2-5-Fe sample, which could be due to instrumental artifacts, most probably presence of small amount of Cu in the cavity.

The overall reduction in the signal intensities of the GLX 2-5-Zn spectrum is supported by the low amount of Fe in the metal analysis (Table 2.3). However, all three
metal centers are present in GLX 2-5-Zn even at the low Fe levels (Figure 2.9). The Fe(II)–Fe(III) signal at \( g < 2 \) is very weak compared to the other two signals in the GLX 2-5-Zn spectrum.

The EPR data on GLX 2-5 enzyme preparations show that GLX 2-5 contains (1) a Fe(III) center possibly coupled with Zn(II) resulting a Fe(III)–Zn(II) dimetal center, (2) a Fe(III)–Fe(II) metal center, (3) a quantitatively less significant Mn(II) center possibly coupled with Zn(II) and (4) a catalytically less significant adventitious Fe(III) center. The EPR study on GLX 2-2 also indicated the presence of a Fe(III)–Zn(II) center (18). It has been reported that the native purple acid phosphatase from red kidney bean contains a catalytically active Fe(III)–Zn(II) metal center (40, 59). Further, Beck et al. prepared the Fe(III)–Fe(II) analog of red kidney bean purple acid phosphatase, demonstrating that Zn(II) can be replaced by Fe(II) with catalytic recovery (40). Even though EPR does not provide direct evidence for the presence of Zn(II), it is highly probable that the existence of the Zn(II) shown in the metal analysis forms dimetal centers with Fe. Dimetal centers similar to those of glyoxalase II are well precedented in the literature (18, 22, 40, 60). Therefore, it is reasonable to propose from the EPR results that our GLX 2-5 preparations predominantly contain at least two forms of catalytically active enzyme, containing a Fe(III)–Zn(II) and Fe(III)–Fe(II) metal centers. In addition to these two forms, there may be other forms of enzyme, including Zn(II)–Zn(II), which is EPR silent, and Zn(II)–Mn(II). Additionally, GLX 2-5 contains some amount of surface-bound Fe(III).

Paramagnetic \(^1\)H-NMR spectroscopy is one of the useful spectroscopic techniques made available to probe a metalloprotein that contains a protein-bound paramagnetic metal ion. A protein-bound paramagnetic metal ion, such as Fe(II), Co(II), and Ni(II), would isotropically shift the proton resonances of the bound and nearby ligands from other protons (42, 61). This technique has been used very often to identify and quantitate histidine residues bound to metal ions, because the solvent exchangeable N—H protons of histidine residues typically yield peaks at 30-70 ppm, and these peaks disappear when the H$_2$O in the sample is replaced with D$_2$O (42, 62). However, the slow relaxing properties of high-spin Fe(III) broaden any resonances from protons of metal bound ligands beyond detection and any visible peak is expected to appear > 80 ppm (42, 63, 64).
All of our data so far indicate the presence of Fe(II)- or Fe(III)-containing metal centers in GLX 2-5. In order to further strengthen our conclusion about the metal centers we conducted paramagnetic $^{1}$H NMR spectroscopy on GLX 2-5-FeZn.

The proposed active site for GLX 2-2 contains a total of five histidine residues. It has been suggested that two or three of the five histidine residues are bound to iron (21). The sequence similarity suggests that the metal-binding residues of GLX 2-5 and GLX 2-2 are identical (Figure 2.12). As observed in other paramagnetic iron proteins (63, 65), the $^{1}$H-NMR spectrum of GLX 2-5-FeZn exhibits paramagnetically-shifted peaks (Figure 2.10) (63, 65). Because the metal analysis of the GLX 2-5-FeZn sample was found to contain no other possible paramagnetic ions, such as Co(II), the presence of paramagnetically-shifted peaks confirms the presence of protein-bound Fe in GLX 2-5.

Peaks A, B, C, D, and E are the most probable candidates for protons of amino acid residues directly bound to the iron center (63). The chemical shift values of these shifted peaks rule out a possible antiferromagnetically-coupled dinuclear iron center through an oxo-bridge. Typically an oxo-bridged Fe(III)-Fe(III) or Fe(II)-Fe(III) system result in poorly shifted peaks, mostly in the region of 10-20 ppm (39, 66). The chemical shift values of the isotropically-shifted peaks are comparable to the chemical shift values of Fe(II)-imidazole model complexes and various other Fe(II)-containing proteins (64, 65, 67). Tentative assignments of the resonances to specific protons of the amino acids are given in Table 2.9. The two solvent-exchangeable peaks (A, D) may arise from Fe(II)-bound histidine NH protons (65, 68). This indicates the presence of a Fe(II) center with two histidines as ligands. Additional assignment of the resonances can be proposed by consulting the literature. Peaks B, and C can be assigned to the two $C_\delta$ H protons of the Fe(II) bound histidines as observed in bovine spleen purple acid phosphatase (65, 69, 70). The most probable candidates for the peaks F and G are $C_\beta$ H of the Fe(II) bound histidines (68). A broad peak (E) at 33.3 ppm, which may be composed of two peaks appears to be originating from the $C_\beta$ H of a metal bound Asp ligand as observed in the Fe(II)-Zn(II) form of superoxide dismutase (68). The origin of the two peaks observed in the most upfield region of -10 to -25 is uncertain. Sorkin et. al observed that the $^{1}$H NMR spectra of amino acid specific isotopic labeled Fe(II)-Zn(II) form of superoxide dismutase exhibited peaks at a similar chemical shift values. They assigned these peaks to protons of amino acid residues (Ala and Trp), which are not metal
<table>
<thead>
<tr>
<th>Resonance Peak</th>
<th>Chemical shift (ppm)</th>
<th>Tentative proton assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*</td>
<td>62.5</td>
<td>His NH</td>
</tr>
<tr>
<td>B</td>
<td>50.3</td>
<td>His C_8H</td>
</tr>
<tr>
<td>C</td>
<td>46.8</td>
<td>His C_8H</td>
</tr>
<tr>
<td>D*</td>
<td>40.0</td>
<td>His NH</td>
</tr>
<tr>
<td>E</td>
<td>33.3</td>
<td>Asp C_βH</td>
</tr>
<tr>
<td>F</td>
<td>20.3</td>
<td>His C_βH</td>
</tr>
<tr>
<td>G</td>
<td>18.4</td>
<td>His C_βH</td>
</tr>
<tr>
<td>H</td>
<td>-10.3</td>
<td>Ala C_βH (?)</td>
</tr>
<tr>
<td>I</td>
<td>-20.6</td>
<td>Trp (?)</td>
</tr>
</tbody>
</table>

Table 2.9. ¹H- NMR proton resonance assignments of GLX 2-5-FeZn spectra

![Histidine](image1)

![Aspartate](image2)
bound but in close proximity (~ 4 Å) to the metals (68). It is possible that the two resonances observed between -10 to -25 originating from protons of non-metal bound amino acid residues.

These NMR results indicate a Fe(II) system with two His and an Asp. However, EPR results suggested the presence of Fe(III)-Zn(II) and Fe(III)-Fe(II) systems. Further, if a Fe(III)-Zn(II) system is present, it would be very hard to observe paramagnetically shifted peaks with sufficient intensities due to the slow relaxing properties of Fe(III) as mentioned above (63, 66). Zinc being spectroscopically silent, it is not possible to observe proton signals with considerable intensity. Further, there were no peaks above 80 ppm due to shifting by Fe(III). On the other hand, in an Fe(III)-Fe(II) system the fast-relaxing properties of Fe(II) would influence the Fe(III) to relax faster and should result in observable NMR signals for the protons of amino acids bound to Fe(III) (42). If this is the situation, then with the possible ligands of both Fe(III) and Fe(II), even in the presence of weak antiferromagnetic coupling, there should be more resonances than what we observed in the spectrum of GLX 2-5-FeZn. Furthermore, any oxidation of Fe(II) to Fe(III) would produce a spectrum without any discernable peaks due to the antiferromagnetic coupling or an unfavorable relaxation time (64).

Our other data, especially EPR suggests the presence of Fe(III). To resolve this conflict, we collected the $^1$H NMR spectrum of H$_2$O$_2$ treated GLX 2-5-FeZn (Figure 2.11). This spectrum was identical to the GLX 2-5-FeZn without H$_2$O$_2$ treatment, except for the absence of a weak peak observed at 33.3 ppm in the GLX 2-5-FeZn spectrum (Figure 2.10). Two plausible explanations for this observation are (1) the GLX 2-5-FeZn sample contains Fe(III), or (2) the oxidation of GLX 2-5-FeZn did not take place by adding H$_2$O$_2$. Based on the data provided here, we can not unequivocally decide the oxidation state of the Fe in the dimetal center. Future experiments are necessary to resolve this conflict and more accurately assign the NMR proton resonances. Nonetheless, it is evident that GLX 2-5-FeZn contains predominantly a Fe(II) or Fe(III)–Zn(II) dimetal center, and that the Fe center has at least two histidines and an aspartate as ligands. This data allows us to propose the structure of the primary dimetal center that is present in the GLX 2-5-FeZn preparation (Figure 2.13).

While this thesis was being written, we received the crystal structure of GLX 2-5-FeZn (Figure 2.14). The data confirmed that GLX 2-5 contains a Fe-Zn dimetal center. Fe
and Zn centers are bridged by a water ligand. The Fe center has 2 histidines and 2 aspartates, and the Zn center has 3 histidines (Table 2.7). The crystal structure shows that the single tryptophan residue is at a distance of ~ 20 Å from Fe and Zn centers. Therefore we were unable to unambiguously attribute the difference in fluorescence spectra of GLX 2-5 preparations to the binding of Fe and Zn at the active site. It is possible that the changes in fluorescence emission in GLX 2-5 preparations may be due to folding difference in the local environment of the tryptophan residue or any adventitious metal binding closer to the tryptophan site.
Figure 2.13. Proposed dinuclear active site model for *A. thaliana* GLX 2-5. The model is generated based on spectroscopic and preliminary crystal structure data. The numbering is based on the amino acid sequence numbering of the recombinant *A. thaliana* GLX 2-5.
Figure 2.14. The dinuclear active site for *A. thaliana* GLX 2-5. The numbering is based on the amino acid sequence numbering of the recombinant *A. thaliana* GLX 2-5. The bridging water ligand is not shown. The figure was generated using the RasMol software on the X-ray crystal structure.
References


Chapter 3
Concluding Remarks

Glyoxalase 2-5 (GLX 2-5) is a mitochondrial glyoxalase II from *A. thaliana*. In order to characterize a plant mitochondrial glyoxalase II, GLX 2-5 has been cloned and overexpressed. A GLX 2-5/pT7-7 plasmid was constructed with the N-terminal sequence MQIELVP, based on the N-terminal sequences of *A. thaliana* cytoplasmic GLX 2-2. GLX 2-5 was over-expressed and produced soluble protein. This is the first time a plant mitochondrial glyoxalase II has been cloned and heterologously over-expressed in *E. coli*. Though no reports show that SLG transports across cellular membranes, GLX 2-5 utilizes SLG as a substrate. GLX 2-5 binds iron and zinc in various amounts depending on the availability of the metal ions in the culture media and the enzyme was active at this varying iron and zinc composition. The catalytic efficiency, $k_{cat}/K_M$, of GLX 2-5 variants containing different compositions of metal, spans in the range of 0.33 - 0.73 s$^{-1}$ µM$^{-1}$, with the substrate S-D-lactoylglutathione. The catalytic activity of GLX 2-5-wt and GLX 2-5-FeZn were higher than those of the GLX 2-5-Fe and GLX 2-5-Zn enzymes.

CD spectral analysis revealed that GLX 2-5 belongs to the $\alpha/\beta$ class of proteins. GLX 2-5 contains a single tryptophan that is responsible for its fluorescence property. Tryptophan excitation at 295 nm yielded overlap of more than one emission spectra in GLX 2-5 variants. This indicates the presence of more than one species with different fluorescent properties.

EPR spectroscopy of GLX 2-5 revealed a number of possible metal centers including Fe(III)-Zn(II) and Fe(III)-Fe(II) together with non-specific binding of Fe(III), as observed in GLX 2-2. However, spin quantitation is necessary to determine the composition of different dimetal centers in each enzyme variant. GLX 2-2 prepared in minimal media contained only one equivalent of metal. EPR and EXAFS data revealed that GLX 2-2 contains a mixture of fully metal-loaded and apo GLX 2-2. Since GLX 2-5-wt contained only one equivalent of total metal, as seen in GLX 2-2, it is possible that GLX 2-5-wt may contain a mixture of fully metal-loaded and apo GLX 2-5.

$^1$H NMR spectroscopy indicated the binding of Fe(II) to two histidines and an aspartate residue. We were able to assign most of the peaks that appeared in the $^1$H
paramagnetic NMR spectrum of GLX 2-5-FeZn. We demonstrated that the GLX 2-5 enzyme can be stable at room temperature for several hours to collect interpretable $^1\text{H}$ NMR data. However, the similarity between the $^1\text{H}$ NMR spectra of the $\text{H}_2\text{O}_2$ treated and non-treated GLX 2-5 is puzzling. In order to confirm the oxidation state of the iron in GLX 2-5 and verify our assignment of protons to the observed resonances, future NMR experiments are necessary. $^1\text{H}$ NMR experiments of GLX 2-5 in oxidizing and reducing conditions are necessary. Further NOE and $T_1$ measurements would be helpful in the accurate assignment of the proton resonances. Amino acid specific isotopic labeling would be an ideal approach to get a complete picture of the active site using NMR. These studies can be extended to inhibitor design studies in the future.

Preliminary X-ray crystallographic data confirmed that GLX 2-5 is a FeZn protein and contains five histidines, two aspartates and a bridging water as ligands in the metal binding site. With the help of this X-ray data, we were able to confirm our proposed model for the active site of $A.\ thaliana$ GLX 2-5.

Future studies should focus on the preparation of apo-GLX 2-5 and substitution with various combinations of Fe and Zn, such as addition of one equivalent of Fe(II) followed by one equivalent of Zn(II) to generate a mixed metal active site and characterize the protein by steady state kinetics, EPR, EXAFS, and $^1\text{H}$ paramagnetic NMR spectroscopic studies to elucidate the metal binding and catalytic properties of the metal reconstituted GLX 2-5. Similar characterization should be performed after reconstitution of GLX 2-5 with Fe(III), Fe(II): Fe(III), Zn(II) as well. In the event of differing metal-binding affinity, i.e. if each site has different $K_D$ values, of the metal binding sites the order of metal addition to the apo protein should also be altered. These experiments would allow us to determine the specific nature of the active site and what effect the structure of the metal center has on catalytic activity.