ABSTRACT

GLYOXALASE 2-2: OVER-EXPRESSION AND CHARACTERIZATION OF A METALLOHYDROLASE FROM ARABIDOPSIS THALIANA

By Nathan F. Wenzel

In order to investigate the metal binding properties of *A. thaliana* glyoxalase 2-2, recombinant GLX 2-2 was over-expressed in rich or minimal media containing various amounts of added Zn(II), Fe(II), or Mn(II). The resulting enzymes were purified, characterized, and compared. Kinetic studies and metal analyses of the samples indicated that no significant catalytic differences existed between the enzymes, in spite of the enzymes binding different types and amounts of metal ions. EPR and EXAFS spectroscopies strongly indicated the presence of positive cooperativity of metal binding and of numerous possible dinuclear metal centers, including Fe(III)Zn(II), Fe(III)Fe(II), and Mn(II)Mn(II) centers. The ability to bind various amounts of manganese, iron, and zinc with no apparent change in catalytic activity and the possibility of positive cooperativity in metal binding make *A. thaliana* glyoxalase 2-2 unique to the family of proteins containing the β-lactamase fold.
Glyoxalase 2-2: Over-expression and Characterization of a Metallohydrolase from *Arabidopsis thaliana*

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Chapter 1

1.1 Introduction: The Glyoxalase System

The glyoxalase system consists of two enzymes that convert cytotoxic 2-oxoaldehydes into 2-hydroxy acids in the presence of reduced glutathione (Figure 1) (1). These two enzymes are lactoylglutathione lyase (GLX 1, 4.4.1.5) and hydroxyacylgulathione hydrolase (GLX 2, 3.1.2.6). GLX 1 is a Zn(II) containing (or Ni (II) (2)) metalloenzyme that catalyzes the formation of S-D-lactoylglutathione (SLG) from the thiohemiacetal intermediate, which is spontaneously and nonenzymatically formed by methylglyoxal (MG) and reduced glutathione (3). MG, thought to be the primary physiological substrate of GLX 1, is formed in many biological processes including glycolysis, acetone and threonine catabolism, and nonenzymatically from triose phosphates (3-6). MG is both cytotoxic and mutagenic because of its ability to reversibly, or in some cases irreversibly, modify arginine, lysine, and cysteine residues in proteins (7), form modified guanylate residues in DNA and RNA (4), and form inter-strand DNA crosslinks (8). GLX 2 catalyzes the hydrolysis of SLG to form D-lactic acid and regenerate reduced glutathione (3). SLG is cytotoxic because of its ability to inhibit DNA synthesis (4, 9). SLG is formed in the cytosol of cells and cannot readily cross cell membranes (3). However, SLG metabolism by γ-glutamyltransferase and dipeptidase generates N-D-lactoylcysteine, which can cross cell membranes and lead to nucleotide synthesis inhibition (3). Because of the toxicity of MG and SLG, the glyoxalase system is thought to play a major physiological role in cellular detoxification (3, 9).
**Figure 1:** The Glyoxalase System catalyzes the isomerization of the hemithioacetal intermediate into SLG. GLX 2 then catalyzes the hydrolysis of SLG into \( D \)-lactate and reduced glutathione.
1.2 Physiological Roles of the Glyoxalase System

Increased glyoxalase activity has been observed in a number of organisms and has been associated with many cellular processes. Elevated GLX 1 and MG levels have been observed in rapidly dividing cells such as plant root tips and seedlings (10), tumor cells such as breast carcinoma cells (11), and human embryo cells (3). In addition, GLX 2 has been suspected of being involved in the regulation of rat spermatogenesis (12). Glyoxalase activities in leukemia and lymphoma cells have been shown to be related, at least partly, to cell differentiation and the cell cycle (3). Increased MG and glyoxalase system activity has been observed in certain complications of diabetes mellitus, in the malaria parasite *Plasmodium falciparum*, and in tumors (16). Multiple studies have shown increased levels of MG in animals with diabetes mellitus, including in the blood stream, and kidneys of diabetic rats, and 2-6 fold increases of MG in the blood of human diabetic patients (17). The malaria parasite, as with tumor cells, is highly dependent on anaerobic glycolysis and requires higher glyoxalase activity to detoxify the correspondingly higher MG concentrations (17). The glyoxalase enzymes and MG are also found in other organisms such as *Escherichia coli* and yeast (13). Although the primary role of the glyoxalase system is thought to be in the cellular detoxification of 2-oxoaldehydes, especially MG, the above studies suggest that the glyoxalase enzymes may participate in a number of different processes and may fulfill important functions for all life.

1.3 Inhibitors of the Glyoxalase Enzymes

The role of the glyoxalase enzymes in cellular detoxification has led to a large amount of attention being devoted to designing inhibitors of the system’s two enzymes in the hopes that new anti-tumor and/or anti-malarial drugs may be found (14-16). Because of the toxicity and high concentrations of MG in tumors and the malaria parasite, the anti-tumor/anti-malarial properties of MG were studied. MG has been shown to effectively inhibit tumor growth by up to 91-99% by inducing apoptosis through DNA adducts and advanced glycation endproducts (AGE) proteins formation (3, 17). Unfortunately, near fatal levels of MG were needed to overcome the glyoxalase detoxification pathway, and tumors rapidly grew back once MG
treatment was stopped (3). These results have led to efforts to design inhibitors for the glyoxalase enzymes (1, 15, 16, 18-20). The theory is that if the glyoxalase system is inhibited, MG should reach lethal concentrations in tumors and in the malaria parasite, while leaving healthy cells relatively unaffected. To this end, several inhibitors of GLX 1 were developed that were able to inhibit tumor cell growth in vitro (1, 15) and in tumor-bearing mice (19). However, other enzymes such as aldose reductase (EC 1.1.1.21) (3) and α-oxoaldehyde dehydrogenase (EC 1.2.1.23) (4) metabolize MG and limit the efficacy of GLX 1 inhibitors.

Inhibitors for GLX 2 have also been developed. Unlike MG, SLG is detoxified primarily through just one enzyme, GLX 2. Additionally, with only one pathway to detoxify SLG, GLX 2 inhibitors should be more efficient than GLX 1 inhibitors at causing a buildup of cytotoxic compounds in tumors and in the malaria parasite. Several inhibitors were designed with very limited information on the active site structure and kinetic mechanism of GLX 2. One GLX 2 inhibitor, \(N, S\)-bis-fluorenlymethoxycarbonyl-glutathione (DiFMOC-G), strongly inhibited mammalian GLX 2 in vitro with a \(K_i\) value of 0.08 \(\mu\)M (20). Unfortunately the in vivo effectiveness of thiocarbonate GLX 2 inhibitors were limited due to problems with the inhibitors not being able to cross cell membranes (20). Lipophilic derivatives of DiFMOC-G had increased membrane permeability but had decreased inhibition against GLX 2 as compared with DiFMOC-G (20). With more information on the active site of GLX 2, it is hoped that more effective in vivo inhibitors can be developed.

### 1.4 Glyoxalase 1

GLX 1 is thought to exist in all organisms, and GLX 1 from a number of sources has been characterized. Some of the organisms from which GLX 1 has been studied include \textit{Escherichia coli}, \textit{Pseudomonas putida}, \textit{Saccharomyces cerevisiae}, \textit{Candida albicans}, \textit{Plasmodium falciparum}, and humans (21). Human GLX 1 is a homodimeric protein of roughly 44 kDa, while various micro-organisms such as \textit{S. cerevisiae} and \textit{E. coli} have monomeric GLX 1 ranging between 20-38 kDa (21). Isoelectric points have been found to be 4.8-5.1, 7.0, and 4.0 for human, yeast, and \textit{P. putida} enzymes, respectively (21).
Both yeast and mammalian enzymes require one Zn(II) per subunit for activity (22). The enzyme-bound metal ion in each subunit is in a distorted octahedral coordination geometry based on optical and EPR studies of the cobalt-substituted enzymes and extended X-ray absorption fine structure (EXAFS) studies on the native enzymes (22). X-ray crystallographic measurements performed on various forms of *E. coli* GLX 1 suggested that an octahedral coordination geometry with two water molecules coordinated to the metal ion is needed for activity (22). *E. coli* GLX 1 is catalytically inactive when Zn(II) is bound in the active site, perhaps due to the fact that Zn(II) was found to be bound in a five-fold, trigonal bipyramidal coordination geometry with only one water molecule coordinated to Zn(II) (22). These studies suggested that two open (solvent) coordination positions on the metal ion are required for catalysis (22).

As stated above, the substrate for GLX 1 is a hemithioacetal formed non-enzymatically from α-oxoaldehydes and reduced glutathione (21). Studies have shown that while GLX 1 has a broad substrate specificity for α-oxoaldehydes, the $V_{\text{max}}$ and $K_M$ values of GLX 1 decrease as the hydrophobicity of the side chain of the α-oxoaldehyde increases (21). The primary physiological substrate of GLX 1 is considered to be the hemithioacetal adduct of MG, and GLX 2 is needed to detoxify the SLG produced from the isomerization of the hemithioacetal substrate of GLX 1.

### 1.5 Glyoxalase 2

GLX 2, which has been purified from many sources, is a monomeric protein that varies from 18-30 kDa, depending on the source of the enzyme (23). Interestingly, GLX 2 from animal sources exhibits basic pI’s, while plant sources and yeast produce GLX 2’s with acidic pI’s (24, 25). Studies on the cytosolic form of GLX 2 from human liver has shown that GLX 2 has a broad pH optimum between pH 6.8–7.5 with higher pH’s giving decreased activities (23). GLX 2 has a broad substrate specificity for thiol esters of glutathione, but the greatest activities have been observed when using 2-hydroxyacyl thiol esters of glutathione as substrates (23). SLG has been shown to be the preferred substrate for cytoplasmic GLX 2 from a variety of sources including human liver, *Zea mays*, *Aloe vera*, spinach, *C. albicans*, and recombinant GLX 2 from *S. cerevisiae* (23-28).
Information on the mitochondrial forms of GLX 2 is more limited than for cytoplasmic enzymes. Since SLG cannot cross the mitochondrial membrane, the preferred substrate for mitochondrial GLX 2 remains unknown (3). However, acyl transfers from coenzyme A thiol esters and a mitochondrial pool of glutathione could produce the thiol esters of glutathione that would necessitate a mitochondrial GLX 2 enzyme (27). Mitochondrial GLX 2 has been purified from spinach (27) and from \textit{S. cerevisiae} (28), and SLG was reported to be the preferred \textit{in vitro} substrate for both enzymes. Results on these mitochondrial GLX 2’s were compared to the corresponding cytosolic enzymes in each species, and it was found that, depending on the species, mitochondrial GLX 2 may or may not exhibit similar kinetic properties to cytosolic GLX 2 (27, 28).

Earlier studies suggested that GLX 2 does not require bound metal ions for activity (23, 29, 30). EDTA, EGTA, phosphoamidon (an inhibitor of metalloproteinases), and 8-hydroxyquinoline had no immediate effect on the activity of human liver GLX 2, and addition of metal ions did not activate the enzyme (23, 29). In addition, rat erythrocyte GLX 2, which was inactivated by dialysis, was not reactivated by the addition of metal ions (31). No inhibition of GLX 2 was observed with E64 and antipain, suggesting that GLX 2 is not a cysteine-dependent hydrolase (23). Evidence for an active-site amine was indicated when GLX 2 from human liver and rat erythrocytes was inactivated by 2,4,6-trinitrobenzenesulphonate, and GLX 2 was protected from inactivation by \textit{N}-acetyl-S-(p-bromobenzyl)glutathione, suggesting an active site nucleophile (23). An active-site arginine was indicated when GLX 2 from rat erythrocytes was inactivated by phenylglyoxal at pH 8, and the enzyme was protected from inactivation by \textit{S}-(\textit{p}-chlorophenacyl)glutathione (31). Also, an active-site histidine was suggested when a reagent specific for histidines, diethylpyrocarbonate at pH 6, inactivated rat-erythrocyte GLX 2 (31).

Further characterization of GLX 2 has been done with the GLX 2’s from \textit{Arabidopsis thaliana}. Five isozymes of GLX 2 have been proposed in \textit{Arabidopsis thaliana} (32). GLX 2-1, 2-4, and 2-5 appear to be localized in mitochondria, GLX 2-2 in the cytosol, and GLX 2-3 has been found in the cytoplasm (Makaroff, unpublished). The amino acid sequence of \textit{Arabidopsis} GLX 2-2 was found to have 55% identity and 71% similarity to the human cytoplasmic GLX 2 (32). In addition, GLX 2 enzymes in general have been found to contain amino acids known to bind Zn(II) in the metallo-\textit{β}-lactamases (32, 33), prompting Crowder \textit{et al.} to predict that \textit{Arabidopsis} GLX 2-2 requires Zn(II) (32). Amino acid sequences from cytosolic GLX 2 in humans, yeast,
and *A. thaliana* were found to have a highly conserved zinc-binding domain (THXHXDH) that is present in the metallo-β-lactamases, suggesting that all GLX 2 enzymes may bind Zn(II) (Figure 2) (34). In contrast to earlier studies suggesting that GLX 2 does not require bound metal ions (23, 29, 30), *Arabidopsis* GLX 2-2 was found to bind Zn(II) and the enzyme was predicted, based on its similarity to metallo-β-lactamases, to bind two Zn(II) ions, utilizing five histidines, two aspartic acids, and a bridging water molecule as metal binding ligands (34). These predictions were confirmed by the crystal structure of recombinant human GLX 2 (35). The crystal structure showed that the active site of GLX 2 is most similar to *Stenotrophomonas maltophilia* metallo-β-lactamase L1 (33). In addition, the crystal structure showed that GLX 2 is a member of the family of enzymes with the metallo-β-lactamase fold. More recently, *Arabidopsis* GLX 2-2 was found to bind both zinc and iron (37), and mutations within and outside of the metal-binding ligands of the enzyme influenced the relative amounts of metal that was bound in the active site (37). The metallo-β-lactamase fold has been found to be able to bind various metals. Besides metallo-β-lactamases, which bind Zn (II) (33), and GLX 2, which binds Zn(II), Fe, and Mn (37, this thesis), rubredoxin:oxygen oxidoreductase (ROO), which contains a metallo-β-lactamase fold, binds iron (38). In addition, subtle differences in metal-binding ligands have been suggested to possibly be responsible for differences in metal-binding properties between different family members (39). Human GLX 2 has been reported to be a zinc metalloenzyme although iron was found to be bound (35). These studies suggest that GLX 2 may be able to bind various metals.

1.6 Summary

In this thesis, GLX 2-2 from *A. thaliana* was characterized using spectroscopic and kinetic studies to address the contradictory data in the literature concerning the metal center(s) in GLX 2. To accomplish this goal, recombinant GLX 2-2 was over-expressed in rich and minimal media and in the presence of added Zn(II), Fe(II), and Mn(II). Kinetic analyses on the resulting purified enzymes were conducted to determine how metal content affects enzyme activity. Metal analyses were used to determine the quantity and identity of metals bound in the active site and to correlate metal content with the activity of the enzymes. Electron paramagnetic resonance
Figure 2: Aligned Amino Acid Sequences of GLX 2. *A. thaliana* GLX 2-2
zymes are labeled as *Arab* GLX 2-X (X=1-5). The sequences were aligned by
the clustal method as described by Higgins and Sharp (36). The shaded area shows
the majority amino acid at a specific residue position.
(EPR), UV-VIS, and extended X-ray absorption fine structure (EXAFS) spectroscopies were performed on these enzymes to determine the nature of metal centers present in GLX 2-2. Furthermore, circular dichroism (CD) and fluorescence spectroscopic studies were conducted to determine if the secondary structure of the enzyme was changed when various metals were in the active site of GLX 2-2. Finally, an *in vivo* competition study between zinc, manganese, and iron was performed in order to see which metals were preferentially incorporated into GLX 2-2. The results from these studies demonstrate that GLX 2 can utilize a number of different metal ions and metal centers to be an active enzyme.
1.7 References


2.1 Introduction

Past studies have identified five putative GLX 2 isozymes in *A. thaliana* (1). To further investigate the structure/function of GLX 2, GLX 2-2, a cytosolic isozyme in *A. thaliana*, was characterized. GLX 2-2 is a small monomeric protein of 28.8 kDa in mass (2) and has a predicted pI value of 6.2. Early studies suggested that GLX 2 did not require Zn(II) for activity (3, 4); however, Makaroff and coworkers discovered that GLX 2 contains a THXHXD motif, which is found in metallo-β-lactamases and is known to bind Zn(II) (1, 2, 5, 6). Subsequently, Crowder et al. demonstrated that GLX 2-2 requires Zn(II) for catalytic activity (2). GLX 2-2 has been shown to have more than 50% sequence identity with human GLX 2 (1). The crystal structure of human GLX 2 showed the characteristic overall structure and dinuclear zinc-binding active site for members of the metallo-β-lactamase superfamily (7). The human GLX 2 crystal structure showed that the GLX 2 dinuclear metal-containing active site is most similar to that in metallo-β-lactamase L1 from *S. maltophilia* (5). Both enzymes share three conserved histidines coordinating the first Zn(II) site, and both have a bridging water between the Zn(II) ions. GLX 2 also has a bridging aspartate. The second metal, in both L1 and GLX 2, is coordinated by two histidines and two water molecules. L1 has only one aspartate coordinating the second Zn(II), while GLX 2 has two metal binding aspartates. Despite having a metal content of 1.5 zinc and 0.7 iron, Cameron and workers predicted that human GLX 2 contains a dinuclear Zn(II) metal binding site (7). In more recent studies, Zang et al. have shown that *A. thaliana* GLX 2-2 can bind zinc and iron (8). Other members of the metallo-β-lactamase family have been reported to bind iron only, raising questions as to the metal binding properties of the family members (9).

Based on the ability of proteins with the metallo-β-lactamase fold to bind different metals and the demonstrated ability of *A. thaliana* GLX 2-2 to bind both zinc and iron, several
spectroscopic techniques were employed in order to determine which metals bind to the active site of the recombinant enzyme. Recombinant *A. thaliana* GLX 2-2 was over-expressed in *E. coli* in order to obtain large amounts of protein. The purified enzyme was characterized using kinetic studies, metal analyses, and EPR, circular dichroism (CD), EXAFS, and fluorescence spectroscopies. Kinetic studies were used to probe whether proteins containing different metal contents exhibit different catalytic activities. Metal analyses were performed in order to determine the identity and content of the metal ions that are bound to the proteins. To probe the structure of the dinuclear metal containing active site in GLX 2-2, EPR and EXAFS spectroscopies were used. Minimal media samples were made in order to test how catalytic activity, type and enrichment of metals bound, and active site structure changed in relation to GLX 2-2 samples made in rich media. Metal competition studies in minimal media were performed in order to determine the ratio of zinc, manganese, and iron that are preferentially bound when all the metals have equal bioavailability. CD spectroscopy was employed to determine how the secondary structure of the enzyme changed when different metals were bound into the enzyme’s active site. Finally, fluorescence spectroscopy was utilized to determine whether a change in the protein structure occurred when different metals were bound.

2.2 Materials and Methods

2.2.1 Materials

S-δ-lactoylglutathione (SLG) was purchased from Sigma. All chromatographic steps were conducted on a Pharmacia FPLC operating at 4 °C. Metal standards were purchased from Fisher and were diluted with Nanopure purified H$_2$O. All other chemicals used in these studies were purchased commercially and were of highest quality available. The tryptone and yeast extract used in making ZY media for Research Organics GLX 2-2 were purchased from Research Organics. Components in the ZY media used for all other experiments were purchased from Fisher Scientific.
2.2.2 Methods for Rich Media Preparations

2.2.2.1 Over-expression and purification

The over-expression plasmid, pT7-7/GLX 2-2, was transformed into BL21(DE3) pLysS E. coli cells as described previously (2). Cultures for no metals added (nma) GLX 2-2 were grown at 30 °C in ZY media containing 150 µg/mL ampicillin and 8 mL sterile glycerol/ L of media. ZY media was made of 20 g tryptone, 15 g yeast extract, and 5 g of sodium chloride per liter of media. The ZY media had a pH of 6.83 and was used as the growth media in all of the rich media experiments. Metal-added enzymes were prepared similarly as the nma enzyme except that the ZY media also contained 250 µM ZnCl₂, MnCl₂, or [Fe(NH₄)₂](SO₄)₂·6H₂O. The cultures were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and harvested as previously published except induction occurred at an A₆₀₀nm range of 0.5 to 0.8 and harvested after 30 hours (8). Harvested cell pellets were stored at –80 °C until further steps. Cell pellets were resuspended in 10 mL of 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS), pH 7.2, and the cells were lysed by passage through a French press at 16,000 psi. The lysed cell sample was then centrifuged for 45 minutes at 12,500 rpm, and the resulting supernatant was purified using fast protein liquid chromatography (FPLC) with a Q-Sepharose column as described previously (2). Kinetic data and metal content were obtained and averaged from at least three preparations of each enzyme, except for the nma sample over-expressed in Research Organics media GLX 2-2 (RO GLX 2-2) and the samples over-expressed at different pH’s. Nma GLX 2-2 over-expressed in buffered media was prepared as described above except that the ZY growth media also contained 150 mM MOPS, and the pH of the media was adjusted to 6.5 or 7.5 with NaOH and HCl.

2.2.2.2 PIXE sample preparation

All GLX 2-2 samples were purified as described previously (2). The samples were dialyzed against 2 X 1 L of 10 mM tris(hydroxymethyl)aminomethane (TRIS), pH 7.2, for eight hours and the buffer was exchanged every 4 hours. The pH of the TRIS buffer was adjusted
using acetic acid and sodium hydroxide to avoid introducing chloride into the samples. Samples were sent to Dr. Wolfram Meyer-Klaucke in Hamburg, Germany for PIXE studies.

2.2.2.3 Steady-state kinetics

The steady-state kinetic parameters of purified nma and metal-added GLX 2-2 were determined by measuring the hydrolysis rate of S-D-lactoylglutathione (SLG) at 240 nm in 10 mM MOPS buffer, pH 7.2, at 25 °C with a HP8453 diode array UV-Vis spectrophotometer, as previously described (2). Substrate concentrations ranged between 50 and 600 µM. Higher substrate concentrations were not used due to an observed substrate inhibition.

2.2.2.4 Metal analysis

The metal content of purified nma and metal-added GLX 2-2 was measured with a Varian inductively coupled plasma spectrometer with atomic emission spectroscopy (ICP-AES) detection as previously described (2). Purified enzyme was diluted with 10 mM MOPS, pH 7.2, to a concentration of 10 µM and analyzed for zinc, manganese, iron, and copper.

2.2.2.5 EPR spectroscopy

EPR spectra were obtained using a Bruker Instruments ER 300 spectrometer equipped with ER 035M NMR gaussmeter and a Hewlett Packard 5352B microwave frequency counter. EPR spectra were double integrated with the ESP 300 software package from Bruker Instruments. As isolated, air-oxidized nma or metal-added GLX 2-2 samples were pipetted into 4 mm o.d. quartz EPR tubes and frozen by slow immersion of the EPR tubes into liquid nitrogen.

2.2.2.6 EXAFS sample preparation

All EXAFS samples were prepared by flash freezing the GLX 2-2 samples in liquid nitrogen and then lyophilizing the samples overnight. All GLX 2-2 samples were lyophilized
within 3 days of purification except for the samples over-expressed in buffered media, which were 7 days old. The lyophilized samples were sent to Dr. Wolfram Meyer-Klaucke in Hamburg, Germany for the EXAFS studies.

### 2.2.3 Methods for Minimal Media Preparations

#### 2.2.3.1 Dehua Pei minimal media preparations

All glassware was acid washed with concentrated HNO₃ and rinsed four times with Nanopure H₂O. Dehua Pei minimal media was prepared as described previously (10). Dehua Pei minimal media had a starting pH of 6.86 and was buffered by phosphate. Each liter of media contained 2.5 g D-(+)-glucose, 5 g casamino acids, 10.8 g K₂HPO₄, 5.5 g KH₂PO₄, 10 g NaCl, 1 g (NH₄)₂SO₄, 2 mg thiamine, and 1 mg (+)-biotin. All media was poured directly into acid-washed flasks or culture tubes and then autoclaved. In addition, a minimal metal mix, containing 124 mg MgSO₄·7H₂O, 74 µg CaCl₂·2H₂O, 20 µg MgCl₂·6H₂O, 31 µg H₃BO₃, 1.2 µg (NH₄)₆Mo₇O₄₄·4H₂O, and 1.6 µg CuSO₄ per liter, was added to the media after autoclaving and before addition of the overnight (O/N) culture. The production and purification of nma and metal-added GLX 2-2 samples was identical to the procedure used for nma GLX 2-2 preparation in rich media.

#### 2.2.3.2 Hepes minimal media preparations

All glassware was acid washed with concentrated HNO₃ and rinsed four times with Nanopure H₂O. All media was chelexed and filtered through a 0.22-micron membrane. Dehua Pei minimal media was prepared as described previously (10). Hepes minimal media was prepared as Dehua Pei minimal media except that 11.92 g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/L media replaced K₂HPO₄ and KH₂PO₄ in the Dehua Pei recipe. The pH of Hepes minimal media was adjusted to 6.83 with NaOH and HCl before the media was filtered. All media was poured directly into acid-washed flasks or culture tubes and
then autoclaved. The production of nma and metal-added GLX 2-2 samples was identical to that for nma GLX 2-2 preparations except that precultures were grown in Dehua Pei minimal media, containing 150 µg/mL ampicillin, minimal metal mix in the same proportions as in Dehua Pei minimal media preps, and at 37 °C for 16-17 hours. Cells from the preculture were pelleted by centrifugation and resuspended in 1 mL of Dehua Pei minimal media per 5 mL of preculture. This suspension was transferred to 250 mL of Dehua Pei minimal media containing 150 µg ampicillin/mL and minimal metal mix in the same proportions as the Dehua Pei minimal media procedure. This culture was shaken at 37 °C for 2-3 hours. Cells were then centrifuged at 7,000 g for 10 minutes and resuspended in 10 mL of Heps minimal media. Cultures containing the resuspended cells were grown in Heps minimal media containing 150 µg ampicillin/mL, minimal metal mix in the same proportions as in the Dehua Pei media procedure, and 8 mL sterile glycerol/L media. The culture was shaken at room temperature until reaching an optical density (OD$_{600nm}$) of 0.6-0.8; protein production was induced by making the culture 0.1 mM in IPTG, and the culture was then shaken at 15 °C for 30 hours. Metal-added, minimal media samples of GLX 2-2 were prepared by making the growth cultures 250 µM in Zn(II), Mn(II), or Fe(II).

2.2.3.3 Steady-state kinetics and metal analysis

The same protocol as for the rich media preparations was followed except the data shown are for each preparation.

2.2.3.4 Circular Dichroism (CD) and Fluorescence Spectroscopy

CD samples were prepared from freshly-prepared samples of GLX 2-2, which were dialyzed against a total of 2 X 1 L of 5 mM monobasic phosphate buffer, pH 7.2, for 8 hours (samples placed in fresh phosphate buffer after 4 hours) at 4 °C. The CD samples were made to be as close to 50 µg/ mL as possible. Fluorescence emission spectra of the nma and metal-added GLX 2-2 enzymes were measured as described by Dragani et al. (11) with the instrumentation.
and enzyme concentrations used by Zang et al. (8) except that the emission spectra were taken from only one enzyme preparation.

2.2.3.5 EPR spectroscopy

The same procedures as for the rich media preparations were used to prepare samples and to acquire EPR spectra.

2.2.3.6 EXAFS spectroscopy

The same protocol as for the rich media preparations was followed.

2.3 Results

2.3.1 Rich Media GLX 2-2

2.3.1.1 Over-expression, purification, and characterization of nma and metal-added GLX 2-2 samples

2.3.1.1.1 Over-expression and purification of GLX 2-2

Rich media is a nutrient-rich broth that provides peptides, vitamins, and trace elements. A typical SDS-PAGE gel showing FPLC purification of recombinant GLX 2-2 is shown in Figure 1. Fractions 7-9 (Figure 1) would typically be combined, concentrated, and used in these studies.
Figure 1: SDS-PAGE of a typical column elution profile for recombinant *A. thaliana* GLX 2-2 (from rich media). Lane 1, molecular weight markers; Lane 2, previously purified recombinant GLX 2-2; Lane 3, Q-Sepharose column flow through; Lanes 4-14, column fractions from Q-sepharose (Thick band is at roughly 27 kDa).
All GLX 2-2 preparations produced 20 mg or more of active GLX 2-2 from 1 L of culture except for Mn-added GLX 2-2, which produced roughly half that amount. *E. coli* grown in rich media with or without added metals typically exhibited no differences in the incubation times to reach an OD$_{600nm}$ of 0.6 to 0.8.

2.3.1.1.2 Determining molar absorptivity ($\varepsilon_{280 \text{ nm}}$) of GLX 2-2

Others have used a molar absorptivity of 69,040 M$^{-1}$cm$^{-1}$ to determine the concentration of *A. thaliana* GLX 2-2 samples (2, 8). This number was obtained from an average of sixteen independent amino acid analyses by outside laboratories (Crowder, unpublished). However, the metal ion equivalents calculated when using this molar absorptivity were roughly double what were predicted to be bound to *A. thaliana* GLX 2-2. In order to further evaluate the $\varepsilon$ of GLX 2-2, the method used by Gill and von Hippel for determining $\varepsilon$ was employed (12) and predicted that $\varepsilon_{280\text{nm}} = 37,584$ M$^{-1}$cm$^{-1}$. Particle induced X-ray emission (PIXE) was employed by a collaborating lab in Germany, and this technique determined that the $\varepsilon_{280\text{nm}}$ of *A. thaliana* GLX 2-2 is 34,175 M$^{-1}$ cm$^{-1}$ (Crowder, Makaroff, Meyer-Klaucke, unpublished). The PIXE-determined $\varepsilon$ was determined by quantitating the total sulfur in the GLX 2-2 samples and using this experimentally-determined enzyme concentration with the sample’s $A_{280\text{nm}}$ to calculate $\varepsilon_{280\text{nm}}$. Since the von Hippel calculation is a theoretical model and PIXE is an experimental method to determine $\varepsilon$, $\varepsilon_{280\text{nm}}=34,175$ M$^{-1}$ cm$^{-1}$ was used in this thesis work to determine the concentration of GLX 2-2 samples.

2.3.1.1.3 Steady-State Kinetics

Steady-state kinetic studies were performed on the nma and metal-added GLX 2-2 samples in order to determine the activity of the enzymes and to test the effect of different metal ions on activity. The steady-state kinetic constants obtained for the nma and metal-added GLX 2-2 samples are shown in Table 1. Also shown are comparative values for recombinant human liver GLX 2 (13), native human red blood cell GLX 2 (13, 14), and recombinant *A. thaliana* GLX 2-2 from a past study (8). Due to *A. thaliana* GLX 2-2 having an identical metal binding
site and 55% amino acid sequence identity to cytosolic mammalian GLX 2 (1), comparisons can be made between the enzymes. There is < 2-fold difference in the $K_M$ values for the enzymes. The $k_{cat}$ of GLX 2-2 is also similar to that of human GLX 2, although the Arabidopsis enzyme is slightly more active than the human enzymes when temperature differences are taken into account. The catalytic efficiencies of GLX 2-2 also are comparable when temperature differences are considered. Since a 10 °C increase in temperature corresponds to a doubling in activity, a 12 °C increase should give a 2.4-fold increase in kinetic activity. Therefore at 37 °C, A. thaliana GLX 2-2 theoretically has a catalytic efficiency of $4.2 \times 10^6$ M$^{-1}$ s$^{-1}$, which is of the same order of magnitude as human GLX 2.

Zang et al. previously reported that the $k_{cat}$ for A. thaliana nma GLX 2-2 was 3,930 s$^{-1}$ (8). However, this study used the $\varepsilon$ of 69,040 M$^{-1}$ cm$^{-1}$ to quantitate the enzyme. As shown in Table 2, when the $k_{cat}$ is corrected for the $\varepsilon$ used in this thesis, the reported $k_{cat}$ is still 4-5 fold higher than that of the nma GLX 2-2 presented here. In order to address this difference in enzyme activity, two nma GLX 2-2 samples were prepared in a similar manner and from the same media used by Zang et al. (8). The steady-state kinetic constants and metal analyses of these two preparations were averaged and are shown in Table 2 as RO nma. The $K_M$ of RO (Research Organics) nma GLX 2-2 is closest to the values reported for human GLX 2, but they are a factor of ~ 2 higher than those reported by Zang et al. (8). The $k_{cat}$ of RO nma GLX 2-2, however, is most similar to the $k_{cat}$ of nma GLX 2-2. In addition, the catalytic efficiency reported by Zang et al. (8) is an order of magnitude higher than the other catalytic efficiencies reported in Tables 1 and 2. Table 2 also shows steady-state kinetic constants from studies performed on the same Cary UV-VIS spectrophotometer used by Zang et al. (8). As shown by the data for RO nma (CARY), the steady-state kinetic constants were most similar to RO nma GLX 2-2. These results indicate that the $k_{cat}$ reported by Zang et al. is too large for A. thaliana GLX 2-2. Such a large difference in activity may be due to differing metal content of the enzymes or to an error in the calculation of enzyme concentration. In addition, Zang performed all of her kinetic studies right after column chromatography (Makaroff, personal communication). For the studies reported here, steady-state kinetics were not performed the same day as the chromatographic
Table 1: Steady-state kinetic data and metal content for rich media GLX 2-2 samples

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$s$^{-1}$)</th>
<th>Mn$^d$</th>
<th>Fe$^d$</th>
<th>Zn$^d$</th>
<th>Cu$^d$</th>
<th>Total Metal$^d$</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nma$^a$</td>
<td>430±71</td>
<td>245±65</td>
<td>1.76</td>
<td>0.31±0.06</td>
<td>0.88±0.08</td>
<td>0.40±0.15</td>
<td>0</td>
<td>1.60±0.07</td>
<td>25°C</td>
</tr>
<tr>
<td>GLX 2 ref 14$^b$</td>
<td>780</td>
<td>187</td>
<td>4.17</td>
<td>0</td>
<td>0.7</td>
<td>~1.5</td>
<td>0</td>
<td>~2.2</td>
<td>37°C</td>
</tr>
<tr>
<td>GLX 2 ref 14$^c$</td>
<td>755</td>
<td>172</td>
<td>4.39</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>37°C</td>
</tr>
<tr>
<td>GLX 2 ref 15$^c$</td>
<td>727+16</td>
<td>146+9</td>
<td>5.00</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>37°C</td>
</tr>
</tbody>
</table>

$^a$ No-metals added GLX 2-2
$^b$ Recombinant human adult liver GLX 2
$^c$ Native human GLX 2 from red blood cells
$^d$ Metals are shown as equivalents of metal per enzyme
Table 2: Steady-state kinetic data and metal content for rich media GLX 2-2 samples

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$ s$^{-1}$)</th>
<th>$10^6$</th>
<th>Mn$^c$</th>
<th>Fe$^c$</th>
<th>Zn$^c$</th>
<th>Cu$^c$</th>
<th>Total Metal$^c$</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nma</td>
<td>430±71</td>
<td>245±65</td>
<td>1.76</td>
<td>0.31±0.06</td>
<td>0.88±0.08</td>
<td>0.40±0.15</td>
<td>0.00</td>
<td>1.60±0.07</td>
<td>25°C</td>
<td></td>
</tr>
<tr>
<td>GLX 2-2 ref. 8</td>
<td>3,930±138</td>
<td>63±10</td>
<td>62</td>
<td>N/A</td>
<td>1.5</td>
<td>0.8</td>
<td>?</td>
<td>~2.3</td>
<td>25°C</td>
<td></td>
</tr>
<tr>
<td>GLX 2-2 ref. 8 $^a$</td>
<td>1,945±68</td>
<td>63±10</td>
<td>31</td>
<td>N/A</td>
<td>0.7</td>
<td>0.4</td>
<td>?</td>
<td>~1.1</td>
<td>25°C</td>
<td></td>
</tr>
<tr>
<td>R.O. nma $^b$</td>
<td>252±37</td>
<td>171±50</td>
<td>1.47</td>
<td>0.54±0.01</td>
<td>0.48±0.17</td>
<td>0.16±0.02</td>
<td>0.00</td>
<td>1.13±0.10</td>
<td>25°C</td>
<td></td>
</tr>
<tr>
<td>R.O. nma (CARY)</td>
<td>305±21</td>
<td>171±37</td>
<td>1.78</td>
<td>N/A</td>
<td>1.5</td>
<td>0.4</td>
<td>?</td>
<td>~1.1</td>
<td>25°C</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Zang et al. (8) GLX 2-2 data adjusted for different $i$'s
$^b$ Nma GLX 2-2 grown in media from Research Organics (RO)
$^c$ Metals are shown as equivalents of metal per enzyme
step; instead, the steady-state kinetic studies were normally conducted the following day but always within 24 hours of purification. It is possible that any redox-active metal ion bound to GLX 2-2 could oxidize over time and lead to different kinetic activities. Table 3 shows that the metal-added GLX 2-2 preparations exhibited different activities than nma GLX 2-2. The Fe-added GLX 2-2 kinetic constants were, within error, the same as nma GLX 2-2. Zn-added GLX 2-2 exhibited roughly double the $k_{\text{cat}}$ values of the nma and Fe-added preparations. The $K_M$ value for Zn-added GLX 2-2 was slightly lower than those for nma and Fe-added GLX 2-2. Mn-added GLX 2-2 exhibited less than half the $k_{\text{cat}}$ and $K_M$ of nma GLX 2-2. These results indicate that over-expression in media with added metal does affect enzyme activity, although the changes are surprisingly small.

2.3.1.1.4 Metal Analyses

The metal content of each of the different GLX 2-2 preparations was measured using ICP-AES in an effort to determine if a correlation existed between activity and amount or type of metal ions bound. Tables 1-4 show the metal equivalents for each GLX 2-2 preparation, and these data support the hypothesis given by Zang et al. (8) that *A. thaliana* GLX 2-2 has a dinuclear metal active site. Nma GLX 2-2 binds 0.88 moles of iron and roughly equal amounts of manganese and zinc (Table 1). This result contrasts work on human GLX 2 that was reported to bind 1.5 zinc ions, 0.7 iron ions, and no manganese (7). As can be seen in Table 2, the equivalents of iron and zinc for the nma GLX 2-2 from this study and those reported by Zang et al. (8) are very similar when differences in the molar absorptivities used are taken into account. Fe-added GLX 2-2 has virtually the same metal content as nma GLX 2-2, indicating that iron uptake into the enzyme active site is not due to bioavailability (Table 3). Multiple preparations of Fe-added GLX 2-2 were completed; interestingly, the resulting purified enzymes were found to bind widely varying amounts of metals despite using the same growth media and column buffers. Nonetheless, this result allowed us to evaluate the effect of iron content on activity. The amount of iron in the active site of GLX 2-2 does not seem to significantly affect catalytic efficiencies, although there does appear to be some small correlation with higher efficiency and having roughly 0.7 equivalents of iron in the enzyme active site (Figure 2). FPLC fractions with Fe-added GLX 2-2 from rich media came in two pale colors: purple and yellow-brown,
Table 3: Steady-state kinetic data and metal content for rich media GLX 2-2 samples

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$ s$^{-1}$)</th>
<th>Mn$^a$</th>
<th>Fe$^a$</th>
<th>Zn$^a$</th>
<th>Cu$^a$</th>
<th>Total Metal$^a$</th>
<th>Notes</th>
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<td>245±65</td>
<td>1.76</td>
<td>0.31±0.06</td>
<td>0.88±0.08</td>
<td>0.40±0.15</td>
<td>0.00</td>
<td>1.60±0.07</td>
<td>25°C</td>
</tr>
<tr>
<td>Fe-added</td>
<td>328±97</td>
<td>262±102</td>
<td>1.25</td>
<td>0.24±0.08</td>
<td>0.89±0.11</td>
<td>0.33±0.06</td>
<td>0.00</td>
<td>1.47±0.08</td>
<td>25°C</td>
</tr>
<tr>
<td>Zn-added</td>
<td>658±58</td>
<td>188±31</td>
<td>3.50</td>
<td>0.19±0.02</td>
<td>0.65±0.06</td>
<td>0.76±0.04</td>
<td>0.01±0.01</td>
<td>1.61±0.05</td>
<td>25°C</td>
</tr>
<tr>
<td>Mn-added</td>
<td>152±35</td>
<td>115±25</td>
<td>1.32</td>
<td>0.95±0.16</td>
<td>0.35±0.09</td>
<td>0.04±0.03</td>
<td>0.00</td>
<td>1.29±0.14</td>
<td>25°C</td>
</tr>
</tbody>
</table>

$^a$ Metals are shown as equivalents of metal per enzyme
suggested different iron oxidation states. The purple FPLC fraction is indicative of Fe(II) and became yellow-brown in color after 24 hours, probably due to air oxidation. This result indicates that a change in the oxidation state of one or more metals in the active site occurred and might provide a reason why the steady-state kinetics between Zang et al. and this thesis are so different.

Zn-added GLX 2-2 is similar to nma and Fe-added GLX 2-2 in that over half an equivalent of iron is bound (Table 3). Unlike nma or Fe-added GLX 2-2 though, the Zn-added enzyme does not have a nearly equal ratio of manganese to zinc. Instead, slightly less manganese was bound while almost a full equivalent of zinc was bound. This result suggests that zinc uptake is dependent on bioavailability. As observed with the Fe-added and nma GLX 2-2 samples, multiple preparations of nma and Zn-added GLX 2-2 resulted in samples that contained 0.0 to ~1.0 equivalents of Zn(II). A plot of $k_{\text{cat}}/K_M$ vs. zinc equivalents showed little dependence of catalytic efficiency on zinc content at low equivalents of zinc; however, a 4-5 fold increase in $k_{\text{cat}}/K_M$ was observed in samples with ~1.0 equivalents of zinc as compared to those with no zinc (Figure 3). Unlike nma and Fe-added GLX 2-2, Zn-added GLX 2-2 FPLC fractions are colorless. Like the other GLX 2-2 preparations, Zn-added GLX 2-2 eluted from the Q-Sepharose column at about 20% salt. Results for Mn-added GLX 2-2 also indicate manganese uptake is dependent on bioavailability; this enzyme bound 0.95 equivalents of manganese, 0.35 of iron, and almost no zinc (Table 3). Interestingly, Mn-added GLX 2-2 exhibits a lower $k_{\text{cat}}$ and $K_M$ than all of the other GLX 2-2 samples (Table 3). It is unclear whether the decrease in $k_{\text{cat}}$ is due to higher manganese or to a lack of zinc in the active site, but the enzyme efficiency ($k_{\text{cat}}/K_M$) is not significantly different from nma GLX 2-2. Similar to other metal-added GLX 2-2 samples, multiple preparations of Mn-added GLX 2-2 resulted in enzymes with varied amounts of manganese (Figure 4). Although there is considerable error in the $k_{\text{cat}}/K_M$ values for samples with identical manganese content (as shown by the error bars), there does appear to be a correlation between $k_{\text{cat}}/K_M$ and manganese content. FPLC fractions of Mn-added GLX 2-2 eluted from the Q-Sepharose column at 20% salt and were a transparent, pale yellow-brown color that remained even after several days outside of a glovebox. To study further why *A. thaliana* GLX 2-2 from Zang et al. (8) had such a high activity (Table 2), the RO nma GLX 2-2 samples were prepared with the same media (Research Organics) as used by Zang et al. The resulting enzyme contained about twice as much manganese and one half as much zinc as nma GLX 2-2 grown in media procured from Gibco BRL (Table 2). Based on the trends seen in Figures 3 and 4 regarding the
Figure 2: Plot of $k_{cat}/K_M$ vs iron equivalents. This figure was rendered by plotting the $k_{cat}/K_M$ versus iron content data from multiple preps of nma and metal-added GLX 2-2. The steady-state kinetic studies to determine $k_{cat}/K_M$ were conducted as described in Materials and Methods. Data points with error bars reflect the error in $k_{cat}/K_M$ from multiple samples with the same metal content.
**Figure 3**: Plot of $k_{\text{cat}}/K_M$ vs zinc equivalents. This figure was rendered by plotting the $k_{\text{cat}}/K_M$ versus zinc content data from multiple preps of nma and metal-added GLX 2-2. The steady-state kinetic studies to determine $k_{\text{cat}}/K_M$ were conducted as described in Materials and Methods. Data points with error bars reflect the error in $k_{\text{cat}}/K_M$ from multiple samples with the same metal content.
catalytic efficiency of GLX 2-2 versus its manganese and zinc content, RO nma GLX 2-2 over-expressed in media from Research Organics is most similar to Mn-added GLX 2-2. A possible explanation of these data could be that the $k_{\text{cat}}$ values reported by Zang et al. have a factor of 10 error. Correcting for this error would result in similar $k_{\text{cat}}$ values for nma GLX 2-2 prepared in Research Organics media and Mn-added GLX 2-2. Another possible reason for the difference in enzyme activity may be from air oxidation of the metal active site, as stated previously.

Since the metal content of purified GLX 2-2 appeared to be due to the bioavailability of the metal ions in the growth media, nma GLX 2-2 was over-expressed in ZY media buffered at pH 6.5 and 7.5. We reasoned that at pH 7.5, insoluble metal hydroxides would form and reduce the bioavailability of certain metal ions for uptake by GLX 2-2. Initially, 50 mM MOPS was used to control the pH of the media. However after 2.5 hours of *E. coli* cell growth in 250 mL of media, the pH had dropped one half of a pH unit. Use of 100 mM MOPS buffer for 2.5 hours of cell growth in 250 mL of media resulted in a drop of 0.13 pH units. Therefore, the media was buffered with 150 mM MOPS. As shown in Table 4, the sample of nma GLX 2-2 over-expressed at pH 7.5 had almost a full equivalent each of iron and zinc with a lower amount of bound manganese than nma and metal-added GLX 2-2. The metal content and kinetics of the pH 7.5 preparation were most similar to Zn-added GLX 2-2, although the $K_M$ was about double that of the Zn-added GLX 2-2. The pH 7.5 preparation exhibited the same purple and yellow-brown FPLC fractions as Fe-added GLX 2-2. The pH 6.5 preparation bound slightly more manganese, a full equivalent of iron, and a third less zinc than the pH 7.5 preparation. There was a small drop in enzyme activity that may be due to reduced zinc binding, but the enzyme efficiency was still in the same magnitude as for nma and metal-added GLX 2-2 (Table 4). The FPLC fractions of the pH 6.5 preparation were a transparent, yellow-brown color and were most similar to Mn-added GLX2-2.

### 2.3.1.1.5 Electron Paramagnetic Resonance Spectroscopy

EPR spectroscopy yields information about the type and oxidation state of paramagnetic metal ions as well as the presence of spin-coupled dinuclear metal centers (15-19). Given the metal content of GLX 2-2 (Tables 1-4), there were potentially a large number of possible metal centers present in the samples. In this study, EPR spectra were collected at ~5 K and on samples
Figure 4: Plot of $k_{cat}/K_M$ vs manganese equivalents. This figure was rendered by plotting the $k_{cat}/K_M$ versus manganese content data from multiple preps of nma and metal-added GLX 2-2. The steady-state kinetic studies to determine $k_{cat}/K_M$ were conducted as described in Materials and Methods. Data points with error bars reflect the error in $k_{cat}/K_M$ from multiple samples with the same metal content.
with concentrations of ~ 150 µM; we found that these conditions allowed for optimal signal to noise in our studies. The EPR spectra of nma GLX 2-2 showed at least three distinct sets of signals (Figure 5): (I) two rhombic signals with apparent g values of 4.6 and 4.3, (II) a broad signal centered at g = 2.1 that exhibits hyperfine splitting, and (III) a broad, rhombic signal at g < 2 with apparent g values of 1.9, 1.8, and 1.6. Multiple samples of nma GLX 2-2 showed the same features (Figure 5); however, the relative signal intensities were different and most likely due to variations in the metal contents of the enzymes (Table 3). The g = 4 signals in Figure 5 are attributed to isolated, high-spin Fe(III) centers (15). Spin-quantitation versus an Fe(III)-EDTA standard shows that the major component of the g = 4 signals is the g = 4.6 signal. This signal arises from ground state \( m_s = \pm 3/2 \) transitions (20, 21). A very small feature at g = 9.7 is attributed to \( m_s = \pm 1/2 \) transitions, which are only detected at very low temperatures (20, 21). This feature at g = 9.7 is missing at 20 and 30 K (data not shown). These features are characteristic of uncoupled, high-spin Fe(III) centers in rhombically-distorted environments and for Fe(III) in dinuclear Fe(III)Zn(II) centers (20, 21). Spin-quantitation of the g = 4.6 and 9.7 signals identified 0.1 equivalents of iron per protein involved with those signals, and with nma GLX 2-2 binding 1 iron per protein and nearly 2 equivalents of total metal (Figure 3), this result suggests that 10% of the nma GLX 2-2 samples contains a dinuclear Fe(III)Zn(II) center. The g = 4.3 signal is attributed to adventitious Fe(III) with greater rhombic distortion (15) and accounts for only 5% of the total iron present. The signals at g < 2 are attributed to a mixed-valent, antiferromagnetically-coupled Fe(III)Fe(II) center (S=1/2) with rhombic distortion, and are similar to those of the mammalian purple acid phosphatases (21, 22) and ribonucleotide reductase (23). The prediction of an antiferromagnetically-coupled Fe(III)Fe(II) center is supported by EPR spectra of GLX 2-2 with added dithionite and hydrogen peroxide (Figure 6). The reductant and oxidant were expected to reduce Fe(III) in FeZn or FeFe centers or oxidize any Fe(II) in any Fe(III)Fe(II) centers, respectively, resulting in EPR silent centers. The redox behavior of GLX 2-2 is identical to the FeZn center in plant purple acid phosphatases and to the FeFe center in uteroferrin (21, 22). These signals are very temperature dependent and disappear at temperatures higher than 25 K (data not shown). Spin-quantitation against a Cu(II) standard gave 0.15 spins per mole of protein, which indicates that 0.3 equivalents of the iron (31% of the total iron) in the nma GLX 2-2 EPR samples are involved in the Fe(III)Fe(II) metal center. Combining the amounts of iron
Table 4: Steady-state kinetic data and metal content for rich media GLX 2-2 samples

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( k_{\text{cat}} \text{(s}^{-1}\text{)} )</th>
<th>( K_M \text{ (\muM)} )</th>
<th>( k_{\text{cat}}/K_M \text{ (M}^{-1}\text{s}^{-1}) \times 10^6 )</th>
<th>Mn(^a)</th>
<th>Fe(^a)</th>
<th>Zn(^a)</th>
<th>Cu(^a)</th>
<th>Total Metal(^a)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>nma</td>
<td>430±71</td>
<td>245±65</td>
<td>1.76</td>
<td>0.31±0.06</td>
<td>0.88±0.08</td>
<td>0.40±0.15</td>
<td>0.00</td>
<td>1.60±0.07</td>
<td>25°C</td>
</tr>
<tr>
<td>pH 7.5</td>
<td>904±36</td>
<td>361±31</td>
<td>2.50</td>
<td>0.11</td>
<td>0.83</td>
<td>0.78</td>
<td>0.00</td>
<td>1.73</td>
<td>25°C</td>
</tr>
<tr>
<td>prep.</td>
<td>pH 6.5</td>
<td>542±29</td>
<td>337±39</td>
<td>1.61</td>
<td>0.19</td>
<td>0.86</td>
<td>0.54</td>
<td>1.59</td>
<td>25°C</td>
</tr>
</tbody>
</table>

\(^a\) Metals are shown as equivalents of metal per enzyme
Figure 5: X-Band EPR spectra of three nma GLX 2-2 samples at 4.7 K. The concentration of nma GLX 2-2 in these samples was 128 µM. These spectra were taken with the following conditions: 2 mW microwave power, 9.48 GHz microwave frequency, 10 G modulation amplitude, 100 kHz modulation frequency, sweep time of 41.9 s, receiver gain of 1X10^5, 41 ms conversion time, 82 ms time constant, the center field was at 3350 G, and the sweep width was 6400 G.
Figure 6: X-band EPR spectra of nma GLX 2-2 samples at 4.7 K with a: 1 equivalent H\textsubscript{2}O\textsubscript{2}, b: 1 equivalent dithionite, and c: nma GLX 2-2. These spectra were taken with the following conditions: 2 mW microwave power, 9.48 GHz microwave frequency, 10 G modulation amplitude, 100 kHz modulation frequency, sweep time of 41.9 s, receiver gain of 1X10\textsuperscript{5}, 41 ms conversion time, 82 ms time constant, the center field was at 3350 G, and the sweep width was 6400 G.
calculated in the Fe(III)Zn(II) and Fe(III)Fe(II) centers, 41% of the iron in nma GLX 2-2 is EPR active. The rest of the iron may exist as part of S = 0, spin-coupled centers (Fe(III)Fe(III)) or as sites containing ferrous iron. The EPR spectra of nma GLX 2-2 also show a broad signal centered at $g = 2.1$ that is split into at least 6 lines with a hyperfine constant $A = 38$ G. This signal is indicative of $^{55}$Mn(II) ($I = 5/2$). The $g = 2.1$ signal rules out mixed-valent, dinuclear manganese centers, which would give overlapping, multiline signals (24). The observed hyperfine constant is similar to those of Mn(II)Fe(II) or Mn(II)Mn(II) centers, like in the spin-coupled iron-manganese center in $[\text{Fe}^{II}\text{Mn}^{II}\text{BPMP}(\text{O}_2\text{CCH}_2\text{CH}_3)_2](\text{BPh}_3)$ (25) or the ferromagnetically coupled, Mn(II)Mn(II) center in Mn(II)Mn(II) catalase ($S = 5$) (26, 27). The hyperfine constant does not indicate a mononuclear Mn(II) or Mn(II)Zn(II) center, but a much smaller six-line pattern with larger hyperfine splitting ($A \sim 90$ G) is possibly present and buried underneath the major features at $g = 2.1$ and $g < 2$. Therefore, mononuclear Mn(II) or Mn(II)Zn(II) centers can not be confirmed or ruled out. To determine whether the Mn(II) signal of nma GLX 2-2 is due to Fe(II)Mn(II) or Mn(II)Mn(II) centers, the temperature dependence properties of the $g = 2.1$ signal were explored. The EPR signal of $[\text{Fe}^{II}\text{Mn}^{II}\text{BPMP}(\text{O}_2\text{CCH}_2\text{CH}_3)_2](\text{BPh}_3)$ is attributed to transitions in the ground state $M_s = \pm \frac{1}{2}$ spin levels (25) and at 20 K, there was a new resonance at $g = 5.5$, which corresponds to transitions in the excited $M_s = \pm \frac{3}{2}$ spin level. The GLX 2-2 Mn(II) signal showed no new features at 30 K (data not shown), suggesting vastly differing zero-field splittings between the model complex and the Mn(II) in GLX 2-2 (25). The GLX 2-2 samples therefore do not contain Fe(II)Mn(II) centers, and thus the $g = 2.1$ signal is attributed to a Mn(II)Mn(II) center. Support for this comes from comparing Figure 5 with those of Mn(II)Mn(II) catalase (26, 27). The catalase EPR spectrum shows a broad, multiline signal extending over 4000 G with peak separations of 430 and 730 G. One or more of the lines are split into 6-11 lines due to hyperfine coupling (26, 27). Figure 5 also shows a broad, low-intensity signal extending over 4000 G. At 30 K, the hyperfine splitting signal at the $g = 2.1$ signal remains present (data not shown). This center will be discussed in more detail below. As observed with the nma GLX 2-2 spectra (Figure 5), there are differences in the relative intensities of the signals, which is consistent with the different metal contents observed from prep to prep (Table 3).

EPR spectra were also collected on Fe-added, Zn-added, and Mn-added GLX 2-2 samples (Figures 7-9). The spectra for the Fe-added and Zn-added samples are virtually identical.
to those of nma GLX 2-2, except the relative intensities of the signals attributed to iron are larger for the Fe-added samples (Figure 5, Table 3). These spectra indicate that the same metal centers that appear in nma GLX 2-2 also appear in these samples. The EPR spectra for the Mn-added samples reveal the predominance of a different metal center (Figure 8). Those spectra show broad, multiline signals that extend over 4000 G with peak separations of 440 and 750 G. These features are similar to those of Mn(II)Mn(II) catalase (26, 27) and suggest the presence of a predominant, ferromagnetically coupled Mn(II)Mn(II) center in Mn-added GLX 2-2.

2.3.1.1.6 EXAFS Spectroscopy

X-ray absorption spectroscopy (XAS) allows for element specific characterization of metal sites in biological systems and yields information about the types and distances of coordinating ligands as well as the oxidation state of metal centers (28, 29). For multi-nuclear, metal containing proteins, the metal-metal distance can also be determined (30). EXAFS integrates the individual coordination spheres for one element in the case of multiple binding sites. All EXAFS experiments and analyses were performed by Dr. Wolfram Meyer-Klaue and coworkers at the EMBL Outstation in Hamburg, Germany. The position of the absorption edge can help to deduce the oxidation state of the absorbing metal with higher oxidation states absorbing at higher energies. For nma GLX 2-2, Fe-added GLX 2-2, and Mn-added GLX 2-2, the inflection point of the iron absorption edge was determined to be 7123 eV (data not shown), corresponding to a mixture of Fe(II) and Fe(III). The edge position was exclusively compared to iron-protein samples of which the X-ray absorption spectra were recorded at the EMBL beamline D2 to assure identical energy calibration (31, 32). The shape of the absorption edge for manganese is a more distinctive marker for the oxidation state, especially for Mn(II) (33). The typical Mn(II) feature is a narrow but intense absorption maximum directly at the absorption edge. For higher oxidation states, this peak broadens to a great extent and is shifted to higher energies. The absorption edge shape clearly indicates Mn(II) for the nma, Fe-added, and Mn-added GLX 2-2 samples (Figure 10). Also, the manganese absorption edge position of 6547-6548 eV (inflection point method) correlates well with Mn(II) (34). Both EPR and XAS identify a mixture of Fe(II) and Fe(III) and the presence of Mn(II) in all GLX 2-2 samples tested.
Figure 7: X-Band EPR spectra of three Fe-added GLX 2-2 samples at 4.7 K. These spectra were taken with the following conditions: 2 mW microwave power, 9.48 GHz microwave frequency, 10 G modulation amplitude, 100 kHz modulation frequency, sweep time of 41.9 s, receiver gain of 1X10^5, 41 ms conversion time, 82 ms time constant, the center field was at 3350 G, and the sweep width was 6400 G.
Figure 8: X-Band EPR spectra of three Mn-added GLX 2-2 samples at 4.7 K. These spectra were taken with the following conditions: 2 mW microwave power, 9.48 GHz microwave frequency, 10 G modulation amplitude, 100 kHz modulation frequency, sweep time of 41.9 s, receiver gain of $1 \times 10^5$, 41 ms conversion time, 82 ms time constant, the center field was at 3350 G, and the sweep width was 6400 G.
Figure 9: X-Band EPR spectra of three Zn-added GLX 2-2 samples at 4.7 K. These spectra were taken with the following conditions: 2 mW microwave power, 9.48 GHz microwave frequency, 10 G modulation amplitude, 100 kHz modulation frequency, sweep time of 41.9 s, receiver gain of $1 \times 10^5$, 41 ms conversion time, 82 ms time constant, the center field was at 3350 G, and the sweep width was 6400 G.
Figure 10: Normalized iron, manganese and zinc absorption edges. a: nma GLX 2–2; b: Fe-added GLX 2–2; c: Mn-added GLX 2–2; a. u., arbitrary units. The normalized K$_\alpha$–fluorescence is shown. Spectra for component a and b have an offset of +0.6 and +0.3 a.u., respectively.
EXAFS data for \( k_{\text{max}} > 10 \text{ Å}^{-1} \) were obtained at the iron, manganese, and zinc K-edge for nma, Fe-added, and Mn-added GLX 2-2 preparations except for manganese with the nma GLX 2-2 sample and zinc with the Mn-added GLX 2-2 sample. The different iron, manganese, and zinc ratios of the various GLX 2-2 preparations obviously do not alter the specific EXAFS spectra (Figure 11), which indicates that the coordination environment is conserved despite variations in the metal composition. Constrained refinement leads to a collective model of the iron, manganese, and zinc coordination with specific metal-ligand distances (Table 5 and Figure 12). For all three metals, this model consists of \( 2.5 \pm 0.5 \) histidine ligands and \( 2.5 \pm 0.5 \) oxygen ligands as part of water/hydroxyl molecules or carboxylate groups of amino acid residues. Coordination numbers determined by EXAFS analysis typically have an error range of 20% (35), preventing a distinction between the two binding sites that essentially differ by one histidine. For manganese and zinc, the distances to oxygen and nitrogen were collectively refined. The first shell distances are \( 2.19 \pm 0.01 \text{ Å} \) for manganese and \( 1.99 \pm 0.01 \text{ Å} \) for zinc. The first shell distances for iron were refined independently, yielding \( 2.15 \pm 0.01 \text{ Å} \) for nitrogen and \( 1.98 \pm 0.01 \text{ Å} \) for oxygen. All metal-ligand distances fall without exception within the error margins of reference values taken from a comprehensive analysis of high resolution structures found in the Cambridge Structural Database (36) and the Protein Data Bank (37), including the different iron-oxygen and iron-nitrogen distances. The iron, manganese, and zinc EXAFS spectra clearly show a neighboring metal at about 3.2 Å distance (Figure 12). All three element-specific metal-metal distances were refined independently and overlap within their uncertainties (Table 5), yielding an average distance of \( 3.19 \pm 0.06 \text{ Å} \). This is within the range of dinuclear zinc sites with bridging monodentate carboxylate residues (38). EXAFS analysis does not distinguish between iron, manganese, or zinc for the neighboring metal. In addition, the iron coordination number can be deduced from the size of the iron pre-edge peak (39, 40). Its area was determined to be 6.2 units that is typically in the range for 6-fold iron coordination. However, 6-fold coordination results in EXAFS fits with elevated R-factors\(^3\) (data not shown) in comparison to 5-fold coordination. Since EXAFS analysis determines coordination numbers with a 20% accuracy (35), 5- and 6-fold coordination overlap within the error range. The iron, manganese, and zinc coordination models were confirmed by bond valence sum analyses (41) (data not shown).
Table 5: Coordination spheres for iron, manganese, and zinc as derived by EXAFS analysis\textsuperscript{a}.

<table>
<thead>
<tr>
<th></th>
<th>Iron EXAFS</th>
<th>Manganese EXAFS</th>
<th>Zinc EXAFS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermi energy offset (eV)</td>
<td>–1.7 (5)</td>
<td>–6.7 (6)</td>
<td>–2.5 (9)</td>
</tr>
<tr>
<td>R–factor\textsuperscript{3}</td>
<td>27.8 %</td>
<td>23.7 %</td>
<td>36.5 %</td>
</tr>
<tr>
<td>ligand atom</td>
<td>r (Å) σ\textsuperscript{2} (Å\textsuperscript{2})</td>
<td>r (Å) σ\textsuperscript{2} (Å\textsuperscript{2})</td>
<td>r (Å) σ\textsuperscript{2} (Å\textsuperscript{2})</td>
</tr>
<tr>
<td>Imidazole group\textsuperscript{b}, representing histidine residue</td>
<td>(\angle_{\text{(Met–N–C3)}}): 115 (7) °</td>
<td>(\angle_{\text{(Met–N–C3)}}): 130 (9) °</td>
<td>(\angle_{\text{(Met–N–C3)}}): 125 (15) °</td>
</tr>
<tr>
<td>N</td>
<td>2.14 (1) 0.008 (1)</td>
<td>2.26 (1) 0.002 (1)</td>
<td>2.00 (2) 0.010 (3)</td>
</tr>
<tr>
<td>C</td>
<td>3.21       3.18</td>
<td>2.96</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>2.5 (5) 2.98</td>
<td>0.006 (2) 0.004 (4)</td>
<td>3.00        0.009 (6)</td>
</tr>
<tr>
<td>C</td>
<td>4.25       4.33</td>
<td>4.08</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>4.18       4.40</td>
<td>4.13</td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>2.5 (5) 1.96 (1)</td>
<td>0.008 (1) 2.11 (1)</td>
<td>0.004 (2) 1.98 (4)</td>
</tr>
<tr>
<td>Second metal\textsuperscript{c} in dinuclear site</td>
<td>3.15 (5) 0.007 (2)</td>
<td>3.20 (4) 0.006 (3)</td>
<td>3.19 (6) 0.009 (3)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} N is the coordination number, r is the mean interatomic distance, and \(\sigma^2\) the Debye–Waller factor. \(\angle_{\text{(Met–N–C3)}}\) describes the planar angle between the metal, the coordinating nitrogen and the C3 atom of the imidazole group. Numbers in brackets indicate the uncertainty of the last digit. The error range of the coordination number is based on a reference study (35).

\textsuperscript{b} Histidine residues are represented by an imidazole group as provided by EXCURV98 and used as a rigid body for constrained refinement. Only the distance to the ligating nitrogen atom is refined, the uncertainty of the distance is not calculated for outer shell imidazole atoms as indicated by italic letters. The coordination number of outer shell imidazole atoms is the same as for the coordinating first shell nitrogen atom. Debye–Waller factors of outer shell imidazole atoms with similar distances to the metal center are refined collectively. The “Met–N–C3” angles are refined to partially compensate for inaccuracies of the rigid imidazole body.

\textsuperscript{c} The type of the neighboring metal is set to be 1/3 Fe, 1/3 Mn and 1/3 Zn as this EXAFS analysis cannot distinguish between a neighboring iron, manganese or zinc atom. The refined metal–metal distance only marginally depends on the type of the neighboring metal (0.01 – 0.03 Å), this is included in the respective error margin. As a consequence of the correlation between the Debye–Waller parameter and the coordination number, the metal–metal coordination number is 1.0 (2) despite substoichiometric metal occupancy (Table 3).
Figure 11: EXAFS spectra and Fourier transform from GLX 2–2 preparations with different ratios of iron, manganese and zinc.
a: nma GLX 2–2, offset + 5 Å⁻³; b: Fe-added GLX 2–2, no offset; c: Mn-added GLX 2–2, offset – 5 Å⁻³. The metal content of the individual EXAFS samples are: a) 0.5 iron, 0.3 manganese and 0.4 zinc per protein; b) 0.7 iron, 0.3 manganese and 0.2 zinc per protein; c) 0.5 iron, 0.6 manganese and 0.0 zinc per protein (error range: ± 10%). Manganese EXAFS for nma GLX 2–2 is not presented due to a very high noise level; zinc EXAFS for Mn–added GLX 2–2 has not been recorded due to the low zinc content. $\chi(k)$: EXAFS amplitude. Fourier transform was performed with the EXCURV98 software applying the Fermi energy offset values stated in Table 5 and phase shift corrected for the first shell, yielding the reduced distance $r'$. Dotted lines indicate the zero for each Fourier transform.
Figure 12: Model fits of the EXAFS spectra.
Experimental data are represented by thin black lines while thick black lines represent spectra calculated for the metal specific models given in Table 5. Gray lines represent spectra calculated for refined models without a neighboring metal. The experimental spectra shown in Figure 11 are averaged to reach a better signal to noise ratio. $\chi(k)$ is the EXAFS amplitude; $r'$ is the metal-ligand distance corrected for first shell phase shifts; a. u., arbitrary units; FT, Fourier transform amplitude.
Together, EXAFS and bond valence sum analysis as well as comparison with the crystal structure of human GLX 2 (7) suggest an overall 5-6 fold coordination. The EXAFS based model of the *A. thaliana* nma GLX 2-2 dinuclear site is in good agreement with the crystal structure of human GLX 2 (7). In the human GLX 2 crystal structure, one zinc is coordinated by three histidines, one bridging water, and one bridging aspartate. The second zinc is coordinated by two histidines, two aspartates (one is bridging), and one bridging water. In addition to this 5-fold coordination, both zinc ions are ligated by either the substrate analogue $S$-($N$-hydroxy-$N$-bromophenylcarbamoyl)-glutathione or by cacodylate buffer. Neither compound was present in the GLX 2-2 samples studied here, but the sixth binding place is probably occupied by an additional water ligand. The integration of the EXAFS spectra for both binding sites yielded a model with $2.5 \pm 0.5$ histidines and $2.5 \pm 0.5$ oxygen ligands for iron, manganese, and zinc with all metal-ligand bond distances falling within their specific distances. The simultaneous binding of three different metals with their characteristic coordination bond lengths demonstrates that the nma GLX 2-2 active site is structurally very flexible.

### 2.3.2 Minimal Media GLX 2-2

#### 2.3.2.1 Over-expression, purification, and characterization of nma and metal-added GLX 2-2 samples from minimal media

**2.3.2.1.1 Over-expression and purification**

Minimal media is a broth containing only the essential nutrients needed for *E. coli* growth, and the use of this media allows for a more stringent control of the bioavailability of metal ions during over-expression. Over-expression levels of GLX 2-2 samples grown in minimal media were comparable to those grown in rich media (data not shown). In addition, over-expressed proteins from cultures grown in minimal media eluted from a Q-Sepharose column similarly to cultures over-expressed in rich media, suggesting similar structures of the minimal and rich media proteins. The growth times of all *E. coli* cultures grown in minimal media, both Dehua Pei and Hepes minimal medias, were longer by a factor of 1-2 compared to cultures grown in rich media.
2.3.2.1.2 Steady-State Kinetics

The steady-state kinetic constants obtained for nma and metal-added GLX 2-2 samples grown in Dehua Pei minimal media are shown in Table 6. These experiments were performed by Anne Carenbauer and Mary Pam Pfister at Miami University. A comparison of kinetic constants between nma GLX 2-2 preparations from Dehua Pei minimal media (Table 6) and rich media (Table 3) indicate no significant differences. Mn-added GLX 2-2 from Dehua Pei minimal media also does not exhibit a significant difference in activity from Mn-added GLX 2-2 produced from rich medium. However, the \( k_{\text{cat}} \) of Zn-added GLX 2-2 from Dehua Pei minimal media was 3-fold less than that of the comparable rich media sample (Table 3), while the \( K_M \) values for the Zn-added samples were similar. Fe-added GLX 2-2 from Dehua Pei minimal media exhibited a 2.6-fold drop in \( k_{\text{cat}} \) and a 2-fold increase in \( K_M \) as compared to Fe-added GLX 2-2 from rich media. These drops in activity may be due to the decreased equivalents of metal bound (Table 3). The nma and metal-added GLX 2-2 samples in Dehua Pei minimal media exhibited the same general trends as the corresponding samples in rich media, and over-expression in media with added metal does affect enzyme activity although the changes are small and within an order of magnitude. Metal competition studies in Dehua Pei minimal media were undertaken in order to determine which metals recombinant *A. thaliana* GLX 2-2 bound when there was equal bioavailability of manganese, iron, and zinc. Table 7 shows the steady-state kinetic constants and metal content of three preparations of metal competition GLX 2-2 produced from Dehua Pei minimal media. Interestingly, the Dehua Pei minimal media metal competition samples exhibit kinetic constants roughly equivalent to Zn-added GLX2-2 from rich media (Table 3). Unfortunately, all of the experiments with Dehua Pei minimal media produced a white, flaky precipitate whenever manganese, iron, or zinc was added to the media. The precipitate was most likely Zn-, Mn-, and Fe-phosphates, which are rather insoluble with \( K_{sp} \)'s on the order of \( 10^{-22} \) to \( 10^{-37} \) (42). To remedy this problem, the Dehua Pei minimal media was modified to exclude \( K_2\text{HPO}_4 \) and \( \text{KH}_2\text{PO}_4 \) and include 50 mM HEPES as the buffer. The pH of Hepes minimal media was adjusted to 6.83, thereby matching the initial pH of Dehua Pei minimal media.
Table 6: Steady-state kinetic data and metal content for Dehua Pei minimal media GLX 2-2 samples

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$(s$^{-1}$)</th>
<th>$K_M$ (μM)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$s$^{-1}$) *10$^6$</th>
<th>Mn$^a$</th>
<th>Fe$^a$</th>
<th>Zn$^a$</th>
<th>Cu$^a$</th>
<th>Total Metal$^a$</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>nma</td>
<td>428+20</td>
<td>209+24</td>
<td>2.05</td>
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<td>0.50+0.27</td>
<td>0.46+0.27</td>
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<td>1.01</td>
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<tr>
<td>Fe-added</td>
<td>124+19</td>
<td>600+77</td>
<td>0.21</td>
<td>0.03+0.01</td>
<td>0.76+0.27</td>
<td>0.11+0.02</td>
<td>0.00</td>
<td>0.90</td>
<td>25°C</td>
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<tr>
<td>Mn-added</td>
<td>173±45</td>
<td>110±13</td>
<td>1.57</td>
<td>0.86±0.18</td>
<td>0.14±0.05</td>
<td>&lt;0.01</td>
<td>0.00</td>
<td>1.00</td>
<td>25°C</td>
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<tr>
<td>Zn-added</td>
<td>218+127</td>
<td>139+16</td>
<td>1.57</td>
<td>0.03+0.01</td>
<td>0.18+0.09</td>
<td>0.73+0.22</td>
<td>0.00</td>
<td>0.94</td>
<td>25°C</td>
</tr>
</tbody>
</table>

$^a$ Metals are shown as equivalents of metal per enzyme.
Growth media was made 250 μM in the metal of choice.
Table 7: Steady-state kinetic data and metal content for Metal Competition GLX 2-2 samples

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{\text{cat}}/K_M$ (M$^{-1}$·s$^{-1}$) *10$^6$</th>
<th>Mn$^a$</th>
<th>Fe$^a$</th>
<th>Zn$^a$</th>
<th>Cu$^a$</th>
<th>Total Metal$^a$</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dehua Pei media</td>
<td>782±20</td>
<td>187±13</td>
<td>4.18</td>
<td>0.88</td>
<td>0.31</td>
<td>0.47</td>
<td>0.02</td>
<td>1.68</td>
<td>25°C</td>
</tr>
<tr>
<td>Dehua Pei media</td>
<td>646±24</td>
<td>148±20</td>
<td>4.36</td>
<td>0.93</td>
<td>0.27</td>
<td>0.38</td>
<td>0.02</td>
<td>1.60</td>
<td>25°C</td>
</tr>
<tr>
<td>Dehua Pei media</td>
<td>566±26</td>
<td>129±19</td>
<td>4.39</td>
<td>0.93</td>
<td>0.30</td>
<td>0.37</td>
<td>0.00</td>
<td>1.60</td>
<td>25°C</td>
</tr>
<tr>
<td>Hepes minimal media</td>
<td>750±38</td>
<td>214±30</td>
<td>3.50</td>
<td>0.96</td>
<td>0.35</td>
<td>0.58</td>
<td>0.00</td>
<td>1.89</td>
<td>25°C</td>
</tr>
</tbody>
</table>

$^a$ Metals are shown as equivalents of metal per enzyme
$^b$ Growth media was made 250 µM in Mn, Fe, and Zn
Cultures grown in Hepes minimal media grew to a maximum $OD_{600nm} = 0.37$. This bacteriostasis was believed to be due to a lack of phosphorus in the growth media. This problem was solved by growing the preculture for roughly 17 hours in Dehua Pei minimal media to provide phosphorus and then upscaling the culture into Hepes minimal media where zinc, manganese, and iron could be added without precipitation. This modified media was used for all of the subsequent minimal media experiments. Table 7 shows a metal competition GLX 2-2 sample prepared with Hepes minimal media that exhibits similar kinetic constants to three metal competition GLX 2-2 samples prepared in Dehua Pei minimal media. The metal competition GLX 2-2 samples were grown in media that was made 250 $\mu$M in Mn, Fe, and Zn in order to see if any metals were preferentially bound over the other metals. This indicates that the metal precipitation exhibited in Dehua Pei minimal media did not significantly affect the activity or metal uptake of recombinant *A. thaliana* GLX 2-2.

Table 8 shows the kinetic constants for the metal-added GLX 2-2 samples grown in Hepes minimal media. Fe-added GLX 2-2 grown in Hepes minimal media exhibited a $k_{cat}$ that closely resembled the $k_{cat}$ of Fe-added GLX 2-2 grown in Dehua Pei minimal media. However, the abnormally high $K_M$ value of Fe-added GLX 2-2 grown in Dehua Pei media (Table 6) was not exhibited by Fe-added GLX 2-2 grown in Hepes minimal media. Zn-added GLX 2-2 grown in Hepes minimal media exhibited a $>3$-fold increase in $k_{cat}$ as compared to that exhibited by Zn-added GLX 2-2 grown in Dehua Pei minimal media (Table 6), and this sample exhibits steady-state kinetic constants similar to those of Zn-added GLX 2-2 grown in rich media (Table 3). The $K_M$ values of Zn-added GLX 2-2 grown in Hepes minimal media is similar to that of Zn-added GLX 2-2 grown in Dehua Pei minimal media (Table 6) and in rich media (Table 3). The catalytic efficiency of Zn-added GLX 2-2 in Hepes minimal media is roughly 2-fold higher than that of Zn-added GLX 2-2 grown in rich media (Table 3) and was 3-fold higher than that grown in Dehua Pei minimal media alone (Table 6). The catalytic efficiency of Mn-added GLX 2-2 in Hepes minimal media was comparable to those grown in Dehua Pei minimal media (Table 6) and rich media (Table 3). Interestingly, Mn-added GLX 2-2 from Hepes minimal media was more active than Fe-added GLX 2-2. We hypothesized that the relatively low $k_{cat}$ values of Fe-added GLX 2-2 grown in Hepes minimal media were due to the samples being contaminated with other proteins. The contaminating proteins would cause errors in protein quantitation and in lower values for $k_{cat}$. Figure 13 shows the SDS-PAGE gels of a single Fe-added GLX 2-2 sample.
Table 8: Steady-state kinetic data and metal content for Hpes minimal media GLX 2-2 samples

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_M$ (µM)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$ s$^{-1}$) *10$^6$</th>
<th>Mn$^c$</th>
<th>Fe$^c$</th>
<th>Zn$^c$</th>
<th>Cu$^c$</th>
<th>Total Metal$^c$</th>
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<td>Fe-added$^a$</td>
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<td>141±16</td>
<td>0.91</td>
<td>0.05</td>
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<td>0.07</td>
<td>0.00</td>
<td>1.14</td>
<td>25°C</td>
</tr>
<tr>
<td>Fe-added$^b$</td>
<td>99±2</td>
<td>202±12</td>
<td>0.49</td>
<td>0.01</td>
<td>1.10</td>
<td>0.00</td>
<td>0.00</td>
<td>1.11</td>
<td>25°C</td>
</tr>
<tr>
<td>Zn-added</td>
<td>682±23</td>
<td>121±14</td>
<td>5.64</td>
<td>0.22</td>
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<td>1.15</td>
<td>0.00</td>
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</tr>
<tr>
<td>Zn-added</td>
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<td>111±11</td>
<td>7.43</td>
<td>0.00</td>
<td>0.24</td>
<td>1.33</td>
<td>0.00</td>
<td>1.57</td>
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<tr>
<td>Mn-added</td>
<td>210±5</td>
<td>151±12</td>
<td>1.39</td>
<td>1.27</td>
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<td>0.00</td>
<td>1.38</td>
<td>25°C</td>
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<tr>
<td>Mn-added</td>
<td>355±15</td>
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<td>N/A</td>
<td>N/A</td>
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<td>0.60</td>
<td>0.47</td>
<td>0.07</td>
<td>1.22</td>
<td>25°C</td>
</tr>
</tbody>
</table>

$^a$ Purified with a Q-Sepharose FPLC column

$^b$ Sample from $^a$ was further purified with an affinity column containing S-carbobenzoxyglutathione

$^c$ Metals are shown as equivalents of metal per enzyme
purified by FPLC through a Q-Sepharose column and after further purification through a S-carbobenzoxyglutathione affinity column (43). FPLC purification of Fe-added GLX 2-2 through the affinity column removed essentially all of the impurities not removed by Q-Sepharose (Figure 13). The kinetic constants of Fe-added GLX 2-2 did not significantly change after further purification through the affinity column, which indicates that impurities remaining after FPLC purification through a Q-Sepharose column do not significantly affect the kinetic activity of GLX 2-2.

2.3.2.1.3 Metal Analyses

Tables 6-8 show the metal equivalents for the GLX 2-2 samples grown in minimal media. In general, the GLX 2-2 samples grown in minimal media take up slightly less total metal than those samples grown in rich media (Tables 3, 6, and 8). In fact, the samples grown in Dehua Pei minimal media contained only roughly 1 equivalent of total metal (Table 6), and not surprisingly, these samples exhibited lower $k_{\text{cat}}$ values than the comparable samples grown in rich media (Table 3). To test whether the lower total metal content of the GLX 2-2 grown in Dehua Pei minimal media was due to lower bioavailability of metal, GLX 2-2 samples were also grown in Hepes minimal media with added metal (Table 8). The resulting samples contained more total metal than the samples grown in Dehua Pei minimal media, but less than those grown in rich media (Tables 3 and 8). The $k_{\text{cat}}$ values exhibited by the Hepes media samples are similar to those volumesexhibited by the rich media samples. Tables 6 and 8 also show that even though minimal media GLX 2-2 samples did not bind their full complement of metals, these samples had improved enrichment of specific metal ions. For example, Fe-added GLX 2-2 grown in Hepes minimal media (Table 8) was 90% enriched with Fe, compared to 61% enrichment in Fe-added GLX 2-2 grown in rich media (Table 3). Mn-added and Zn-added GLX 2-2 grown in Hepes minimal media samples also exhibited greater enrichment of the metal ion of choice (Table 8). As with rich media GLX 2-2 samples (Table 3), certain trends highlighting the relationship between the types of metals bound and enzyme activity exist. Zn-added GLX 2-2 grown in Hepes minimal media has a much higher $k_{\text{cat}}$ than either Mn-added or Fe-added GLX 2-2 grown in the same media (Table 8). Fe-added GLX 2-2 from Hepes minimal media exhibited a lower activity than Mn-added GLX 2-2 and Zn-added GLX 2-2, although this may be due to a lack of
Figure 13: SDS-PAGE gels of Fe-added GLX 2-2 (Hepes minimal media) purification. Top Gel: Fe-added GLX 2-2 purified by FPLC using a Q-Sepharose column. Lane 1, Q-Sepharose column flow through; Lanes 2-9, Q-Sepharose fraction; Lanes 10-12, boiled cell fraction taken pre-induction; Lane 13, boiled cell fraction taken after E. coli cells were french-pressed; Lane 14, boiled cell fraction taken after centrifugation of sample at 12,500 rpm; Lane 15, molecular weight markers. Bottom Gel: Fractions 4 and 5 of top gel were pooled and run on a S-CBZ glutathione affinity column. Lane 1, molecular weight markers; Lanes 2-11, fractions from affinity column. GLX 2-2 was eluted by gravity; column dimensions were 15 cm X 1.2 cm, 10 mL volume.
any appreciable amount of manganese or zinc in the active site. This hypothesis is supported by metal competition GLX 2-2 in minimal media (Table 7), which binds manganese, iron, and zinc and exhibits kinetic constants most similar to Zn-added GLX 2-2. Table 7 also indicates that manganese is bound in preference to iron and zinc, although recombinant *A. thaliana* GLX 2-2 still binds around one half of an equivalent of iron and zinc each. However, metal analyses on the growth media prior to cell growth indicate that manganese was more bioavailable than zinc or iron by 30 µM. Zinc and iron ions were equally bioavailable. This may have influenced metal uptake by GLX 2-2. The colors of the FPLC fractions of each of the metal-added GLX 2-2 samples grown in Hepes minimal media were the same as for their counterparts in rich media. For example, purple fractions of Fe-added GLX 2-2 grown in Hepes minimal media eluted right before yellow-brown colored fraction(s), and all GLX 2-2 samples grown from Hepes minimal media were eluted at roughly 20-25% salt. Figure 14 shows a UV-Vis spectra of a purple Fe-added GLX 2-2 FPLC fraction within three hours of elution. The peak (\( \varepsilon_{566nm} = 369 \text{ M}^{-1} \text{ cm}^{-1} \)) may be due to a d-d transition of an Fe\(^{2+} \) ion, which is supported by the fact that within 24 hours the purple Fe-added GLX 2-2 oxidizes to become a yellow-brown color.

Since the metal competition GLX 2-2 samples bound close to two equivalents of total metal when grown in minimal media containing 750 µM total metal (Table 7), the possibility was considered that the bioavailability of these metals was too low for GLX 2-2 to bind its full complement of metals when grown in the presence of 250 µM metal. In order to address this issue, GLX 2-2 samples were grown in Hepes minimal media containing 750 µM manganese, iron, or zinc. Cell growth was 50%-75% of the yield of cell growth in Hepes minimal media made 250 µM in the metal ion of choice. Table 9 shows the metal equivalents bound by GLX 2-2 in high metal bioavailability conditions and shows that a total of only 1.3 equivalents of manganese, 0.7 equivalents of iron, or 1.2 equivalents of zinc were bound to GLX 2-2 after purification. This result suggests that both binding sites in GLX 2-2 can bind manganese or zinc but not iron. This result was surprising because EPR studies demonstrated the presence of an Fe(III)Fe(II) center. The fact that none of these samples bound 2 equivalents of total metal suggests that the bioavailability of metal may still not be high enough at 750 µM metal for full enrichment. However, since cell growth was inhibited, the cells may have been unhealthy and incorporated less metal. It is difficult to reconcile these results from the metal competition studies.
Figure 14: UV-VIS spectrum of 54.2 µM purple Fe-added GLX 2-2, from Hepes minimal media.
2.3.2.1.4 Electron Paramagnetic Resonance Spectroscopy

To determine which metal centers were present in the minimal media GLX 2-2 samples, EPR spectra were collected on all of the samples. In order to address whether the Dehua Pei minimal media GLX 2-2 samples (Table 6), which contained roughly one equivalent of metal, were made up of mononuclear enzymes, dinuclear enzymes, or mixtures of both, EPR spectra were collected from nma GLX 2-2, Mn-added GLX 2-2, Fe-added GLX 2-2, and Zn-added GLX 2-2 (Figure 15). The EPR spectrum of the minimal media GLX 2-2 with no added metal ions at 4.7 K shows three distinct sets of signals: (a) two rhombic signals with g values of 4.6 and 4.3, (b) a broad signal centered at g = 2.1 exhibiting hyperfine splitting, and (c) a broad, rhombic signal at g < 2, with apparent g values of 1.9, 1.8, and 1.6 (Figure 15B). As previously interpreted in Figures 5-9, this EPR spectrum is consistent with this sample having numerous possible metal centers, including an antiferromagnetically-coupled Fe(III)Fe(II) center (g < 2 signals), a mononuclear Mn(II) or Mn(II)Zn(II) center (multi-line g = 2.1 signal), a Fe(III)Zn(II) center, and several other metal centers that are EPR silent. The EPR spectrum of minimal media GLX 2-2 over-expressed with added Mn(II) shows a broad, multi-line signal, extending over 4000 G with peak separations of 440 and 750 G, and a weaker signal centered near g = 2.1 that is split into at least 6 lines with a hyperfine constant of A ≈ 38 G (Figure 15A). As argued previously for EPR spectra of GLX 2-2 over-expressed in rich media, this EPR spectrum for minimal media GLX 2-2 arises predominantly from a ferromagnetically-coupled Mn(II)Mn(II) center. The minimal media GLX 2-2 sample over-expressed in the presence of added iron yielded an EPR spectrum with new g ≈ 4 signals and a different g < 2 signal (Figure 15D). The latter signal is similar to that of high-pH bovine spleen purple acid phosphatase (22). The EPR spectrum of minimal GLX 2-2 over-expressed in the presence of Zn (II) shows a weak g < 2 signal and a relatively more intense g = 4.3 signal. The latter signal is similar to those of FeZn metalloenzymes such as calcineurin and plant purple acid phosphatases (20, 44). The EPR data indicate that spin-coupled dinuclear metal centers are present in the samples over-expressed in Dehua Pei minimal media, even though the samples contain only one equivalent of total metal (Table 6).
Table 9: Metal content for High Metal Bioavailability
Hepes minimal media GLX 2-2 samples

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mn$^b$</th>
<th>Fe$^b$</th>
<th>Zn$^b$</th>
<th>Cu$^b$</th>
<th>Total Metal$^b$</th>
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<tr>
<td>Zn$^a$</td>
<td>0.00</td>
<td>0.12</td>
<td>1.25</td>
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<td>Fe$^a$</td>
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<td>0.00</td>
<td>0.79</td>
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<tr>
<td>Mn$^a$</td>
<td>1.34</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>1.34</td>
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</table>

$^a$ Growth media was made 750 μM with the metal ion of choice
$^b$ Metals are shown as equivalents of metal per enzyme
Figure 15: X-Band EPR spectra of minimal media GLX 2-2 samples at 4.7 K: A. Mn-added GLX 2-2, B. nma-added GLX 2-2, C. Zn-added GLX 2-2, and D. Fe-added GLX 2-2. These spectra were taken with the following conditions: 2 mW microwave power, 9.48 GHz microwave frequency, 10 G modulation amplitude, 100 kHz modulation frequency, sweep time of 41.9 s, receiver gain of 1X10^5, 41 ms conversion time, 82 ms time constant, the center field was at 3350 G, and the sweep width was 6400 G.
The EPR spectra from Hepes minimal media (Figures 16-19) also indicate the presence of numerous possible metal centers. The Mn-added GLX 2-2 EPR data (Figure 16) shows similar features to Mn-added GLX 2-2 from Dehua Pei minimal media (Figure 15). One difference, however, is the increased size of the feature at \( g = 1.8 \). Metal analyses indicate this peak is due to a roughly \( \frac{1}{2} \) equivalent increase in manganese in the Hepes minimal media sample (Table 8), since the amounts of iron, zinc, and copper are nearly identical to the Dehua Pei minimal media EPR samples (Table 6). In addition, the EPR spectra for the high manganese bioavailability study (Figure 16C) indicates no change between GLX 2-2 grown in the presence of 250 \( \mu \text{M} \) Mn (Figure 16 A, B) and 750 \( \mu \text{M} \) Mn. Since \( E. \ coli \) cells grown in the presence of 750 \( \mu \text{M} \) metal had much lower yields of recombinant GLX 2-2 than with 250 \( \mu \text{M} \) metal, it is unlikely that not enough metal was available to completely fill the GLX 2-2 active site. Possibly, the \( \varepsilon \) of GLX 2-2 may be low, which would account for less than two equivalents of metal appearing to be bound. The EPR spectra for Fe-added GLX 2-2 produced in Hepes minimal media (Figure 17 B, C) also shows similar features to the Dehua Pei minimal media samples (Figure 15). The EPR spectrum of high iron bioavailability GLX 2-2 (Figure 17A) exhibits similar features to Fe-added GLX 2-2 grown from Hepes and Dehua Pei minimal medias. This supports the conclusion made with the EPR spectra of high manganese bioavailability GLX 2-2 that no observable change occurs when GLX 2-2 is grown in the presence of 250 \( \mu \text{M} \) metal or 750 \( \mu \text{M} \) metal. The EPR spectra for Zn-added GLX 2-2 grown in Hepes minimal media (Figure 18) look most similar to nma GLX 2-2 in rich media (Figure 5). The EPR spectra (Figure 19) for the metal competition studies exhibit features similar to that of Mn-added GLX 2-2 from minimal media. As with Fe-added GLX 2-2 grown in rich media, the small features at \( g = 4.6 \) are attributed to isolated, high spin Fe(III) centers. The \( g = 2.1 \) feature is attributed to a ferromagnetically coupled Mn(II)Mn(II) center as for the Mn-added GLX 2-2 EPR spectra from rich media (Figure 8). The Hepes minimal media EPR samples, along with the samples from Dehua Pei minimal media, do indicate the presence of spin-coupled metal centers in GLX 2-2.
Figure 16: X-Band EPR spectra of Mn-added GLX 2-2 from Hepes minimal media at 4.7 K. A. Mn-added GLX 2-2, B. Mn-added GLX 2-2, C. Mn-added GLX 2-2 grown in presence of 750 µM Mn. These spectra were taken with the following conditions: 2 mW microwave power, 9.48 GHz microwave frequency, 10 G modulation amplitude, 100 kHz modulation frequency, sweep time of 41.9 s, receiver gain of $1 \times 10^4$, 41 ms conversion time, 82 ms time constant, the center field was at 3350 G, and the sweep width was 6400 G.
Figure 17: X-Band EPR spectra of Fe-added GLX 2-2 from Hepes minimal media at 12 K. A. Fe-added GLX 2-2 (High metal bioavailability study), B. Fe-added GLX 2-2, C. Fe-added GLX 2-2 (2 mW microwave power). These spectra were taken at the following conditions: 1 mW microwave power, 9.48 GHz microwave frequency, 10 G modulation amplitude, 100 kHz modulation frequency, sweep time of 41.9 s, receiver gain of $1 \times 10^4$, 41 ms conversion time, 82 ms time constant, center field at 3350 G, and sweep width of 6400 G.
Figure 18: X-Band EPR spectra of Zn-added GLX 2-2 from Hepes minimal media at 4.7 K. The spectra are separated to put them on the same scale as other EPR spectra. These spectra were taken with the following conditions: 2 mW microwave power, 9.48 GHz microwave frequency, 10 G modulation frequency, sweep time of 41.9 s, receiver gain of 1X10^4, 41 ms conversion time, 82 ms time constant, center field of 3350 G, and sweep width of 6400 G.
Figure 19: X-Band EPR spectra of metal competition GLX 2-2 at 4.7 K. A. Metal Competition GLX 2-2 grown from Dehua Pei minimal media, B. Metal Competition GLX 2-2 grown from Hepes minimal media. These spectra were taken at the following conditions: 2 mW microwave power, 9.48 GHz microwave frequency, 10 G modulation amplitude, 100 kHz modulation frequency, sweep time of 41.9 s, receiver gain at $1 \times 10^4$, 41 ms conversion time, 82 ms time constant, center field was at 3350 G, and sweep width of 6400 G.
2.3.2.1.5 EXAFS Spectroscopy

As discussed in the section on rich media GLX 2-2 samples, X-ray absorption spectroscopy (XAS) has been applied to characterize the coordination of manganese, iron, and zinc in GLX 2-2. All EXAFS experiments were run by Dr. Wolfram Meyer-Klauke and coworkers at the EMBL Outstation in Hamburg, Germany. EXAFS spectra were collected on GLX 2-2 samples prepared in Dehua Pei minimal media by Anne Carenbauer and coworkers. The zinc K-edge EXAFS spectra of GLX 2-2 over-expressed in Dehua Pei minimal media with added iron and added zinc are very similar (Figure 20) to those grown in rich media (Figure 10). This indicates conserved zinc coordination despite significant variations in the zinc content and total metal composition. EXAFS analysis yielded a model consisting of $2.5 \pm 0.5$ histidines and $2.5 \pm 0.5$ oxygen ligands at $1.99 \pm 0.01$ Å (Table 10, Figure 21), being in very good agreement with typical zinc-ligand distances derived from small molecule crystallography and high-resolution protein crystallography (36, 37). While this model theoretically includes 4-6 ligands, comparison with the crystal structure of human GLX 2 (5) excludes 4-fold coordination, as shown with EXAFS studies on rich media GLX 2-2 samples. Also as shown with EXAFS studies on rich media GLX 2-2 samples, five- and six-fold coordination with $2.5 \pm 0.5$ histidines and $2.5 \pm 0.5$ oxygen ligands is consistent with the zinc coordination found in the crystal structure of human GLX 2 (7). The GLX 2-2 samples over-expressed in Dehua Pei minimal media with added Fe and added Zn show evidence for a second metal atom at $3.14-3.19$ Å (Table 10, Figure 20). The presence of a neighboring metal atom is supported by the similarity to EXAFS spectra from rich media GLX 2-2 samples. Therefore, despite an average content of about one metal per protein (Table 6), zinc is present in dinuclear sites that are fully loaded with two metals. Both the X-ray absorption near edge structure (XANES) and the EXAFS show some differences between GLX 2-2 with high and low iron content (Figures 20, 21). The double peak feature at $k = 4$ Å$^{-1}$ is more prominent for GLX 2-2 with 0.2 iron equivalents (Figure 21, arrow). This feature arises from multiple scattering of amino acid ligands, mainly histidine residues. The two metal binding sites of GLX 2 essentially differ by one histidine residue (7, 2, 8). EXAFS analysis, with an error range of 20% for coordination numbers (35), can not distinguish between the two sites. However, the higher intensity of the double peak feature leads to the assumption that iron is mainly located at the three-histidine site in the case of GLX 2-2 with low iron content. This
Figure 20: Normalized iron- and zinc-$K$-absorption edges of GLX 2-2. Black lines: Fe-added GLX 2-2; gray lines: Zn-added GLX 2-2. The inset represents the iron pre-edge peak.; a.u.: arbitrary units
Figure 21: Iron- and zinc-$K$-edge EXAFS of GLX 2-2. Samples are Fe-added GLX 2-2 (a: black lines), Zn-added GLX 2-2 (b: gray lines), and GLX 2-2 from a previous study (c: thin lines) (Schilling et al., in press). The arrow indicates the double-peak at $k = 4 \text{ Å}^{-1}$ for the iron $K$-edge EXAFS.
**Table 10: EXAFS model fits:**

N is the coordination number, r is the mean interatomic distance and \(2\sigma^2\) the Debye–Waller factor. Number in brackets indicates the uncertainty of the last digit. The error range of the coordination number is based on a reference study (35). Fermi energy offset values are \(0.7 \pm 0.5\) eV for the iron– and \(-2.0 \pm 0.5\) eV for the zinc– spectrum of the sample with 0.2 iron and 0.9 zinc per protein; \(1.3 \pm 0.4\) eV for the iron– and \(-2.4 \pm 0.5\) eV for the zinc– spectrum of the sample with 0.9 iron and 0.2 zinc per protein. b Histidine residues are represented by an imidazole as provided by EXCURV98 and used for rigid body refinement. Only the distance to the ligating nitrogen atom is refined. Accordingly, the uncertainty of the distance is not calculated for outer shell imidazole atoms. b For the zinc spectra, the distances of the first shell ligands and Debye–Waller factors of atoms with similar distances to the metal center are refined collectively. This is indicated by equal subscripts (1, 2, 3). In the case of iron the distances of the first shell ligands nitrogen / imidazole and oxygen have to be refined independently. c The type of the neighboring metal is set to zinc for samples with higher zinc content and to iron in the case of higher iron content.

assumption is supported by the low intensity of the g < 2 signals in the GLX 2-2 samples over-

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<td>Zinc K–edge</td>
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<td></td>
<td>25.8%</td>
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<td></td>
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expressed with added zinc (Figure 15C). A high degree of similarity is obvious for EXAFS spectra from GLX 2-2 with 0.84 iron equivalents and EXAFS spectra for GLX 2-2 loaded with iron, manganese, and zinc in the rich media section. EXAFS data for low iron GLX 2-2 were fitted with $3.0 \pm 0.5$ histidine ligands at $2.16 \pm 0.01$ Å and $2.0 \pm 0.5$ oxygen ligands at $2.00 \pm 0.01$ Å; data from GLX 2-2 with elevated iron content were fitted with $2.5 \pm 0.5$ histidine ligands at $2.17 \pm 0.01$ Å and $2.5 \pm 0.5$ oxygen ligands at $1.99 \pm 0.01$ Å (Table 10, Figure 22). All distances match reference values from small molecule crystallography and high-resolution protein crystallography (36, 37). The size of the iron pre-edge peak (Figure 20, inset) is a measure for the coordination number. Its area was determined to be 11.3 units for low-iron, Zn-added GLX 2-2, indicative of 5-fold coordination; and 6.5 units for high-iron, Fe-added GLX 2-2, indicative of 6-fold coordination. Due to its error range of 20% (35), the EXAFS model does not distinguish 5- and 6-fold coordination. Both coordination numbers are within the error range of the model, the difference most likely arises from an additional water ligand. Both iron EXAFS spectra clearly indicate the presence of a neighboring metal atom at 3.13-3.18 Å (Table 10, Figure 22), therefore confirming the above findings for zinc. Hence, the majority of both iron and zinc is located in fully occupied dinuclear active sites. At an average content of about one metal per protein (Table 6), this implies the simultaneous presence of fully loaded and metal-free GLX 2-2. The iron K-edge EXAFS for GLX 2-2 with an average content of 0.84 iron and 0.12 zinc per protein unambiguously identifies a neighboring metal atom. With only 0.12 zinc equivalents, this signal must be due at least in part from the presence of two iron ions in both binding sites. However, for GLX 2-2 with low iron content, it appears that most of the iron is located in the three histidine site. Decreased intensity of the double peak feature at $k = 4$ Å$^{-1}$ supports the distribution of iron between the two binding sites; even at a total iron content of about one equivalent per protein, which theoretically prevents the exclusive binding of iron to one site. The presence of zinc in di-metal centers was equally demonstrated. For GLX 2-2 with an average content of 0.2 iron and 0.8 zinc per protein, this indicates the occurrence of di-zinc centers and hence the distribution of zinc between the two binding sites. This is also assumed for low zinc GLX 2-2 as all GLX 2-2 samples have virtually identical EXAFS spectra.
Figure 22: Model fits of GLX 2-2 EXAFS spectra. Experimental data are represented by thin, black lines, while thick, gray lines represent spectra calculated for the models stated in Table 10. $\chi(k)$: EXAFS amplitude; $r'$: metal-ligand distance corrected for first shell phase shifts; a.u.: arbitrary units; FT: Fourier transform amplitude.
2.3.2.1.6 Circular Dichroism

CD spectroscopy was employed to determine how the secondary structure of the enzyme was changed when various metals were bound at the enzyme’s active site. The CD spectra shown in Figure 23 show that Mn-added and Zn-added GLX 2-2 have very similar secondary structures. Fe-added GLX 2-2 exhibited a significant difference in ellipticity, which may be due to either a difference in secondary structure or from a difference in the concentration of sample used for Fe-added GLX 2-2 versus the Zn-added and Mn-added GLX 2-2 samples. Fe-added GLX 2-2 samples were not examined by PIXE, so a concentration error due to a difference in \( \varepsilon \) cannot be ruled out. The ratio of molar ellipticity of 205 nm to 222 nm has been used previously to estimate the helical content of CD samples by Vijayakumar, et al. (45). This ratio was calculated for each of the metal-added GLX 2-2 CD samples and then compared to each other. The ratio comparison determined that the helical content of the metal-added GLX 2-2 samples were within 5% of each other, and therefore that the difference in CD spectral intensity was not due to differences in helical content.

2.3.2.1.7 Fluorescence Spectroscopy

Fluorescence spectroscopy was also utilized to determine whether a change in the protein structure occurs when different metals are bound. The fluorescence spectra shown in Figure 24 showed that the fluorescence of Zn-added and Mn-added GLX 2-2 samples were virtually identical. The fluorescence spectroscopy data (Figure 24), along with the CD spectroscopy experiments (Figure 23), suggest that the GLX 2-2 protein structure is unchanged when mainly zinc or manganese is bound. However, the fluorescence data also showed that Fe-added GLX 2-2 exhibited about half the fluorescence intensity shown by Zn-added and Mn-added GLX 2-2. These data agree with the CD spectroscopy experiments that suggested that a concentration difference exists between Fe-added GLX 2-2 and Zn-added or Mn-added GLX 2-2. Zang, et al. measured the fluorescence intensity of wt GLX 2-2 (nma GLX 2-2 in this thesis) (8) which, when corrected for the \( \varepsilon \) difference, was very similar in magnitude and shape to the Zn-added and Mn-added GLX 2-2 fluorescence spectra in Figure 24. This result, along with kinetic data.
Figure 23: CD spectra of Hepes minimal media GLX 2-2 samples. A. Zn-added GLX 2-2, B. Mn-added GLX 2-2, C. Fe-added GLX 2-2
Figure 24: Fluorescence spectra of Hepes minimal media GLX 2-2 samples. All samples were made to be 6.9 µM and were buffered by 5 mM phosphate. The excitation wavelength was 280 nm. A. Zn-added GLX 2-2, B. Mn-added GLX 2-2, C. Fe-added GLX 2-2
from Table 2, supports the hypothesis that *A. thaliana* GLX 2-2 from Zang et al. contained a significant amount of manganese, since a high amount of iron decreases fluorescence intensity significantly (Figure 24). The fluorescence decrease in Fe-added GLX 2-2 may be due to a quenching of a tryptophan in the active site, thus causing the molar extinction coefficient ($\varepsilon$) and calculated concentration of Fe-added GLX 2-2 to be different from Zn-added and Mn-added GLX 2-2. This result supports the possibility of a sample concentration error causing a difference in the CD spectra shown in Figure 23. In addition, an error in sample concentration may suggest that some of the variance in the $k_{\text{cat}}$ values and metal content in nma and metal-added GLX 2-2 is due to the variable iron content of the samples from preparation to preparation (Tables 3, 6, and 8). Human and *A. thaliana* GLX 2-2 have two conserved tryptophan residues (Figure 2, Chapter 1). Figure 25 shows the positions of Trp 57 and Trp 199 with respect to the metal ions in human GLX 2-2. Although W199 is unlikely to contribute significantly to quenching because of the large distance between the residue and the active site, W57 is close enough to the active site to allow for possible quenching by an iron ion. Any tryptophan quenching is most likely to occur primarily from metal site 2, since the ligand-metal distance is shortest from there. All of the GLX 2-2 samples from rich and minimal medias have significant amounts of Fe, which could cause variable amounts of quenching in each preparation depending on to which site(s) iron is bound. Future studies need to be performed on multiple preparations of nma and metal-added GLX 2-2 in order to determine the variability in fluorescence between preparations.

2.4 Conclusions

Recombinant *A. thaliana* GLX 2-2, over-expressed in rich media, has been found to bind iron, manganese, and zinc in varying ratios without significantly affecting the enzyme’s kinetic activity. Zn-added GLX 2-2 exhibited a higher catalytic turnover than nma GLX 2-2, while Mn-added GLX 2-2 exhibited a lower catalytic turnover. Nonetheless, the changes in $k_{\text{cat}}$ were within an order of magnitude. *A. thaliana* GLX 2-2 appears to allow for significant activity with different types of metal centers and ratios of bound metal ions. While site-specific binding of individual metals was not found with the above experiments, EPR spectroscopy revealed a number of possible metal centers. Spin-quantitation indicated that about 50% of the metal centers
Figure 25: Rasmol plot of ligand-metal distance in *A. thaliana* GLX 2-2. EMBL accession no. Y08357; Ligand-metal distances were measured from zinc ion to center axis of the specific tryptophan residue.
in *A. thaliana* GLX 2-2 were EPR active. EPR and EXAFS studies demonstrated that both metal sites can bind Fe, Mn, or Zn. In addition, the detection of an Fe(III)Fe(II) center raises the possibility that one binding site could specifically bind a trivalent iron while the other site binds a divalent iron ion, reminiscent of the di-iron sites found in the mammalian acid phosphatases (21, 22). The *in vivo* iron oxidation state is likely to be altered by the oxidative experimental conditions, which could explain the absence of an EPR-active Fe(II)Mn(II) center. In addition to these EPR-active metal centers, there may be many EPR-silent metal centers that are likely present as well. Zang *et al.* reported that *A. thaliana* GLX 2-2 required both zinc and iron for full activity, but the manganese content was not investigated (8). None of the rich media preparations of GLX 2-2 contained more than one zinc equivalent, but a preference for iron and manganese binding was exhibited. The EXAFS experiments showed that all three metals are coordinated at their typical coordination bond lengths, which indicates a high degree of structural flexibility within the GLX 2-2 active site. Note that the metal binding is strong enough to limit significant metal loss during purification, as indicated by an overall metal content of 1.4-1.9 metals per protein.

The dinuclear metal containing proteins with a β-lactamase fold are a diverse family that have been shown to bind a number of metal ions (46). *A. thaliana* GLX 2-2 is unique to this family since it can bind various ratios of iron, zinc, and manganese with no significant effect on its catalytic efficiency (this thesis). The physiological relevance of this is that GLX 2-2 likely binds metal ions and is active regardless of the bioavailability of zinc, iron, or manganese during different stages of cell growth. We have attempted to prepare apo-GLX 2-2, add two equivalents of zinc, iron, or manganese, and characterize the resultant enzymes (Crowder, unpublished). However, GLX 2-2 precipitates during dialysis and in the presence of chelating agents like EDTA. GLX 2-2 was over-expressed in minimal media in the presence of added iron, zinc, or manganese in an effort to enrich GLX 2-2 samples in one metal ion. These samples could be enriched with the metal ion of choice, but this procedure did not yield samples containing only one particular metal ion. Interestingly, GLX 2-2 samples over-expressed in Dehua Pei minimal media exhibited less bound total metal than those over-expressed in Hepes minimal media. This result is most likely due to iron, zinc, and manganese precipitation in Dehua Pei minimal media. This is supported by the results in Tables 8 and 9 showing that Zn-added GLX 2-2 and Mn-added GLX 2-2 bound roughly 1.3 equivalents of their respective metal ion of choice. In
addition, these results suggest that *A. thaliana* GLX 2-2 preferentially binds zinc and manganese over iron since more equivalents of the former metals are bound than iron, even when the growth medium is made to be 750 μM iron (Tables 8 and 9).

Since the GLX 2-2 samples prepared in Dehua Pei minimal media contained 1 equivalent of total metal, we initially expected that our samples contained roughly half-filled metal binding sites. For example, β-lactamase II, a metallo-β-lactamase from *Bacillus cereus* and is another enzyme with the β-lactamase fold, can be isolated containing 0, 1, or 2 metal ions (47). EPR and EXAFS spectroscopic studies were used to probe the metal binding sites of the GLX 2-2 samples. Both techniques strongly indicate that the samples contain primarily dinuclear metal centers. These results suggest that our samples are a mixture of fully metal-loaded GLX 2-2 and essentially metal-free GLX 2-2. This result is in contrast to the previous speculation by Zang *et al.* (8) that apo-GLX 2-2 does not fold properly and cannot be purified in a soluble state. The presence of dinuclear centers in proteins that contain an average of one metal per protein is a strong indication for positive cooperativity. Recent reports on several metallo-β-lactamases indicate that 1 Zn(II) analogs of these enzymes can be prepared and characterized, suggesting vastly differing $K_D$'s for the two metal sites in these enzymes and negative cooperativity of metal binding (48-52). Positive cooperativity of metal binding to proteins has been reported for several proteins: (a) Co(II) binding to ribonucleotide reductase (53), (b) Zn(II) and Cd(II) binding to several metallothioneins (54), (c) copper binding to ATPase 1 (55), and (d) Co(II) binding to bacterioferritin (56). By exhibiting positive cooperativity, GLX 2-2 is unique among the members of the metallo-β-lactamase family. This characteristic may have physiological implications since fully-loaded and active GLX 2-2 can be obtained at lower intracellular metal ion concentrations than if the enzyme exhibited negative cooperativity of metal binding.
2.5 References


