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Arabidopsis Glyoxalase II Contains a Zinc/Iron Binuclear Metal Center That Is Essential for Substrate Binding and Catalysis*[S]

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Glyoxalase II participates in the cellular detoxification of cytotoxic and mutagenic 2-oxoaldehydes. Because of its role in chemical detoxification, glyoxalase II has been studied as a potential anti-cancer and/or anti-protozoal target; however, very little is known about the active site and reaction mechanism of this important enzyme. To characterize the active site and kinetic mechanism of the enzyme, a detailed mutational study of *Arabidopsis* glyoxalase II was conducted. Data presented here demonstrate for the first time that the cytoplasmic form of *Arabidopsis* glyoxalase II contains an iron-zinc binuclear metal center that is essential for activity. Both metals participate in substrate binding, transition state stabilization, and the hydrolysis reaction. Subtle alterations in the geometry and/or electrostatics of the binuclear center have profound effects on the activity of the enzyme. Additional residues important in substrate binding have also been identified. An overall reaction mechanism for glyoxalase II is proposed based on the mutational and kinetic data from this study and crystallographic data on human glyoxalase II. Information presented here provides new insights into the active site and reaction mechanism of glyoxalase II that can be used for the rational design of glyoxalase II inhibitors.

The glyoxalase system consists of two enzymes that convert cytotoxic 2-oxoaldehydes into hydroxy acids utilizing the cofactor glutathione. Under physiological conditions, glyoxalase I (lactoylglutathione lyase) catalyzes the formation of *S*-D-lactoylglutathione (SLG)[1] in the presence of methylglyoxal and glutathione (1). Methylglyoxal, a cytotoxic compound that can inactivate proteins, modify guanylate residues in DNA, and create interstrand cross-links (2–4), is formed as a by-product of several biochemical reactions including that of triose-phosphate isomerase (2). Glyoxalase II (hydroxyacylglutathione hydrolase) hydrolyzes SLG to form D-lactic acid and regenerate glutathione (1). SLG is also known to exhibit cytotoxic effects through the inhibition of DNA synthesis (5). Therefore, the glyoxalase system is thought to play a major role in chemical detoxification (5, 6).

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[S] The on-line version of this article (available at *http://www.jbc.org*) contains Figs. 1–3).

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[1] The abbreviations used are: SLG, *S*-D-lactoylglutathione; PCR, polymerase chain reaction; MOPS, 4-morpholinepropanesulfonic acid; ICP, inductively coupled plasma.

The glyoxalase system has been the subject of intense study because of its potential implications in anti-protozoal and anti-tumor drug design (6–8). Increased cellular levels of methylglyoxal and glyoxalase activity are associated with tumor cells, the malaria parasite, and several complications associated with diabetes (6). Inhibitors of glyoxalase I and glyoxalase II have been designed as potential anti-tumor agents; however, all inhibitors tested to date exhibited problems that limited their therapeutic usefulness (6–9). It should be noted that these inhibition studies were all conducted with little or no information on the active site structure or the kinetic mechanism of the enzyme. Therefore, it is clear that a more detailed understanding of the active site of glyoxalase II and its reaction mechanism is essential to the rational design and implementation of clinically useful inhibitors.

Glyoxalase II has been purified and cloned from a number of sources (10–15), including *Arabidopsis thaliana* (16, 17). Five glyoxalase II isozymes have been identified in *Arabidopsis* including three, *GLX2-1*, *2-4*, and *2-5*, which appear to be mitochondrial forms; *GLX2-2*, a cytoplasmic form; and *GLX2-3*, which has not yet been localized (16). The *GLX2-2* gene has been successfully cloned and overexpressed in *Escherichia coli* yielding protein concentrations as high as 100 μM (18). Early biochemical studies suggested that glyoxalase II, unlike glyoxalase I, does not require bound metal ions for activity (13, 15, 17, 19). However, through the analysis of recombinant GLX2-2, we demonstrated that glyoxalase II is a Zn(II)-requiring enzyme (18). All species of glyoxalase II, including human, yeast, and *Arabidopsis*, contain a highly conserved metal binding domain (TH*X*H*X*DH) that is also present in the family of metallo-β-lactamases, which are known to require Zn(II) (16, 18, 20, 21). Based on its similarity to the metallo-β-lactamases, we predicted that glyoxalase II binds two Zn(II) ions, utilizing five histidines, two aspartic acids, and a bridging water molecule (18). These predictions have been restated by Melino *et al*. (22) and supported through the recent determination of the human glyoxalase II crystal structure (23). The crystallographic data indicates that the metal binding and active sites of glyoxalase II resemble those in the metallo-β-lactamase L1 from *Stenotrophomonas maltophilia* (24).

Early chemical modification, inhibition, and pH dependence studies on glyoxalase II suggested that an arginine, an amine, and a critical histidine are involved in the active site (13, 25). Given that Zn(II) is critical for activity (18), the critical histidine is most likely one of the metal ligands. In addition to the highly conserved metal-binding ligands, several other highly conserved residues, including arginine and lysine residues, have been identified (18). To probe the functionality of some of these residues, we have generated and analyzed a series of site-directed mutants of glyoxalase II. Conserved residues predicted to participate in substrate and metal binding were mu-

tated, and the altered forms of the enzyme were analyzed for conformation, metal content, and kinetic properties. Most of the mutations did not lead to a significant drop in substrate affinity; however, one mutation, R248W, did cause a 10-fold increase in K_m. All of the mutations affecting metal ligands resulted in a large drop in activity, suggesting that the metals must be bound in an optimal geometry for catalysis. Evidence was also obtained that the binuclear metal site of glyoxalase II is able to bind both zinc and iron. These results provide significant new insight into the metal binding and active sites of glyoxalase II and its mechanism of catalysis.

EXPERIMENTAL PROCEDURES

Materials

Deep Vent polymerase and other overlap PCR reagents were purchased from New England Biolabs (Beverly, MA); the oligonucleotides were synthesized by Integrated DNA Technologies, Inc (Coralville, IA). All of the chromatographic reagents, columns, and fast protein liquid chromatography system were obtained from Amersham Pharmacia Biotech. The SLG was purchased from Sigma. All other molecular biology reagents were purchased commercially and were of highest quality.

Methods

Site-directed Mutagenesis—Site-specific mutations were introduced into the glyoxalase II gene (*GLX2–2*) through overlap extension PCR (26). The wild type template, pT7-7/GLX2-2, was previously described by Maiti *et al*. (16). Numbering of amino acids corresponds to the *Arabidopsis* GLX2-2 sequence (GenBank™ accession number O24496). One mutation, R248W, found in the wild type cDNA template, was corrected through overlap PCR (TGG to CGC). The forward and reverse primers were between 26 and 32 bases long with the mutation in the center. The mutations generated were as follows: H54N, CAT to AAC; D58C, GAT to TGT; H59S, CAT to AGT; H59C, CAT to TGC; K74A, AAA to GCA; C140A, TGT to GCG; K142A, AAG to GCA; H172R, CAT to CGC; N178A, AAC to GCA; and R225A, CGT to GCA. All mutated PCR products were cloned into pT7-7 (27) and sequenced.

Protein Production and Purification—The expression plasmids were transformed into BL21(DE3)pLysS *E. coli* cells and overexpressed as described previously (18). The induction time was extended to 30 h, and the induction temperature was lowered to 15 °C to increase the yield of soluble protein. The wild type and mutant proteins, except for H59C and H172R, were purified using a Q-Sepharose column as described previously (18). The H59C and H172R enzymes could not be purified; therefore, these proteins were assessed in crude soluble extracts. Kinetic data and metal content were obtained and averaged from three preparations of each enzyme.

Steady State Kinetics and Crude Assays—The steady state kinetic parameters of purified wild type and mutant enzymes were determined by measuring the rate of hydrolysis of SLG at 240 nm using a Cary IE UV-visible spectrophotometer as previously described (18).

To prepare crude soluble protein extracts of the wild type, H59C, and H172R enzymes, 50-ml cultures of each strain were grown at 30 °C in ZY media (2% (w/v) tryptone, 1.5% yeast extract, 0.5% NaCl, 1% (v/v) glycerol) supplemented with 500 μM $ZnSO_4$ and 150 μg/ml ampicillin. The cultures were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at A_{600} of 0.7 and transferred to a shaker at 15 °C. After 24 h, the cultures were harvested and washed three times with Nanopure water. The cell pellets were resuspended in 10 ml of 10 mM MOPS, pH 7.2, and lysed by two passes through a French press at 16,000 pounds/square inch. A soluble extract was obtained after centrifugation at 12,500 rpm for 45 min. The amount of crude protein in the soluble extract was quantitated through SDS-polyacrylamide gel electrophoresis and the BCA protein assay. The total activity of crude soluble protein was determined from the rate of hydrolysis of 100 nmol of SLG in a 1-ml reaction volume over a 2-min period at 25 °C and standardized relative to the amount of glyoxalase II in the extract.

End Product Inhibition Studies of SLG Hydrolysis—Inhibition studies were performed on glyoxalase II using glutathione and D-lactic acid, the products of SLG hydrolysis. Steady state kinetic studies of purified wild type glyoxalase II in the presence of varying concentrations of glutathione (0.8, 1.7, 5, and 6.7 mM) or lithium D-lactate (103, 205, and 410 mM) were conducted by measuring the rate of hydrolysis of SLG. Glutathione and lithium D- lactate were prepared in 10 mM MOPS, pH 7.2; the pH of the lithium D-lactate solution was adjusted to 7.2 after dissolution of the salt. The enzyme and inhibitor were added first to allow the inhibitor time to interact with the enzyme. The substrate was added at time 0. For all reactions Lineweaver-Burk plots were generated to determine the mode of inhibition (28). Greater experimental error is associated with the results obtained from assays conducted at high inhibitor concentrations (low activity) than those at lower inhibitor concentrations, resulting in some lines that do not cross at a common point. Slope replots were generated to confirm the mode of inhibition and the K_I for each product (28).

Solvent Isotope Effects—Solvent isotope effect studies were performed on the wild type and D58C mutant enzymes to assess the presence of a rate-limiting proton transfer. Steady state kinetic studies of purified wild type or D58C mutant enzymes were conducted by measuring the rate of SLG hydrolysis at 240 nm in 0, 25, 50, 75, and 100% D_2O. The k^H/k^D and $(k/K_m)^H/(k/K_m)^D$ ratios were then calculated from the steady state kinetic constants obtained in 10 mM MOPS, pH 7.2, made with 0 and 100% D_2O.

Metal Analysis—The metal content of purified wild type and mutant proteins was measured with a Varian inductively coupled plasma spectrometer (ICP) with atomic emission spectroscopy detection as previously described (18). Purified protein was diluted in 10 mM MOPS, pH 7.2, to a concentration of 10 μM and analyzed for zinc, cobalt, and iron.

Whole Cell Metal Analysis—Whole cell metal analysis was performed on soluble extracts from wild type and mutant cultures through a modification of the method of D'souza and Holz (29). Fifty-milliliter cultures of BL21 (DE3) pLysS *E. coli* cells containing pT7-7, *GLX2-2*/pT7-7, and the *GLX2-2*/pT7-7 plasmids containing the H59C and H172R mutations were grown at 30 °C in ZY media minus $ZnSO_4$ with 150 μg/ml ampicillin. The cultures were induced with 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at A_{600} of 0.7 and transferred to a shaker at 15 °C. After 24 h, the cells were harvested, washed three times with Nanopure water and three times with 10 mM MOPS, pH 7.2, and a crude protein extract was obtained as described above. The extracts were diluted with Nanopure water to a concentration of 2 mg/ml and analyzed for zinc, cobalt, and iron content using ICP.

Fluorescence Emission—The intrinsic fluorescence of the wild type and mutant proteins was measured as described by Dragani *et al*. (30). Protein solutions of 0.2 mg/ml were prepared in 10 mM MOPS buffer and analyzed with a PerkinElmer Life Sciences Luminescence Spectrometer. The blank-corrected emission spectra were measured at 2 nm/s with excitation wavelengths of 280 and 295 nm from three enzyme preparations and were averaged.

RESULTS

Overexpression and Purification of Wild Type and Mutant Glyoxalase II—Several highly conserved amino acids were mutated, and the effect of these mutations on enzyme activity was analyzed to elucidate the roles of metal ligand and substrate-binding residues in the hydrolysis of SLG by glyoxalase II. Four metal-binding ligands of glyoxalase II (His-54, Asp-58, His-59, and His-172) were mutated to assess the sensitivity of metal binding and activity to changes in polarity, electrostatics, and geometry of the metal ions. In addition, several other highly conserved residues (Lys-74, Asn-178, Cys-140, Lys-142, and Arg-225) were replaced to evaluate their role in substrate binding.

Wild type and mutant proteins were overexpressed in *E. coli* and purified through fast protein liquid chromatography. All but three of the enzymes were produced at high levels and readily purified. Typically 40–80 mg of protein with 95–99% purity was obtained from a 1-liter culture. Two of the enzymes, metal ligand mutants H59C and H172R, could not be purified. Although the proteins accumulate to relatively high levels in the cell, neither enzyme bound efficiently to Q-Sepharose, preventing purification at levels appropriate for Michaelis-Menten kinetics and metal analysis. The H59C and H172R mutations appear to destabilize the structure of the proteins, which may result in denaturation during purification. The loss of column affinity as a result of mutations in metal ligands of other proteins has previously been reported (31). The K74A mutation results in a highly insoluble enzyme. It could not be purified, and therefore was not analyzed. Lysine 74 is located on the outside of the protein in the middle of a β strand (23). The drastic change in protein solubility likely results from dramatic

TABLE I
Activity of crude extracts of wild type and mutant glyoxalase II

Enzyme	Activity
	$M\ s^{-1}/mg/ml$ protein
Wild type	9.77E-06
H59C	1.38E-07
H172R	7.01E-09
R225A	1.03E-05

structural changes. All other mutant proteins were successfully expressed in soluble, active forms.

Analysis of Crude Protein Extracts of Wild Type and Mutant Glyoxalase II—Because the H59C and H172R proteins could not be purified, crude protein extracts were analyzed for glyoxalase II activity. Both enzymes could be produced at levels relatively comparable to wild type glyoxalase II and were found at least in part in the soluble protein fraction (see Fig. 1 of Supplemental Material). Levels of soluble enzyme with the H172R mutation were, however, consistently lower than that observed for the wild type enzyme. Therefore, the relative amount of soluble glyoxalase II was determined in all experiments. When the relative amounts of glyoxalase II present in the extracts were taken into consideration, the H59C and H172R proteins were found to exhibit ~1 and 0.05% of the crude protein activity seen in wild type GLX2–2, respectively (Table I). The reduced activity seen in the H59C and H172R enzymes is similar to that seen in the other metal ligand mutants (see below).

The *in vivo* metal content of crude protein extracts of cells overexpressing the H59C and H172R enzymes were also compared with cells expressing the wild type enzyme using ICP with atomic emission spectroscopy detection to determine if the mutant enzymes are still able to bind metal. A variation of this method has been used to determine the physiologically relevant metal ions present in methionyl aminopeptidase (29). Zinc and iron uptake was calculated by measuring the difference between the metal content of cells containing the empty expression vector (pT7-7) and those overexpressing the *GLX2-2* enzymes. Since glyoxalase II is expressed at high levels after induction, comparison of the whole cell metal concentrations in the presence and absence of glyoxalase II expression should reflect the relative ability of the enzymes to bind metal. This method is not, however, able to give the stoichiometry of the metal in the enzymes. Cells overexpressing wild type GLX2-2 contain approximately twice as much zinc (3.79 ± 0.66 μM/mg/ml protein) and iron (3.75 ± 0.37 μM/mg/ml protein) as control cells with no GLX2-2 expression (1.75 ± 0.23 μM Zn/mg/ml protein and 1.84 ± 0.57 μM Fe/mg/ml protein). Cells expressing the H59C enzymes contain iron levels (3.96 ± 0.42 μM/mg/ml protein) similar to those observed in cells overexpressing the wild type enzyme, whereas cells expressing the H172R enzyme did not accumulate iron above control levels (1.77 ± 0.45 μM/mg/ml protein). Cells expressing both the H59C and H172R enzymes failed to accumulate zinc and contained essentially the same levels of zinc as the control cells (1.71 ± 0.37 and 1.67 ± 0.47 μM/mg/ml protein, respectively).

These results suggest that the H59C mutation alters the binding site for metal 2, which is bound by Asp-58, His-59, Asp-133, His-172, and a bridging water molecule (23), such that it prevents metal binding. Although cysteine should be capable of binding either zinc or iron, the change in charge caused by this substitution may significantly alter the electrostatic environment of the active site thus preventing metal ion binding. Overexpression of the H172R enzyme did not result in a detectable uptake of either metal. Therefore, the H172R mutation seems to cause significant changes in the protein, which affect the binding of both metal 2 and metal 1, which is bound by His-54, His-56, His-110, Asp-133, and a bridging water molecule (23).

Metal Analysis of Purified Wild Type and Mutant Glyoxalase II—The total metal content of purified wild type and mutant proteins was measured using ICP with atomic emission spectroscopy detection to determine whether any of the mutations affect the ability of glyoxalase II to bind metal ions. All of the proteins, with the exception of the C140A and R225A enzymes, contained approximately two metal ions as shown in Table II. Previous data suggested that glyoxalase II binds two zinc ions; however, the GLX2-2 enzyme analyzed in this earlier study contained the mutation R248W (18). As shown in Table II, enzyme containing the R248W mutation binds approximately 2 mol of zinc and 0.4 mol of iron. In contrast, the wild type protein binds ~0.8 mol of zinc and 1.5 mol of iron. Therefore, it appears that both zinc and iron can occupy the metal sites of fully active glyoxalase II.

Although the total amount of metal bound by different forms of the enzyme was relatively constant at 2 mol of metal/mol of enzyme, the ratio of zinc to iron in glyoxalase II appears to be influenced by growth conditions and the environment around the metal-binding ligands. The metal content of wild type enzyme preparations ranged from 1:1 to 1:2 (Zinc:iron) with the average Zinc:iron ratio being 1:2. The D58C, K142A, and N178A enzymes bind iron and zinc at levels and in ratios nearly identical to wild type (1:1.7, 1:1.7, and 1:2.5, respectively). The R225A enzyme binds approximately half of the total metal seen in wild type GLX2-2 and seems to have a higher affinity for iron than does wild type (1:4.3). The C140A and R248W enzymes have higher affinities for zinc than wild type, with ratios of 1.6:1 and 5.1:1, respectively. The increased Zinc:iron ratio seen in the C140A enzyme is the result of increased zinc binding without a corresponding decrease in the amount of iron bound. Therefore, this enzyme binds twice as much total metal as does wild type glyoxalase II. Data presented in Tables I and II suggest that metal site 1 has a preference for iron, whereas metal site 2 binds zinc. The H59C mutation in metal site 1 results in an enzyme that binds normal levels of iron and no zinc, whereas the H54N mutation in metal site 2 results in an enzyme that binds zinc but little iron. Whereas further experiments are required to confirm these observations, these results provide preliminary insights into the nature of the metal-binding sites of glyoxalase II.

Steady State Kinetic Analysis of Purified Wild Type and Mutant Glyoxalase II—Steady state kinetic studies on the wild type and mutant proteins were performed to determine the effects of the mutations on substrate binding and catalysis. The steady state constants, k_{cat} and K_m, extracted from kinetic data fitted to the Michaelis-Menten equation, for the wild type and mutant enzymes are shown in Table II. The K_m value obtained for wild type enzyme was similar to data previously obtained for *Arabidopsis* glyoxalase II (17). Very few of the mutations affected the K_m values when using SLG as substrate. Amino acid residues Asn-178, Cys-140, Lys-142, and Arg-248 were previously proposed to interact with the glutathione portion of SLG in human glyoxalase II (23). The K_m values for the N178A and C140A enzymes are similar to that of wild type, suggesting that these residues do not play an important role in substrate binding. In contrast, the K142A and R248W substitutions did result in 3–10-fold higher K_m values, respectively. Removing the positive side chains of these two amino acid residues most likely impaired their affinity for the carbonyl groups on glutathione. Replacement of Arg-248 with a tryptophan residue results in an enzyme with a K_m value that is 10-fold higher than wild type. This suggests that Arg-248 plays an important role

TABLE II
Kinetic constants and metal content of purified wild type and mutant glyoxalase II

Enzyme	k_{cat}	K_m	Zinc	Iron	Total metal
	s^{-1}	μM	*mol Zn/mole enzyme*	*mole Fe/mole enzyme*	*mole metal/mole enzyme*
Wild type	3930 ± 138	63 ± 10	0.77 ± 0.19	1.47 ± 0.40	2.23
H54N	10.9 ± 0.7	130 ± 30	1.81 ± 0.14	0.15 ± 0.02	1.96
D58C	10.6 ± 0.5	76 ± 22	0.89 ± 0.15	1.52 ± 0.16	2.40
C140A	6780 ± 298	62 ± 10	2.43 ± 0.61	1.52 ± 0.13	3.95
K142A	1760 ± 170	170 ± 53	0.75 ± 0.31	1.30 ± 0.10	2.05
N178A	1190 ± 77	98 ± 30	0.50 ± 0.04	1.25 ± 0.07	1.75
R225A	600 ± 38	60 ± 13	0.15 ± 0.07	0.64 ± 0.38	0.79
R248W[a]	484 ± 92	600 ± 100	2.10 ± 0.50	0.41 ± 0.24	2.60

[a] Data are from Crowder *et al.* (18).

in substrate binding. The H54N enzyme also displayed a reduced affinity for SLG. Histidine 54 was not previously implicated with substrate binding. It does, however, bind metal 1, which is located within binding distance of the lactoyl carbonyl. Therefore, changes in this residue can be expected to affect substrate binding.

Most of the mutations examined had an effect on the activity of the enzyme. All of the metal ligand substitutions, H54N, D58C, H59C, and H172R, caused a significant reduction in the k_{cat} values (~99% reduction) of the enzymes. The K142A mutation, which is predicted to alter the position of a second shell metal ligand (23), caused a less severe reduction in activity (~55% loss). Given that the H54N, D58C, and K142A enzymes bind normal amounts of metal, these data suggest that enzyme activity depends not only on the correct metal stoichiometry but also on the geometry of the metals bound within the active site. The N178A mutation caused a 70% reduction in activity. The change in polarity generated by the asparagine to alanine substitution may alter the local electrostatics of the active site thus reducing activity.

Purified enzyme containing the R225A mutation displayed a significant reduction in activity and metal content. However, crude protein extracts from cells expressing the R225A enzyme displayed activity comparable to wild type (Table I). This suggests that the enzyme is initially correctly folded and active. The low enzymatic activity and metal content of the purified enzyme suggest that the mutation may cause structural alterations that result in denaturation during purification. Consistent with this hypothesis is the observation that preparations of the R225A enzyme displayed highly variable fluorescence patterns, suggesting that the preparations contained protein at varying stages of denaturation (see below).

The C140A mutation yielded an enzyme that is more active than wild type. In contrast to all of the other mutations, which reduced enzyme activity, the k_{cat} of the C140A enzyme is ~170% of the wild type enzyme, whereas the K_m values of the two enzymes are identical. The possibility that the increased metal content and activity was a result of an error in enzyme concentration caused by a change in the molar absorptivity was considered. To evaluate this possibility the concentration of the purified enzyme was analyzed visually through SDS-polyacrylamide gel electrophoresis with other glyoxalase II enzymes, spectroscopically through UV absorption at 280 nm, and chemically through the BCA assay. All methods yielded similar protein concentrations indicating that the concentration value used in k_{cat} and metal content determinations is correct. Therefore, the C140A mutation results in an increase in metal binding and enzyme activity.

Fluorescence Emission Spectra of Purified Wild Type and Mutant Glyoxalase II—Fluorescence emission spectra were obtained for purified wild type and mutant proteins using excitation wavelengths of 280 and 295 nm to determine the effect of the mutations on the general conformation of the enzyme. GLX2-2 contains two tryptophan residues at amino acid residues 57 and 198. It has previously been demonstrated that mutations that alter the general structure of glyoxalase II change the environment around the two tryptophan residues and result in an increased fluorescence emission (30). Most of the mutant enzymes, including the D58C enzyme, exhibited a fluorescence emission spectrum similar to that of wild type GLX2-2, suggesting that no significant change in the general structure of the proteins occurred as a result of the mutations (Fig. 1*C*). As seen in Fig. 1, *A* and *B*, two of the enzymes, H54N and C140A, exhibited a much greater intrinsic fluorescence intensity than the wild type enzyme. This suggests that these mutations, which are both at the active site of the enzyme, cause a significant change in the structure of the proteins. In contrast to the other enzymes, the fluorescence emission pattern for the purified R225A enzyme varied significantly from preparation to preparation, suggesting that the preparations may contain varying amounts of misfolded and/or denatured protein.

Solvent Isotope Effects—The reaction mechanism of glyoxalase II, specifically if a proton transfer occurs during the rate-limiting step of SLG hydrolysis, was investigated by conducting solvent isotope effect kinetics on the wild type and D58C mutant enzymes. The hydrolysis of SLG by wild type glyoxalase II shows very little solvent isotope effect, k^H/k^D = 1.4 ± 0.2; $(k/K_m)^H/(k/K_m)^D$ = 1.0 ± 0.5. These data are similar to that previously determined for human liver glyoxalase II (13). If there is a rate-limiting proton transfer occurring between the bridging water and aspartic acid 58, its replacement with a cysteine should prevent this proton transfer from occurring, which would lower the solvent isotope effect. The D58C enzyme exhibited solvent isotope effects similar to that determined for the wild type enzyme, k^H/k^D = 1.5 ± 0.1; $(k/K_m)^H/(k/K_m)^D$ = 1.3 ± 0.2. Although we cannot completely rule out the possibility, our data suggest that the rate-limiting step of *Arabidopsis* GLX2-2 does not involve a proton in flight. Typically a k^H/k^D = 1.11 is indicative of a carbonium ion in the transition state, as seen with lysozyme (32). In contrast the metallo-β-lactamases, which utilize a rate-limiting proton transfer exhibit a k^H/k^D = 2.5–3.5 (33, 34). The fact that the D58C mutation did not significantly lower the solvent isotope effect indicates that Asp-58 does not participate in a rate-limiting proton transfer in the catalytic mechanism of glyoxalase II.

End Product Inhibition Studies—To determine the order of product release after the hydrolysis of SLG, end product inhibition studies were performed. Steady state kinetic studies, using the substrate SLG, were conducted on wild type GLX2-2 in the presence of varying concentrations of glutathione (0.8–6.7 mM) or lithium D-lactic acid (100–410 mM). Inhibition by glutathione was seen at concentrations as low as 0.8 mM, whereas inhibition by D-lactic acid was seen at concentrations as low as 20 mM. Steady state kinetics was conducted at 10, 30, 50, and 70% inhibition by glutathione and at 18, 33 and 50%

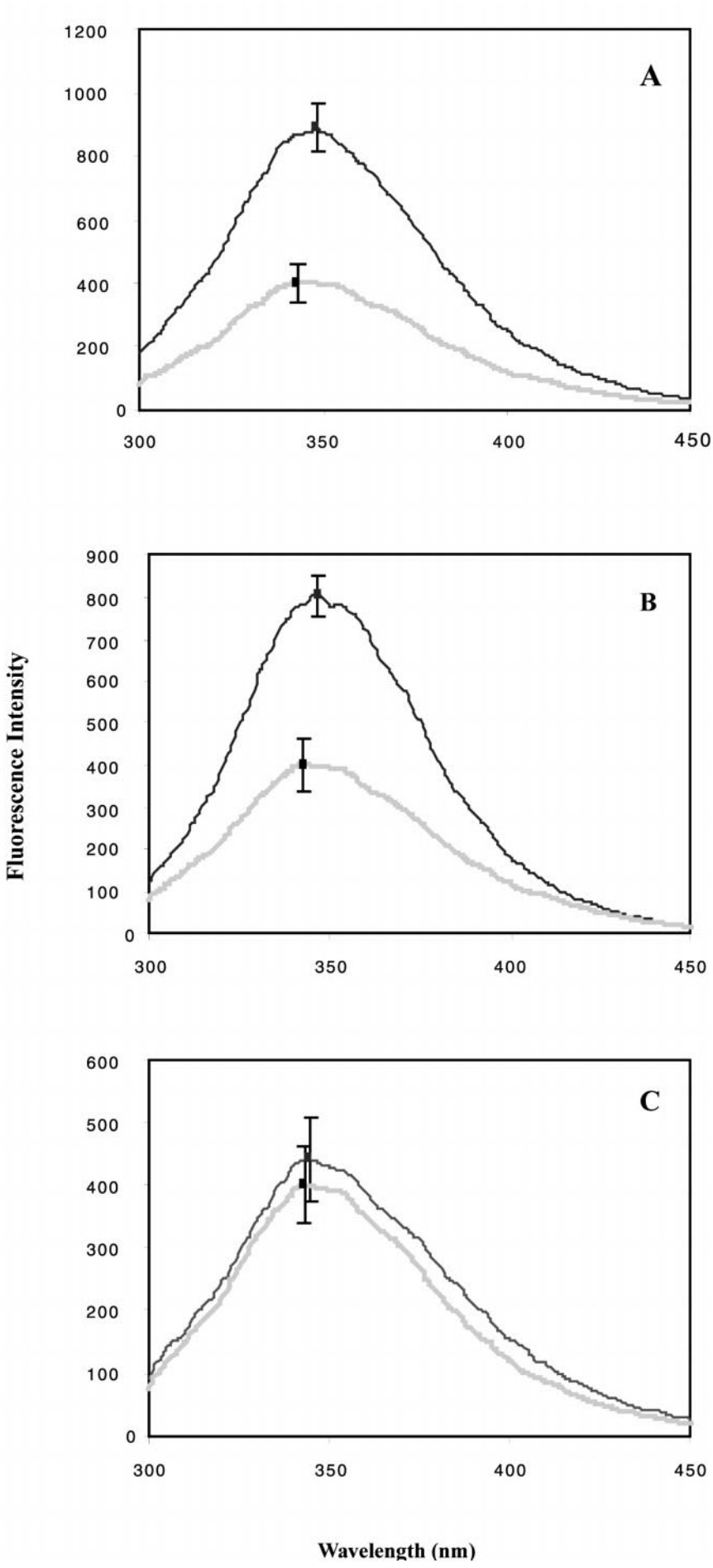

FIG. 1. **The fluorescence emission spectra of wild type and mutant glyoxalase II obtained with an excitation wavelength of 280 nm.** *A,* H54N (—) and wild type (···); *B,* C140A (—) and wild type (···); *C,* D58C (—) and wild type (···). The *error bar* represents the error at the maximum point of emission observed for three trials.

inhibition by D-lactic acid. Lineweaver-Burk plots for glutathione were typical of a mixed mode of inhibition, whereas D-lactic acid exhibited weak competitive inhibition (Supplemental Figs. 2 and 3, respectively). The slope replots resulted in K_I values of 0.4 and 122 mM for glutathione and D-lactic acid, respectively. These data differ from earlier biochemical studies on human liver glyoxalase II that suggest glutathione is a weak competitive inhibitor with a K_I = 4.0 mM, and D-lactic acid is not an inhibitor (19). The significant differences in the K_I values of glutathione and D-lactic acid suggest that product release after the hydrolysis of SLG is ordered (28). The low K_I for glutathione suggests that it is bound more tightly into the active site and therefore is released after D-lactic acid.

DISCUSSION

In this report we present results of mutational and kinetic studies that were conducted on glyoxalase II to gain insight into the role of highly conserved amino acids in metal ion and substrate binding. Early studies suggested that glyoxalase II is not a metalloenzyme (13, 15, 17, 19). However, in 1997 we showed that glyoxalase II in fact requires 2 mol of Zn(II) per mol of enzyme for catalytic function (18). The protein used in this study was subsequently found to contain a mutation, R248W. Evidence presented here demonstrates that wild type *Arabidopsis* glyoxalase II contains a binuclear metal-binding site that is able to bind both zinc and iron. As shown in Table II, enzyme preparations containing the R248W mutation bind 2 mol of zinc and 0.4 mol of iron, whereas wild type *Arabidopsis* glyoxalase II binds an average of 0.8 mol of zinc and 1.5 mol of iron. Furthermore, data on several of the mutations presented here indicate that the relative stoichiometry of the metals bound by glyoxalase II is influenced by the environment around the metal ligands. The R248W and C140A mutant enzymes bind more zinc than iron, whereas the wild type enzyme has a preference for iron. The observation that *Arabidopsis* glyoxalase II can bind both zinc and iron is consistent with recent results on human glyoxalase II, which was found to contain ~1.5 mol of zinc and 0.7 mol of iron (23). In this study the authors concluded that the enzyme is a binuclear zinc protein. However, the resolution of the crystal structure was not sufficient to distinguish between zinc and iron in the protein.

Binuclear iron-zinc proteins have been found throughout nature, including the kidney bean purple acid phosphatases and protein phosphatases 1, 2A, and 2B (35, 36). Purple acid phosphatases can have Fe(III)-Fe(II), Fe(III)-Mn(II), or Fe(III)-Zn(II) binuclear metal centers. In the mammalian dinuclear iron purple acid phosphatases zinc can readily replace iron to form functional Fe-Zn enzymes (37), whereas iron can replace zinc in the Zn-Fe purple acid phosphatases from plants (36). Our current results indicate that glyoxalase II can utilize both zinc and iron. Further experiments are required to determine the exact nature of the binuclear metal center in the enzyme and to determine whether it is able to utilize other metals, such as manganese. The ability to utilize both zinc and iron is not seen in the metallo-β-lactamases, which only bind zinc (38), even though the metal-binding ligands of glyoxalase II most closely resemble those of the metallo-β-lactamase L1 from *S. maltophilia* (24). It is interesting that two enzymes with essentially the same metal-binding ligands can exhibit such different metal-binding preferences.

Early chemical modification, inhibition, and pH dependence studies suggested that an arginine, an amine, and a critical histidine are involved in the active site of glyoxalase II (13, 25). The critical arginine may be Arg-240, which is essential for substrate binding, as shown by the 10-fold reduction in SLG affinity when Arg-248 is substituted by tryptophan. The critical amine is not Lys-142; changing this residue to an alanine did not result in a large loss of activity. There are no other conserved amines in the active site with the exception of the imidazole rings of the metal ligand histidines (16).

The essential histidine residue previously identified is most likely one or more of the metal ligands. Our finding that any alteration in the metal-binding ligands of glyoxalase II results in a significant reduction in activity clearly demonstrates the essential role of the binuclear metal site in the catalysis of this enzyme. Substitutions of metal-binding ligands that are expected to alter the geometry of the metal ions but still allow metal binding (H54N, D58C, and H59C) all resulted in a dramatic decrease in enzyme activity. In addition, the structure of glyoxalase II cannot accommodate the loss of one metal, as knocking out one metal ligand through the H172R mutation yielded a near inactive enzyme with virtually no bound metal.

It has been proposed that the active site of human glyoxalase II is also dependent on a GCG hairpin; the cysteine of this hairpin was proposed to be involved with substrate binding and stabilizing several highly conserved active site residues (23). Replacing this cysteine (140) with alanine in GLX2-2 results in dramatic structural changes, as shown by the significant increase in the intrinsic fluorescence of the enzyme. Interestingly, this alteration does not reduce enzymatic activity. Instead the activity increases almost 2-fold. It was also noted that the metal content of the C140A enzyme is twice that of wild type glyoxalase II. Quantification of the C140A protein using several methods indicated that the 2-fold increases in activity and metal content are not due to errors in enzyme concentration. The increased fluorescence of the C140A mutant enzyme indicates that the environment around the active site tryptophan residues (57 and 198) has been changed by this mutation. It has been suggested previously that substrate binding is the rate-limiting step for glyoxalase II (39). It is possible that the C140A mutation alters the structure of the enzyme to facilitate access of substrate to the active site. Further experiments, including crystallographic studies, are necessary to confirm this proposal.

Many binuclear metalloenzymes, such as the metallo-β-lactamases and leucine aminopeptidase, appear to utilize a hydroxide, arising from a bridging water molecule in the nucleophilic attack of substrate (33, 40). Residues that hydrogen-bond to the nucleophilic hydroxide usually play a critical role in orienting the hydroxide and holding it in a fixed position that reduces the entropic barrier for nucleophilic attack on the substrate (41). We predict that Asp-58 is the critical residue for hydroxide positioning in *Arabidopsis* glyoxalase II. The aspartic acid at position 58, in human glyoxalase II, has also been predicted to hydrogen-bond to the bridging hydroxide through its carbonyl oxygen (23). There are several other metalloenzymes, including the metallo-β-lactamases, that have a homologous aspartic acid in their binuclear metal-binding sites (33). In addition to removing the possibility of a proton transfer, changing an aspartic acid to cysteine removes the hydrogen bond partner of the nucleophilic hydroxide. The D58C substitution caused a reduction in activity greater than 99%, while still maintaining wild type levels of metal. This suggests an important role for Asp-58 in orienting the nucleophilic hydroxide for attack. The fact that the enzyme binds normal levels of metal and exhibits wild type fluorescence emission patterns suggests that the change in activity is not due to major structural changes. The solvent isotope effect data do not support a role for this residue in a rate-limiting proton transfer event but do not rule out an orientation role for Asp-58. The homologous aspartic acid (residue 103) in the metallo-β-lactamase from *Bacteroides fragilis* has been proposed to play an identical role in orienting the nucleophilic hydroxide for attack of the sub-

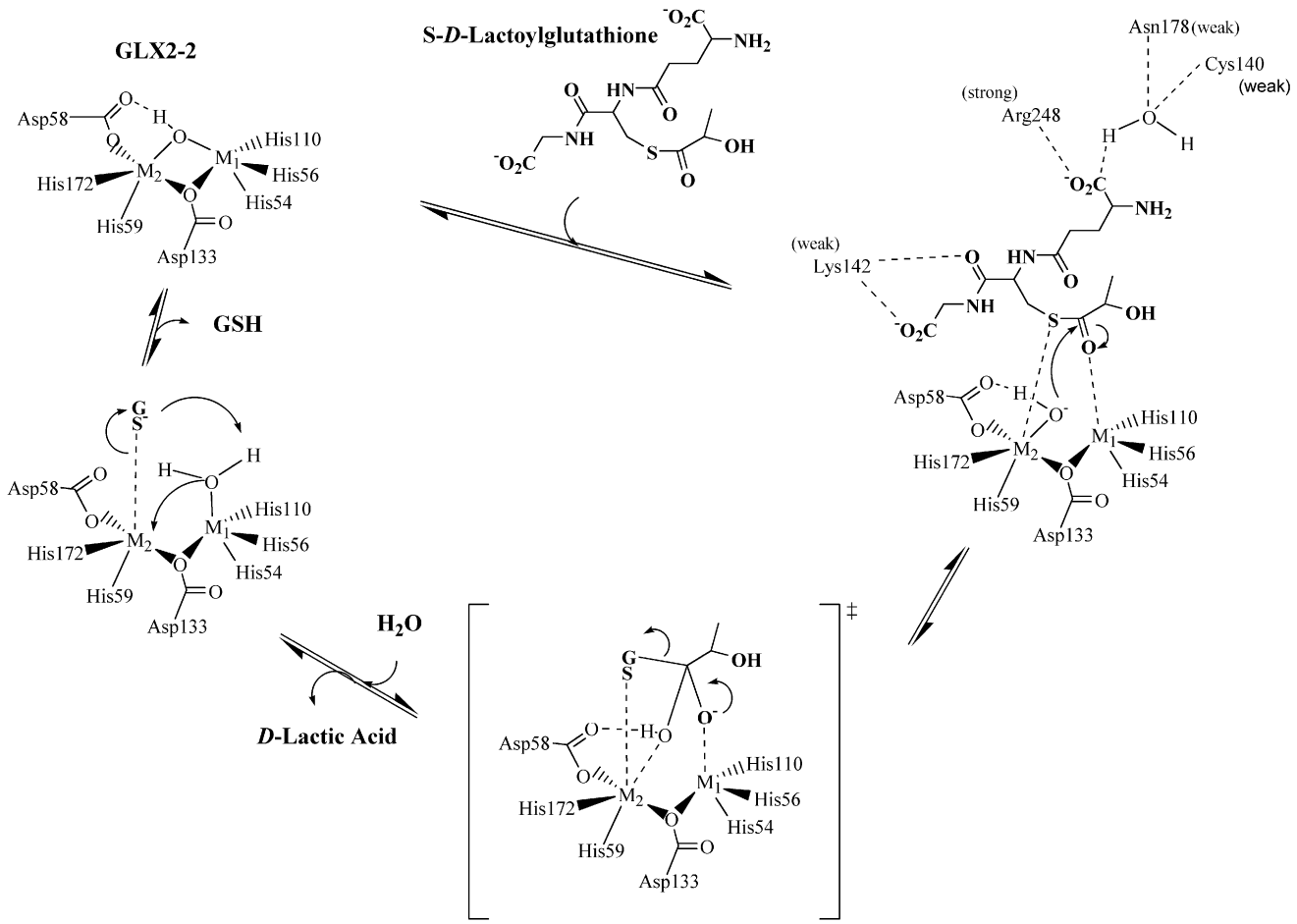

FIG. 2. **The proposed mechanism for SLG hydrolysis by glyoxalase II.** Metal 1 (M_1), metal 2 (M_2), Asn-178, Lys-142, Cys-140, and Arg-248 participate in the initial binding of SLG. The nucleophilic hydroxide, held in position by Asp-58, attacks the lactoyl carbonyl, yielding a tetrahedral transition state. Ligand exchange occurs at metal 1, where D-lactic acid is replaced by water, and a water on metal 1 protonates the glutathione leaving group. Numbering of amino acids corresponds to the *Arabidopsis* GLX2-2 sequence (GenBank™ accession number O24496). *GS*, glutathione.

strate nitrocefin, rather than participating in the reaction (33, 34).

By using data from this study, the crystallographic data on human glyoxalase II, and results from earlier biochemical studies, an overall reaction mechanism can be offered (Fig. 2). Arginine 248 apparently participates in the initial binding of *S*-lactoylglutathione; Lys-142 and Asn-178 also participate in substrate binding but to a lesser extent. In contrast to the proposal of Cameron and coworkers (23), Cys-140 seems to have little to no initial binding interaction with SLG; the K_m of the C140A enzyme is identical to wild type. The binuclear metal center also plays a role in substrate binding, with both metal ions potentially helping to orient SLG. Metal 2 is proposed to be in close proximity to the sulfur of the glutathione portion of SLG, whereas metal 1 is proposed to be in close proximity to the lactoyl carbonyl of SLG (23). The metals may help orient SLG and stabilize the excess negative charge formed in a tetrahedral transition state. The participation of metal ions in orienting substrate for nucleophilic attack has been proposed in several other binuclear metalloenzymes, including the metallo-β-lactamases and leucine aminopeptidase (33, 38, 40). Once SLG has been bound and oriented for nucleophilic attack, the hydroxide, now bound only to metal 2 and held in position by Asp-58, can attack the lactoyl carbonyl. After initial nucleophilic attack, a tetrahedral transition state is formed.

The tetrahedral transition state is predicted to be stabilized by interactions with metal 2, at the sulfur of glutathione and metal 1 at the former lactoyl carbonyl. In leucine aminopeptidase, a lysine residue homologous to Lys-142 of glyoxalase II has been shown to participate in transition state stabilization (40). Replacing this lysine with alanine in leucine aminopeptidase resulted in a 99% reduction in activity (40). Substituting Lys-142 with alanine in glyoxalase II did not lead to as drastic a reduction in activity (55%), suggesting that Lys-142 is not stabilizing the transition state. However, it is possible that the binuclear metal center in GLX2-2 might accommodate for the loss of Lys-142. Glyoxalase II may complete the hydrolysis reaction in a manner similar to the *B. fragilis* metallo-β-lactamase. In this enzyme, it was predicted that Zn_2-OH_2, serving as an acid, protonates the negatively charged nitrogen of the leaving group, inducing product release. Ligand exchange occurs at Zn_1 as the acyl group of the product is replaced with a solvent water molecule, regenerating the active site of the metallo-β-lactamase (33). In glyoxalase II, metal 2, serving as an acid, may protonate the negatively charged sulfur of the leaving group, inducing glutathione release. Ligand exchange may occur at metal 1 as lactic acid is replaced with the hydroxide on metal 2, regenerating the active site.

In conclusion, the characterization of site-directed mutations of metal ligand and substrate-binding residues has provided important new information about the nature of the binuclear metal center and the mechanism of SLG hydrolysis of glyoxalase II. They have identified critical active site residues of glyoxalase II and helped differentiate the roles of several highly conserved residues in metal binding, substrate binding,

and catalysis. Specifically, we have shown for the first time that the binuclear metal center of glyoxalase II can readily accommodate different metal ions, including both zinc and iron and that metal preference is readily influenced by both first and second shell metal ligands. The two metal ions are absolutely essential for full enzymatic activity. Both metal ions appear to participate in substrate binding, transition state stabilization, and the hydrolysis reaction. We provide strong evidence to support the hypothesis that Asp-58 plays a critical role in orienting the hydroxide for attack of SLG. In addition we have confirmed the role of residues Arg-248, Lys-142, and Asn-178 in substrate binding, and we demonstrated that contrary to predictions made based on the crystal structure that Cys-140 does not have a major role in substrate binding.

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