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) in the presence of methylglyoxal and glutathione (1). Methylglyoxal, a cytotoxic compound that can cause interstrand cross-links (2-4), is formed as a by-product during cellular metabolism. GLY, the mammalian form of glyoxalase I, is most likely one of the metal ligands. In addition to the above residues, we have 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 mutated (5, 6).

Received for publication, June 13, 2000, and in revised form, November 16, 2000
Published, JBC Papers in Press, November 20, 2000, DOI 10.1074/jbc.M005090200

Trinity M. Zang, Denise A. Hollman, Patrick A. Crawford, Michael W. Crowder, and Christopher A. Makaroff‡

From the Department of Chemistry and Biochemistry, Miami University, Oxford, Ohio 45056

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 isoforms 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 μg/ml (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 (THXHDXH) that is also present in the 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 indicate 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 mutated...
tated, and the altered forms of the enzymes 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 (Corvaline, IA). All of the chromatographic reagents, columns, and fast protein liquid chromatography system were obtained from Amersham Pharmacia Bio- tech. 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, pTT-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 TCT). 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 GCC; K142A, AAG to GCA; H172R, CAT to GCC; N178A, AAC to ACA; and R248W, CTG to GTA. All mutant PCR products were cloned into pT7-7 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 from a 1-liter culture. Two of the enzymes, H59C and H172R, could not be purified. Typically 40–80 mg of protein with 95–99% purity was obtained from a 1-liter culture. 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 purity was previously 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 b strand (23). The drastic change in protein solubility likely results from dramatic

**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 b strand (23). The drastic change in protein solubility likely results from dramatic
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, 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.
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 \( k_{\text{cat}} \) 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. The N178A mutation caused a 70% reduction in 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 \( k_{\text{cat}} \) 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 absorbivity 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, spectrophotoscopically 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 \( k_{\text{cat}} \) 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. GLX-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 GLX-2, suggesting that no significant change in the general structure of the proteins occurred as a result of the mutations (Fig. 1C). 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, \( k_H/k_D = 1.4 \pm 0.2; (k/K_m)^{1H}/(k/K_m)^{1D} = 1.0 \pm 0.5 \). 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, \( k_H/k_D = 1.5 \pm 0.1; (k/K_m)^{1H}/(k/K_m)^{1D} = 1.3 \pm 0.2 \). Although we cannot completely rule out the possibility, our data suggest that the rate-limiting step of *Arabidopsis* GLX-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 GLX-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% inhibition by d-lactic acid.

---

**Table II**

| Enzyme     | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( K_m \) (μM) | Zinc (mol Zn/mole enzyme) | Iron (mol Fe/mole enzyme) | Total metal (mol metal/mole enzyme) |
|------------|---------------------------------|----------------|---------------------------|--------------------------|-----------------------------------|
| Wild type  | 3930 ± 128                      | 62 ± 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 ± 70                       | 170 ± 52       | 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      | 900 ± 38                        | 82 ± 13        | 0.15 ± 0.07               | 0.64 ± 0.58              | 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).
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-248, 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 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-
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-$\beta$-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-$\beta$-lactamase. In this enzyme, it was predicted that Zn$_2$-$\text{OH}_2$, 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-$\beta$-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.

Acknowledgment—We thank Daniel N. Sobieski for helping with the solvent isotope effect studies on wild type glyoxalase II.

REFERENCES

1. Thornalley, P. (1990) Biochem. J. 269, 1–11
2. Rahman, A., Shahabuddin, A., and Hadi, S. (1990) J. Biochem. Toxicol. 5, 161–166
3. Lo, T., Westwood, M., McLellan, A., Selwood, T., and Thornalley, P. (1994) J. Biol. Chem. 269, 32299–32305
4. Papoulis, A., Al-Abed, Y., and Bucala, R. (1995) Biochemistry 34, 648–655
5. Thornalley, P. (1993) Mol. Aspects Med. 14, 287–371
6. Thornalley, P. J. (1996) Gen. Pharmacol. 27, 565–573
7. Allen, R., Lo, T., and Thornalley, P. J. (1993) Biochem. Soc. Trans. 21, 535–540
8. Elia, A. C., Chyna, M. K., Principato, G. B., Giovannini, E., and Rosi, G., and Norton, S. J. (1995) Biochem. Mol. Biol. Int. 35, 763–771
9. Norton, S. J., Elia, A. C., Chyna, M. K., Gillis, G., Frenzel, C., and Principata, G. B. (1995) Biochem. Soc. Trans. 21, 545–549
10. Allen, R., Lo, T., and Thornalley, P. (1993) Eur. J. Biochem. 213, 1261–1267
11. Talesa, V., Uotila, L., Koivusalo, M., Principato, G. B., Giovannini, E., and Rosi, G. (1999) Biochem. Biophys. Res. Comm 258, 7–11
12. Murata, K., Inoue, Y., Watanabe, K., Fukuda, Y., Saikusa, T., Shimoseka, M., and Kimura, A. (1986) Agric. Biol. Chem. 50, 135–142
13. Vander Jagt, D. L. (1993) Biochem. Soc. Trans. 21, 522–527
14. Talesa, V., Rosi, G., Contenti, S., Mangiapane, C., Lupattelli, M., Norton, S. J., Giovannini, E., and Principato, G. B. (1999) Biochem. Int. 22, 1115–1120
15. Ridderstrom, M., Saccucci, F., Hellman, U., Bergman, T., Principato, G., and Mannervik, B. (1996) J. Biol. Chem. 271, 319–323
16. Maiti, M. K., Krishnasamy, S., Owen, H. A., and Makaroff, C. A. (1997) Plant Mol. Biol. 35, 471–481
17. Ridderstrom, M., and Mannervik, B. (1997) Biochem. J. 322, 449–454
18. Crowder, M. W., Maiti, M. K., Banovic, L., and Makaroff, C. A. (1997) FEBS Lett. 418, 351–354
19. Uotila, L. (1973) Biochemistry 12, 3944–3951
20. Crowder, M. W., Wang, Z., Franklin, S. L., Zovinka, E. P., and Benkovic, S. J. (1996) Biochemistry 35, 12126–12132
21. Concha, N. O., Rasmussen, B. A., Bush, K., and Herzberg, O. (1996) Structure 4, 823–836
22. Melino, S., Capo, C., Dragani, B., Aceto, A., and Petruzelli, R. (1998) Trends Biochem. Sci. 23, 381–382
23. Cameron, A. D., Ridderstrom, M., Olin, B., and Mannervik, B. (1999) Structure 7, 1067–1074
24. Ullah, J. H., Walsh, T. R., Taylor, I. A., Emery, D. C., Verma, C. S., Gamblin, S. J., and Spencer, J. (1998) J. Mol. Biol. 284, 125–136
25. Ball, J., and Vander Jagt, D. (1991) Biochemistry 20, 889–905
26. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 77, 51–59
27. Tabor, S., and Richardson, S. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1074–1078
28. Segel, I. H. (1993) Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems, pp. 544–555, John Wiley & Sons, Inc., New York
29. D'Souza, V. M., and Holz, R. C. (1999) Biochemistry 38, 11079–11085
30. Dragani, B., Cocco, R., Ridderstrom, M., Stenberg, G., Mannervik, B., and Aceto, A. (1999) J. Mol. Biol. 289, 481–490
31. Geeganage, S., and Frey, P. A. (1999) Biochemistry 38, 13398–13406
32. Schwenk, K., and Schowen, R. (1998) Methods Enzymol. 307, 551–566
33. Wang, Z., Fast, W., and Benkovic, S. J. (1999) Biochemistry 38, 10013–10023
34. Yanchak, M., Taylor, R., and Crowder, M. (2000) Biochemistry 39, 11390–11399
35. Mondragon, A., Griffith, E. C., Sun, L., Xiong, F., Armstrong, C., and Liu, J. O. (1997) Biochemistry 36, 4934–4942
36. Twitchett, M. B., and Sykes, A. G. (1999) Eur. J. Inorg. Chem. 12, 2105–2115
37. Wang, X., Randall, C. R., True, A. E., and Que, J., L. (1998) Biochemistry 37, 1034–10354
38. Crowder, M. W., and Walsh, T. R. (1999) in Antimicrobial Agents and Chemotherapy, Vol. 3, Part 1 (Pandalai, S. G., ed) pp. 105–102, Research Signpost, Trivandrum, India
39. Creighton, D. J., Migliorni, M., Pourmotabbed, T., and Guha, M. K. (1988) Biochemistry 27, 7376–7384
40. Sträter, N., Sun, L., Kantronowitz, E. R., and Lipscomb, W. N. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11151–11155
41. Christianson, D. W., and Cox, J. D. (1999) Annu. Rev. Biochem. 68, 33–57

Mutational and Mechanistic Studies on Glyoxalase II 4795