Reaction Mechanism of Glyoxalase I Explored by an X-ray Crystallographic Analysis of the Human Enzyme in Complex with a Transition State Analogue†

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ABSTRACT: The structures of human glyoxalase I in complexes with S-(N-hydroxy-N-p-iodophenylcarbamoyl)glutathione (HIPC-GSH) and S-p-nitrobenzylcarbonylglutathione (NBC-GSH) have been determined at 2.0 and 1.72 Å resolution, respectively. HIPC-GSH is a transition state analogue mimicking the enediolate intermediate that forms along the reaction pathway of glyoxalase I. In the structure, the hydroxycarbamoyl function is directly coordinated to the active site zinc ion. In contrast, the equivalent group in the NBC-GSH complex is approximately 6 Å from the metal in a conformation that may resemble the product complex with S-d-lactoylglutathione. In this complex, two water molecules occupy the liganding positions at the zinc ion occupied by the hydroxycarbamoyl function in the enediolate analogue complex. Coordination of the transition state analogue to the metal enables a loop to close down over the active site, relative to its position in the product-like structure, allowing the glycine residue of the glutathione moiety to hydrogen bond with the protein. The structure of the complex with the enediolate analogue supports an “inner sphere mechanism” in which the GSH—methylglyoxal thiohemiacetal substrate is converted to product via a cis-enediyl intermediate. The zinc ion is envisioned to play an electrophilic role in catalysis by directly coordinating this intermediate. In addition, the carboxyl of Glu 172 is proposed to be displaced from the inner coordination sphere of the metal ion during substrate binding, thus allowing this group to facilitate proton transfer between the adjacent carbon atoms of the substrate. This proposal is supported by the observation that in the complex with the enediolate analogue the carboxyl group of Glu 172 is 3.3 Å from the metal and is in an ideal position for reprotation of the transition state intermediate. In contrast, Glu 172 is directly coordinated to the zinc ion in the complexes with S-benzylglutathione and with NBC-GSH.

Glyoxalase I (EC 4.4.1.5) catalyzes the first of two enzymatic steps in the conversion of 2-oxoaldehydes to the corresponding 2-hydroxycarboxylic acids by way of the glyoxalase system, which uses glutathione as a cofactor (1–3). Physiologically, the reaction seems most relevant in the detoxication of methylglyoxal, which is continuously generated as a side product of glycolysis (4). The substrate of glyoxalase I is not methylglyoxal itself, but rather the thiohemiacetal formed when it reacts with glutathione. Glyoxalase I catalyzes the isomerization of this thiohemiacetal to S-d-lactoylglutathione, which is later hydrolyzed by glyoxalase II to d-lactic acid and glutathione. The reaction is thought to proceed via an enediolate intermediate with a base on the protein catalyzing the proton transfer between C1 and C2 (see Figure 1) (5–7). The enzyme has an absolute requirement for metal ions. The zinc ion in the human enzyme can be replaced by other bivalent metal ions to yield partially active enzyme (8, 9). The metal ion has been proposed to indirectly facilitate catalysis via a metal-bound water molecule that stabilizes the enediolate intermediate and flanking transition states (5, 6).

Recently, we determined the structure of human glyoxalase I, a homodimer with a molecular mass of 43 kDa, in a complex with S-benzylglutathione (B-GSH)† (10). The two active sites of the homodimer are located at the dimer interface and are characterized by a glutathione binding site, a zinc binding site, and a large hydrophobic pocket. Two amino acid residues from each subunit and one water molecule coordinate the zinc ion in a square pyramidal arrangement. These results confirmed previous conclusions

† Abbreviations: HIPC-GSH, S-(N-hydroxy-N-p-iodophenylcarbamoyl)glutathione; NBC-GSH, S-p-nitrobenzylcarbonylglutathione; B-GSH, S-benzylglutathione; rms, root-mean-square; NCS, nomenclatural symmetry.

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about the nature of the active site deduced from inhibitor binding and spectroscopic studies. From the structure, however, it was not obvious which residue could act as the catalytic base in the proton-transfer reaction, since the only reasonable candidates were zinc ligands. However, site-directed mutagenesis, in conjunction with the X-ray crystal structure, indicated that Glu 172, one of the zinc ligands, is critical for catalysis and is possibly the catalytic base (11).

Glyoxalase I has been suggested as a possible target for novel antitumor and antiprotozoal drugs, where inhibition of the enzyme would cause the buildup of cytotoxic methylglyoxal (12). In this regard, a number of inhibitors have been designed. The tightest binding are the S-p-nitrophenylcarbamoylglutathione, and (C) S-(N-hydroxy-N-p-iodophenylcarbamoyl)glutathione.

**MATERIALS AND METHODS**

**S-p-Nitrobenzylcarboxyloxyglutathione Complex.** The protein was expressed and purified, and native crystals were grown as described previously (10,15). A crystal was then soaked for a period of 4 days in an equivalent mother liquor containing 10 mM NBC-GSH before being transferred briefly to a similar solution containing 5% ethylene glycol. The crystal was then flash-frozen under a nitrogen gas stream at 90 K. The NBC-GSH was a gift from G. Principato (Università` di Ancona, Ancona, Italy). Data were collected on beamline X11 at DESY (Hamburg, Germany) on a MAR Research image plate detector and integrated and merged using the HKL suite of programs (16). Further processing was carried out using programs from the CCP4 package (17).

Data collection statistics are presented in Table 1.

As the starting model for refinement, the structure of the protein that had been determined in a complex with B-GSH (10) was used after removal of the B-GSH and all water molecules. The reflections set aside for use in the calculation of Rfree (18) were equivalent to those used during the refinement of the B-GSH complex. Rigid-body refinement in X-PLOR (19, 20) resulted in lowering the initial Rfactor and Rfree values of 45.1 and 47.1%, respectively (10–3.0 Å), to 32.7 and 32.8%, respectively. Further refinement in X-PLOR was carried out using the slow cool procedure (21) using all data between 7.5 and 1.75 Å and maintaining strict noncrystallographic symmetry (NCS) constraints between the four molecules of the asymmetric unit. No geometrical restraints were applied to the zinc coordination. Rebuilding of the model and inclusion of water molecules were carried out in O (22), using 2Feo – Fe maps averaged with RAVE (23). The glutathione moiety of the NBC-GSH was included.

**Table 1: Data Collection and Refinement Statistics**

<table>
<thead>
<tr>
<th></th>
<th>NCB-GSH</th>
<th>HIPC-GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>space group</td>
<td>P4_1</td>
<td>P2_1</td>
</tr>
<tr>
<td>cell dimensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a = b = 67.3 Å</td>
<td>28–17.2</td>
<td>29–2.0</td>
</tr>
<tr>
<td>c = 167.7 Å</td>
<td>(1.75–1.72)</td>
<td>(2.11–2.0)</td>
</tr>
<tr>
<td>no. of measured reflections</td>
<td>311697</td>
<td>78648</td>
</tr>
<tr>
<td>no. of unique reflections</td>
<td>99.9 (100)</td>
<td>16.4 (4.1)</td>
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<tr>
<td>completeness (%)</td>
<td>93.6 (93.5)</td>
<td>8.9 (3.2)</td>
</tr>
<tr>
<td>resolution range (Å)</td>
<td>27–1.72</td>
<td>29.3–2.0</td>
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<tr>
<td>Rfree (%)</td>
<td>17.4</td>
<td>18.0</td>
</tr>
<tr>
<td>Rsym (%)</td>
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<tr>
<td>no. of protein atoms</td>
<td>5697</td>
<td>2800</td>
</tr>
<tr>
<td>no. of non-protein atoms</td>
<td>1058</td>
<td>256</td>
</tr>
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<td>rmsd from ideal values (Å)</td>
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<td>0.007</td>
</tr>
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<td>bonds (Å)</td>
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<td>planes (Å)</td>
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<tr>
<td>rmsd B values, bonded atoms (Å²)</td>
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<td>1.0</td>
</tr>
<tr>
<td>no. of residues in disallowed regions of Ramachandran plot (%)</td>
<td>0.15</td>
<td>0.0</td>
</tr>
<tr>
<td>average B value (protein) (Å²)</td>
<td>18, 19, 18</td>
<td>26, 26</td>
</tr>
<tr>
<td>average B value (inhibitor) (Å²)</td>
<td>33, 29, 32, 30</td>
<td>21, 21</td>
</tr>
</tbody>
</table>

* A low-resolution sweep was carried out to compensate for detector overloads. Values in parentheses are for the highest-resolution shells. As defined by Engh and Huber (49). Using a stringent boundary as defined by Kleywegt and Jones (50). Values are for molecules A–D of the asymmetric unit, respectively.
at this time. Since the two dimers are situated in similar crystallographic environments (the symmetry operation to move one dimer onto the other involves a 2-fold rotation around the [110] axis and translations of \( \frac{1}{2} \) and \( \frac{1}{4} \) along \( \mathbf{a} \) and \( \mathbf{b} \), respectively), further refinement cycles were carried out with NCS constraints or restraints only between the two dimers and not between the monomers of each dimer. This was first done in X-PLOR with strict NCS constraints between the two dimers and then in REFMAC (24) using all data between 28 and 1.72 Å with these constraints replaced by restraints. During the final cycle, no NCS restraints were applied between molecules. The final coordinates and the structure factors have been deposited in the Protein Data Bank and assigned entry code 1QIP.

S-(N-Hydroxy-N-p-iodophenylcarbamoyl)glutathione. The expression, purification, and crystallization protocols were based on those described previously (10, 15). However, the protein was eluted from the S-hexylglutathione affinity column with HIPC-GSH rather than with S-hexylglutathione, and 2.5 mM HIPC-GSH was added to the protein solution prior to crystallization. The HIPC-GSH was prepared by a method analogous to those described previously (13). Hexane-1,6-diol (3%) was also added to the protein mixture. Though the HIPC-GSH complex crystals were grown under conditions similar to those of the native crystals and had a similar appearance, indexing of the diffraction images showed that they belonged to space group \( \text{P}2_12_12_1 \) and \( \text{P}4_1 \) along \( \mathbf{a} \) and \( \mathbf{b} \), respectively), further refinement cycles were carried out with NCS constraints or restraints only between the two dimers and not between the monomers of each dimer. This was first done in X-PLOR with strict NCS constraints between the two dimers and then in REFMAC (24) using all data between 28 and 1.72 Å with these constraints replaced by restraints. During the final cycle, no NCS restraints were applied between molecules. The final coordinates and the structure factors have been deposited in the Protein Data Bank and assigned entry code 1QIP.

RESULTS

Complex with S-p-Nitrobenzoxycarbonylglutathione

Overall Structure. The refined structure has an \( R \) factor of 17.4% (27–1.72 Å), a corresponding \( R \) free of 21.4%, and reasonable stereochemistry (Table 1). This model contains two protein dimers, four zinc ions, four molecules of NBC-GSH, four molecules of 2-mercaptoethanol covalently linked to each protein monomer at Cys 60, and 906 water molecules.

As a consequence of the pseudosymmetry (see Materials and Methods), the two dimers are more similar to one another than the monomers of each dimer. For residues 8–180, the rms deviation in the corresponding \( C_\alpha \) positions after superposition is 0.2 Å for one dimer compared to the other and 0.4 Å for each monomer compared to the other monomer of the same dimer. Slight differences in the main chain of the molecules occur at the N-terminus where residues 2–7 can only be seen in one molecule of each dimer, in the loops, and at the C-terminus. The three terminal residues are not well defined in any of the molecules. Overall, the structure is very similar to that of the B-GSH complex (10) with the rms deviation in the \( C_\alpha \) positions calculated between this structure and each molecule of the superposed NBC-GSH complex being ~0.3 Å. The largest differences between the structures occur at the C-terminus which, although not well-ordered in either complex, does appear to be affected by the inhibitor.

Inhibitor Binding. In each of the four molecules, the NBC-GSH is clearly defined in the associated electron density (Figure 2A). Superpositioning of the four molecules of the asymmetric unit, based on a least-squares fit of the corresponding \( C_\alpha \) atoms, shows that the position of the NBC-GSH with respect to the protein differs only slightly among the four molecules. The rms deviation of all NBC-GSH atoms lies between 0.4 and 0.8 Å, depending on which molecules are compared. The crystal environment presumably influences this variation, since the carboxylate group of the glycine moiety of the glutathione is involved in crystal contacts in three out of four molecules. Despite these slight differences, the interactions between the glutathione moiety of the inhibitor and the protein dimer to which it is bound are very similar both to each other and to the equivalent moiety in the B-GSH complex. The similarity between the glutathione moiety in the NBC-GSH and B-GSH complexes is particularly apparent when maps averaged over the four molecules of the asymmetric unit are compared. In addition to the conservation of the interactions between the respective inhibitors and the side chains of Arg 37, Asn 103, and Arg 122, the water-mediated hydrogen bond from the amido nitrogen of the cysteine moiety of the NBC-GSH to Thr 101 \( O_\beta \) is also present in both structures (Figure 3). Another conserved water molecule is situated between the carbonyl oxygen of the cysteine moiety and the carboxylate oxygen of the \( \gamma \)-glutamyl moiety.

In none of the four molecules of the asymmetric unit is the S-substituent of the glutathione as well defined in the electron density as the glutathione moiety itself, perhaps reflecting incomplete substitution of the NBC-GSH in addition to some flexibility (Figure 2A). Nevertheless, it can clearly be seen that the carboxyl group of the thioester linkage is not within the inner coordination sphere of the zinc ion but instead is situated approximately 6 Å from the zinc ion with an intervening water molecule. The nitrobenzylcarbonyl group points down into the hydrophobic pocket and interacts with the side chains of Glu 58, Cys 60, Ile 88, and Leu 92. Although the electron density is suggestive of some flexibility of this group, the benzene ring always lies in the same plane. Cys 60 forms a mixed disulfide with 2-mercaptoethanol, which was included in the crystallization mixture. Relative to the B-GSH complex, there are no significant changes in the conformations of the side chains.
in the hydrophobic pocket other than for Met 183, the terminal residue. If this residue were to be modeled unchanged in the B-GSH complex, it would clash with the benzene ring of the NBC-GSH. The small variations that are seen may be due to the higher resolution of the NBC-GSH data and the difference in the refinement protocols.

**Zinc Coordination.** The zinc ion and its respective ligands are very similar among the four molecules of the asymmetric unit. A positional deviation of \(~0.1–0.2\) Å was observed after superposition of the respective molecules, on the basis of a least-squares fit of the corresponding Cα atoms of residues 8–180. The distance from each of the coordinating atoms to the zinc ion is given in Table 2. There are two major differences between the geometry of coordination of the zinc ion in this complex and that seen in the complex with B-GSH (Figure 4A). In the B-GSH structure, the zinc ion has a square pyramidal coordination and is bound by the Oα atoms of Gln 33, Glu 99, and Glu 172, the Nα2 atom of His 126, and a water molecule, with His 126 as the axial ligand (10). In the NBC-GSH complex, there is a second water molecule on the opposite side of the zinc ion from His 126 that is \(~2.6–2.9\) Å from the metal (B-factor \(~20–28\) Å\(^2\) as compared to \(15–25\) Å\(^2\) for the first water molecule). In addition to interacting with the zinc ion, this water molecule is within hydrogen bonding distance of Gln 33 Nα2, Glu 172 Oα2, and the other zinc-bound water molecule (see Figure 4). Although this water molecule is a little far for coordination, the zinc ion has an approximately octahedral arrangement of ligands with an average angle of 94° between any two atoms coordinating the zinc ion.

The second difference between the two complexes is the orientation of the carboxyl functions of the glutamate residues. Relative to the B-GSH complex, Glu 99 and Glu 172 are rotated 85° and 60°, respectively, around their Cα–Cα bonds (see Figure 4A). The other residues are in very similar positions. In two of the four zinc sites of the asymmetric unit, single conformations of Glu 99 and Glu 172 are observed. In the other two, the electron density is
more rounded. It is in fact possible to model a second position of Glu 99 into the electron density with the same conformation as observed in the B-GSH complex. On the other hand, the conformation seen in the NBC-GSH complex is not consistent with the electron density associated with the B-GSH structure. The difference in the orientation of the carboxylates means that the zinc ion lies within 0.2 Å of the planes of the functional groups of each of these two residues. This is much closer than those seen in the B-GSH complex [0.5 Å from Glu 99 and 1.0 Å from Glu 172 (10)].
Complex with S-(N-Hydroxy-N-p-iodophenylcarbamoyl)-glutathione

Overall Structure. The refined structure has an \( R \text{-factor} \) of 18\% (30–2.0 Å) and a corresponding \( R\text{free} \) of 21\%. The model contains one dimer of glyoxalase I (with residues from 8 to 183 of each monomer), two zinc ions, two molecules of HIPC-GSH, and a total of 192 water molecules. Due to the noncrystallographic restraints applied during refinement, the rms deviation in the positions of the corresponding C\(_\alpha\) atoms of all residues between positions 8 and 180 after superposition of the two molecules is very low (0.04 Å). Overall, the structure is similar to those of the B-GSH and NBC-GSH complexes (rms deviation in the positions of the corresponding C\(_\alpha\) atoms after superposition being ~0.4–0.5 Å for all residues between positions 8 and 180). Most of the differences between this and the other structures arise from variations in the crystal environment. There is, however, a small but significant conformational change involving the loop, including residues 152–159 (Figure 5). The loop is situated between the two outermost \( \beta \)-strands (\( \beta \)7 and \( \beta \)8) of the barrel that forms the active site and helps to shield the substrate from the surrounding solvent. Relative to the other structures, the C\(_\alpha\) atom of Lys 156, which is situated at the tip of the loop, has moved ~3 Å toward the active site. This conformational change was observed to occur in both molecules of the dimer when the NBC-GSH model was subjected to a cycle of refinement against the HIPC-GSH complex data using simulated annealing and without non-crystallographic symmetry restraints. Again the positions of the C-terminal residues deviate from those seen in the other structures. The electron density associated with these residues is much better defined than that seen in the other complexes.

**Inhibitor Binding.** In both molecules of the dimer, the HIPC-GSH is extremely well-defined in the electron density (Figure 2B). This compound has a much lower average B value relative to the protein than was observed for the inhibitors in either the B-GSH or the NBC-GSH complex (see Table 1). The \( \gamma \)-glutamyl moiety makes contacts with the protein very similar to those seen in the other structures, and even here, the two water-mediated interactions mentioned above are present (Figure 3). On the other hand, an approximately 15° change in the torsion angle around the bond between the amide nitrogen and C\(_\alpha\) atom of the cysteine residue of the glutathione moiety, together with other smaller changes, causes a shift in the relative positions of the glycine and the S-substituent (Figures 3 and 5). This change, in conjunction with the movement of the loop between \( \beta \)7 and

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**Table 2: Zinc Ligand Distances (Å) in the Glyoxalase I Crystal Structures**

<table>
<thead>
<tr>
<th>Distance</th>
<th>NCB-GSH</th>
<th>HIPC-GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn–Gln 33 O2</td>
<td>2.0</td>
<td>2.1</td>
</tr>
<tr>
<td>Zn–Glu 99 O2</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Zn–His 126 N2</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Zn–Glu 172 O1</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Zn–Wat 1/O</td>
<td>2.1</td>
<td>2.3</td>
</tr>
<tr>
<td>Zn–Wat 2/OH</td>
<td>2.6</td>
<td>2.8</td>
</tr>
</tbody>
</table>

a The four molecules of the asymmetric unit are denoted A–D for the NCB-GSH structure and A and B for the HIPC-GSH structure.
8° toward the active site, brings the carboxylate oxygens of the glycine moiety to within hydrogen bonding distance of the main chain nitrogens of Met 156 and Lys 157 (Figure 3). In the other structures, the distance between the corresponding atoms is approximately 7 Å.

For the S-substituent, the conformational change of the glutathione moiety serves to bring the hydroxycarbamoyl group toward the zinc ion so that the two oxygen atoms are within coordination distance of the metal ion. The p-iodophenyl group is completely shielded from the solvent with the iodine atom situated in a similar position with respect to the center of the benzene ring of bound NBC-GSH (Figure 5). Relative to the other structures, there are a number of side chains that have moved, but these are either on the flexible loop (Met 157 and Leu 160) that packs onto the inhibitor or on the C-terminal helix.

**Zinc Coordination.** The O1 atoms of Gln 33 and Glu 99 and the Nε2 atom of His 126 coordinate the zinc ion (see Figure 4A). However, the distance between the zinc ion and the O1 atom of Glu 172 in this complex is 3.3 Å, which is too far for direct coordination. This increased separation of 1.2 Å, relative to the other structures, is caused partly by a small conformational change of the side chain of Glu 172 and partly by the movement of the two glutamic acid residues away from each other with the zinc ion following Glu 99. The two oxygen atoms of the hydroxycarbomoyl group take the place of the water molecules seen in the NBC-GSH structure, giving the metal a square pyramidal coordination. The mean angle between any two of the atoms coordinated to the zinc ion via the metal is 97°. The ligand–zinc distances are given in Table 2. The amido group of Gln 33, the hydroxycarbamoyl group of the HIPC-GSH, and the Nε2 atom of His 126 lie in a plane with the zinc ion ~0.5 Å below this plane. The rms deviation of the atoms making up the plane from the plane is 0.05 Å. The zinc lies ~0.2 Å out of the plane of the functional group of the axial ligand, Glu 99, which adopts a conformation in which its carboxylate group is rotated by ~16° with respect to that seen in the NBC-GSH complex (see Figure 4B). Gln 33 and His 126 adopt conformations similar to those observed in the other structures.

**DISCUSSION**

**Inhibitor Binding and Conformational Changes.** Over the past three decades, many S-substituted glutathione derivatives have been tested as inhibitors of glyoxalase I. The earliest studies demonstrated that binding is a function of the hydrophobicity of the S-substituent, presumably reflecting the presence of a hydrophobic binding pocket in the active site (30–32). The strongest inhibitor of human erythrocyte glyoxalase I was S-p-bromobenzylglutathione ($K_i = 0.08 \, \mu\text{M}$) (8). More recently, S-(N-hydroxy-N-methylcarbamoyl)-glutathione has been proposed as a transition state analogue mimicking the enediolate intermediate that forms along the reaction pathway of the enzyme. In support of this assertion, the $K_i$ value with yeast glyoxalase I is 30-fold lower than that of S-d-lactoylglutathione and 7-fold lower than the $K_m$ of the glutathione–methylglyoxal thiohemiacetal substrate (33). The enediol analogue is a moderately strong competitive inhibitor of the human enzyme ($K_i = 1.7 \, \mu\text{M}$) (33). The S-(N-aryl-N-hydroxycarbamoyl)glutathione derivatives were subsequently discovered to be much stronger inhibitors, with $K_i$ values that decrease with increasing hydrophobicity of the aryl substituent. The strongest inhibitor that was tested was the p-bromophenyl derivative ($K_i = 0.014 \, \mu\text{M}$) (13).

Fluorescence quenching experiments indicate that binding of glutathione, S-p-bromobenzylglutathione (5), and the S-(N-aryl-N-hydroxycarbamoyl)glutathione derivatives (34) causes a small conformational change in the enzyme. In contrast, binding of the product S-d-lactoylglutathione produces no change in the intrinsic fluorescence of the protein (5, 34). The fluorescence quenching experiments, together with proteolytic susceptibility studies, led Creighton and co-workers to propose the presence of a flexible loop near the active site that closes over the bound enediolate analogue. They further proposed that the loop is stabilized in an open conformation in the presence of bound S-d-lactoylglutathione (34). Paramagnetic nuclear relaxation studies, using the Mn$^{2+}$-substituted enzyme, indicated that the carbonyl and
hydroxyl oxygens of bound S-\textit{d}-lactoylglutathione are in the second coordination sphere of the metal, precluding direct coordination interactions (6, 7).

The recently determined crystal structure of the human enzyme in complex with B-GSH confirmed some of the predictions of the inhibitor binding studies (10). The active site includes a large hydrophobic pocket containing the benzyl function of the bound ligand. In addition, a water molecule was observed between the sulfur atom of the ligand and the metal ion. From a single structure, no information about conformational changes could be gained. However, the specific residues that were proposed to form the flexible loop, on the basis of sequence comparisons with the "catalytic loop" of triosephosphate isomerase, belong to the $\beta$-sheet forming the core of the protein.

A comparison of the crystal structures of the binary complexes of the enzyme and the transition state analogue S-(\textit{N}-hydroxy-\textit{N}-p-iodophenylcarbamoyl)glutathione and product analogue S-p-nitrobenzoxycarbonylglutathione shows that there is an intimate relationship between the nature of the S-substituent and the conformation of the bound ligand. Both ligands have bulky S-substituents, and like the S-substituent and the conformation of the bound ligand.

Zinc Coordination. Previous EXAFS studies with human glyoxalase I (37) and EPR experiments with the Co$^{2+}$- and Cu$^{2+}$-substituted enzymes (38, 39) indicated distorted octahedral coordination geometry for the metal ion. Interestingly, zinc ions in proteins generally have tetrahedral coordination geometries (40). NMR studies of glyoxalase I indicate that two of the zinc ligands are rapidly exchanging water molecules (5). One of these is either immobilized or displaced from the metal ion when S-p-bromobenzylglutathione or the product binds to the enzyme (5). In the X-ray structure of the binary B-GSH–enzyme complex, the zinc ion has a square pyramidal coordination geometry (10). The ligands to the Zn$^{2+}$ include four protein residues (Glu 33, Glu 99, His 126, and Glu 172) and one water molecule. Introduction of an additional water molecule, to form an octahedral coordination field, appears to be precluded by steric interactions with the benzyl group of bound B-GSH. In the NBC-GSH complex, a water molecule does indeed interact with the zinc ion in this position, giving the metal ion an approximately octahedral coordination. The zinc to water distance is, however, larger than expected for a zinc ligand (40, 41). A water molecule a similar distance from the zinc ion was also seen in the crystal structure of the double mutant Q33E/E172Q crystallized with S-hexyl-GSH present in the active site (11). In the HIPC-GSH complex, the oxygen atoms of the hydroxycarbamoyl group of the inhibitor replace the two water molecules. In addition, Glu 172 is displaced from the inner coordination sphere of the metal ion, presumably by a combination of stereochemical and electrostatic effects. Despite these differences, the zinc ion has again a square pyramidal coordination.

On the basis of the comparison of the three structures, it would seem that whereas His 126 and Glu 33 interact tightly with the metal ion in a fixed conformation, the other ligands are reasonably flexible and can move to accommodate the different inhibitors. These results are consistent with an ionic rather than a covalent nature of the interactions between the carboxylates and the zinc ions. EXAFS studies also predicted that the nature of the inhibitor might affect the orientation of two of the zinc ligands (37). Whereas it was inferred from the EXAFS experiment that two imidazole rings would rotate, our results show that it is the two carboxylates bound a significant (80-fold) decrease in binding affinity, while there is little change in affinity when the C-OH group of S-\textit{d}-lactoylglutathione is substituted with C-H (13). Second, ethylation of the glycyl residue of the glutathione moiety decreases the binding affinity of the enediolate analogue, but has little effect on the binding affinity of S-\textit{d}-lactoylglutathione (13). Ethylation of the glycyl residue of the glutathione also affects the binding of S-p-bromobenzylglutathione, suggesting that this compound hydrogen bonds to the closed loop (36). This suggestion is reinforced by the results of the fluorescence experiments, which indicate that the loop closes when S-p-bromobenzylglutathione binds to the enzyme.

Both NBC-GSH and HIPC-GSH have larger S-substituents than S-benzylglutathione. Nevertheless, these bulky groups are easily accommodated in the hydrophobic pocket of the protein with minimal movement of the side chains lining the pocket. Apart from the differences caused by the position of the flexible loop, only the C-terminal helix seems to vary among the three complexes. This helix appears to be fairly mobile.
to the zinc ion that differ in conformation between the structures. What causes the difference, however, is a matter of speculation. On the basis of the out-of-plane distances of the zinc ions from the carboxylate functional groups, it could be argued that the geometry of coordination seen in the NBC-GSH complex is the more favorable. In the B-GSH complex, the carboxylates may have altered their positions to counteract the loss of the water molecule occluded by the binding of the B-GSH.

**Reaction Mechanism.** An enediol proton-transfer mechanism is now generally accepted for glyoxalase I. The fact that the native enzyme depends on Zn$^{2+}$ for activity implies that the metal ion plays an important role in catalysis. On the basis of NMR studies of the binding of the product, S-Dlactoylglutathione, and the apparent substrate analogue S-acetonylglutathione, the metal was proposed not to interact directly with the substrate or intermediate but rather to facilitate catalysis through outer sphere effects (5–7, 42). This is consistent with a report that replacement of Zn$^{2+}$ with V$^{3+}$ or Ga$^{3+}$ results in enzyme species with $k_{cat}$ values that are larger than the ligand exchange rates of these metals in aqueous media (3). The structural results reported here, however, do not support such a mechanism.

In the structure of the enzyme in a complex with HIPC-GSH, the inhibitor is coordinated to the metal ion. HIPC-GSH is a tight binding competitive inhibitor of human glyoxalase I ($K_i = 10$ nM) that mimics the stereoelectronic features of the enediolate reaction intermediate. Its conformation in the structure strongly implies that the reaction proceeds through a metal-coordinated cis-enediolate intermediate. Previously, we proposed a reaction mechanism in which Glu 172, one of the zinc ligands, may act as the base in the reaction (10, 11). The complex with HIPC-GSH provides further support for this mechanism since in this structure Glu 172 is not coordinated to the zinc ion as in the other complexes. Moreover, its O$_2$ carboxylate oxygen is $\sim 3.3$ Å from the C1 and N atoms of the intermediate analogue in an ideal position for the residue to act as the base.

It is well established that glyoxalase I produces D-lactoylgluthathione exclusively from the racemic thiohemiacetal substrate (43). Pulse-chase isotope-trapping studies and NMR measurements indicate that both the S- and R-diastereomers of the substrate are used by the enzyme (44–46). Two different types of mechanisms have been proposed to account for this observation. On one hand, it has been suggested that the enzyme catalyzes the interconversion of the two bound diastereomers before only one of the diastereomers is converted to product (44). On the other hand, others have proposed a mechanism wherein each diastereomer is separately processed to the product via a cis-enediolate intermediate without prior interconversion of the different substrate forms (47). Modeling studies indicate that the configuration of the S-diastereomer of GSH–methylglyoxal thiohemiacetal is ideally suited for abstraction of the C1 proton by Glu 172 to form the cis-enediolate intermediate. Reprotonation of the zinc-bound intermediate by Glu 172 would result in D-lactoylgluthathione. However, it is much harder to model the R-diastereomer so that Glu 172 would be in a position to abstract the proton to form the cis-enediolate. It is tempting to suggest that Glu 99, diametrically opposite Glu 172 in the zinc coordination sphere, could act as the base when the R-diastereomer of the substrate is present in the active site. Though this residue is coordinated
to the metal ion in all the structures, it is flexible and in the complex with HICP-GSH its O$_2$ atom is close to the C1 and N atoms of the intermediate (3.1 and 3.2 Å, respectively). Site-directed mutagenesis studies, though ambiguous, do indicate that the residue may play some role in the reaction mechanism (11). However, its position with respect to the metal-coordinated endiolate intermediate excludes it from reprotonation at C2 since this would result in l-lactoylgluthione. In the above discussion, we have not mentioned the transfer of the hydrogen that is situated on the oxygen at C1 of the substrate (Figure 1) and the oxygen at C2 of the product. With respect to the oxygens of the intermediate, both Glu 172 and Glu 99 are in suitable positions to aid in the transfer.

A simplified reaction mechanism with the S-diastereomer of the substrate would be as follows (Figure 6). Substrate first enters the active site and is anchored by its γ-glutamate moiety. It then swings in toward the zinc ion, displacing the metal-bound water molecules and Glu 172. This interaction presumably stabilizes the closed conformation of the peptide loop over the active site. The glycyl moiety of the substrate is important for transition state stabilization since the value of $k_{cat}/K_m$ for the GSH–methylglyoxal hemithioacetal is 10-fold higher than the value for its glycyl ethyl ester (48). Glu 172 then abstracts the proton from C1, forming a cis-enediolate intermediate that is coordinated to the zinc ion. It should be emphasized that displacement of Glu 172 from the inner coordination sphere of Zn$^{2+}$ during substrate binding should cause a dramatic increase in the $pK_a$ of the carboxyl group. At the same time, direct coordination of the substrate to the metal ion should decrease the $pK_a$ of C1-H. These shifts in $pK_a$ are in the right direction to promote catalysis. Reprotonation at C2 results in the formation of the product, which will then dissociate from the zinc ion. This will cause the flexible loop to open and facilitate the diffusion of the product from the active site. In this mechanism, we have omitted any discussion of the transfer of the proton from the oxygen at C1 to the oxygen at C2 that must occur.

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