Involvement of an Active-site Zn$^{2+}$ Ligand in the Catalytic Mechanism of Human Glyoxalase I*

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The Zn$^{2+}$ ligands glutamate 99 and glutamate 172 in the active site of human glyoxalase I were replaced, each in turn, by glutamines by site-directed mutagenesis to elucidate their potential significance for the catalytic properties of the enzyme. To compensate for the loss of the charged amino acid residue, another of the metal ligands, glutamine 33, was simultaneously mutated into glutamate. The double mutants and the single mutants Q33E, E99Q, and E172Q were expressed in Escherichia coli, purified on an S-hexylglutathione matrix, and characterized. Metal analysis demonstrated that mutant Q33E/E172Q contained 1.0 mol of zinc/mol of enzyme subunit, whereas mutant Q33E/E99Q contained only 0.3 mol of zinc/mol of subunit. No catalytic activity could be detected with the double mutant Q33E/E172Q (<10⁻⁴ of the wild-type activity). The second double mutant Q33E/E99Q had 1.5% of the specific activity of the wild-type enzyme, whereas the values for mutants Q33E and E99Q were 1.3 and 0.1%, respectively; the E172Q mutant had less than 10⁻⁸ times the specific activity of the wild-type.

The crystal structure of the catalytically inactive double mutant Q33E/E172Q demonstrated that Zn$^{2+}$ was bound without any gross changes or perturbations. The results suggest that the metal ligand glutamate 172 is directly involved in the catalytic mechanism of the enzyme, presumably serving as the base that abstracts a proton from the hemithioacetal substrate.

The catalytic mechanism of glyoxalase I has been proposed to involve a base responsible for the abstraction of a proton from carbon 1 of the hemithioacetal moiety of the substrate and deliver it to carbon 2 of the same molecule (9, 10). This shielded proton transfer has support from NMR and other spectroscopic studies. However, in the crystal structure no amino acid residue that could serve as a base is present in the active site close to the expected position of the carbon 1 proton of the substrate, with the exception of the metal ligands glutamate 99, glutamate 172, and the metal-bound water. Glutamate 172 is closest to carbon 1, and based on modeling, the distance could be less than 4 Å (8). In the presence of bound S-benzylglutathione and S-b-lactoylglutathione, only one molecule of water could be detected (9). In agreement with the NMR data, the second water molecule is not seen in the crystal structure of glyoxalase I with S-benzylglutathione as ligand. If present, the coordination of Zn$^{2+}$ would be octahedral (8, 11). The substrate is bound adjacent to the Zn$^{2+}$ via its glutathione moiety with the S-substituent in a hydrophobic pocket.

The possible involvement of glutamate 99 in catalysis cannot be excluded. This residue is positioned 6 Å from the Zn$^{2+}$ upon binding of the substrate the carboxylate would be in a favorable position for abstraction of the proton.

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The atomic coordinates and structure factors (code 1BHG) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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Glyoxalase I (EC 4.4.1.5) catalyzes the formation of S-b-lactoylglutathione from the hemithioacetal formed nonenzymatically from methylglyoxal and reduced glutathione (1–3). Methylglyoxal is produced as a by-product of the triosephosphate isomerase reaction in glycolysis (4). Because this 2-oxoaldehyde readily reacts with proteins and nucleic acids, its inactivation by glyoxalase I is an important detoxication reaction. Glyoxalase I has attracted interest in research on diabetes and cancer because of the toxicity of the 2-oxoaldehyde substrate (2).

Human glyoxalase I is a homodimeric protein of 43 kDa. Its catalytic activity is dependent on Zn$^{2+}$, one metal atom located in each of the two active sites of the enzyme (5). The Zn$^{2+}$ has been replaced by other divalent ions of metals such as magnesium, manganese, cobalt, and copper (3, 6, 7). The replacement with magnesium is the only one that fully restores the high $k_{cat}$ value of the wild-type enzyme.

The two active sites are located at the dimer interface, and residues from both subunits contribute to each of the binding pockets (8). The Zn$^{2+}$ is coordinated to four amino acid residues, two from each subunit (Fig. 1). The crystal structure obtained with S-benzylglutathione as ligand shows a square pyramidal arrangement with glutamine 33, glutamate 99, glutamate 172 and one molecule of water in the plane, and histidine 126 as the axial ligand. NMR studies of the manganese-substituted enzyme showed a second molecule of water to be present in the first coordination sphere of the active-site metal when glutathione derivatives were absent from the active site (9, 10). In the presence of bound S-p-bromobenzylglutathione and S-p-lactoylglutathione, only one molecule of water could be detected (9). In agreement with the NMR data, the second water molecule is not seen in the crystal structure of glyoxalase I with S-benzylglutathione as ligand. If present, the coordination of Zn$^{2+}$ would be octahedral (8, 11). The substrate is bound adjacent to the Zn$^{2+}$ via its glutathione moiety with the S-substituent in a hydrophobic pocket.
The two models were superposed (thin lines; crosses for the zinc and water molecules) that obtained before the release of the noncrystallographic symmetry constraints. The wild-type structure (thick lines; solid spheres for the zinc and water molecules) has previously been published (8). The two models were superposed based on the least squares fit of their respective Cz atoms (residues 30–173). The glutathione derivatives bound (to the left) in the active site were S-benzylglutathione (wild-type) and S-hexylglutathione (Q33E/E172Q mutant).

in catalysis, the residues were replaced sequentially by glutamine using site-directed mutagenesis. To compensate for the loss of the charged residue, a third metal ligand, glutamine 33, was replaced by glutamate. The mutant enzymes were purified and characterized by metal analysis and activity measurements. The crystal structure of the Q33E/E172Q mutant in complex with S-hexylglutathione was determined.

### EXPERIMENTAL PROCEDURES

**Materials**—Enzymes used for PCR and recombinant DNA work were obtained from Boehringer Mannheim (Mannheim, Germany). Oligonucleotides were custom-synthesized by Operon Technologies (Alameda, CA). 5′-d(TTCAATACGGGAGCTTTGGCACT) and whole cDNA for the 3′-end were incorporated as primers, for 25 cycles under standard conditions. The primer pair was digested with HindIII present in the cDNA sequence (underlined in the primer HGIE99Q), ligated, and used as template in the second PCR with the primers pKkFor and GIE2STOP. The cDNA sequences containing the desired mutations were cut with EcoRI and SauI and ligated to the expression vector pN-301 (14). All constructed mutants were sequenced for verification that no unwanted mutations were present.

**Purification**—The mutant forms of glyoxalase I and the wild-type enzyme were purified from 1- to 3-liter cultures of *E. coli* grown in the presence of 1 mM ZnSO₄ at 30 °C as described previously (15). Human glyoxalase I and the mutant variants were purified on S-hexylglutathione affinity gel and eluted with 5 mM S-hexylglutathione in 10 mM Tris, pH 7.8, 0.2 mM diithiothreitol. The purified enzymes were dialyzed against 10 mM Tris-HCl, pH 7.8, 0.1 mM diithiothreitol.

**Characterization**—The relative molecular mass of the purified glyoxalase I mutants was determined on SDS-polyacrylamide gel electrophoresis (12.5% w/v) with Coomassie Brilliant Blue or silver staining for visualization of the protein bands.

Specific activity was measured in a 1-ml reaction with 2 mM methylglyoxal and 2 mM reduced glutathione in 0.1 M sodium phosphate, pH 7.0, at 30 °C. The extinction coefficient used for the formation of the thiolester at 240 nm was 3.370 M⁻¹ cm⁻¹ (16). The enzyme was chromatographed on a PD-10 column prior to measurements of specific activity as well as for the kinetic analysis to remove any S-hexylglutathione remaining from the purification. The earlier estimate of the extinction coefficient (1.68 mg⁻¹ cm⁻¹) (17) was used to determine the protein concentration (15).

**Measurement of steady-state kinetic saturation curves** for Q33E/E99Q, Q33E, and E99Q were made in 1 M sodium phosphate, pH 7.0, at 30 °C. The concentration of free glutathione was kept constant at 0.3 mM. The data were analyzed by nonlinear regression analysis using the SimFit program package (17).

### Analysis of metal content in all of the mutant variants of glyoxalase I (11 metals/sample) was made by inductively coupled plasma atomic emission spectrometry.
The stoichiometry of moles of metal/mol of dimer of enzyme.

| Metal          | Wild-type | Q33E/E99Q | Q33E | E99Q | Q33E/E172Q | E172Q |
|----------------|-----------|-----------|------|------|------------|-------|
| Calcium        | 0.16      | ND        | 0.08 | <0.07| 0.22       | 0.15  |
| Cobalt         | <0.005    | <0.02     | <0.02| <0.05| <0.01      | <0.01 |
| Copper         | <0.02     | 0.04      | 0.03 | <0.03| <0.01      | <0.01 |
| Iron           | 0.73      | 0.33      | 0.91 | 0.25 | <0.04      | <0.03 |
| Magnesium      | 0.02      | <0.02     | <0.03| <0.07| <0.01      | <0.01 |
| Manganese      | 0.04      | <0.01     | <0.03| <0.03| <0.004     | <0.004|
| Zinc           | 2.07      | 0.56      | 1.57 | 0.09 | 2.03       | 0.32  |

*5 From Ref. 15.

Methods of Crystal Structure Analysis—Crystals of the mutant Q33E/E172Q were obtained as described for the wild-type protein (8). X-ray diffraction data were collected at 100 K on a Rigaku R-axis imaging plate mounted on a rotating anode source. The crystals were prepared for freezing by a short soak in an equivalent mother liquor containing 5% (v/v) ethylene glycol. The crystals were of space group P41, as opposed to P42, in which the wild-type enzyme crystallized, but had similar cell dimensions (P41: a = b = 67.3 Å, c = 164.7 Å, P42: a = b = 68.0 Å, c = 169.4 Å). Data were integrated using MOSFLM (18) and scaled and processed using the CCP4 suite of programs (19). The data set extends to a resolution of 2.2 Å, has an R-merge of 4.8% (12.8%), has a redundancy of 3.5 (1.8), and has an R-factor of 25.0% (28–2.2 Å). Anisotropic scaling of the data was used. Because this refinement was carried out in REFMAC (27) after resetting the noncrystallographic symmetry constraints were kept between the 4 subunits but not between the two monomers of each dimer. The coordinates were applied only between the two dimers of the asymmetric unit but not between the two dimers of the asymmetric unit, during the last cycle of refinement, re-

The mutant forms of glyoxalase I were expressed in *E. coli* cultured in the presence of 1 mM ZnSO₄. Affinity for the S-hexylglutathione matrix was retained in all of the mutant proteins, indicating structural integrity of the active site. The amounts obtained from the purification of mutants Q33E, E99Q, E172Q, and the double mutants Q33E/E99Q and Q33E/E172Q were 11, 10, 32, 8, and 45 mg, respectively, from a 3-liter culture. These yields are somewhat lower than the yield of the wild-type enzyme (60 mg/liter).

Structural Properties—DNA sequencing verified that the mutant forms did not contain any unwanted mutations in their sequences. The relative molecular mass of the corresponding purified proteins was estimated by SDS-polyacrylamide gel electrophoresis to 22 kDa/subunit.

Metal analysis of the mutant enzymes revealed a stoichiometry of 1:1 zinc/subunit of the double mutant Q33E/E172Q and 0.8:1/subunit for Q33E (Table II). The second double mutant Q33E/E99Q and the single mutants of the glutamates contained considerably lower amounts of zinc. Ten other divalent metal ions were analyzed by inductively coupled plasma atomic emission spectrometry and were not found to substitute for the Zn²⁺ (Table II, values for aluminum, chromium, molybdenum, and nickel were <0.08 atoms/subunit and are not shown in the table). In all of the analyzed samples the amount of iron and calcium appear to be because of unspecific binding to the protein and presence in the buffer in the different batches.

Catalytic Properties—The specific activities of the mutant variants were significantly lower than that of the wild-type enzyme. The double mutant Q33E/E172Q had no detectable activity with the hemithioacetal adduct of methylglyoxal and glutathione. Based on the sensitivity of the assay method and the amount of enzyme used, any remaining activity was estimated to be less than 3·10⁻⁶ μmol/min·mg⁻¹, which corresponds to a >10⁴-fold decrease in activity compared with 2·340 μmol/min·mg⁻¹ for wild-type human glyoxalase I (Table III). The Q33E/E99Q mutant had a specific activity of 34 μmol/min·mg⁻¹, which corresponds to 1.5% of the wild-type value. The single-point mutants Q33E and E99Q displayed 1.3 and 0.1% of the activity of the wild-type enzyme, respectively. The mutant E172Q had less than 10⁻⁵·times the specific activity of the wild-type enzyme.

The kcat/Km, kcat/Km values for the mutant variants (Q33E, E99Q, and Q33E/E99Q) with detectable activity with the glutathione adduct of methylglyoxal are also given in Table III. The Q33E/E99Q mutant had a specific activity of 34 μmol/min·mg⁻¹, which corresponds to 1.5% of the wild-type value. The single-point mutants Q33E and E99Q displayed 1.3 and 0.1% of the activity of the wild-type enzyme, respectively. The mutant E172Q had less than 10⁻⁵·times the specific activity of the wild-type enzyme.

The kcat/Km, kcat/Km values for the mutant variants (Q33E, E99Q, and Q33E/E99Q) with detectable activity with the glutathione adduct of methylglyoxal are also given in Table III. The Kₘ values determined for all of these mutants are 3–4-fold higher than for the wild-type enzyme. The kcat values range between 0.1 and 2% compared with the wild-type. Hence, the calculated kcat/Km values are in a range of 100–2500 times lower than for the wild-type. The estimated kcat/Km values of the double mutant Q33E/E99Q and the single mutant Q33E are similar, although their Zn²⁺ contents differ by a factor of 1.3.

Structure Determination—The structure of the catalytically inactive Q33E/E172Q mutant was solved using molecular replacement and refined to a resolution of 2.2 Å. The refined model has an R-factor of 18.8% based on all data between 28 and 2.2 Å and a corresponding R-free of 25.4% (see “Experimental Procedures”). It includes all residues from 2 to 183 of the protein and presence in the different batches.

Expression and Characterization of Mutant Glyoxalase I—The mutant forms of glyoxalase I were expressed in *E. coli* cultured in the presence of 1 mM ZnSO₄. Affinity for the S-hexylglutathione matrix was retained in all of the mutant proteins, indicating structural integrity of the active site. The amounts obtained from the purification of mutants Q33E, E99Q, E172Q, and the double mutants Q33E/E99Q and Q33E/E172Q were 11, 10, 32, 8, and 45 mg, respectively, from a 3-liter culture. These yields are somewhat lower than the yield of the wild-type enzyme (60 mg/liter).

Structural Properties—DNA sequencing verified that the mutant forms did not contain any unwanted mutations in their sequences. The relative molecular mass of the corresponding

*2 M. Richardström, A. D. Cameron, T. A. Jones, and B. Mannervik, unpublished data.*
Specific activities and kinetic constants determined for human glyoxalase I and the mutant variants

Measurements were carried out at 30 °C in 1 ml of 0.1 M sodium phosphate, pH 7.0. The concentration of free glutathione was fixed at 0.3 mM. The $k_{cat}$ values are calculated per dimer (43 kDa). The values in parentheses correspond to the relative value compared with the wild-type. The data were fitted by nonlinear regression analysis. The determined stoichiometry of zinc per protein dimer (43 kDa) is given for the glyoxalase I variants.

| Glyoxalase I | Specific activity | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ | $[Zn^{2+}]/[dimer]$ |
|-------------|------------------|----------|-------|--------------|------------------|
| Wild-type   | $2340 \pm 1$ | $1500 \pm 30$ | $66 \pm 5$ | $2.3 \cdot 10^5$ | 2.0 |
| Q33E/E99Q  | 34 (0.01) | 36 ± 2 | 236 ± 22 | $1.5 \cdot 10^5$ | 0.6 |
| Q33E       | 30 (0.01) | 34 ± 4 | 175 ± 47 | $1.9 \cdot 10^5$ | 1.6 |
| E99Q       | 3 (0.001) | 2 ± 0.2 | 219 ± 40 | $9.1 \cdot 10^3$ | 0.1 |
| Q33E/E172Q | (<10$^{-8}$) | | | | 2.0 |
| E172Q      | (<10$^{-5}$) | | | | 0.3 |

*The data for the wild-type enzyme are from Ref. 38.

![Fig. 2. Structure of the zinc site in the Q33E/E172Q mutant of human glyoxalase I](image)

The electron density is of a 2$F_o - F_c$ map calculated using phases derived from this model but without the inclusion of water 1 and then subjected to 10 cycles of averaging (contoured at 1σ). There is little electron density associated with W1, but a water molecule can be located at all four sites in the unaveraged maps. The figure shows the model before the release of the noncrystallographic symmetry constraints.

**DISCUSSION**

The isomerization reaction catalyzed by glyoxalase I is believed to involve a base responsible for the abstraction of a proton from carbon 1 and its delivery to carbon 2 of the substrate. However, in the crystal structure of the active site of human glyoxalase I, no residue that could act as a base seems to be present in close proximity to the chemical groups undergoing reaction. The strongest candidate seems to be one of the ligands to the active-site zinc ion, glutamate 172, which is situated close to the expected position of the carbon 1 atom of the substrate. It is also possible that another of the zinc ligands, glutamate 99, could be involved in the reaction mechanism. Based on the out of plane distance of the zinc ion from the carboxylates of these 2 residues, glutamate 172 and glutamate 99 may be more weakly bound to the zinc ion than are the other two ligands (8) and therefore could dissociate from the zinc ion during the reaction.
To investigate the possible contribution of glutamate 172 to the reaction mechanism, the residue was changed into the isosteric amino acid glutamine by site-directed mutagenesis. Because the affinity for the zinc ion would be expected to become severely reduced by the removal of the charged residues, glutamine 33 was simultaneously replaced by glutamate.

Both the double mutant Q33E/E172Q and the single mutant E172Q were drastically impaired in their catalytic functions. The double mutant Q33E/E172Q has no detectable activity (>10^6-fold lower than the wild-type, Table III). Its metal-binding site is fully occupied by zinc, as determined by chemical analysis. The crystal structure of the mutant in complex with S-hexylglutathione shows the zinc to be bound and there to be only minor changes of the zinc-binding site (Fig. 1). The most significant differences appear to arise from the substitution of glutamine 33 with a glutamic acid. In the wild-type structure this residue is hydrogen-bonded to glutamine 32. In the mutant, however, because this hydrogen bond cannot occur, glutamine 32 is displaced. Instead it hydrogen bonds to a water molecule, which in turn hydrogen bonds to the NE2 atom of glutamine 172. In the wild-type structure the position of this water molecule is occupied by the side chain of leucine 174, and glutamate 172 is in a completely hydrophobic environment. In water molecule is occupied by the side chain of leucine 174, and glutamine 172. In the wild-type structure the position of this water molecule is occupied by the side chain of leucine 174, and glutamate 172 is in a completely hydrophobic environment. 

Regardless of the reason for the very slight differences in activity of the two mutants, there is a drastic drop in activity when the carboxylate is removed. Other investigators have shown that on replacement of catalytically important amino acid residues, the mutant proteins can retain detectable activity. For example, the replacement of serine (to alanine) in the catalytic triad of trypsin results in a reduced catalysis by a factor of 2.5*10^-5 (30). This taken together with the approximate conservation of structure between the wild-type and mutant enzymes strongly suggests that glutamate 172 is acting as the base in catalysis (Fig. 3). The exact mechanism is not clear. The currently accepted reaction mechanism is one where the enediol intermediate does not bind to the zinc ion. Based on the structure, however, it is also possible to propose a reaction mechanism where the intermediate is coordinated to the metal. One of the arguments that has been used against this scenario is that the enzyme is still active when the zinc ion is replaced by either Ga^3+ or V^5+, which are relatively inert to ligand exchange (3). However, this argument may no longer be valid, because ligand exchange may be necessary whether the reaction intermediate binds to the zinc ion or not. First, glutamate 172 must dissociate from the zinc to abstract the proton from the substrate, and second, to model the C1 atom of the substrate sufficiently close to the carboxylate of glutamate 172 may also require the removal of the zinc-coordinated water molecule seen in the wild-type structure. Therefore, whether the enediol intermediate does bind to the zinc ion is still an open question.

Based on the metal analysis, the mutations introduced in E99Q and Q33E/E99Q seem to distort the zinc-binding site (Table II). Why the double mutant involving glutamate 99 should disrupt the zinc-binding site more than the corresponding Q33E/E172Q mutant is not clear. In the wild-type structure in complex with S-benzylglutathione, neither glutamate 99 nor glutamate 172 are involved in hydrogen bonding networks. It is possible that in the Q33E/E99Q double mutant the hydrogen bonding pattern involving glutamine 33 and glutamate 172 seen in the Q33E/E172Q mutant is retained. This would mean that the Q33E/E99Q double mutation would affect three of the four zine ligands. The double mutant Q33E/E99Q has 1% of the wild-type enzyme activity, showing that although impaired the enzyme is still catalytically competent (>10^6 times the estimate for Q33E/E172Q), which supports the suggestion that glutamate 99 is not the base responsible for the abstraction of the C1 hydrogen. Structural information of this mutant would be needed for explanation of the loss of metal and activity. However, no crystals have yet been obtained.

The single mutant Q33E has lower activity than expected, either because the absence of the glutamate results in a decreased zinc content. Because this residue is far from the position of the C1 hydrogen, it is pre-
sumed that it cannot be involved directly in the reaction. Moreover, in most glyoxalase I amino acid sequences the structurally equivalent residue is histidine. The low specific activity of mutant Q33E might be a result of an additional negative charge introduced by glutamate 33 in the active-site cavity or may be a result of the altered hydrogen bonding network described previously.

The involvement of glutamate 172 in catalysis needs further clarification by structural information with substrate analogues and this is in progress. However, the present results show that glutamate 172 is a strong candidate as the proton abstracting base in the catalytic mechanism of glyoxalase I.

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