Yeast Glyoxalase I Is a Monomeric Enzyme with Two Active Sites*

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The tertiary structure of the monomeric yeast glyoxalase I has been modeled based on the crystal structure of the dimeric human glyoxalase I and a sequence alignment of the two enzymes. The model suggests that yeast glyoxalase I has two active sites contained in a single polypeptide. To investigate this, a recombinant expression clone of yeast glyoxalase I was constructed for over-production of the enzyme in Escherichia coli. Each putative active site was inactivated by site-directed mutagenesis. According to the alignment, glutamate 169 and glutamate 318 in yeast glyoxalase I correspond to glutamate 172 in human glyoxalase I, a Zn(II) ligand and proposed general base in the catalytic mechanism. The residues were each replaced by glutamine and a double mutant containing both mutations was also constructed. Steady-state kinetics and metal analyses of the recombinant enzymes corroborate that yeast glyoxalase I has two functional active sites. The activities of the catalytic sites seem to be somewhat different. The metal ions bound in the active sites are probably one Fe(II) and one Zn(II), but Mn(II) may replace Zn(II). Yeast glyoxalase I appears to be one of the few enzymes that are present as a single polypeptide with two active sites that catalyze the same reaction.

Glyoxalase I and glyoxalase II are the two enzymes of the glyoxalase system. Glyoxalase I is an isomerase that catalyzes the formation of S-lactoylglutathione from the hemimercaptoalde-
Two Active Sites in Yeast Glyoxalase I

X-100, 0.1 mg of nuclease-free bovine serum albumin/ml, 0.8 μM of each primer, 125 μM dNTP, 125 μM MgCl₂, and 2.5 units of Pfu DNA polymerase (Stratagene). The reaction was incubated at 94 °C for 7 min and then 35 cycles were carried out with denaturation at 95 °C for 30 s, annealing at 50 °C for 45 s, and elongation at 72 °C for 1 min 30 s. The PCR product was digested with PstI and XhoI and ligated to the PstI and SalI restriction sites of the pGEM-3z vector (Promega Corp.).

Construction of the Wild-type Expression Clone—To eliminate an internal EcoRI site a silent mutation was introduced using PCR. Here, the gene was amplified in two pieces, changing the EcoRI site with internal primers before these two pieces were put together in a final PCR. The pGEM vector primers M13CCV (5′-AACAGCTATGACCATG-3′) and M13FOR (5′-CACCAGGTITTCCTCCAGTCGACG-3′) were used in combination with the primers 5′-CCACCCCGTCTTTAGAATTCCCTACACCCAGAAG-3′ and 5′-CATATTGTAACCATCTAAAGACCGGGTTGG-3′, respectively, to produce the two mutated fragments. By changing one base pair (underlined) the EcoRI site was eliminated (bold). Equal amounts of these fragments together with primer 1 and primer 2 (above) were used to amplify the final mutated full-length DNA. The PCR reactions were the same as above except for the template, where now ~10 ng of pGEM-3z vector carrying DNA encoding for yeast glyoxalase I was used in the first PCRs, and about 70 ng of each fragment was used in the final PCR. Twenty-five cycles were carried out with denaturation at 95 °C for 2 min, annealing at 62 °C for 1 min, and elongation at 72 °C for 2 min. The final PCR product was digested with EcoRI and PstI and ligated into the pGEM-3z vector to give the construct pGYGlxI. The DNA encoding yeast glyoxalase I was then subcloned into the EcoRI and PstI sites of the expression vector pKK-D (12) which is a modified pKK223-3 vector (Amersham Pharmacia Biotech). This construct was named pKYGlxI.

Construction of Expression Clones of the Mutants E163Q, E318Q, and E163Q/E318Q—The DNA sequences encoding the mutants E163Q and E318Q were constructed using inverted PCR with pGYGlxI as template. For the E163Q mutant the 5′-phosphorylated primers 5′-GGATACTGATTTCCAGTCGACG-3′ and 5′-ATCAGGTCTAAGCAAAGCCATGT-3′ were used in a PCR run under similar conditions as described above. Twenty-five cycles were run with denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and elongation at 72 °C for 11 min. The E318Q mutant was mutated using the 5′-phosphorylated primers 5′-GGTTATTCCATTCAGGTCGTTCCT-3′ and 5′-ATCAGGTCTAAGCAAAGCCATGT-3′. The PCR products were blunt-end ligated at room temperature to give the constructs pGE163Q and pGE318Q. To construct the double mutant E163Q/E318Q, both pGE163Q and pGE318Q were digested with EcoRI and XhoI, an internal restriction site of the yeast glyoxalase I gene. The fragments were combined and ligated at room temperature in such a way that both mutations were present in the construct pGE163Q/E318Q. The DNA sequences encoding the three mutants were subcloned into the EcoRI and PstI sites of the expression vector pKK-D to give the expression clones pKE163Q, pKE318Q, and pKE163Q/E318Q.

Heterologous Expression and Purification of Recombinant Wild-type Yeast Glyoxalase I and the Mutants—Medium containing 16% (w/v) tryptone, 10% (w/v) yeast extract, and 5% (w/v) NaCl supplied with 50 μg of ampicillin/ml was used for expression. A 3-liter culture of bacteria harvested by centrifugation at 2,500 × g for 10 min, resuspended in 10 mM Tris/HCl, pH 7.8, containing 0.1 mM dithiothreitol (buffer A), sonicated for 3 × 20 s and centrifuged at 25,000 × g for 10 min. The cells were again resuspended in buffer A, sonicated, and centrifuged. The lysates were pooled and DNase I was added before a final centrifugation at 25,000 × g for 60 min. The lysate was applied to an S-hexylglutathione affinity matrix (2 cm × 2 cm) equilibrated with buffer A. The column was first washed with buffer A supplemented with 0.2 mM NaCl and then with 5 mM glutathione in 10 mM Tris/HCl, pH 7.8. The enzyme was eluted with 3 mM S-hexylglutathione and 5 mM glutathione in 10 mM Tris/HCl, pH 7.8 (7). To purify the E163Q/E318Q and E163Q mutants the same protocol as above was followed. The E318Q mutant was also purified like the wild-type with the addition of a washing step with 20 mM glutathione in 10 mM Tris/HCl, pH 7.8. To check that the proteins were pure, sodium dodecyl sulfate-polyacrylamide (12.5% w/v) gel electrophoresis was run and the gel was stained with Coomassie Brilliant Blue to visualize the protein band.

Kinetic Studies—Steady-state kinetic measurements were conducted by varying the adduct concentration at a constant concentration of free glutathione (0.1 mM) in 0.1 M sodium phosphate buffer, pH 7.0, at 30 °C, and monitoring the formation of S-n-lactoylglutathione spectrophotometrically at 240 nm using an extinction coefficient of 3.37 mM⁻¹ cm⁻¹ (13). The enzyme concentration was 0.1–0.5 μM in the cuvette, except in experiments with the E163Q/E318Q double mutant where the concentration was 0.5 μM. The equilibrium constant used to calculate the concentration of hemimercaptal adduct from methylglyoxal and glutathione (Mn(II), Co(II)) added to the bacterial growth medium. Cells were harvested by centrifugation at 2,500 × g for 10 min, resuspended in 10 mM Tris/HCl, pH 7.8, containing 0.1 mM dithiothreitol (buffer A), sonicated for 3 × 20 s and centrifuged at 25,000 × g for 10 min. The cells were again resuspended in buffer A, sonicated, and centrifuged. The lysates were pooled and DNase I was added before a final centrifugation at 25,000 × g for 60 min. The lysate was applied to an S-hexylglutathione affinity matrix (2 cm × 2 cm) equilibrated with buffer A. The column was first washed with buffer A supplemented with 0.2 mM NaCl and then with 5 mM glutathione in 10 mM Tris/HCl, pH 7.8. To purify the E163Q/E318Q and E163Q mutants the same protocol as above was followed. The E318Q mutant was also purified like the wild-type with the addition of a washing step with 20 mM glutathione in 10 mM Tris/HCl, pH 7.8. To check that the proteins were pure, sodium dodecyl sulfate-polyacrylamide (12.5% w/v) gel electrophoresis was run and the gel was stained with Coomassie Brilliant Blue to visualize the protein band.
thione was 3.0 mM (14). Inhibition studies with S-hexylglutathione were performed by varying the methylglyoxal-glutathione adduct concentration and the inhibitor concentration at a free glutathione concentration of 0.1 mM. The kinetic data were analyzed using the Prism 2.0 program (GraphPad, San Diego, CA) or the SIMFIT program (15).

Metal Analysis—The amount of different metals in the pure enzymes was determined by Dr. Jean Pettersson (Department of Analytical Chemistry, Uppsala University) using Inductively Coupled Plasma-Emission Spectrometry.

Modeling of Yeast Glyoxalase I—A structural model was built using InsightII, Modeler, and Discover (Molecular Simulations Inc., San Diego, CA). The sequence alignment made by Ridderstrom and Mannervik (9) was used as input. The coordinates of human glyoxalase I (5) were obtained from the Protein Data Bank (16) and used to structurally align the enzymes. A combined molecular dynamics and energy minimization run was performed to yield the final structure. The pictures were made using Molscript (17) and Raster 3D (18).

RESULTS

Homology Modeling—The modeling of yeast glyoxalase I was based on a primary structure alignment of human and yeast glyoxalase I (9), and the crystal structure of human glyoxalase I (5). The sequence identity and similarity were found to be ~44 and 64%, respectively. The matching segments allowed the building of a model of the tertiary structure of yeast glyoxalase I (Fig. 2) that is very similar to the crystal structure of the human enzyme. However, the human glyoxalase I dimer consists of 372 amino acid residues and is larger than the 326-amino acid yeast enzyme. Consequently, there are gaps in the sequence alignment that are reflected in the model. For example, residues B8 to B30 and B76 to B86 of the human enzyme form extra loops compared with the model of yeast glyoxalase I. Residues 174 to 183 in the monomeric yeast glyoxalase I connect the two sequences that correspond to the two subunits of the human enzyme. The superposition of the crystal structure of human glyoxalase I with the model of the yeast enzyme gives a root mean square deviation of 1.75 Å, when 279 amino acids of yeast glyoxalase I are included. The value was calculated using the program O (19).

According to the modeled structure it is clearly possible that yeast glyoxalase I has two active sites (Fig. 2). Both putative active sites have several amino acid residues in common with the human glyoxalase I active site and can thus be assumed to function according to a similar mechanism. Residues His-25, Glu-89, Glu-269, and Glu-318 build up one putative metal-binding site. Residues His-185, Glu-242, His-117, and Glu-163 form another one. His-25, Glu-89, and Glu-318 in the first and His-185, Glu-242, and Glu-163 in the second site occupy three corners of the respective square in the two pyramidal binding sites. His-269 and His-117 in turn are the axial ligands (Fig. 3, A and B). The backbone peptide bonds of Lys-156 and Met-157 in human glyoxalase I probably donate hydrogen bonds to the glycol carboxylate of the substrate, but the residues do not play a direct role in catalysis (20, 21). Corresponding residues are found in the yeast enzyme; Lys-156 in human glyoxalase I corresponds to Arg-302 in the C-terminal half of yeast glyoxalase I and to Arg-147 in the N-terminal half. Met-157 corresponds to Met-303 in the C-terminal half and Glu-148 in the N-terminal half. Replacing Met-157 with glutamine in human glyoxalase I results in a fully functional enzyme (21).

Cloning and Sequencing of Yeast Glyoxalase I—Sequencing revealed that the DNA coding for yeast glyoxalase I amplified from the genomic DNA differed in five positions compared with the previously published sequence (6). This resulted in three changes in the deduced amino acid sequence. The changes are presented in Table I. None of the variant residues affect the putative binding sites of metal but the His-322→Tyr mutation may affect the binding of the glutathione moiety in one of the active sites. Amplification of yeast glyoxalase I DNA from genomic DNA preparations was performed twice and the five mutations were present in both clones. Hence, it is unlikely that the differences resulted from artifacts that arose in the PCR amplifications.

Expression of Recombinant Wild-type and Mutant Yeast Glyoxalase I—Purification of recombinant wild-type enzyme resulted in 6–20 mg of protein from a 3-liter culture. Purification of the three mutants E163Q, E318Q, and E163Q/E318Q each yielded less protein than the wild-type. Based on activity measurements it can be concluded that the different yields were due to differences in the expression levels and not to differences in binding to the affinity matrix. The similar affinities for the S-hexylglutathione matrix and the stoichiometries of metal binding (see below) indicate that the physical properties of the mutants are similar to those of the wild-type enzyme. The extinction coefficient was determined as 1.4 ml mg⁻¹ cm⁻¹ at 280 nm by amino acid analysis of the wild-type and the mutants. Pure wild-type glyoxalase I was frozen at ~80 °C, where it was found to be stable. When stored on ice, the wild-type slowly lost activity with a half-life of ~6 days. The mutants, on the other hand, were subjected to kinetic analyses immediately after the purification since they lost activity somewhat more rapidly. Their half-lives when stored on ice were 2 to 3 days.

Kinetic Properties—Steady-state kinetic constants for wild-

### Table I

Differences found in the DNA encoding yeast glyoxalase I as compared to the sequence previously published (6)

| Position | Triplet with changed nucleotide | Deduced amino acid |
|----------|---------------------------------|--------------------|
| 106/108  | ACC → GCT                     | Thr → Ala          |
| 467      | GGC → GAC                     | Gly → Asp          |
| 822      | TGT → TGC                     | Cys                |
| 964      | CAT → TAT                     | His → Tyr          |

**FIG. 3.** Close-up of the two putative metal-binding sites of yeast glyoxalase I.
Two Active Sites in Yeast Glyoxalase I

The concentration of free glutathione was maintained at 0.1 mM for all measurements. The adduct concentration was varied between 0.02 and 2.6 mM. The reaction temperature was 30 °C.

The double mutant has a very low $k_{\text{cat}}$ value as compared with the wild-type and the single mutants. Thus, it can be concluded that the mutated active site in E163Q/E318Q and E163Q/E318Q displayed an increased zinc content as compared with the other enzymes.

**DISCUSSION**

Yeast glyoxalase I is a monomeric enzyme (7). However, it has not been clear how many active sites it possesses. In a previous study one zinc per enzyme molecule was found, and yeast glyoxalase I was thus regarded as having one active site (8). A recent sequence alignment (9) with the dimeric human glyoxalase I showed that the N-terminal part of yeast glyoxalase I is homologous to the C-terminal part. This suggested the possibility of two active sites in the monomeric yeast glyoxalase I.

Residue 172 in the human enzyme, a glutamate, is proposed to act as the base in the catalyzed reaction (20, 24). The corresponding amino acid residues in the two portions of yeast glyoxalase I are Glu-163 and Glu-318. Therefore, Glu-163 and Glu-318 were mutated into glutamines to substantially decrease the activity of each potential active site (Table II). The mutations might cause a loss of the metal ion in the active sites and thus result in unwanted structural changes. In human glyoxalase I it was possible to make a compensatory mutation of a glutamine into a glutamate in the active site to counterbalance the loss of charge and thus maintain the zinc (24). Since apparently no glutamates or asparagines (only histidines and glutamates) coordinate the zinc in the yeast enzyme it was not possible to accomplish such a charge balance. However, the metal analyses (Table III) show that the mutants still bind two metal ions stoichiometrically. Also, it was possible to purify the mutants on an S-hexylglutathione column suggesting that the mutated enzymes still bind and largely retain the binding characteristics of the active site and the tertiary structure.

The double mutant has a very low $k_{\text{cat}}$ value as compared with the wild-type and the single mutants. Thus, it can be concluded that the mutated active site in E163Q and E318Q, respectively, do not contribute significantly to the $k_{\text{cat}}$ and $k_{\text{cat}}/K_{m}$ values of the respective mutants. This, and the fact that the double mutant, but not the single mutants, has such a low activity, strongly suggest that yeast glyoxalase I has two cat-

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### Table II

| Recombinant yeast glyoxalase I | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ | $K_i^a$ |
|-------------------------------|----------------|-----|------------------|-------|
| | s$^{-1}$ | mM | mM$^{-1}$ s$^{-1}$ | μM |
| Wild-type$^b$ | 1,700 ± 100 | 0.41 ± 0.04 | 4,200 ± 200 | |
| Wild-type$^c$ | 1,100 ± 100 | 0.37 ± 0.05 | 2,900 ± 200 | |
| Wild-type$^d$ | 1,600 ± 50 | 0.45 ± 0.05 | 3,500 ± 300 | 15 ± 4 |
| Wild-type$^e$ | 2,700 ± 100 | 0.24 ± 0.03 | 11,000 ± 1000 | |
| E163Q$^f$ | 990 ± 50 | 0.30 ± 0.05 | 3,300 ± 400 | 27 ± 5 |
| E318Q$^g$ | 280 ± 20 | 0.24 ± 0.05 | 1,200 ± 200 | 32 ± 10 |
| E163Q/E318Q$^h$ | 0.31 ± 0.01 | 0.14 ± 0.03 | 2.3 ± 0.4 |
| Native enzyme | 1,817$^i$ | 0.52$^i$ | 700$^i$ | 0.32 ± 0.02$^i$ |

$^a$ $K_i$ values for S-hexylglutathione were determined by varying methylglyoxal-glutathione adduct and S-hexylglutathione concentrations at a constant free glutathione concentration of 0.1 mM.

$^b$ Prepared without addition of metals to the growth medium.

$^c$ Prepared with addition of 1 mM Mn(II) to the growth medium.

$^d$ Prepared with addition of 1 mM Mn(II) to the growth medium.

$^e$ Prepared with addition of 1 mM Mn(II) to the growth medium.

$^f$ Prepared with addition of 1 mM Co(II) to the growth medium.

$^g$ Prepared with addition of 1 mM Co(II) to the growth medium.

$^h$ Prepared with addition of 1 mM Co(II) to the growth medium.

$^i$ Prepared without addition of metals to the growth medium and purified with Chelex-treated buffers.

$^j$ Prepared without addition of metals to the growth medium.

$^k$ Prepared without addition of metals to the growth medium.

$^l$ Prepared with addition of 1 mM Mn(II) to the growth medium.

$^m$ Prepared with addition of 1 mM Mn(II) to the growth medium.

$^n$ From Ref. 22. Free glutathione concentration maintained at 2.0 mM.

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### Table III

| Recombinant yeast glyoxalase I | Enzyme concentration$^a$ | Fe | Zn | Mn | Total metal content |
|-------------------------------|----------------|----|---|---|------------------|
| | μM | | | | |
| Wild-type$^b$ | 43 | 0.8 | 1.0 | 0.05 | 1.9 |
| Wild-type$^c$ | 19 | 1.2 | 0.3 | 0.3 | 1.8 |
| Wild-type$^d$ | 14 | 0.9 | 0.2 | 0.9 | 2.0 |
| Wild-type$^e$ | 7 | <0.1 | 1.3 | 0.08 | 2.9$^a$ |
| E163Q$^f$ | 31 | 0.8 | 0.2 | 0.6 | 1.6 |
| E318Q$^g$ | 12 | 0.8 | 0.9 | <0.1 | 1.8 |
| E163Q/E318Q$^h$ | 11 | 0.3 | 1.4 | 0.2 | 1.9 |

$^a$ The enzyme concentration of the sample used in the metal analysis.

$^b$ Prepared without addition of metals to the growth medium.

$^c$ Prepared with addition of 1 mM Mn(II) to the growth medium.

$^d$ Prepared with addition of 1 mM Mn(II) to the growth medium.

$^e$ Prepared without addition of metals to the growth medium and purified with Chelex-treated buffers.

$^f$ Prepared with addition of 1 mM Co(II) to the growth medium.

$^g$ Prepared with addition of 1 mM Co(II) to the growth medium; The Co content was determined as 1.5 per enzyme molecule, which is included in the total metal content.

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The kinetic parameters of yeast glyoxalase I with the adduct between methylglyoxal and glutathione as substrate are presented in Table II. The kinetic data were well described by the Michaelis-Menten equation and the data did not give evidence for a more complex rate behavior that could have indicated cooperativity. The previously published $k_{\text{cat}}$ and $K_m$ values for native yeast glyoxalase I (22, 23) are in the same range as those determined for wild-type recombinant enzyme in the present study. The E318Q mutant clearly is less active than the wild-type. The E163Q mutant also appears to be less active, having a $k_{\text{cat}}$ value that is almost 4 orders of magnitude lower than that of the wild-type. The corresponding amino acid residues in the two portions of yeast glyoxalase I (E172Q) had an activity of $\sim 10^{-5}$ times that of the wild-type (24). The affinity for S-hexylglutathione appears to be slightly higher for the wild-type than for the two single mutants as shown by the $K_i$ values.

**Metal Contents**—All enzyme variants were found to have approximately two metals per enzyme molecule (Table III). The wild-type, E163Q, and E318Q contain at least one iron per enzyme molecule. In the wild-type, the second metal is zinc or manganese, apparently dependent on the growth conditions. Inside the living cell, the second metal could even be an additional iron; when wild-type glyoxalase I was purified with Chelex-treated buffers, the enzyme displayed an increased iron content. Only if the wild-type was expressed in medium supplemented with Co(II), no iron was detected by metal analysis, but instead cobalt. The second metal in E163Q seems to be manganese with some zinc, while E318Q contains only zinc as second metal. E163Q/E318Q displayed an increased zinc content as compared with the other enzymes.
alytically competent active sites. According to the kinetic parameters in Table II, the active site with His-25, Glu-89, His-269, and Glu-318 contributes about 75% to the total activity, while the active site with His-185, Glu-242, His-117, and Glu-163 contributes some 25%. The activities of the two single mutants do not completely account for all of the activity of the wild-type. However, the nature of the mutation of one site may influence the catalytic function of the other. Also, the differences in metal substitutions of the two single mutants could affect their respective activities. Hence, the kinetic properties of the mutants are not necessarily additive.

The nature of the metal ions utilized by yeast glyoxalase I is not completely clear. It seems as if a number of bivalent ions in different combinations can be used, and that these give catalytically competent enzymes with similar activities (Table II and Table III). It is well established that mammalian glyoxalase I is active with a variety of bivalent metal ions (25, 26). In most cases the yeast enzyme shows a 1:1 ratio between one metal on the one hand, and one or two metals on the other (Table III). This suggests a preference for a distinct type of metal distinguishing the two somewhat different active sites. However, it cannot be ruled out that each active site uses alternative metals, even in the same enzyme preparation. Iron, most probably present as Fe(II), clearly binds to one of the metal-binding sites in wild-type yeast glyoxalase I. The other site seems to be occupied by a Zn(II) ion. Zinc was also found in the previous analysis (8). Nevertheless, Mn(II) appears to function equally well and may be preferred if present in the growth medium. Also Co(II) may be utilized and gives an enzyme with a slightly increased $k_{cat}/K_m$ value. However, the availability of cobalt under "natural" conditions is probably limited.

Yeast glyoxalase I is one of few enzymes that have two functionally similar and independent active sites per one polypeptide. Other examples are protein-disulfide isomerase (27) and isomaltase (28, 29) from different sources. Also, carbonic anhydrase from the red alga Porphyridium purpureum has two active sites (30). Interestingly both active sites of the latter enzyme contain zinc, and, similarly to yeast glyoxalase I, the enzyme seems to have evolved by gene duplication and subsequent gene fusion. Although it is not common, there are several possible advantages of having two active sites in one monomer. Cooperativity could be evolved, and it would also be possible to acquire a totally new function in one of the active sites, if necessary. One could also imagine that the enzyme has as yet undiscovered functions in vivo and that the active sites actually have different functions. However, due to the high structural similarity of the two active sites in yeast glyoxalase I, it is most likely that they have evolved to catalyze the same isomerization reaction.

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