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Accelerated Publications

Active Monomeric and Dimeric Forms of *Pseudomonas putida* Glyoxalase I: Evidence for 3D Domain Swapping†

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ABSTRACT: 3D domain swapping of proteins involves the interconversion of a monomer containing a single domain−domain interface and a 2-fold symmetrical dimer containing two equivalent intermolecular interfaces. Human glyoxalase I has the structure of a domain-swapped dimer [Cameron, A. D., Olin, B., Ridderström, M., Mannervik, B., and Jones, T. A. (1997) *EMBO J. 16*, 3386−3395] but *Pseudomonas putida* glyoxalase I has been reported to be monomeric [Rhee, H.-I., Murata, K., and Kimura, A. (1986) *Biochem. Biophys. Res. Commun. 141*, 993−999]. We show here that recombinant *P. putida* glyoxalase I is an active dimer (k_{cat} ∼500 ± 100 s^{-1}; K_M ∼0.4 ± 0.2 mM) with two zinc ions per dimer. The zinc is required for structure and function. However, treatment of the dimer with glutathione yields an active monomer (k_{cat} ∼115 ± 40 s^{-1}; K_M ∼1.4 ± 0.4 mM) containing a single zinc ion. The monomer is metastable and slowly reverts to the active dimer in the absence of glutathione. Thus, glyoxalase I appears to be a novel example of a single protein able to exist in two alternative domain-swapped forms. It is unique among domain-swapped proteins in that the active site and an essential metal binding site are apparently disassembled and reassembled by the process of domain swapping. Furthermore, it is the only example to date in which 3D domain swapping can be regulated by a small organic ligand.

3D domain swapping (*1*−*3*) has been proposed as a mechanism by which stable dimeric proteins could evolve from monomeric proteins in a single step. In this mechanism, the monomer contains two domains linked covalently by a flexible hinge and tightly associated with each other via noncovalent interactions; the term "domain" may refer to any element of secondary or tertiary structure. Opening of the hinge allows the two domains to dissociate from each other and to reassociate with equivalent domains from a different monomer giving rise to a 2-fold symmetrical dimer

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[1] Abbreviations: 3D, 3 dimensional; BRP, bleomycin resistance protein; CD, circular dichroism; C-domain, C-terminal globular domain of Glx I; DHBD, dihydroxybiphenyl dioxygenase; DT, diphtheria toxin; EDTA, ethylenediaminetetraacetic acid; Glx I, *Pseudomonas putida* glyoxalase I; GSH, reduced glutathione; LB, Luria broth; MALDI, matrix-assisted laser desorption ionization; N-domain, N-terminal globular domain of Glx I; Ni−NTA, nitrilotriacetic acid; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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containing two domain−domain interfaces, each identical to the interface that existed in the original monomer.

The strongest support for the 3D domain-swapping mechanism comes from a few proteins that can exist in both monomeric and domain-swapped dimeric forms. These include diphtheria toxin (DT)[1] (*1*, *4*), RNase A (*5*) and bovine seminal RNase (*6*), α-spectrin α14 fragment (*7*), the cell cycle regulatory proteins CksHs2 and Suc1 (*8*), the N-terminal domain of CD2 (*9*, *10*), and single-chain Fv fragments (*11*, *12*). The 3D domain-swapping hypothesis is also supported by several pairs of proteins that have similar sequences and structures except that one is a monomer and the other is a domain-swapped dimer. Examples include wild-type and mutant staphylococcal nucleases (*13*), wild-type and mutant chorismate mutases (*14*), γB- and βB-crystallins (*15*), helical growth factors (*16*), and odorant binding proteins (*17*, *18*).

The recent X-ray crystal structure (*19*) of the dimeric zinc-dependent human enzyme glyoxalase I indicated that this enzyme resembles a domain-swapped dimer. Each monomer consists of an N-terminal βαββαβ domain (N-domain; residues 31−104), a C-terminal βαβββα domain (C-domain; residues 124−183), an extended linker (residues 105−123), and an N-terminal tail with one additional α-helix (residues 12−18). The 2-fold symmetrical dimer is formed by the N-domain of each monomer associating with the C-domain of the other monomer. An active site cleft is situated at each of these interfaces and includes a divalent zinc ion ligated in square pyramidal geometry to one water molecule and two amino acid side chains from each domain (E33, E99, H126′, and Q172′; the prime indicates that these residues are from the second monomer). The crystal structure includes a molecule of the inhibitor *S*-benzylglutathione bound at each active site. The N-terminal tail of each monomer stretches across the N-domain of the other monomer, and the linker regions of the two monomers lie on the same face of the dimer.

Glyoxalase I catalyzes the interconversion of the glutathione thiohemiacetal of methylglyoxal and *S*-D-lactoylglutathione, thus facilitating the clearance of cytotoxic methylglyoxal (*20*, *21*). Remarkably, the glyoxalase I structure does not resemble other glutathione binding enzymes but instead exhibits similarities to two functionally unrelated proteins, the bleomycin resistance protein (BRP) (*22*) and dihydroxybiphenyl dioxygenase (DHBD) (*23*, *24*). BRP is a dimer with the same domain organization and pairing as glyoxalase I and antibiotic binding sites located in each of the two domain−domain interfaces. In contrast, DHBD is a monomer containing four similar domains in which the N-terminal and C-terminal pair of domains each fold onto each other; a single active site is located between the C-terminal pair and includes an iron binding site.

Considering that DHBD involves consecutive domains forming intramolecular pairs while the domains in glyoxalase I and BRP form intermolecular pairs, Cameron et al. (*19*) have proposed that this protein family is a novel example of 3D domain swapping. To date, no member of this family has been shown to interconvert between monomeric and dimeric structures. However, Rhee et al. (*25*) have reported that glyoxalase I from *Pseudomonas putida* (hereafter referred to as Glx I), which is ∼55% identical to the human enzyme (*27*), is monomeric, whereas Ridderström and Mannervik (*26*) have suggested that it is a dimer. Here we show that Glx I is an active dimeric enzyme containing two zinc ions per dimer but that treatment with glutathione converts it to an active monomeric enzyme containing one zinc ion; the monomer is metastable in the absence of glutathione. Our results suggest that Glx I is a new example of 3D domain swapping and is particularly unusual in that the active site and zinc binding site are cradled between the two swapped domains resulting in both monomeric and dimeric forms being enzymatically active. Furthermore, this is the first example of regulation of 3D domain swapping by a small organic molecule.

EXPERIMENTAL PROCEDURES

Materials. Reduced L-glutathione was purchased from Alexis Corp. (Läufelfingen, Switzerland), and oxidized L-glutathione was purchased from Sigma (St. Louis, MO). Methylglyoxal was synthesized by acid-catalyzed hydrolysis of the corresponding dimethylacetal (*38*) and purified by codistillation with water under reduced pressure. Ni−NTA agarose was purchased from Qiagen Inc. (Santa Clarita, CA). Source 15Q anion-exchange media and PD-10 columns were purchased from Pharmacia Biotech (Piscataway, NJ). The Bio-Rad assay kit was obtained from Bio-Rad laboratories (Hercules, CA).

Expression and Purification of the Zn_2 Dimer. The gene encoding *P. putida* glyoxalase I was excised from the plasmid M13mp8/GlxI (*27*, *28*) and amplified by PCR using Taq polymerase; the oligonucleotide primers used were 5′-CGTCTCTGGAGCTCATATGAGCCTGAACGACC-3′ and 5′-AAAAGGATCCGCTGTACTTACAA-GGGCGTTGGC-3′. The 0.6 kb fragment isolated from the PCR mixture was digested by *Nde*I and *Bam*HI and inserted between the corresponding restriction sites of the pET-15b expression vector (Novagen, Madison, WI). The resulting vector was designed to express the Glx I protein with a His tag linked by a thrombin cleavage site to its N-terminus. The sequence of the Glx I protein used in this study differs from reported sequences in two positions. Residue 110 is Asp in the current sequence; it has been reported to be either Asp (*41*) or His (*27*). Residue 152 is Asp in the current sequence but has been reported to be Asn (*41*, *27*). We have repeated the DNA sequence determination for the gene in the original plasmid sequenced by both Lu et al. (*27*) and Rhee *et al.* (*41*) and have found residue 110 to be Asp and residue 152 to be Asn. *Escherichia coli* BL21(DE3) cells (Novagen, Madison, WI) transformed with the recombinant vector were inoculated into 10 mL of LB medium containing 50 μg/mL ampicillin, grown to stationary phase at 37 °C, and then diluted into 1 L of LB/ampicillin medium. The culture was grown to an absorbance at 550 nm (OD_{550}) of 0.7−0.8. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and growth was continued for a further 4 h. The cells were collected by centrifugation, resuspended in 30 mL of 5 mM imidazole, 0.5 mM NaCl, 20 mM Tris, pH 8.0 (Ni−NTA buffer), containing phenylmethanesulfonyl fluoride (1 mM) and lysozyme (1 mM), and lysed by sonication. After centrifugation and removal of the supernatant, the pellet was resuspended in 15 mL of Ni−NTA buffer, sonicated, and centrifuged again. A third cycle of sonication and centrifugation was performed, and then the combined supernatants were loaded onto a Ni−NTA

affinity column. The column was washed with Ni−NTA buffer containing 5 mM imidazole and then with Ni−NTA buffer containing 30 mM imidazole, and the fusion protein was eluted with Ni−NTA buffer containing 100 mM imidazole. Pooled fractions containing fusion protein were dialyzed against 20 mM Tris, pH 8.0, and then NaCl and $CaCl_2$ were added to final concentrations of 150 and 2.5 mM, respectively. Human thrombin was added to a final concentration of 30 ng/mL, and the solution was incubated at room temperature for 40 h and then loaded onto a ResourceQ anion-exchange column. The protein was eluted with 20 mM bis-Tris, pH 6.0, containing a gradually increasing concentration of NaCl. Purified Glx I eluted at ∼220−230 mM NaCl. The concentration of Glx I was determined by UV absorbance at 280 nm ($\epsilon_{280} = 16\,500$ cm^{-1} M^{-1}; calculated from amino acid sequence) or using the Bio-Rad colorimetric protein assay; these two methods were in excellent agreement. The typical yield from 1 L of culture medium was 60−120 mg of pure protein. The isolated enzyme was stable for weeks, without any significant loss of activity, stored at 4 °C with 0.02% sodium azide added to the ion-exchange elution buffer. The amino acid composition was consistent with the expected sequence. The N-terminal sequence was Gly-Ser-His-Met-Ser-Leu, as expected. The molecular mass was determined by MALDI time-of-flight mass spectrometry to be 19809 ± 10 Da. (cf. 19 802 Da calculated for M + H^+).

Preparation of the Apo Monomer. The apo monomer was prepared by dialysis of the purified Zn_2 dimer (∼10−50 μM) against 20 mM bis-Tris, 0.23 M NaCl, and 30 mM EDTA, pH 6.0, at room temperature. Nearly complete conversion to the apoenzyme occurred after 96 h. The apo monomer was isolated by size exclusion chromatography.

Reconstitution of the Zn_2 Dimer from the Apo Monomer. The Zn_2 dimer was reconstituted by dialysis of the apo monomer (∼25 μM) against 20 mM bis-Tris, 0.23 M NaCl, and 10 mM $ZnCl_2$, pH 6.0, at room temperature. Excess zinc caused severe aggregation. Conversion was 90% complete after ∼96 h. The Zn_2 dimer was isolated by size exclusion chromatography.

Preparation of the Zn Monomer. The Zn monomer was prepared by dialysis of the Zn_2 dimer (∼25 μM) against oxidized or reduced glutathione (5 mM) in 20 mM bis-Tris 0.23 M NaCl, pH 6.0, at room temperature; the half-life for interconversion was approximately 24 h, with >90% conversion after 96 h, independent of whether oxidized or reduced glutathione was used. The Zn monomer was isolated by size exclusion chromatography. The N-terminal amino acid sequence was found to be (Arg or Gly)-Ser-His-Met-Ser-Leu, the expected sequence except for the ambiguity at position 1, which should be Gly. The molecular mass by MALDI mass spectrometry was 19 809 ± 10 Da (cf. 19 802 Da calculated for M + H^+).

Reconstitution of the Zn_2 Dimer from the Zn Monomer. Conversion of the Zn monomer to the Zn_2 dimer was achieved by concentrating the Zn monomer to ∼0.1−1 mM using a stirred ultrafiltration cell pressured with nitrogen gas and then dialyzing against 20 mM bis-Tris and 0.23 M NaCl, pH 6.0, to remove the glutathione. Approximately 80% conversion was achieved after 120 h. The reconstituted Zn_2 dimer was purified by size exclusion chromatography.

Size Exclusion Chromatography. Analytical and preparative size exclusion chromatographies were performed using a Pharmacia Biotech HiLoad 16/60 Superdex column, a flow rate of 0.5 mL/min, and the buffer in which Glx I eluted from the ion-exchange column. Molecular masses were determined from a standard curve (Figure 1I) constructed using RNase A (13 700 Da), chymotrypsinogen A (25 000 Da), ovalbumin (43 000 Da), bovine serum albumin (67 000 Da), and blue dextran 2000 (2 000 000 Da).

Zn^{2+} Determination. The zinc content of the enzyme was determined by atomic absorption using a Varian Techtron Model AA-6 spectrometer with an acetylene−oxygen flame ($\upsilon = 213.86$ nm). The samples were desalted on a PD-10 column containing Sephadex G-25M (Pharmacia Biotech) before analysis. Control experiments showed that protein digestion with nitric acid did not alter the results, confirming that the bound zinc was released quantitatively in the flame.

Mass Spectrometry. Matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry was performed using a Voyager RP-DE instrument (PerSeptive Biosystems, Framingham, MA) operating in positive ion mode. The matrix used was 3,5-dimethoxy-4-hydroxycinnamic acid.

Secondary Structure Determination. CD spectra were recorded on a Jasco J-715 spectropolarimeter. Each spectrum is the sum of 64 scans recorded at a resolution of 0.1 nm and a scan speed of 50 nm/min. The data were converted to residue molar ellipticity. Secondary structures were estimated from the CD spectra using the SELCON program (*29*, *30*). The secondary structure of human glyoxalase I was taken from the assignments listed in the Protein Data Bank file (accession code: 1fro).

Enzymatic Assays. Kinetic measurements at 25 °C were obtained with a Shimadzu UV 160U spectrophotometer equipped with a Brinkmann RM6 Lauda circulating water bath. GSH−methylglyoxal thiohemiacetal (0.1−2 mM) was prepared in situ by addition of reduced glutathione to methylglyoxal in 20 mM phosphate, pH 7.0, and allowing a minimum of 10 min for equilibration before initiation of the assay. For a desired concentration of thiohemiacetal (H), the required total concentrations of methylglyoxal (M_t) and glutathione (G_t) were calculated from the equations: $[M_t] = [H](1 + K_d/[G])$ and $[G_t] = [G] + [H]$, in which the dissociation constant (K_d) is 2.2 mM (*39*) and the concentration of free glutathione (G) is 0.1 mM. Upon addition of enzyme (final concentration ∼20−200 ng/mL), the initial rate of product formation was monitored at 240 nm ($\epsilon_{240} = 3300$ cm^{-1} M^{-1}). The magnitudes of the kinetic constants (k_{cat}, K_m) were obtained from Lineweaver−Burk plots of the initial rate data. Uncertainties quoted are the ranges observed for enzyme samples from three to five different preparations. One unit of enzyme is defined as the amount required to turn over 1 μmol of substrate per minute under V_{max} conditions.

RESULTS

Expression and Characterization of the Zn_2 Dimer. Glx I was expressed as a His tag fusion protein and purified by nickel affinity chromatography. The His tag leader was removed by thrombin digestion, and subsequent anion-exchange purification yielded Glx I with the expected amino

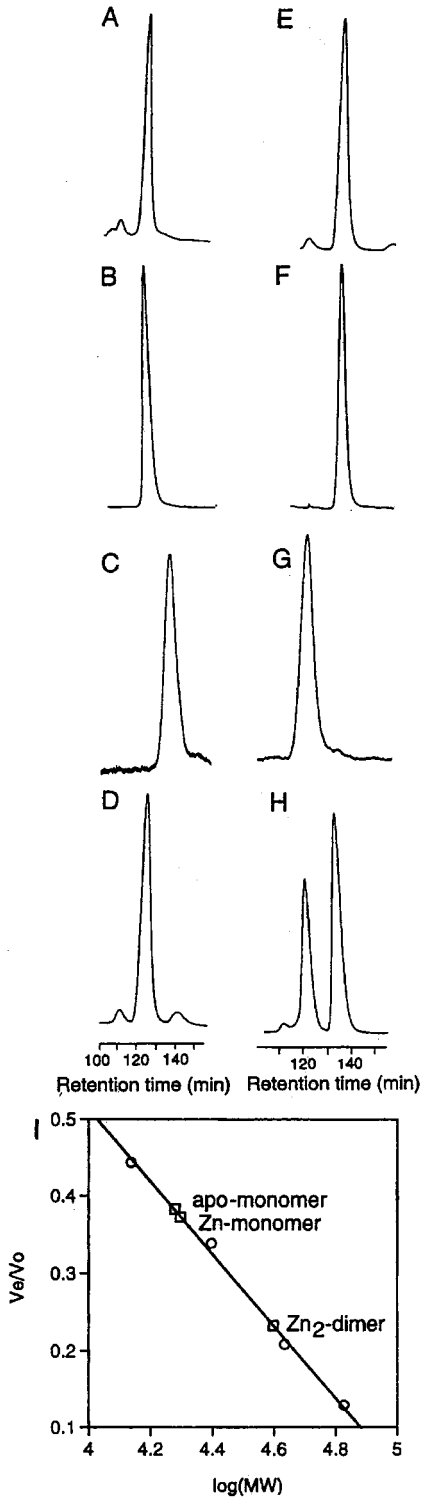

FIGURE 1: Size exclusion chromatography traces for recombinant Glx I. (A) Recombinant enzyme (predominantly Zn_2 dimer) after ion-exchange purification. (B) Zn_2 dimer isolated from trace A and reinjected onto the column. (C) Apo monomer obtained by treatment of the Zn_2 dimer in trace B with EDTA. (D) Zn_2 dimer reconstituted from the apo monomer in trace C. (E) Zn monomer obtained by treatment of the Zn_2 dimer with reduced glutathione (oxidized glutathione gives the same result). (F) Zn monomer isolated from trace E and reinjected onto the column. (G) Zn_2 dimer reconstituted from the Zn monomer in trace F. (H) A mixture of Zn_2 dimer and Zn monomer (~4:6) indicating that these species do not interconvert significantly during the chromatographic run. (I) Calibration curve for the size exclusion column showing the elution volumes of the three Glx I species (squares) and the four standards (circles). Details are given in Experimental Procedures.

acid composition and N-terminal amino acid sequence and the correct molecular mass by both SDS−PAGE and mass spectrometry. Purified Glx I was enzymatically active in the isomerization of methylglyoxal−glutathione thiohemiacetal and exhibited Michaelis−Menten kinetics with a k_{cat} of 500 ± 100 s^{-1} and a K_M of 0.4 ± 0.2 mM (Figure 2). Previously, the k_{cat} and K_M have been reported to be ~400 ± 300 s^{-1} and ~0.44 ± 0.05 mM (*27*, *28*), in good agreement with the present results.

X-ray crystallography has shown that human glyoxalase I is a dimeric protein containing two zinc ions, each coordinated to two amino acid side chains from each monomer (*19*). To determine whether our purified Glx I was also dimeric, we performed size exclusion chromatography. The protein eluted as a single peak with a retention time of 120 ± 3 min, corresponding to a molecular mass of 40 000 ± 2000 Da (Figure 1A,I). The monomer molecular mass of Glx I is 19 740 Da so this result indicated that the isolated Glx I was indeed a dimer. This species remained dimeric upon isolation and reinjection onto the size exclusion column (Figure 1B). Atomic absorption spectroscopy showed that the protein contained 1.01 zinc atoms per protein molecule (two zincs per dimer), in excellent agreement with the crystal structure of the human enzyme. The CD spectrum (Figure 3) indicated that the protein contains ~18% α-helix, ~50% β-sheet, and ~33% random coil. In comparison, the human enzyme contains ~23% α-helix, ~26% β-sheet, and ~51% random coil (*19*). The higher apparent β-sheet content in our protein may be because non-hydrogen-bonded extended regions may contribute to the β-sheet estimate of our sample whereas only hydrogen-bonded extended regions in the crystal structure were considered to be β-sheet. It should also be noted that secondary structure estimation from CD spectra is only approximate, particularly for predominantly β-structures. In summary, all of our structural information is consistent with the recombinant bacterial Glx I that we isolated having a very similar structure to the human enzyme. We refer to this species as the Zn_2 dimer.

Isolation and Characterization of the Apo Monomer. To test whether the zinc ions in the Zn_2 dimer are required for dimerization and enzymatic activity, we removed the zinc by extensive dialysis against EDTA. Complete removal of zinc took approximately 4 days and yielded a protein that was indistinguishable from the Zn_2 dimer by SDS−PAGE but monomeric by size exclusion with a retention time of 142 ± 3 min (Figure 1C). This corresponds to an apparent molecular weight of 19 500 ± 2000 Da (Figure 1I), close to the theoretical value for a monomer. This species contained 0.09 zinc atom per protein molecule and was enzymatically inactive (Figure 2A). We conclude that EDTA dialysis was effective at removing the metal ions from the protein. We refer to this species as the apo monomer. Analysis of the CD spectrum (Figure 3) indicated that this protein was ~4% α-helix, ~50% β-sheet, and ~47% random coil. Thus, removal of zinc resulted in a dramatic loss of α-helical secondary structure and of quaternary structure and a complete loss of enzymatic activity. To prove that this result was not due to protein degradation, we attempted to reconstitute the holoenzyme by dialyzing the sample against zinc chloride. After ~96 h, the sample contained ~90% dimeric enzyme (Figure 1D) with identical size exclusion retention time, zinc content, enzymatic activity, and second-

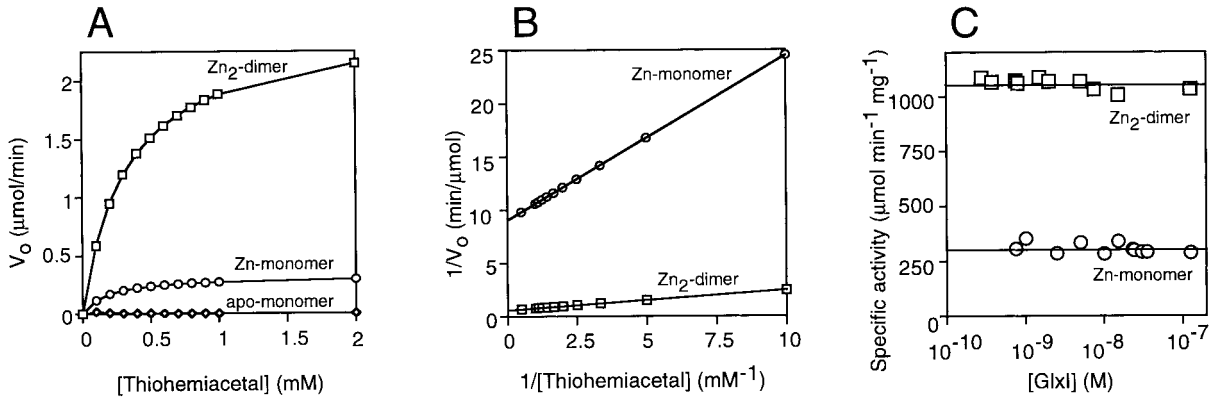

FIGURE 2: Enzymatic activity of recombinant Glx I. (A) Initial velocity versus substrate concentration for the Zn_2 dimer (squares), the Zn monomer (circles), and the apo monomer (diamonds). (B) Lineweaver−Burk plots for the Zn_2 dimer (squares) and the Zn monomer (circles). (C) Specific activity (V_{max}/mg of enzyme) versus enzyme concentration for the Zn_2 dimer (squares) and the Zn monomer (circles). Horizontal lines are drawn at the average specific activity for each species (1057 μmol·min^{-1}·mg^{-1} for dimer and 306 μmol·min^{-1}·mg^{-1} for monomer).

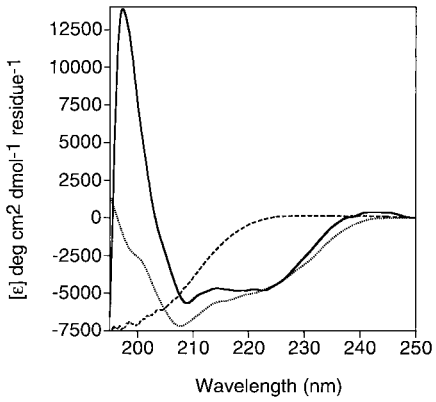

FIGURE 3: Circular dichroism spectra of the Zn_2-dimer (solid line), the Zn monomer (dotted line), and the apo monomer (dashed line).

ary structure to the above Zn_2 dimer. Thus, deactivation by removal of zinc was reversible and was not due to protein degradation.

Isolation and Characterization of the Zn Monomer. Rhee et al. (*25*) have reported that *P. putida* glyoxalase I is a monomer. However, the above data clearly demonstrate that the active species that we isolated was a dimer. We noticed that Rhee et al. had used glutathione in several of their purification buffers. Therefore, we tested the effect of glutathione on the oligomerization of Glx I. After extensive dialysis of the Zn_2 dimer against either reduced or oxidized glutathione for 96 h, the predominant species present eluted from the size exclusion column with a retention time of 138 ± 3 min, corresponding to an apparent molecular mass of 20 000 ± 2000 Da (Figure 1E,I), i.e., a monomeric species. This species was purified by size exclusion chromatography and was found to be enzymatically active with a k_{cat} of 115 ± 40 s^{-1} and a K_M of 1.4 ± 0.4 mM (Figure 2). The k_{cat} is consistent with the value of 125 s^{-1} reported by Rhee et al. (*25*); the K_M was not reported. However, the k_{cat} is ∼4-fold lower and the K_M is ∼3.5-fold higher than the values we measured for the Zn_2 dimer. This protein migrated identically to the Zn_2 dimer and the apo monomer by SDS−PAGE. The molecular mass determined by mass spectrometry was identical, within error, to the theoretical value and to the value determined for the Zn_2 dimer, and the N-terminal amino acid sequence was as expected for the expressed protein. Amino acid analysis was consistent with the expected sequence but also revealed the presence of ∼20−30 mol equiv of glutathione (based on the Gly and Glu/Gln determinations), indicating that the size exclusion chromatography and dialysis used to prepare the sample for analysis had removed the glutathione reagent from the protein at only ∼98−99% efficiency. Atomic absorption showed that this monomeric species contained 0.99 zinc atom per protein molecule. Thus, we refer to this species as the Zn monomer. The CD spectrum (Figure 3) indicated that the Zn monomer contained ∼54% α-helix, ∼18% β-sheet, and ∼28% random coil, an increased apparent α-helical content and decreased apparent β-sheet content relative to the Zn_2 dimer.

The Zn monomer was metastable. Upon isolation by size exclusion chromatography and reinjection onto the same column, it eluted as a monomer (Figure 1F) with unchanged enzymatic activity, zinc content, and secondary structure (data not shown). However, upon extensive dialysis to remove the glutathione, the protein was converted back to the Zn_2 dimer (Figure 1G) with identical size exclusion retention time, zinc content, enzymatic activity, and secondary structure to the Zn_2 dimer described above. Taken together with the mass spectrometry and amino acid analysis data, this indicates that the Zn monomer does not arise from irreversible changes in the primary structure of the protein.

The observation of activity in both dimeric and monomeric forms of the same enzyme is very unusual. Therefore, we performed several experiments to exclude the possibility that the observed activity of the Zn monomer was due to an impurity of Zn_2 dimer or to interconversion of the Zn monomer and the Zn_2 dimer preceding or during the enzymatic assays. First, a mixture of the freshly isolated Zn monomer and the Zn_2 dimer was injected onto the size exclusion column, a series of small fractions spanning the peaks were collected, and the fractions were assayed for activity. Both the UV absorbance and enzymatic activity traces indicated that the monomer and dimer peaks were baseline separated (data not shown); i.e., the monomer peak did not contain any significant impurity of dimer. Second, the freshly isolated Zn monomer and the Zn_2 dimer were combined in various ratios, the mixtures were incubated for 1 h at room temperature, and the samples were injected onto the sizing column. The ratios of monomer and dimer peaks observed were the same as the ratios used in the mixtures (see, for example, Figure 1H), indicating that the two species

do not interconvert noticeably on a 3 h time scale. Third, the specific activities of the Zn monomer and the Zn_2 dimer were determined as a function of enzyme concentration, over a concentration range of more than 2 orders of magnitude (Figure 2C). The specific activities were independent of enzyme concentration, and the activity of the dimer was consistently ~3−4 times that of the monomer. Thus, there was no significant interconversion of these species during the assay.

Finally, we note that the Zn monomer is accessible by means other than treatment with glutathione. During dialysis of the Zn_2 dimer against EDTA, several intermediate species were observed, by size exclusion, before complete conversion to the apo monomer was accomplished. The exact profile of species was dependent on enzyme concentration, pH, temperature, and EDTA concentration. However, a monomeric species isolated after dialysis of a 1.5 μM enzyme sample against a 30 mM EDTA buffer at pH 6.0 and room temperature for 12 h contained a single zinc atom per protein molecule and had the same k_{cat} and K_M as the Zn monomer described above. Thus, the Zn monomer appears to be an intermediate in the conversion of the Zn_2 dimer to the apo monomer. Similar experiments also provided preliminary evidence for the transient existence of a dimeric species containing one zinc atom (data not shown).

DISCUSSION

We have identified two enzymatically active forms of Glx I, one dimeric species containing two zinc atoms and one monomeric species containing one zinc atom. We propose here that the Zn_2 dimer is a 3D domain-swapped dimer with a structure similar to that of the human glyoxalase I dimer and that the Zn monomer is an alternative conformation of the same protein that has not undergone 3D domain swapping.

Structure of the Zn_2 Dimer. The data reported here indicate that Glx I isolated from our expression system most likely has essentially the same structure as the human glyoxalase I dimer (19). A structural model for the Zn_2 dimer is shown in Figure 4A. The following lines of evidence support this model. First, the Glx I species isolated is clearly a stable dimer. Second, it contains two atoms of zinc per protein dimer. Third, the secondary structure is consistent with that of the human dimer structure. Fourth, removal of the zinc results in loss of secondary structure, indicating that the zinc is an integral part of the stable fold. Fifth, human and *P. putida* glyoxalase I have sequences that are 55% identical; importantly, there is even higher identity (63%) in the linker region. Finally, the time scale of exchange between the dimer and monomer is extremely slow (several days), suggesting that interconversion requires a significant structural rearrangement (see below). We cannot exclude the possibility that the dimer we observe is formed by direct association of two active monomers (without domain swapping). However, we consider this possibility unlikely because (1) the Zn monomer and Zn_2 dimer have a 15-fold difference in k_{cat}/K_M, indicating that the active site structure has been slightly reorganized; (2) the CD spectra of the Zn monomer and the Zn_2 dimer (Figure 3) differ, suggesting some structural rearrangement; and (3) for such a dimer there would most likely be a faster rate of monomer−dimer interconversion than observed because no rearrangement of tertiary structure would be required (see below).

Structure of the Zn Monomer. We have shown here that Glx I can also be induced to exist as a metastable monomer that contains one atom of zinc per protein molecule and that it is enzymatically active, albeit slightly less active than the dimer. These data strongly suggest that the Zn monomer contains an active site that is structurally similar to the active site in the human glyoxalase I crystal structure (*19*). The only way that such an active site could form in a monomer is for the N-domain and C-domain of the same protein molecule to fold directly onto each other, forming a single intramolecular interface that is essentially identical to the two intermolecular interfaces present in the domain-swapped dimer (Figure 4). Formation of such a monomer would require structural rearrangement of the linker but could be achieved with each of the principal domains maintaining precisely the same conformation as in the dimer structure. A modeled structure for the Zn monomer is shown in Figure 4C. In this model, the N- and C-domains have the same structures as in the dimer and the linker is packed against the N-domain. The linker structure varies significantly among our lowest energy families of structures and so cannot be predicted confidently.

The CD spectrum and the enzymatic activity of the Zn monomer differ from those for the Zn_2 dimer. The difference in CD spectra may be rationalized by proposing that the linker region and the N-terminus form extended structures in the Zn_2 dimer but more compact structures with significant α-helical character in the Zn monomer. Such changes may allow for more efficient packing of these regions against the two globular domains. It is not obvious why the k_{cat} and K_M should be different between the monomer and the dimer. It is possible that structural alterations in the linker peptide are responsible for these subtle changes, since part of the linker abuts the active site (see below).

Comparison with Other Proteins. The 3D domain swapping for which we have found evidence has been proposed previously (*3*, *19*) on the basis of comparison of the human glyoxalase I crystal structure (*19*) and the similar dimeric structure of BRP (*22*) with the four domain structure of DHBD (*23*, *24*). In the latter, the first two domains and the last two domains are associated as pairs in a manner similar to the domain pair proposed here for the Zn monomer. Babbit and Gerlt (*31*) have classified these proteins as representatives of a new enzyme superfamily (the vicinal oxygen chelate fold), because they have similar structures but catalyze different, yet mechanistically similar, reactions.

The structures proposed here involve swapping of supersecondary structural elements, each consisting of ~60−70 amino acids. This is in notable contrast to most documented examples of domain swapping which more typically involve swapping of just one or two α-helices or β-strands (*2*). The only exceptions are DT (*1*, *4*), single-chain Fv fragments (*11*, *12*), and the crystallins (*15*), in which a whole globular domain (~100−150 residues) is swapped, and the CD2 N-terminal domain, in which the swapped elements are four β-strands (43 residues) comprising one β-hairpin from each side of a β-sandwich structure (*9*). These comparisons emphasize that the potential for proteins to undergo 3D domain swapping may exist in a wide variety of different structural contexts and may therefore be a rather general

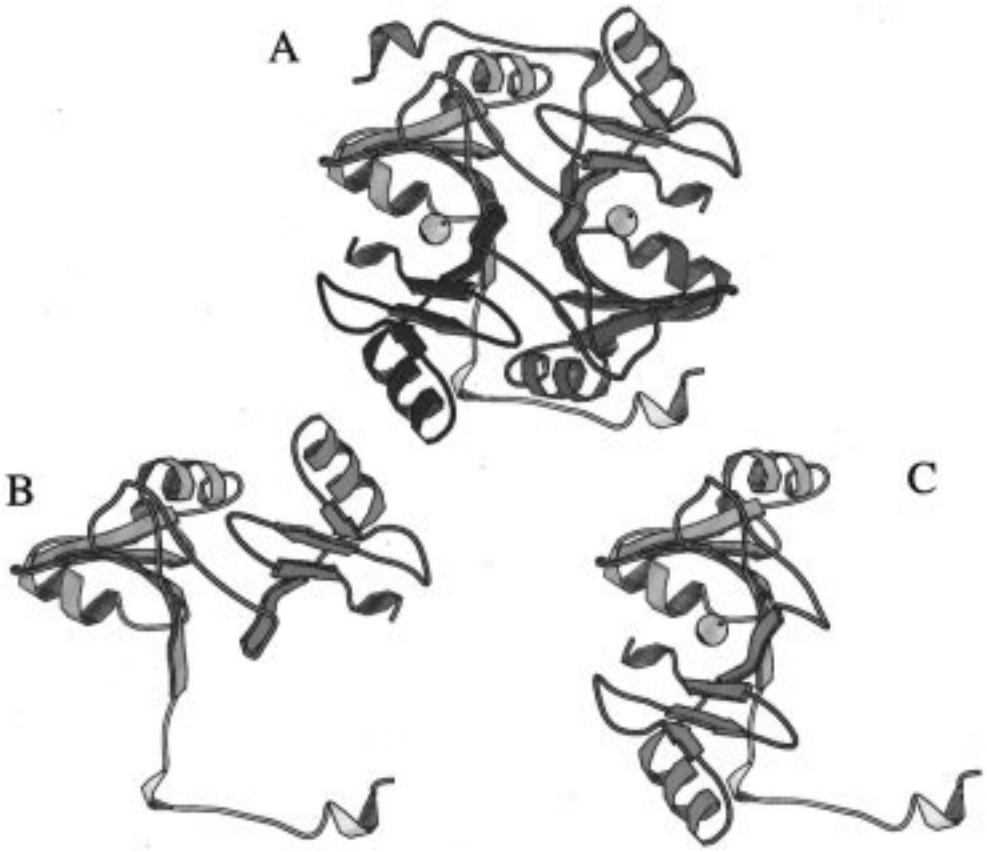

FIGURE 4: Ribbon representations of structural models for Glx I. (A) The Zn_2 dimer obtained by 3D homology modeling based on the coordinates of the human dimer. Residues 1−173 of Glx I are shown, corresponding to residues 8−179 of the human enzyme according to the sequence alignment reported by Lu et al. (*27*). Thus, homology modeling required only a single insertion (Pro-10). Monomer A is shown in orange (N-domain), red (C-domain), and yellow (N-terminal tail and linker) whereas monomer B is shown in green (N-domain), dark blue (C-domain), and light blue (N-terminal tail and linker). The N-terminal tail, N-domain, linker, and C-domain comprise residues 1−24, 25−98, 99−117, and 118−173, respectively. Zinc atoms are represented as gray spheres. The two benzylglutathione molecules bound in the active sites in the model are not shown. (B) A single monomer in the same conformation and orientation as monomer A in the dimer model. (C) The Zn monomer obtained from the Zn_2 dimer model as follows. Molecule A residues 115−173, molecule B residues 1−114, and the zinc atom and benzylglutathione coordinated by these regions were deleted. The carbonyl carbon atom of residue A114 and the amino nitrogen atom of residue B115 were linked by a peptide bond; these atoms were 7.8 Å apart. The linker was energy minimized, and then simulated annealing (200 cycles) was performed on the linker (A99−B117) with the rest of the structure kept invariant. Each cycle consisted of heating from 0 to 1000 K over 2 ps, equilibration at 1000 K for 5 ps, cooling to 100 K over 5 ps, and energy minimization. Structures were stored after each cycle. The 200 structures were grouped into 22 families, each representing a different conformation of the linker. The average of each family was energy minimized. The structure shown is the lowest energy structure of the 22 averaged minimized structures. The average strain energy (the sum of bond stretching, bond angle bending, torsion angle bending, and out-of-plane distortion energies) is 10.2 kcal mol^{-1} per residue in the linker compared to 9.9 kcal mol^{-1} per residue for the remainder of the protein. The color coding in structures B and C is the same as for monomer A in structure A. The single benzylglutathione molecule bound in the active site of model C is not shown. All modeling was performed using the Insight−Discover suite of programs (Molecular Simulations Inc.) and the ESFF force field. This figure was prepared using the program MolScript (*37*).

mechanism for the evolution of symmetrical dimeric structures. However, we note that alternative mechanisms for dimer evolution have also been proposed (*40*).

The proposed 3D domain swapping of Glx I involves a dramatic reorganization of the active site. The active site is lined by residues from both domains, and each domain contributes two ligands to the essential zinc ion. To our knowledge, the complete disruption and reassembly of an enzyme active site and/or a metal binding site by 3D domain swapping of a single protein are unprecedented.

Metastability of the Zn Monomer. Although the Zn_2 dimer is clearly more stable than the Zn monomer in the absence of glutathione, conversion of the Zn monomer to the Zn_2 dimer occurs only slowly, with a half-life on the order of days; conversion of the Zn_2 dimer to the Zn monomer in the presence of glutathione occurs on a similar time scale. Thus, the free energy barrier separating these two species is large (on the order of ~24 kcal·mol^{-1}), and the Zn monomer is metastable in the absence of glutathione. This situation is typical of interconversion between 3D domain-swapped species (*2*) and has been observed for DT (*1*, *4*), Suc1 (*8*), CD2 (*9*), and the α-spectrin α14 fragment (*7*). Apparently, metastability is a common feature of domain-swapped proteins because interconversion of a monomer and a domain-swapped dimer requires the formation of an "open" monomeric intermediate (e.g., Figure 4B) in which all of the stabilizing interdomain contacts are broken, resulting in a high free energy barrier between the two stable forms (*2*). Thus, the slow rate of interconversion of the Glx I Zn monomer and Zn_2 dimer supports the proposal that these two species are related by 3D domain swapping.

Regulation of 3D Domain Swapping by Glutathione. Glutathione stabilizes the Zn monomer of Glx I relative to the Zn_2 dimer. How does glutathione interact with these

two species? Although glutathiolation of cysteine residues can regulate enzyme activity (and presumably structure) (*32*), it is unlikely to be occurring in this case, because (1) mass spectra and N-terminal sequences show no evidence for the addition of glutathione and (2) both cysteines of Glx I (C122 and C132) are buried in our models. Furthermore, the thiol groups of the two cysteine residues are much too distant (∼14 Å apart) in our models to form a disulfide bond, so it is unlikely that the glutathione is influencing the oxidation state of the protein itself. Instead, it is more likely that glutathione binds noncovalently at the active site, probably in a conformation similar to that of the *S*-benzylglutathione in the crystal structure. The preference for binding the Zn monomer is presumably due to improved contacts with the rearranged linker; several linker residues are close to the glutathione moiety in the X-ray structure. In support of this hypothesis, oxidized glutathione is a competitive inhibitor of both the Zn_2 dimer and the Zn monomer (data not shown).

Regulation of 3D domain swapping by glutathione, or by any other small organic molecule, is unprecedented to the best of our knowledge. Domain swapping has been regulated in vitro by other chemical or physical factors including pH (*1*, *2*, *4*, *5*, *33*), counterions (*8*), and a fusion leader (*9*), and pH-induced dimerization may be important for in vivo regulation of DT activity (*4*). Although it is possible that Glx I dimerization is regulated by glutathione concentration in vivo, the slow rate of monomer−dimer interconversion in vitro and the modest difference in enzymatic activity suggest that monomer−dimer exchange is unlikely to be an effective mechanism for in vivo regulation of methylglyoxal metabolism.

In addition to environmental influences, the oligomerization state of domain-swapped proteins may be controlled by varying the length or sequence of the linker between the domains (*11*, *13*, *15*, *16*). In glyoxalase I sequences from different species, there is considerable variation in linker length (*19*, *34*), with several species (e.g., *E. coli*) having much shorter linkers than human and *P. putida*. Shortening the linker may preclude the type of intramolecular domain association present in our Zn monomer model (Figure 4C) but may still allow intermolecular association to give a domain-swapped dimer, as observed for staphylococcal nuclease (*13*). In accord with this possibility, *E. coli* glyoxalase I is dimeric (*26*).

Evolution of Glyoxalase I. The results reported here shed light on the possible evolutionary history of glyoxalase I and on the structures of glyoxalase I enzymes in other species. Glyoxalase I proteins from most species appear to possess the two-domain structure of the human and *P. putida* enzymes (*19*, *34*) although the proteins from yeast contain a total of four domains, two repeats of the two-domain structure (*26*, *34*). Furthermore, there is noticeable sequence and structural similarity between the two domains of the human enzyme, suggesting that they have arisen by gene duplication (*19*). Thus, glyoxalase I appears to have evolved from an early single-domain precursor (Figure 5A) to a two-domain monomeric protein similar to the Zn monomer (Figure 5B) and then to a 3D domain-swapped dimeric form such as the Zn_2 dimer (Figure 5C) (*19*). Our results support the proposed evolutionary mechanism by demonstrating the feasibility of converting an active monomeric protein to an active dimeric protein in a single step. The results suggest

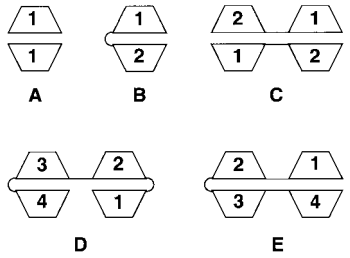

FIGURE 5: Schematic representations of structures occurring in the proposed evolutionary history of glyoxalase I. (A) Homodimer of a single-domain protein. (B) Monomer of a two-domain protein with an intramolecular domain−domain interface; this is the putative structure of the Zn monomer. (C) Domain-swapped dimer of a two-domain protein containing two intermolecular domain−domain interfaces; this is the putative structure of the Zn_2 dimer. D and E Alternative arrangements of a four-domain protein such as yeast glyoxalase I. Arbitrary numerical labels are shown to emphasize the symmetry of the structures and the relationships between them.

that formation of a glyoxalase I active site requires only the association of the two domains rather than a specific conformation of the linker, raising the intriguing possibility that separately expressed domains could be combined to reconstitute an active enzyme.

The existence of 3D domain-swapped variants of Glx I also has a bearing on the possible structures of the yeast enzymes. The evolution of these enzymes from the two-domain species would require a second gene duplication event resulting in a four domain protein (*19*, *34*). Such a protein could potentially exist in one of two monomeric forms (Figure 5D,E), six 2-fold symmetrical domain-swapped dimeric forms, or as a nonsymmetrical dimer or higher order oligomer. Apparently, yeast glyoxalase I adopts one of the monomeric forms (*35*) although at this stage we cannot predict which one is more likely.

Why have higher organisms evolved to possess oligomeric forms of glyoxalase I? Many oligomeric enzymes are allosterically regulated, providing an obvious selectional advantage to the organism. Although there is no evidence of cooperativity for *P. putida* glyoxalase I, Marmstal et al. (*36*) have noted that the mammalian and yeast enzymes display non-Michaelian steady-state kinetics and that the yeast enzyme shows nonlinear inhibition by glutathione. The possibility of cooperative subunit interactions was originally excluded since yeast glyoxalase I is monomeric. However, in light of the current evidence for 3D domain swapping, we suggest that cooperative interactions may indeed exist between the two catalytic units within the same monomer. In any case, our observation that the Zn_2 dimer has a ∼15-fold higher k_{cat}/K_M than the Zn monomer suggests that dimerization may fortuitously lead to improved activity and therefore be favored by natural selection.

Conclusion. We have identified two active forms of *P. putida* glyoxalase I, a dimer containing two zinc atoms and a monomer containing one. It is likely that these species are 3D domain-swapped forms of the same protein. 3D domain swapping within a single protein is very unusual. Glyoxalase I appears to be unique in three respects. First, the active site of the enzyme is formed approximately equally by residues from each of the two swapped domains. Second, an essential metal binding site is formed by association of the two swapped domains. Third, it is the only example in

which a small organic molecule, namely glutathione, is known to regulate the 3D domain swapping. Clearly this system holds great promise for exploring the folding and stabilization of multidomain proteins.

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