A Glutathione-independent Glyoxalase of the DJ-1 Superfamily Plays an Important Role in Managing Metabolically Generated Methylglyoxal in *Candida albicans*∗

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**Background:** Glyoxalases are a varied group of enzymes that detoxify methylglyoxal by converting it to D-lactate.

**Results:** The *Candida albicans* glyoxalase Glx3 is important for yeast growth, especially in glycerol.

**Conclusion:** Many yeasts contain a novel group of glyoxalases that are not redundant with previously characterized enzymes.

**Significance:** This is the first demonstration of physiologically relevant glutathione-independent glyoxalases in fungi.

Methylglyoxal is a cytotoxic reactive carbonyl compound produced by central metabolism. Dedicated glyoxalases convert methylglyoxal to D-lactate using multiple catalytic strategies. In this study, the DJ-1 superfamily member ORF 19.251/GLX3 from *Candida albicans* is shown to possess glyoxalase activity, making this the first demonstrated glutathione-independent glyoxalase in fungi. The crystal structure of Glx3p indicates that the protein is a monomer containing the catalytic triad Cys136-His137-Glu168. Purified Glx3p has an *in vitro* methylglyoxalase activity (*K*<sub>m</sub> = 5.5 mM and *k*<sub>cat</sub> = 7.8 s<sup>-1</sup>) that is significantly greater than that of more distantly related members of the DJ-1 superfamily. A close Glx3p homolog from *Saccharomyces cerevisiae* (YDR533C/Hsp31) also has glyoxalase activity, suggesting that fungal members of the Hsp31 clade of the DJ-1 superfamily are all probable glutathione-independent glyoxalases. A homozygous glx3 null mutant in *C. albicans* strain SC5314 displays greater sensitivity to millimolar levels of exogenous methylglyoxal, elevated levels of intracellular methylglyoxal, and carbon source-dependent growth defects, especially when grown on glycerol. These phenotypic defects are complemented by restoration of the wild-type GLX3 locus. The growth defect of Glx3-deficient cells in glycerol is also partially complemented by added inorganic phosphate, which is not observed for wild-type or glucose-grown cells. Therefore, *C. albicans* Glx3 and its fungal homologs are physiologically relevant glutathione-independent glyoxalases that are not redundant with the previously characterized glutathione-dependent GLO1/GLO2 system. In addition to its role in detoxifying glyoxal, Glx3 and its close homologs may have other important roles in stress response.

Reactive carbonyl species are produced in many organisms as a consequence of central metabolism. Among these, methylglyoxal (MG; H₂C–CO–CHO) has attracted considerable interest dating from the early 20th century for its role as a branch point metabolite for alternative catabolic pathways. MG is an α-ketoaldehyde metabolite produced principally by the spontaneous dephosphorylation of triose phosphates and, in some prokaryotes, by the enzyme-catalyzed dephosphorylation of dihydroxyacetone phosphate (DHAP) (1). A small fraction of MG is also generated by catabolism of acetone and threonine (2, 3) and as a rare elimination side reaction of triose-phosphate isomerase (4). In the model yeast *Saccharomyces cerevisiae*, ~0.3% of all glycolytic carbon flux is converted to MG, making it a potentially abundant metabolite under growth conditions that favor high rates of glycolysis (5).

MG is cytotoxic at elevated concentrations because, as a carbonyl electrophile, it can damage proteins, nucleic acids, and lipids by modification of arginine, lysine, cysteine, adenine, and guanine to form various advanced glycation end products (6, 7). Consequently, many organisms produce glyoxalases that detoxify MG by converting it to D-lactate. These enzymes were initially discovered in rabbit and dog tissues and first reported 100 years ago (8). Curiously for a toxic metabolite, MG is produced enzymatically from DHAP by a dedicated methylglyoxal synthase in certain prokaryotes. In combination with the glyoxalases, methylglyoxal synthase constitutes the initial portion of the methylglyoxal bypass, which converts DHAP to MG, then to D-lactate and, ultimately, to pyruvate (Fig. 1) (9). The methylglyoxal bypass has been proposed to serve an important cat...
abolic role in phosphate-limited conditions, as it permits the oxidation of DHAP to pyruvate without having carbon flux through the more phosphate-demanding Embden-Meyerhof-Parnas (EMP) glycolytic pathway (10).

In contrast to prokaryotes, eukaryotes do not possess a clearly identified methylglyoxal synthase, and therefore MG production from triose phosphates is presumed to be predominantly nonenzymatic in these organisms (11). Enzymes such as semicarbazide-sensitive amine oxidase can produce some MG, although the magnitude of their contribution to total MG pools in eukaryotes is not clear (12). However, the absence of a known methylglyoxal synthase does not necessarily mean that MG serves no metabolic purpose in eukaryotes. Accumulation of triose phosphates is inhibitory to glycolysis and can be partially relieved by their spontaneous, nonenzymatic dephosphorylation to produce MG, although the magnitude of their contribution to total MG pools in eukaryotes is not clear (12). However, the absence of a known methylglyoxal synthase does not necessarily mean that MG serves no metabolic purpose in eukaryotes. Accumulation of triose phosphates is inhibitory to glycolysis and can be partially relieved by their spontaneous, nonenzymatic dephosphorylation to produce MG. Subsequent detoxification of MG by glyoxalases could then facilitate a minor alternative pathway to generate pyruvate and acetyl-CoA under nonoptimal growth conditions. Glyoxalases are required for the detoxification of MG and may serve a metabolic role in producing D-lactate for further catabolism. The best characterized glyoxalase is the glutathione-dependent two enzyme system (glyoxylases I and II) that detoxifies MG by converting it to D-lactate and regenerating free glutathione. In this system, glutathione and MG are converted to R-(S)-lactoylglutathione by the metalloenzyme glyoxylase I (GLO1 in the yeasts) (13). The lactoylglutathione product is then hydrolyzed by a second enzyme, glyoxylase II (GLO2), to give D-lactate and glutathione (14). In addition to the glutathione-dependent system, a less studied glutathione-independent glyoxalase (glyoxalase III) in Escherichia coli can catalyze the conversion of MG to D-lactate without any cofactor (Fig. 1). Although this activity was known to exist in lysates of E. coli for many years (15), it was only recently ascribed to Hsp31 (HchA), a previously characterized chaperone of the DJ-1 superfamily (16). Consistent with its dual role in the heat and carbonyl stress responses, transcription of E. coli Hsp31/glyoxalase III is rpoS-dependent and thus highest in stationary phase (17, 18). An important lingering question is whether the activity of glutathione-independent glyoxalases is physiologically relevant, as it is unclear why this activity would be needed in addition to the established glutathione-dependent system for detoxifying glyoxals. Notably, both the glutathione-dependent and -independent systems are present in many organisms, including the model yeasts S. cerevisiae and Can-
did aleb, suggesting that they may not be functionally redundant.

Investigating the physiological role of putative eukaryotic glutathione-independent glyoxalases is aided by the abundance of organisms that contain homologos from the Hsp31 clade of the DJ-1 superfamily. *S. cerevisiae* is a natural choice for such a study; however, *S. cerevisiae* contains four Hsp31 homologs (YDR533C, YMR322C, YOR391C, and YPL280W), thus presenting the challenge of generating knock-outs targeting multiple and very similar genes and then determining the degree of functional redundancy among them (19, 20). Additionally, *S. cerevisiae* switches from respiratory to fermentative metabolism when presented with fermentable carbon sources such as glucose in aerobic growth conditions (the Crabtree effect) and can survive in fermentative growth conditions with nonfunctional mitochondria (i.e. a “petite positive” yeast). Both of these metabolic features act as serious confounding factors to the study of endogenous MG toxicity as a function of carbon source, as the entire metabolic program of the organism shifts in a carbon source-dependent manner (21). In contrast, the yeast *C. albicans* has only one Hsp31 homolog (ORF19.251), which simplifies the generation of knock-out strains and the characterization of their phenotypes, thereby making *C. albicans* a simpler model for studying the functions of these proteins. Unlike *S. cerevisiae*, *C. albicans* respires in aerobic growth conditions regardless of carbon source (i.e. it is a Crabtree negative yeast) and therefore must retain functional mitochondria, making it a “petite-negative” yeast as well (21). These metabolic features can be used to alter the level of endogenously produced MG in *C. albicans* by growing the cells in carbon sources whose catabolism produces different levels of tricarboxylic acids, the direct precursors to MG. Because *C. albicans* is a Crabtree negative yeast, it will not switch between respiratory and fermentative metabolic programs as a consequence of changing carbon sources, thus eliminating a complication of using Crabtree positive yeasts such as *S. cerevisiae* in this study.

In this study, the *C. albicans* YDR533C/Hsp31 homolog ORF 19.251 is shown to possess glutathione-independent glyoxalase III activity. As a consequence, we propose that this gene be named GLX3. Unlike the homologous proteins from *S. cerevisiae* and *E. coli*, the crystal structure of CalGlx3 indicates that it is a monomer. As expected, the protein possesses a Cys-His-Glu catalytic triad found in other members of the Hsp31 clade. A combination of site-directed mutagenesis and enzyme kinetics indicates that the active site cysteine residue is critical for glyoxalase activity and that a nearby histidine residue is also important. The in vivo relevance of glyoxalase III activity was established by generating a glx3 knock-out mutant of *C. albicans* and testing complementation with the wild-type allele. The homozygous glx3 knockout was significantly more sensitive to both exogenously administered MG and to MG produced endogenously by glucose or glycerol catabolism, especially when grown in glycerol. A rationale for the existence and physiological significance of glutathione-independent glyoxalases in fungi is discussed.

### Experimental Procedures

**Yeast Strains, Media, and Growth Conditions**— *C. albicans* wild-type clinical isolate SC5314 was the generous gift of Dr. Alexander Johnson, University of California at San Francisco. *C. albicans* strains were grown and maintained in yeast extract/pepptide/dextrose (YPD) medium (10 g of yeast extract, 5 g of peptone, and 20 g of glucose per liter) at 30 °C. Growth curves were measured in YPD that had been inoculated with cells washed three times in 50 mM potassium phosphate, pH 6.5, at a density of 1 × 10⁷ cells/ml. Cells were grown aerobically in 250-ml Erlenmeyer flasks containing 50 ml of media that were incubated at 30 °C with 225 rpm rotary agitation. For determination of the role of Glx3p in *C. albicans* metabolism, multiple media were used as follows: YPD medium supplemented with 0–40 mM methylglyoxal, YPG (yeast extract/pepptide medium containing 2% v/v of glycerol), and YP (yeast extract/pepptide medium containing neither glucose nor glycerol). All experiments were done in triplicate and means ± S.D. were calculated.

**Cloning and Purification of Glx3**—The gene encoding Glx3 (ORF19.251) was PCR-amplified from *C. albicans* SC5314 genomic DNA using primers (Table 1) that introduced 5′-Ndel and 3′-Xhol restriction sites. The PCR product was purified, digested with Ndel and Xhol, and cloned between the corresponding restriction sites of the bacterial protein expression vector pET15b (Novagen). The C136S and H137F point mutations were introduced using site-directed mutagenesis, and all constructs were verified using DNA sequencing (Operon). *E. coli* strain BL21 (DE3) (Novagen) was transformed with GLX3-pET15b and grown at 37 °C in terrific broth medium supplemented with 100 μg/ml ampicillin and with shaking at 270 rpm to an OD₆₀₀ ~2.0. Expression of Glx3 was induced by the addition of isopropyl β-d-1-thiogalactopyranoside (Sigma) to a final concentration of 1 mM for 18 h at 25 °C. Two hours

### Table 1

| Sequence | Name | Reference | Purpose |
|----------|------|-----------|---------|
| 5′-TCC GAG GGG GGT ACC TCC TTC-3′ | KpnI | This study | Glx3 knockout |
| 5′-TGG AAA CAC TGC AGG AAA GGG-3′ | Xhol | This study | Glx3 knockout |
| 5′-GGG CAT ACA AAA GCC GGC GAT TTA-3′ | SacI | This study | Glx3 knockout |
| 5′-AAC ATT AAT TGA CTT CCT TTC TCG-3′ | SacII | This study | Glx3 knockout |
| 5′-TAT TGT TGA TGT GAA GAA AAC-3′ | Upstream check | This study | Southern blot |
| 5′-TGT TTA GTA GAT CTT TCT ACA TTC-3′ | Downstream check | This study | Southern blot |
| 5′-TAA AAA TAT CTT ATG GGG AAA GTT TTA TTC-3′ | Ndel | This study | Glx3 insertion in pET15b |
| 5′-TAA TTT CTC GAG TTA ATT ACA TTC AAA AAG-3′ | Xhol | This study | Glx3 (C136S) point mutation |
| 5′-CTT TCT GCT GTC AAT GAT CCT GCC AAT-3′ | Forward C136S | This study | Glx3 (C136S) point mutation |
| 5′-ATG GCC GCA CCA TTA ACA GCA GCA GAA AC-3′ | Forward H137F | This study | Glx3 (H137F) point mutation |
| 5′-GGT TGT TTC GCT CCT GCT ATT TTT-3′ | Reverse C136S | This study | Glx3 (C136S) point mutation |
| 5′-AAA AAT GGC AGG ACC AAA ACA AAC-3′ | Reverse H137F | This study | Glx3 (H137F) point mutation |
before harvesting the cells, chloramphenicol was added to the culture to a final concentration of 100 μg/ml to arrest protein synthesis and enhance the solubility of the expressed protein (22). The cells were harvested by centrifugation at 4 °C and then resuspended in ice-cold lysis buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, and 10 mM imidazole, 1 mM DTT) at a ratio of 5 ml of buffer per g of wet cell mass. Cells were lysed by the addition of lysozyme to a final concentration of 1 mg/ml and incubation for 30 min, followed by sonication. The lysate was clarified by centrifugation at 12,000 × g to remove cellular debris.

Cleared lysate was mixed with His-Select Ni2+ metal affinity resin (Sigma) for 30 min with gentle stirring at 4 °C. The resin was transferred to a glass column and washed extensively with wash buffer (25 mM HEPES, pH 7.5, 300 mM NaCl, 20 mM imidazole, 2 mM DTT) until no protein was detected in the eluate by Bradford’s reagent (Bio-Rad). N-terminal hexahistidine-tagged Glx3 was eluted from the resin with ice-cold elution buffer (25 mM HEPES, pH 7.5, 300 mM NaCl, 200 mM imidazole, 2 mM DTT) and then incubated with bovine thrombin (MP Biomedicals) at a ratio of 1.5 units of thrombin/mg of Glx3 for 3 h at room temperature, followed by overnight dialysis against storage buffer (25 mM HEPES, pH 7.5, 100 mM KCl, and 2 mM DTT). After digestion, any protein retaining the histidine tag was separated from the tag-cleaved protein by a second passage over His-select resin that had been equilibrated in storage buffer. The flowthrough fractions, containing purified tag-cleaved protein Glx3, were then passed over benzamidine-Sepharose (Amersham Biosciences) resin to remove thrombin. Unexpectedly, a considerable amount of Glx3 binds to benzamidine-Sepharose under these conditions. The purified protein was concentrated using a stirred cell concentrator with a nominal molecular mass cutoff of 10 kDa to 20 mg/ml, as determined by UV-visible spectrophotometry with a calculated extinction coefficient at 280 nm (ε280) of 21,555 M⁻¹ cm⁻¹ for Glx3 (ExPASy). The purified protein ran as a single band on Coomassie Blue-stained SDS-PAGE and was aliquoted into 50–100-μl volumes, flash-frozen in liquid nitrogen, and stored at −80 °C until needed.

**Crystal Structure Determination of Glx3—**Glx3 (20 mg/ml in storage buffer) was crystallized using sitting drop vapor equilibration. Initial crystallization conditions were identified using a 96-well plate and commercially available sparse matrix crystallization screens. Conditions delivering crystals were further optimized by fine screening using sitting drop vapor equilibration of drops containing 2 μl of Glx3 with 2 μl of reservoir solution. Obelisk-shaped crystals of wild-type Glx3 measuring ~20 × 20 × 100 μm grew from 100 mM sodium acetate, pH 4.1, 120 mM ammonium acetate, 27% PEG4000, and 3% ethylene glycol at 4 °C after 1–3 days. The crystals were cryoprotected by serial transfer through reservoir solution supplemented with increasing concentrations of ethylene glycol to a final concentration of 20% v/v. Crystals were removed with nylon loops and cryoooled by rapid immersion into liquid nitrogen.

X-ray diffraction data were collected from a single crystal maintained at 100 K at SSRL beamline 9-2 using 13.79 keV incident x-rays and a MarMosaic 325 charge coupled device detector. The crystal was exposed to x-rays during 0.5° oscillations, and 300 images were collected and processed. The data were indexed, integrated, and scaled using HKL2000 (23), and final data statistics are shown in Table 2.

**Phases and an initial model for Glx3** were obtained using maximum likelihood molecular replacement in Phaser (24), part of the CCP4 suite of programs (25). A homology model based on the coordinates of one monomer (chain A) of YDR533C (PDB code 1RW7) (20) was generated by Swiss-Model (26) and used for the rotation and translation searches. A clear top solution was obtained, which was then subjected to stereoechemically restrained refinement with riding hydrogen atoms against an amplitude-based maximum likelihood target in REFMAC5 (27). All measured data were used for the refinement, and a bulk solvent correction was applied. The translation-libration-screw model was used for refinement of anisotropic atomic displacement parameters based on a rigid body displacement model that treated the entire protein as a single rigid group (28, 29). Ordered solvent placement and manual adjustments to the model were performed in COOT (30), and the final model was validated using the validation tools in COOT (30) and MOLPROBITY (31). All structural figures were made with UCSF Chimera (32).

**Gluatatione-independent Glyoxalase Assay—**Glyoxalase activity was assayed in vitro using a previously described fixed time point assay that follows the enzymatic depletion of the substrate methylglyoxal (33). Methylglyoxal (Sigma, 40% solution) is unstable and was thus stored at 4 °C until immediately before use. The reaction was initiated by adding enzyme in reaction buffer (100 mM HEPES, pH 7.5, 50 mM KCl, 2 mM DTT) to the specified initial concentration of methylglyoxal in reaction buffer, followed by incubation at 30 °C. The final enzyme con-

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**TABLE 2**

| Crystallographic data collection and refinement statistics |
|-----------------------------------------------------------|
| Data collection                                           |
| X-ray source: SSRL 9-2                                    |
| X-ray wavelength: 0.90 Å                                   |
| Space group: P4₁,2                                        |
| Cell dimensions:                                          |
| a = b = c = 92.23 Å, 59.37 Å                             |
| α = β = γ = 90°                                           |
| Molecules in asymmetric units: 1                          |
| Wilson B factor: 21 Å²                                      |
| Resolution*: 41 to 1.6 Å                                  |
| No. of reflections: 34,216                                 |
| Completeness: 100.0% (100.0%)                              |
| Redundancy: 12.1 (12.2)                                   |
| Rmergeᵃᵇ: 0.05 (1.00)                                     |
| Rmerge free: 51.1 (2.2)                                    |

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| Refinement                                              |
|--------------------------------------------------------|
| Resolution:                                            |
| Rmerge: 41 to 1.6 Å                                    |
| Rmerge free: 15.8% (15.9%)                             |
| No. of protein atoms: 1843                              |
| No. of solvent atoms: 279                               |
| No. of heteroatoms: 8                                   |
| Root mean square deviations:                           |
| Bond lengths:                                          |
| Bond angles:                                           |
| 1.08                                                 |
| Ramachandran plot favored; allowed; forbidden 97%; 100% |

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*Values in parentheses are for highest resolution shell (1.63 to 1.60 Å).
centration was 10 μM. The methylglyoxal remaining at each time point was determined by reaction with 2,4-dinitrophenylhydrazine (DNPH) to generate the purple chromophore methylglyoxal-bis-2,4-dinitrophenylhydrazine after alkali treatment. As this was a substrate depletion assay, a relatively high concentration of enzyme (10 μM) was used to observe clear differences in remaining substrate concentration over the first 1–2 min of reaction. In all cases, however, substrate was saturating at reaction over the first 1–2 min of reaction. In all cases, however, substrate was saturating at 37 °C.

To this solution, 0.33 ml of a freshly prepared stock of DNPH creating the construct pSFS2A-GLX3-flank. This S71I flipper cassette contains a nourseothricin marker (caSAT1) and an adapted FLIP gene (CaFLP) encoding a site-specific recombinase under the control of C. albicans MAL2 promoter (34). A linear fragment containing the disruption cassette was created by digesting the pSF52A-GLX3-flank construct with KpnI and NcoI. The fragment was purified by agarose gel electrophoresis followed by gel extraction of the excised target band (Qiagen). The fragment was introduced by electroporation into C. albicans according to the procedure described in Reuss et al. (34). Briefly, 1 μg of linear DNA was mixed with 40 μl of electrocompetent SC5314 cells and subjected to electroporation (0.2-cm cuvette, 1.5 kV, 400 ohms). The cells were incubated with a 1:1 ratio of 1 M sorbitol/YPD at 30 °C for 2 h with shaking at 225 rpm followed by spreading onto a YPD plate containing 200 μg/ml nourseothricin, which was incubated at 30 °C overnight. The following day, the resistant colonies were picked and streaked again onto a YPD plate containing 200 μg/ml nourseothricin. Resistant cells were analyzed for the first allelic replacement by PCR and Southern blotting (primers, see Table 1). After confirming that one of the GLX3 alleles had been replaced with the caSAT1 cassette (glx3-a1), the cells were inoculated into YPM liquid medium (10 g of yeast extract, 5 g of peptone, and 20 g of maltose per liter) without selective pressure to allow FLP-mediated excision of the cassette as described by Reuss et al. (34). After 6 h of incubation at 30 °C, serially diluted cells were spread on a YPD plate containing 25 μg/ml nourseothricin to assay for restoration of antibiotic sensitivity resulting from FLP-mediated excision of CaSAT1 (gsk3-a2). These cells were used for the second allelic deletion following the same procedure.

Measurement of Intracellular Methylglyoxal Concentration—Cellular MG concentrations were measured using a modification of the procedure described by Scheijen and Schalkwijk (35).
and McLellan et al. (36). Late log phase cells were harvested from liquid culture by centrifugation and immediately frozen at −80 °C. Cell pellets were resuspended in buffer containing 0.3 M sucrose, 10 mM histidine, and 0.23 mM phenylmethylsulfonyl fluoride, pH 7.4 (2 μl of buffer per mg of pellet). Cell suspensions were then sonicated three times for 5 s (setting 4, Fisher M100 sonicator) within 15-s intervals. Thereafter, the sonicated samples were added to equal volumes of 5.0 M perchloric acid and placed on ice for 10 min. Samples were then centrifuged for 10 min at 3000 rpm (Eppendorf 5417R, EL 129 FA 45-24-11 rotor). Supernatants (50 μl) were then mixed with 150 μl of 0.1 M o-phenylenediamine in 1.6 M perchloric acid and allowed to react for 16 h at room temperature in the dark. After this reaction, mixtures were extracted three times with 2.0 ml of chloroform, and pooled extracts from each sample were dried over anhydrous sodium sulfate. Chloroform was then evaporated from each sample using a gentle stream of air, and samples were resuspended in 500 μl of methanol. High performance liquid chromatography (SCL-Shimadzu 10A) employing a Kinetex 5-μm C18 100R column, a mobile phase of methanol/water/trifluoroacetic acid (52:48:0.1) at a flow rate of 1 ml/min, and a detector wavelength of 312 nm were then used to determine the amount of 2-methylquinoxaline, the product formed following this reaction, mixtures were extracted three times with 2.0 ml of chloroform, and pooled extracts from each sample were dried over anhydrous sodium sulfate. Chloroform was then evaporated from each sample using a gentle stream of air, and samples were resuspended in 500 μl of methanol. High performance liquid chromatography (SCL-Shimadzu 10A) employing a Kinetex 5-μm C18 100R column, a mobile phase of methanol/water/trifluoroacetic acid (52:48:0.1) at a flow rate of 1 ml/min, and a detector wavelength of 312 nm were then used to determine the amount of 2-methylquinoxaline, the product formed following the reaction of MG and o-phenylenediamine. For these experiments 2-methylquinoxaline concentrations ranging from 1 to 100 μM were used to generate the standard curve for absolute concentration determination.

RESULTS

Crystal Structure of C. albicans Glx3p—The crystal structure of Glx3p was determined at 1.6 Å resolution using molecular replacement with a homology model based on S. cerevisiae YDR533C (PDB code 1RW7) (20). Glx3 contains the standard DJ-1 superfamily flavodoxin-like core fold with several noncontiguous secondary structural elements (helices B, H, and I and strands 2 and 3) that form a “cap” region that has been previously called the P domain in other homologs of Hsp31 (Fig. 2A) (20, 37). The structure is very similar to that of YDR533C (core Ca root mean squared deviation = 0.81 Å), as expected based on their 62% sequence identity.

All other structurally characterized members of the Hsp31 clade, including E. coli Hsp31 (PDB code 1N57) (37) and the S. cerevisiae proteins YDR533C (PDB code 1RW7) (20) and YOR391C (PDB code 3MI1) (38), are homodimers. Although all previously characterized Hsp31-like proteins are dimeric, E. coli Hsp31 contains a 45-amino acid N-terminal extension that results in a formation of a different dimer than observed for the yeast proteins YDR533C or YOR391C, which lack this extension (20, 37–41). This N-terminal 45-amino acid region has been shown to be important for chaperone activity of the E. coli Hsp31 (42, 43), and its absence in YDR533C indicates a potential functional difference between these proteins. The structure of C. albicans Glx3 also lacks this 45-amino acid N-terminal extension but, unlike YDR533C and YOR391C, is a monomer in the crystal. The best candidate for a potential dimer interface buries 606 Å² of surface area per monomer as calculated by PISA (44). However, this interface features a cluster of acidic residues (Glu13, Glu48, and Asp117) in which Glu13 makes a short hydrogen bond (2.44 Å) with Glu48 from the other monomer, requiring at least one of the side chains to be protonated as a carboxylic acid (Fig. 2B). Crystals of Glx3 could only be obtained at pH values below 4.1, suggesting that the protonation of these carboxylic acids is essential to crystallize Glx3 in this lattice. Because this lattice contact involves multiple carboxylate side chains that would likely be negatively charged at physiological pH, this intermolecular interface is likely not physiologically relevant.

The active site for C. albicans Glx3 contains the triad residues Cys136, His137, and Glu168, as well as nearby residues Glu28.
and His\textsuperscript{106} (Figs. 2A and 3A). These residues are conserved in Hsp31 proteins from yeast and bacteria, and the Glx3 active site aligns well with that of the \textit{E. coli} enzyme (Fig. 3B). The catalytic Cys\textsuperscript{136} sits at the bottom of a narrow active site pocket that can accommodate only small molecule substrates (Fig. 3C), consistent with a role in detoxifying low molecular mass glyoxals. As observed in many other DJ-1 superfamily crystal structures (20, 45--49), the active site cysteine residue is surrounded by unusual electron density indicative of probable partial oxidation of the reactive thiol(ate). Two oxygen atoms are modeled at 2.25 and 2.06 Å from the S atom of Cys\textsuperscript{136} (Fig. 3A). These distances are shorter than the van der Waals contact distances expected for these atoms (~3.3 Å) but too long to be fully formed covalent bonds, similar to a previous observation in a crystal structure of human DJ-1 (50). Although the cysteine residue of human DJ-1 and its close homologs are easily oxidized to cysteine sulfenic acid (51), the electron density for Cys\textsuperscript{136} in Glx3 is not completely consistent with this covalent modification. Therefore, these two oxygen atoms were conservatively modeled as water molecules in the crystal structure. We speculate that photoreduction and subsequent redox chemistry caused by synchrotron radiation may be responsible for this modification, as noted previously in \textit{Pseudomonas fluorescens} isocyanide hydratase (46). In total, the structural features of the Glx3 active site are consistent with a hydrolase employing a Cys-His-Glu catalytic triad with a reactive cysteine that attacks small electrophilic substrates.

\textit{Glx3 Has Glutathione-independent Glyoxalase Activity in Vitro}—The \textit{in vitro} glyoxalase activity of \textit{C. albicans} Glx3 was assayed using a fixed time point assay that follows the depletion of the substrate methylglyoxal as detected by formation of the purple-colored methylglyoxal-bis-2,4-dinitrophenylhydrazone adduct (see “Experimental Procedures”). Purified \textit{C. albicans} Glx3 possessed robust glyoxalase activity \textit{in vitro} (Table 3), and this activity was confirmed by a complementary assay based on the appearance of \textit{d}-lactate (Table 3). Glutathione was not required for this activity, consistent with previous studies of \textit{E. coli} Hsp31 (16). The kinetic constants for \textit{C. albicans} Glx3 (\(K_m = 5.5\) mM, \(k_{cat} = 7.8\) s\textsuperscript{-1} (see Fig. 4)) are comparable with those reported for \textit{E. coli} Hsp31 (\(K_m = 1.43\) mM, \(k_{cat} = 2.7\) s\textsuperscript{-1}) (16). A notable structural difference between the fungal and prokaryotic enzymes is the more exposed active site in \textit{C. albicans} Glx3 that is partially occluded by an \(\alpha\)-helix in \textit{E. coli} Hsp31 (Fig. 3B). As expected, mutation of the proposed catalytic nucleophile Cys\textsuperscript{136} to serine resulted in an inactive enzyme (Table 3 and Fig. 4). Mutation of His\textsuperscript{137} to phenylalanine in the catalytic triad gave ~15% of wild-type activity (Table 3), suggesting that His\textsuperscript{137} also plays an important role during catalysis. It may serve as a general acid/base for one or more of the proton shuffling steps that are required to convert MG to lactate without a change in redox state. A proposed mechanism for glyoxalase III enzymatic activity is presented under “Discussion.”

\textit{Methylglyoxalase Activity Is Common to Yeast Members of the Hsp31 Clade}—Three recombinant yeast enzymes exhibited significant glyoxalase activity (Table 3), whereas under our assay conditions, we could detect only very weak glyoxalase activity for recombinant human DJ-1 (Table 3). The \textit{S. cerevisiae} protein YDR533C had a comparable specific activity for
both MG consumption and D-lactate production to *C. albicans* Glx3 (Table 3), reflecting the aforementioned structural similarities of these two members of the Hsp31 clade of the DJ-1 superfamily. Interestingly, significant consumption of MG was also measured for *S. pombe* DJ-1 (SPAC22E12.03c), which is a close homolog of human DJ-1 and not a member of the Hsp31 clade (52, 53). However, unlike *C. albicans* Glx3 and *S. cerevisiae* YDR533C, no D-lactate production was detected for the human or *Schizosaccharomyces pombe* enzymes, suggesting either that another product is generated or that the D-lactate assay was not sensitive enough to detect the small quantity of product formed. The physiological relevance of the glyoxalase activities of the human and *S. pombe* proteins are therefore unclear. The Hsp31-like yeast proteins CaGlx3 and ScHsp31/YDR533C, in contrast, have robust glyoxalase activities and produce the expected D-lactate product, suggesting conservation of function for this clade of the DJ-1 superfamily.

Glx3 Confers Protection against Both Exogenous and Endogenously Generated Methylglyoxal—*C. albicans* is a diploid organism, and consequently the biological significance of Glx3 must be assessed via a double knock-out mutant. The double knock-out (glx3-Δ1/glx3-Δ1) and reconstituted strains were created by standard procedures (34), confirmed by Southern blots (Fig. 5), and then tested for their respective sensitivities to exogenous MG in yeast extract/peptone + 2% glucose (YPD) medium under aerobic culture conditions. The glx3-Δ1/glx3-Δ1 mutant was more sensitive to 20 and 40 mM exogenous MG than was its wild-type parent (Fig. 6). In contrast, 10 mM MG had no effect on the growth of any strain (Fig. 6). This experiment establishes that Glx3 can detoxify MG in vivo; however, we note that the high levels of exogenous MG used here are of uncertain physiological relevance.

Endogenous MG is generated as an unavoidable consequence of central carbon metabolism and was modulated by altering the carbon source provided to cultured *C. albicans*. Of the commonly used carbon sources in media, glycerol has been shown to produce the highest levels of triose phosphates, the immediate precursors to MG (54). The metabolic defects caused by Glx3 deficiency are evident when cell yields obtained by growth in yeast extract/peptone (YP), yeast extract/peptone/glucose (YPD), and yeast extract/peptone/glycerol (YPG) media are compared (Fig. 7). Note that following microbiological convention, the OD600 values of all growth curves in Figs. 6–8 are plotted on logarithmic y axes. Both the wild-type and Glx3-reconstituted (glx3-Δ1/glx3-Δ1/GLX3) *C. albicans* had equivalent cell yields (OD600 ~10) on YPD and YPG but achieved only OD600 = 3.5 on YP alone. Thus, cells that expressed Glx3 used glucose and glycerol equivalently, resulting in an ~2.5-fold increased yield compared with YP alone. In contrast, the glx3-Δ1/glx3-Δ1 mutant had markedly reduced OD600 values of ~7 and 2.5 on YPD and YPG media, respec-
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Levels of intracellular methylglyoxal in C. albicans

The experiment was repeated in technical duplicate with replicate values differing by less than 5% for all measurements.

| Media       | Wild type | ΔGlx3 | ΔGlx3+glx3 |
|-------------|-----------|-------|------------|
| YP<sup>a</sup> | 4.5<sup>b</sup> | 13.1  | 12.3       |
| YPD        | 4.5       | 24.9  | 1.3        |
| YPG        | 13.8      | 49.5  | 12.8       |

<sup>a</sup> YP, yeast extract/peptone; YPD, yeast extract/peptone/dextrose; YPG, yeast extract/peptone/glycerol.
<sup>b</sup> All units are in nanomoles of methylglyoxal/g wet cell weight.

FIGURE 7. Glx3 protects against endogenous MG generated by basal catabolism. Cell yields for parental (filled circles), glx3 null mutants (open circles), and reconstituted (filled squares) C. albicans were measured by optical density in multiple media and plotted on semi-log axes as a function of time. A shows that all cell types grow comparably well on yeast extract/peptone (YP) medium. B, glx3 null cells (Δglx3; open circles) show a marked growth defect in YPD (YP + 2% glucose) medium that is complemented by restoration of the wild-type GLX3 locus (Δglx3/GLX3; filled squares). C, glx3 null cells (Δglx3; open circles) show a more pronounced growth defect in YPG (YP + 2% glycerol) medium that is complemented by restoration of the wild-type GLX3 locus (Δglx3/GLX3). YPG medium is expected to produce the highest levels of endogenous MG. Values shown are the average of triplicate experiments ± S.D.

Phosphate Supplementation Partially Complements the Growth Defect of Glx3 Null Cells in Glycerol—Inorganic phosphate (orthophosphate) plays a key role in the metabolism of triose phosphate sugars, and phosphate-rich trioses accumulate when yeasts are grown on glycerol as a carbon source (54). Adding 10 mM of supplemental potassium phosphate to the growth medium had no effect on the cell yields for wild-type C. albicans under any condition (Fig. 8, A–C) and only a slightly substantially increase the production of MG in S. cerevisiae (54). Therefore, Glx3 plays an important role in central carbon metabolism and cellular viability in C. albicans, particularly when glycerol is the principal carbon source.

Because Glx3 has glyoxalase activity in vitro, we tested the hypothesis that the observed growth defects of the knockout are due to elevated levels of endogenously produced MG. The levels of intracellular MG in late log phase cells were directly measured using the chemical capture of MG by α-phenylenediamine to form the UV-absorbing compound 2-methylquinoxaline that was monitored by HPLC (see “Experimental Procedures”). This quantitative method is highly selective for the target α-oxoaldehydes and is more efficient that previous approaches, which are important advantages when measurements are being made in the cellular milieu (35). Greater than 95% of the MG in solution reacted with α-phenylenediamine, and chloroform extraction recovered >96% of 2-methylquinoxaline from solution. The results are shown in Table 4. As expected, wild-type cells produced more endogenous MG in media containing glycerol (YPG) than in media containing glucose (YPD). Cells grown in media lacking either carbon source (YP) have an unexpectedly high level of MG, which is comparable with that of cells grown in YPD medium. The importance of Glx3 for detoxification of endogenously produced MG is shown by the 3–5-fold increase in levels of intracellular MG in glx3-Δ1/glx3-Δ1 cells grown in YPD or YPG media compared with wild-type cells grown in the same media. Re-introduction of the GLX3 gene into the knock-out strain restored intracellular MG concentrations to levels that are slightly below those of wild-type cells grown in either YPD or YPG media, confirming that elevated MG in the knock-out strain is due to the absence of Glx3. In contrast, MG levels in glx3-Δ1/glx3-Δ1 cells grown in YP are elevated and not reduced to base-line levels by restoration of GLX3. This is a topic for future investigation. These experiments allow for the simultaneous measurement of cellular glyoxal, a closely related α-oxoaldehyde. Glyoxal levels were unaffected by the presence or absence of Glx3 in all media, indicating that the enzyme is selective for MG as a substrate in vivo.
ameliorative effect for the \textit{glox3-\Delta1/glox3-\Delta1} mutant in YPD (Fig. 8B), but it doubled the cell yield for the \textit{glox3} null mutant in YPG (Fig. 8C). Because this additional inorganic phosphate has no effect on the yields of the wild-type cells, we conclude that YP medium is not limited in total phosphate. However, we found that the free orthophosphate concentration in this preparation of YP medium is only \(~0.7\) mM using the ammonium molybdate/ascorbate assay (55), indicating that free phosphate levels are low. The selectively positive effect that addition of inorganic phosphate has on the \textit{glox3-\Delta1/glox3-\Delta1} mutant in glycerol suggests that supplemental orthophosphate decreases MG levels in the cell, possibly by favoring more rapid catabolism of the triose phosphates that generate MG.

DISCUSSION

Functionally diverse members of the DJ-1 superfamily are found throughout the evolutionary tree (52, 56), and this diversity allows us to take a comparative approach by examining the enzymatic and physiological functions of these proteins in the lower eukaryotes. The proteins of interest in this study belong to the Hsp31 clade of the DJ-1 superfamily, which is well represented in bacteria and lower eukaryotes but largely absent in plants and animals (52, 56). Among the fungi, \textit{C. albicans}, a well studied human pathogen (57), has only one Hsp31 homolog (ORF 19.251), whereas the model yeast \textit{S. cerevisiae} has four as follows: YDR533C (also called Hsp31) along with the three additional proteins YPL280W (Hsp32), YOR391C (Hsp33), and YMR322C (SNO4). The latter three proteins are 69\% identical at the amino acid level to YDR533C and \(~99\%\) identical to each other (20). In \textit{S. cerevisiae}, Hsp31 has been knocked out, and the mutant is viable but with increased sensitivity to some oxidative stressors (19). \textit{E. coli} Hsp31, a chaperone and the charter member of this clade, is 23\% identical to YDR533C from \textit{S. cerevisiae} and 22\% identical to ORF 19.251 from \textit{C. albicans}. Because \textit{E. coli} Hsp31 was recently shown to have a glutathione-independent glyoxalase activity (16), we sought to determine whether this enzymatic activity was conserved and physiologically relevant in the eukaryotic members of the Hsp31 clade of the DJ-1 superfamily. The results of this work indicate that fungal Hsp31-like proteins are robust glyoxalases with physiologically relevant roles in detoxifying endogenously produced MG, particularly when glycerol is the principal carbon source.

Mechanism of the Glutathione-independent Glyoxalases—The Hsp31-like glutathione-independent glyoxalases possess a Glu-His-Cys catalytic triad, where the cysteine is highly conserved among nearly all DJ-1 superfamily members and is catalytically essential in diverse enzymes in the superfamily (58). From a mechanistic standpoint, the conserved cysteine in the glutathione-independent glyoxalase substitutes for the thiol of glutathione in glyoxalase I, producing a glyoxal-derived thiohemiacetal in Glx3 that obviates the need for glutathione and accounts for the absolute requirement for this conserved cysteine for glyoxalase III activity (16). Moreover, Glx3 enzymes use no cofactor to convert methylglyoxal to lactate, and the catalyzed reaction does not involve a redox component. These constraints are factored into a proposed general mechanism (Fig. 9) that is based on the currently accepted mechanism for glyoxalase I and a prior proposal for \textit{E. coli} Hsp31 (16). In this mechanism, the conserved cysteine residue (Cys\textsuperscript{136} in Glx3) acts as the catalytic nucleophile, and a general base abstracts a proton from the first tetrahedral thiohemiacetal intermediate to form an enediolate intermediate. This enediolate intermediate was first proposed in the reaction mechanism of glyoxalase I (lactoylglutathione lyase) (59, 60) and facilitates the critical C1-C2 proton shift that is, in effect, a keto-enol tautomerization. Subedi \textit{et al.} (16) proposed an enediolate intermediate for the glutathione-independent \textit{E. coli} glyoxalase III, and more recently, Kwon \textit{et al.} (61) have proposed an essentially identical mechanism for the more distantly related DJ-1 superfamily glutathione-independent glyoxalases from \textit{Arabidopsis thaliana}. Therefore, we suggest that this may be a
A recent report indicates that human DJ-1 and its close homologs, *Caenorhabditis elegans* DJR-1.1 and DJR-1.2, also possess glyoxalase activity (62). This result is somewhat surprising, because these proteins are members of a distinct clade of the DJ-1 superfamily and possess active sites and dimeric structures that are substantially different from the Hsp31-like proteins. In particular, there is no histidine residue in human DJ-1, DJR-1.1, or DJR-1.2 that is structurally equivalent to the important His<sup>137</sup> residue in *C. albicans* Glx3. As expected from these major structural differences, we detect only weak methylglyoxalase activity for human DJ-1, although we find somewhat higher activity for its *S. pombe* homolog SPAC22E12.03c. We could not detect production of the expected product D-lactate by either protein. This could be because these enzymes generate L-lactate or some other product or that our assay is insufficiently sensitive to detect the small amounts of D-lactate produced by these less active DJ-1 homologs.

There are two methodological differences that may account for the discrepancies between our observations and previously reported results. First, we used only MG as a substrate, whereas Lee et al. (62) also used glyoxal, which appears to be a superior substrate for some of the DJ-1 proteins. Second, we performed our assays at 30 and 37 °C, which are standard physiological temperatures for *C. albicans*, whereas Lee et al. (62) performed their in vitro assays at 45 °C. We note, however, that Lee et al. (62) were also able to detect glutathione-independent glyoxalase activity in mouse and worm extracts at 22 and 37 °C but not in extracts prepared from DJ-1 knockouts (62). Although the specific reason for these differences is unclear, our results indicate that Hsp31-like proteins are considerably more active methylglyoxalases than human DJ-1. This conclusion is consistent with a recent report on the glyoxalase activities of several *A. thaliana* members of the DJ-1 superfamily, which found that the closest homologs of human DJ-1 in that organism (AtDJ-1A and AtDJ-1B) have only modest glyoxalase activity (61). Therefore, it remains to be established if the glyoxalase activities of human DJ-1 and its close homologs are physiologically relevant. In contrast, the glyoxalase activities of the Hsp31-like members of the DJ-1 superfamily are of clear physiological significance. Functions of Hsp31-like Glx3 Proteins in Yeasts—A persistent question about MG metabolism is what, if any, purpose does this compound serve in the cell? In bacteria, one answer to this question is the MG bypass, which directs carbon flux from DHAP to pyruvate through MG and D-lactate intermediates and thus avoids the phosphate-intensive synthesis of 1,3-bisphosphoglycerate under phosphate-limiting conditions. In many eukaryotes, MG production from triose phosphates is primarily nonenzymatic, and thus no dedicated MG bypass exists. Therefore, eukaryotic glyoxalases may serve simply to detoxify MG that is unintentionally produced by metabolism. However, these glyoxalases may also serve a role in eukaryotes as a metabolic “spillway” for triose phosphate pools that accumulate under nonoptimal growth conditions, including when using glycerol as a primary carbon source. This MG spillway serves two ends. First, it can produce pyruvate from triose phosphates in a GAPDH-independent pathway that includes D-lactate dehydrogenase. Second, it ensures flux through glycolysis by preventing the inhibitory accumulation of large pools of triose phosphates. The accumulation of triose phosphates is a particular risk in inorganic phosphate-limited conditions where GAPDH activity (which requires inorganic phosphate as a substrate) may be compromised. Because this alternative pathway also sacrifices all of the ATP-synthesizing steps of glycolysis, it can only provide an energetic benefit for yeast growing aerobically, as MG-derived pyruvate can only feed into the TCA cycle and produce NADH to drive oxidative phosphorylation in the presence of oxygen. Therefore, in order for cells to use the EMP pathway, they must have evolved a way to address two related issues: avoiding the large scale accumulation of inhibitory triose phosphates and detoxifying the low levels of MG that arise even if the carbon flux and phosphate availability are sufficient to keep the triose phosphate levels low. These evolutionary constraints are both satisfied by glyoxalases such as Glx3, possibly accounting for its wide distribution and high degree of conservation in lower eukaryotes.

In this study, we have shown that the glutathione-independent glyoxalase Glx3 is an important component of central metabolism in *C. albicans*, especially when glycerol is the primary carbon source. *C. albicans* is particularly well suited to the study of endogenously produced MG because it does not switch between respiratory and fermentative growth in aerobic atmosphere as a function of carbon source (i.e. it is a Crabtree negative yeast). *C. albicans* also contains the glutathione-dependent Glo1/Glo2 glyoxalase system, and therefore we conclude that the glutathione-independent Glx3 enzyme studied here is functionally distinct and provides a growth advantage even when the Glo1/Glo2 system is functional. This is somewhat surprising, given that Glx3 is a significantly less efficient enzyme ($k_{cat}/K_m = 1.4 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$) than is Glo1 ($k_{cat}/K_m = 1.2 \times 10^7 \text{ M}^{-1} \text{s}^{-1}$) (63). However, unlike Glx3, Glo1 also requires reduced glutathione as a co-substrate and has complex kinetics...
as a result (64). Therefore, the in vivo rates of MG detoxification by Glo1 and Glx3 may be less divergent than suggested by their differing catalytic efficiencies. Furthermore, Glx3 activity would be particularly valuable to the cell in conditions where the Glo1/Glo2 system is less effective due to diminished levels of reduced glutathione, such as chronic oxidative stress, stationary phase, or sulfur limitation. Importantly, the physiological relevance of Glx3 activity was directly demonstrated by the 3–5-fold elevation of intracellular MG levels in glx3 knock-out cells grown in YPD and YPG media compared with wild-type cells, confirming that Glx3 detoxifies MG in vivo.

As glycerol catabolism produces high levels of triose phosphates (54), our observation that growth of the glx3 null mutant is strongly impaired when glycerol is the primary carbon source is consistent with a spillway hypothesis for MG and Glx3 activity. This hypothesis was confirmed by the marked elevation in the intracellular MG level measured in the glo1/gl2 system is less effective due to diminished levels of phosphate supplementation helps mitigate. A study showed that YDR533C mitigates oxidative stress in S. cerevisiae by catalyzing the reaction between hexose diphosphate and dihydroxyacetone diphosphate.

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