Identification and Phenotypic Analysis of Two Glyoxalase II Encoding Genes from *Saccharomyces cerevisiae*, GLO2 and GLO4, and Intracellular Localization of the Corresponding Proteins*  

(Received for publication, April 9, 1997)

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We have isolated and characterized two genes coding for the glyoxalase II enzyme from *Saccharomyces cerevisiae*. The coding region of the GLO2 gene corresponds to a protein with 274 amino acids and a molecular mass of 31,306 daltons. The open reading frame of the GLO4 gene could be translated into a protein with 285 amino acids and a molecular mass of 32,325 daltons. The amino acid sequences of the deduced proteins are 59.1% identical and show high similarities to the sequence of the human glyoxalase II. When grown on either glucose or glycerol as a carbon source, a *glo2 glo4* double deletion strain contains no glyoxalase II activity at all and shows no obvious phenotype during vegetative growth. However, this strain showed a similar high sensitivity against exogenous methylglyoxal as compared with a glyoxalase I-deficient strain. Whereas the GLO2 gene is expressed on both glucose and glycerol, the GLO4 gene is only active on glycerol. The active Glo2p protein is localized in the cytoplasm and the active Glo4p in the mitochondrial matrix. Heterologous expression of the full-length GLO2 coding region in *Escherichia coli* resulted in an active protein. However, to get an active Glo4p protein in *E. coli*, the putative mitochondrial transit peptide at the N-terminal end had to be removed by shortening the 5' end of the GLO4 open reading frame.

The glyoxalase system (for review see Ref. 1) consists of two enzymes, glyoxalase I and glyoxalase II. Using glutathione as cofactor it converts 2-oxoaldehydes into the corresponding 2-hydroxy acids. The most important substrate seems to be methylglyoxal. In most organisms, this toxic compound is formed as a by-product of glycolysis from triose phosphates through the action of triose-phosphate isomerase (2). But the main source for methylglyoxal in yeast and other microorganisms is dihydroxyacetone phosphate which is converted to methylglyoxal by the methylglyoxal synthase (3, 4). As shown in Reaction 1 (where MG, HTA, and GSH indicate methylglyoxal, hemithioacetal, and glutathione, respectively), the first step in the conversion of methylglyoxal into D-lactic acid through the glyoxalase system is the spontaneous formation of hemithioacetal from the reaction of methylglyoxal with reduced glutathione. Hemithioacetal is metabolized to S-D-lactoylglutathione (SLG) by glyoxalase I. Glyoxalase II catalyzes the reaction of SLG to D-lactate and GSH.

\[
\begin{align*}
\text{CH}_3\text{CHO} + \text{GSH} & \rightarrow \text{CHOH} + \text{GSH} \\
\text{CHO} + \text{GSH} & \rightarrow \text{GSH} + \text{COOH}
\end{align*}
\]

**REACTION 1**

The glyoxalase system is present in all organisms analyzed so far (5–7) and is thought to be ubiquitous. It is clear that the glyoxalase system can detoxify methylglyoxal and other 2-oxoaldehydes, but the exact biological role of the glyoxalase system is still unclear. It has been shown that there is some correlation of the activities of the two enzymes with proliferation and differentiation of eucaryotic cells. In several systems it has been shown that immature, proliferating cells and tissues have relatively high glyoxalase I activities and low glyoxalase II activities, whereas mature, differentiated cells have relatively low glyoxalase I activities and high glyoxalase II activities (reviewed in Refs. 1, 8, and 9). Therefore, it was presumed that in growing cells, there should be an increase of the SLG level compared with resting cells. In accordance with this, inhibitors of glyoxalase I lead to a slowing down or even complete arrest of cell growth of plant or animal cells. However, up to now there is no evidence for a causal relationship between the glyoxalase system and cell proliferation.

Recently, Inoue and Kimura (10) characterized the glyoxalase I gene, GLO1, from *Saccharomyces cerevisiae*. Besides an enhanced sensitivity against the presence of methylglyoxal in the growth medium, they could not detect any obvious phenotype of a glo1 null mutant. Genes encoding glyoxalase I enzymes were isolated and characterized previously also from other species (11–14). However, the first glyoxalase II gene isolated recently (15) was the human glyoxalase II gene, *HAGH1*. It has been shown that glyoxalase II is present in the cytoplasm and the mitochondria of rat liver cells (16). It is not clear if the two isoenzymes are encoded by different genes.

Here we present the cloning and characterization of two genes from *S. cerevisiae* which code for functional glyoxalase II enzymes. Originally, we identified one of the genes (referred herein as GLO4) as a multicopy suppressor gene for a mutant yeast strain which has a reduced efficiency of spore germination (17). The suppressor gene showed a high homology to the

* This work was supported by Grant P10831-MOB from the Austrian Fonds zur Förderung der wissenschaftlichen Forschung FWF. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: SLG, S-D-lactoylglutathione; PCR, polymerase chain reaction; bp, base pair(s); ORF, open reading frame.
human HAGH1 gene. The deduced protein showed also 59% identity to another unidentified protein predicted from an ORF (GenBank/EMBL accession no. U51030) identified in the yeast genome project. Therefore, we cloned this gene (referred herein as GLO2 and GLO4) and/or the glyoxalase I gene. Using these deletion strains constructed which lacked either one or both of the glyoxalase II genes and/or the glyoxalase I gene. This study

Microorganisms and Cultures—The strains and plasmids used in this study are listed in Table I. E. coli cells were grown in Luria broth (LB) at 37 °C. Yeast cells were grown at 28 °C on YPD complex medium (2% glucose, 2% peptone, 1% yeast extract) or on synthetic minimal medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate) containing the appropriate amino acids, adenine and either 2% glucose or 3% glycerol. For the growth experiments in the presence of methylglyoxal, cells were grown up to an optical density at 600 nm (A600) of 1 in synthetic minimal medium as above. 4 ml of fresh medium (in 15 ml tubes) containing various concentrations of methylglyoxal (0, 2, 4, 6, or 8 mM) were inoculated with these cells to an initial A600 between 0.05 and 0.08. The A600 was measured every 5 h over a period of 25 h.

Amplification of DNA by Polymerase Chain Reaction (PCR)—DNA was amplified by PCR (18) using the Taq DNA polymerase. 100 ng of chromosomal yeast DNA or 2 ng of plasmid DNA was used in 100-μl reaction mixtures containing 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 50 mM KCl, 0.2 mM dNTP, and 0.5 μM 5’ and 3’ primers. 3 units of Taq DNA polymerase were added. The mixture was overlaid with 50 μl of mineral oil. After an initial denaturation step at 95 °C for 5 min, 30 cycles of amplification of chromosomal DNA or 15 cycles of amplification of plasmid DNA followed. Each cycle consisted of 1 min of DNA denaturation at 94 °C, 1 min of primer annealing at 48–54 °C (depending on the primers used), and 1 to 2 min of DNA synthesis (depending on the length of the amplified DNA).

DNA Cloning and DNA Sequencing—DNA cloning and sequencing were done as described by Sambrook et al. (19). DNA was sequenced manually by the chain termination method (20). For the sequencing of the GLO2 gene and GLO4 gene, vector primers, cloning primers (see below), and different sequencing primers (not shown) were used.

Construction of pE351G2—The GLO2 gene was PCR-amplified by using the Glu2–5 Eco 5’-CCG AAA TCT CCGGACGATG TGCAAAGC-3’ primers.
Yeast Glyoxalase II Genes, GLO2 and GLO4

Deletion of the Glyoxalase Genes in Yeast—Haploid strains with deletions of the glyoxalase genes were generated by the one-step gene replacement method (24). The deleted regions of the genes are shown in Fig. 5. For the construction of the GLO2 deletion cassette, the GLO2 ORF was cut out from plasmid pE351G2 with the restriction enzymes ClaI and SacI, blunt-ended by Klenow polymerase, and replaced by the URA3 gene, resulting in plasmid pE351G2ΔClaI. This plasmid was digested with BglII and XhoI. The fragment containing the GLO2 deletion cassette was purified by agarose gel electrophoresis and used for the transformation of yeast strain W303Δ. For the deletion of the GLO4 gene from strain W303Δ, the GLO4 deletion cassette was constructed by cloning the GLO4 ORF from plasmid pE351G4 into plasmid pUC18 using the KpnI and BamHI sites. From the resulting plasmid pUCG4, the GLO4 ORF ranging from the PetI to the HindIII site was replaced by the URA3 gene, resulting in plasmid pUCG4Δ. The SfuI-BamHI fragment was used as the GLO4 deletion cassette. For the deletion of the GLO1 gene in the strain W303Δ, the plasmid pE351G1Δ was constructed as follows. The plasmid pE351G1 was cut with BamHI. The fragment that lacked the GLO1 ORF and part of the GLO1 promoter was ligated with the pKanMX4 module which had been taken from plasmid pKanMX4 (25). The SalI-PmlII fragment was used as the GLO1 deletion cassette. All deletions were shown to be correct by PCR and Southern blotting using genomic DNA from the respective deletion strains (see below). Haploid and the corresponding diploid strains missing two or all three glyoxalase genes were generated by crossing.

Genomic Southern Analysis—Genomic DNA from the yeast strains with different deletions of glyoxalase genes was digested with SspI in the case of the glo2 and glo4 deletion loci or with HindIII for the glo1 deletion locus, respectively, and blotted onto ZETA probe membrane by alkaline capillary transfer (26). The blots were hybridized with different probes for the glyoxalase genes. These probes were labeled with 32P-dATP by random priming (27) using the Kit from Boehringer Mannheim. As probes, the following restriction fragments were used (see Fig. 5): the ClaI-EcoRI fragment of the GLO2 gene, the SfuI-DraI fragment of the GLO4 gene, and the HindIII-Ndel fragment of the GLO1 gene, respectively. The wild type GLO2 locus and the wild type GLO4 showed 1100-bp bands, respectively, whereas 1500-bp bands were characteristic for the deletion loci in both cases. The wild type GLO1 locus is characterized by a 1100-bp fragment, and the corresponding deletion locus shows a 700-bp band.

Heterologous Expression of the Glyoxalase II Proteins in E. coli and Preparation of Cell Lysates—10-ml cultures (grown in LB) were induced at an A600 of 0.2–0.4 with 0.2 mM isopropyl-β-D-thiogalactoside and grown for a further 3 h at 37 °C. The bacteria were harvested by centrifugation and resuspended in 50 mM Tris-HCl, pH 7.5, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol. The cells were lysed by several freeze-thaw cycles. The cell lysates were centrifuged at 4 °C for 30 min at 60,000 × g to remove cell debris. The total protein concentration was determined by the method described by Bradford (28). Total protein extracts were prepared by resuspension of pelleted cells in SDS-polyacrylamide gel electrophoresis sample buffer containing 2% SDS and boiling for 5 min. Electrophoretic separation of protein extracts on the SDS-polyacrylamide gel electrophoresis was done as described (29).

Preparation of the Cytoplasmic and Mitochondrial Fractions of Yeast Cells—For the isolation of the cytoplasmic and mitochondrial compartments, yeast strains were cultured in 400 ml of minimal synthetic media with and without 2% galactose in 5-liter Erlenmeyer flasks. For the growth of deletion strains W3G2 and W3G4, the growth medium contained 3% glycerol instead of glucose, and the cells were harvested by centrifugation at an A600 of 1 to 1.5. The strains W3G2ΔpGlo2Δg and W3G2ΔpGlo4Δg were grown in a medium containing 5% glycerol and 0.1% glucose up to an A600 of 0.5. For the induction of the GAL1 promoter 2% galactose was added to the medium, and the strains were grown for further 6 h before the cells were harvested. The cytoplasmic and the mitochondrial compartments of the cells were prepared by differential centrifugation (30). Further subfractionation of the mitochondria was done as described (30).

Determination of the Glyoxalase I and Glyoxalase II Activity and Preparation of Yeast Cell Extracts—For the determination of the specific enzyme activity of glyoxalase I and II, the cultures were grown up to an A600 of 1 to 1.5. The cells were harvested by centrifugation, washed once in water, and pelleted again. The cells were resuspended in 50 mM Tris-HCl, pH 7.5, 0.5 mM N-phenylmethylsulfonyl fluoride, 1 mM dithiothreitol and disrupted by vortexing with glass beads. To remove cellular debris and macromolecular structures, the cell homogenates were centrifuged at 30,000 × g for 30 min at 4 °C. The supernatants were used as cell extracts. The total protein concentration was determined by the method described by Bradford (28).

Glyoxalase I and glyoxalase II activity was measured as described (32, 33). One unit of glyoxalase II is defined as the amount of enzyme catalyzing the hydrolysis of 1 μmol of S-lactoylglutathione (SLG) in 1 min at 37 °C under the assay conditions (300 μg SLG, 50 mM Tris-HCl, pH 7.5, 2% SLG hydrolysis was followed spectrophotometrically at 240 nm (ε = 3.1 nmol−1 cm−1−1). For the determination of the glyoxalase II activity (given in units glyoxalase II per mg total protein) different amounts of extracts were used to measure the same decrease of SLG of approximately 10% of the total SLG content in the assay reaction.

RESULTS

Molecular Cloning and Sequence Determination of the Glyoxalase II Genes—Originally we identified the gene which we propose to name GLO42 as a multicopy suppressor for a spore germination defect of a mutant yeast strain (17). The mutant strain was transformed with a genomic yeast library constructed in the BamHI site of plasmid Yep13 (34). The insert of a complementing plasmid (pE13G4) was sequenced. The sequence (Fig. 1) contained the SNR9 gene coding for the small nuclear RNA 9, the 3’ end of the CK2b gene coding for the β subunit of casein kinase 2, and one complete unknown open reading frame. For a long time, no significant homology of the deduced protein sequence to any protein of known function could be found in the sequence data bases, and we had no indication of the protein function. At the beginning of 1996 the structural gene for the human glyoxalase II (15), HAGH1, was characterized (GenBank/EMBL accession no. X80999). The deduced amino acid sequence of the suppressor gene ORF showed 34% amino acid identity to the sequence of the human glyoxalase II protein. Furthermore, in April, 1996, the yeast genome sequencing project was finished, and the DNA sequence determined by us revealed 100% identity to a region on chromosome XV (EMBL accession no. Z74948 and X87391). Previously, we had also mapped the GLO4 gene to chromosome

2 The gene names GLO2 and GLO4 were reserved in the SGD Gene Name Registry (internet: http://genome-www.stanford.edu.html); the gene name GLO3 was already registered for a gene encoding a zinc finger protein of yet unknown function with similarity to the Gcs1 protein (Gcs1p-like ORF).
XV by chromosomal Southern blotting (35). The predicted Glo4 protein has a length of 285 amino acids and the calculated molecular mass is 32,325 Da.

The yeast genome sequence also contains a second ORF on yeast chromosome IV (GenBank/EMBL accession no. U51030) which is homologous to the human glyoxalase; the deduced amino acid sequence has 59.1% identity to our putative yeast glyoxalase II, and 41.2% identity to the human glyoxalase II sequence. Therefore, we amplified by PCR a genomic fragment encompassing this second hypothetical glyoxalase II gene and 300 bp upstream and 240 bp downstream of the ORF. We propose to name this gene GLO2.

At the 5′ end, the upstream primer contained EcoRI and BspEI restriction sites, and the downstream primer contained a BamHI site at the 5′ ends. The PCR product showed the predicted length of 1430 bp. It was digested with EcoRI and BamHI and cloned into YEp351 vector (21) resulting in plasmid pE351G2. The DNA sequence determined (Fig. 2) was 100% identical to that found in the data base. The GLO2 open reading frame could code for a protein of 274 amino acids and the calculated molecular mass is 31,306 Da.

To verify that the two yeast genes are glyoxalase II genes, the yeast strain W303e was transformed either with the plasmid pE13G4 or pE351G2. Transformants were grown in synthetic medium without leucine for plasmid selection and assayed for glyoxalase II activity. In these experiments glucose was the sole carbon source. The strain harboring the GLO2 gene in high copy number showed an approximately 10 times higher glyoxalase II activity than the untransformed wild type strain. The strain harboring the GLO4 gene in high copy number showed no significant increase in glyoxalase II activity (not shown, similar experiments are described below). So we had the first indication that the GLO2 gene encodes a glyoxalase II protein. On the other hand we had no such indication for the GLO4 gene.

Amino Acid Sequence Comparison of Glyoxalase II Proteins—In the GenBank/EMBL data base we found 16 further proteins predicted from open reading frames that are homologous to the two yeast and the human glyoxalase II proteins. The length of the predicted proteins lies between 206 (the protein from *Methanococcus jannaschii* ) and 285 amino acids (Glo4p from yeast). The alignment of all these sequences (Fig. 3) reveals six regions (box I to VI) that are highly conserved and are distributed over nearly the whole length of the protein.

Cloning and Heterologous Expression of the Yeast Glyoxalase II ORFs in *E. coli*—To verify that both yeast ORFs are coding for glyoxalase II enzymes, the complete ORFs from both genes were amplified by PCR and cloned between the NdeI and the StuI sites of *E. coli* vector pMW172 (22). The inserts of the resulting plasmids, pGlo2-Ec2 and pGlo4-Ec2, were sequenced and found to be correct. The plasmids were transformed into the *E. coli* strain BL21(DE3) that allows the expression of the

![Fig. 1. Nucleotide sequence and deduced amino acid sequence of the GLO4 gene from *S. cerevisiae*.](image)
plasmid inserts after induction with isopropyl-β-D-thiogalacto-
side (36). In total protein extracts of both transformant strains
additional proteins compared with the strain transformed with
pMW172 vector without an insert are clearly visible on the
SDS-polyacrylamide gel electrophoresis (Fig. 4). The apparent
molecular weight of the induced protein of the
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molecular weight of the induced protein of the

GLO2

orf (Fig. 2). Nucleotide sequence and deduced amino acid sequence of the GLO2 gene from S. cerevisiae. Two sequences with homology to the TATA box consensus are underlined. The sequences of the PCR primers used for the cloning of the complete sequence in the yeast vector and for the cloning of the GLO2 ORF in the E. coli vector are marked by arrows; dashes indicate additional nucleotides for the cloning sites at the 5’ end of the primers. The SspI site used for the cloning of the ORF in E. coli is indicated. At the beginning of the sequence there is the end of another
orf (with homology to a copper transporting ATPase) which is shown in italics.

FIG. 2. Nucleotide sequence and deduced amino acid sequence of the GLO2 gene from S. cerevisiae. Two sequences with homology to the TATA box consensus are underlined. The sequences of the PCR primers used for the cloning of the complete sequence in the yeast vector and for the cloning of the GLO2 ORF in the E. coli vector are marked by arrows; dashes indicate additional nucleotides for the cloning sites at the 5’ end of the primers. The SspI site used for the cloning of the ORF in E. coli is indicated. At the beginning of the sequence there is the end of another ORF (with homology to a copper transporting ATPase) which is shown in italics.
FIG. 3. Sequence alignment of the glyoxalase II proteins and homologous ORFs from different organisms. The sequences were aligned by the GCG program. The upper five sequences and the sequence of Arabidopsis thaliana are from eucaryotic organisms; the two lower Yeast Glyoxalase II Genes, GLO2 and GLO4.
but showed approximately 32% activity on glycerol compared with the wild type activity. This activity is due to the GLO4 gene because the glo2 glo4 double deletion strain (W3G24) had no glyoxalase II activity at all when grown on either glucose or glycerol. Both GLO2 and GLO4 code for glyoxalase II enzymes active in vivo. From these data it seems very likely that there are no other glyoxalase II genes in yeast besides GLO2 and GLO4. The data were confirmed by the results obtained with transformants of the double mutant strain W3G24 harboring either the GLO2 multicopy plasmid pE351G2 or the GLO4 multicopy plasmid pGlo4-Ec2. The strain containing multiple copies of the GLO2 gene shows an approximately 7-fold higher glyoxalase II activity than the wild type strain on both carbon sources. The double deletion strain transformed with the GLO4 multicopy plasmid, pE13G4, when grown on glycerol has a 5-fold higher glyoxalase II activity as compared with the glo2 deletion (= single copy GLO4) strain, W3G2. The same strain grown on glucose displays a small residual glyoxalase II activity of 22% compared with the activity present in the wild type. Our interpretation of this finding is that while a chromosomal single copy of GLO4 (W3G2) is completely repressed on glucose, a partial derepression occurs if the gene is present on an episomal multicopy plasmid.

**Effect of the Copy Number of GLO2 and GLO4 on Growth in Methylglyoxal-containing Medium—** Sensitivity to methylglyoxal was tested in the homozygous diploid deletion strains and the strains overexpressing the two glyoxalase II genes and compared with the wild type strain (Fig. 6). The growth rates of all strains decreased with increasing methylglyoxal concentrations in the growth medium. The growth of the wild type strain was completely inhibited at 8 mM methylglyoxal on glucose and at 6 mM methylglyoxal on glycerol. The single mutant strains were sensitive against lower methylglyoxal concentrations. The minimal growth inhibitory concentration was 6 mM methylglyoxal for the glo4 deletion strain on both carbon sources. The growth of the glo2 deletion strain was totally inhibited at 4 mM methylglyoxal on glucose and at 6 mM on glycerol. The minimal inhibitory concentration of the glo2 glo4 double mutant strain was 2 mM methylglyoxal on glucose and at 4 mM on glycerol. All strains lacking the GLO1 gene were inhibited at 2 mM methylglyoxal on both carbon sources (only the glo1 deletion strain, W3G1, is shown). The glo2 glo4 double deletion strains harboring either the GLO2 or the GLO4 multicopy plasmid showed only poor growth in the presence of 4 mM methylglyoxal on both carbon sources and were therefore not able to reach wild type resistance against methylglyoxal. A more detailed interpretation of the results is given under “Discussion.”

**Intracellular Localization of the Glyoxalase II Proteins—** ActGlo4p could be possibly localized in the mitochondria because (i) heterologous GLO4 expression in E. coli showed that the protein has an N-terminal extension which must be removed to get an active enzyme, (ii) the GLO4 expression in yeast is induced on a carbon source (glycerol) which can support growth only in the presence of active mitochondria, and (iii) the PSORT program which uses the algorithm of Nakai and Kanehisa predicts equal probabilities for the localization of the Glo4p in the cytoplasm and in the mitochondria, whereas the Glo2p is predicted to be present mainly in the cytoplasm. The possible cleavage site for the mitochondrial targeting sequence is at position 21 (see Fig. 1).

To find out if the two glyoxalase II proteins are in fact localized in different cellular compartments, we isolated the cytoplasmic and mitochondrial fractions from the two deletion strains, W3G2 and W3G4, which were grown in 400 ml of glycerol-containing synthetic media. Whereas the Glo2p showed a 11.5 times higher activity in the cytoplasm than in the mitochondria, the Glo4p activity was found to be 15.0 times higher in the mitochondria compared with the cytoplasm (Table IV). However, the total amount of the glyoxalase II activity of the W3G2 strain in these experiments was relatively low as compared with the experiment with the small scale cultures (Table III), maybe as a result of the poorer aeration in the medium scale cultures. Therefore, we decided to clone the GLO2 and GLO4 genes under the control of the GAL1p promoter

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**TABLE II**

| Strain/plasmid | Glyoxalase II activity ± S.D.* (units/mg protein) |
|---------------|-----------------------------------------------|
| BL21(DE3)/pMW172 | 0 ± 0.01                                      |
| BL21(DE3)/pGlo2-Ec2 | 168.60 ± 2.26                               |
| BL21(DE3)/pGlo4-Ec | 0 ± 0.00                                      |
| BL21(DE3)/pGlo4-Ec3 | 55.70 ± 8.46                                  |

*Activities are the mean values of three or six independent experiments.

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**FIG. 4.** Heterologous expression of the yeast glyoxalase II genes in E. coli. E. coli strain BL21(DE3) was transformed with the plasmids indicated below. The transformants were cultured in 10 ml of LB medium and induced for glyoxalase II gene expression. The 60,000 x g supernatant (7 µg of protein amount) of lysed cells (lanes 1–5) prepared by several freeze-thaw cycles and the total protein extracts (lanes 6–10) prepared by boiling whole cell pellets in SDS-containing sample buffer were electrophoresed under reducing conditions on a gradient (8–28%) polyacrylamide gel. The separated proteins were visualized by staining with Coomassie Blue. Lane M, molecular mass markers; lanes 1 and 6, pMW172; lanes 2 and 7, pGlo2-Ec2; lanes 3 and 8, pGlo4-Ec3; lanes 4, 5, 9, and 10, pGlo4-Ec2 (two different transformants). Arrows indicate the position of induced glyoxalase II proteins. The molecular mass of the marker proteins is given in kDa.

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The sequences are from archaeabacteria; the other sequences are of eubacterial origin. Only the upper three sequences (Glo4p, Glo2p, from yeast and the human Highlp) represent glyoxalase II proteins that are identified and characterized so far. The other sequences are deduced from unidentified ORFs in the data base. At the end of each sequence the length of the polypeptide and the GenBank/EMBL accession number (in parentheses) are given. Amino acid residues that are identical or similar to the consensus sequence are shown in uppercase letters. Six regions (numbers I to VI) of high similarity are indicated by shaded boxes. In the consensus line, the residues that are identical or similar in 12 or more (out of 19) sequences are given in lowercase letters; totally conserved residues or 18/19 matches (R/K or D/E substitutions were considered as identities) are shown in uppercase letters; those amino acids that are present in 18 or all sequences and that could be involved in the binding and/or processing of the substrate (H, R/K, D/E) are underlined.
(23) to get a higher enzyme activity. The complete ORFs of the genes were cloned behind the \( \text{GAL1} \) promoter into the multicopy plasmid pYES2. From the resulting plasmids the \( \text{URA3} \) gene was replaced by the \( \text{LEU2} \) gene because the \( \text{URA3} \) gene was already used as a marker gene for the \( \text{glo2} \) and the \( \text{glo4} \) deletions. The resulting plasmids, pGlo2g5 and pGlo4g5, were introduced into the double deletion strain W3G24. The transformant strains, W3G24/pGlo2g5 and W3G24/pGlo4g5, were induced for glyoxalase II expression by galactose addition to exponential growth cultures, the cytoplasmic and mitochondrial fractions were isolated, and the corresponding glyoxalase II activities were determined (Table IV). In both cases, the expression of the genes from the \( \text{GAL1} \) promoter was much higher as compared with the expression from the cognate promoters and the localization determined previously was confirmed (Table IV). The Glo2p activity was found to be 44.7 times higher in the cytoplasm than in the mitochondria, and the Glo4p protein activity was 34.0 times higher in the mitochondria compared with the activity found in the cytoplasm. The cytoplasmic and mitochondrial fractions were characterized by Western blotting with antibodies directed to citrate synthase (mitochondrial localized) and hexokinase (cytoplasmically localized), respectively (not shown). For the cytoplasmic fraction a small contamination with the mitochondrial fraction could be detected. The reason seems to be lysis of mitochondria during the preparation of the cytoplasmic fraction which we could not prevent completely.

Further subfractionation of the mitochondrial fraction from the strain W3G24/pGlo4g5 revealed a 6.7-fold higher glyoxalase II activity in the matrix fraction compared with the intermembrane space fraction. The respective activities are 38.44 units/mg for the matrix fraction and 5.75 units/mg for the intermembrane space fraction (not shown). Again, we cannot exclude a small contamination of the intermembrane fraction with the matrix fraction.

**DISCUSSION**

In the present paper, we have shown that in *S. cerevisiae* glyoxalase II is encoded by two nuclear genes, which we propose to name \( \text{GLO2} \) and \( \text{GLO4} \). The \( \text{GLO2} \) gene (yeast ORF number YDR272w) is located on chromosome IV near \( \text{GCN2} \). The molecular mass of the Glo2 protein calculated from the deduced amino acid sequence is 31,306 Da. The \( \text{GLO4} \) gene...
(yeast ORF number YOR040w) is located on chromosome XV adjacent to CKB2. The molecular mass of the Glo4 protein calculated from the deduced amino acid sequence is 32,325 Da.

The heterologous overexpression of the complete GLO2 ORF or of a truncated GLO4 ORF in E. coli leads to high glyoxalase II activities, which is not detectable in E. coli strains transformed with the expression plasmid without an insert. Overexpression on multicopy plasmids in yeast leads to severalfold higher glyoxalase II activities as compared with the untransformed strain. A double deletion yeast strain missing both genes has no glyoxalase II activity at all. The yeast genome doesn’t contain another ORF with significant homology to GLO2 and GLO4. We conclude that the two genes, GLO2 and GLO4, are the only glyoxalase II encoding genes in yeast.

Single, double, or triple (with glo1 deletion) deletion strains were generated and tested for (i) enzyme activity, (ii) growth properties, and (iii) resistance to exogenous methylglyoxal. These experiments were performed on both glucose and glycerol as sole carbon sources. The results show that glyoxalase II, as well as the whole glyoxalase system, is not essential for growth on either carbon source (Fig. 6). Measurements of enzyme activity show that GLO2 is expressed on both carbon sources, whereas GLO4 is expressed exclusively on glycerol. However, the total glyoxalase II activity is increased only slightly on glycerol which is in contrast to the 20-fold increase of the glyoxalase I activity on this carbon source (39).

Localization studies of the glyoxalase II isoenzymes using different deletion strains and the overexpression of the two genes from the GAL1 promoter revealed that the enzymatically active Glo2 protein is located in the cytoplasm, and the enzymatically active Glo4p is located in the mitochondrial matrix. This is in accordance with the finding that the GLO4 expression is induced on glycerol. Utilization of glycerol for growth is possible only in cells with functionally active mitochondria. The

| Gene | Strain tested | Glyoxalase II activity \( \pm \) S.D. | Ratio |
|------|---------------|-------------------------------------|-------|
|      |               | Mitochondria | Cytoplasm | Mit/Cyt<sup>a</sup> | Cyt/Mit<sup>b</sup> |
| Glo2p | W3G4          | 0.016 ± 0.011 | 0.184 ± 0.014 | 11.5          |                   |
|       | W3G24/pGlo2g5 | 0.092 ± 0.009 | 4.108 ± 1.080 | 44.7          |                   |
| Glo4p | W3G2          | 0.060 ± 0.014 | 0.004 ± 0.004 | 15.0          |                   |
|       | W3G24/pGlo4g5 | 23.871 ± 6.357 | 0.703 ± 0.655 | 34.0          |                   |

<sup>a</sup> Activities are the mean values of six separate measurements.

<sup>b</sup> Mit/Cyt, mitochondria and cytoplasm, respectively.

**Fig. 6.** Effect of glyoxalase gene dosage on growth in media containing methylglyoxal. Different strains were cultured in test tubes containing 4 ml of minimal synthetic medium with the appropriate amino acids, 2% glucose or 3% glycerol, and various concentrations of methylglyoxal (●, 0 mM; □, 2 mM; ○, 4 mM; △, 6 mM; ▲, 8 mM). For strains W3G24/pE351G2 and W3G24/pE13G4 synthetic medium without leucine was used. Optical density (OD<sub>600</sub>) was measured every 5 h over a period of 25 h. Gene dosage of each strain is indicated in the upper part of the figure; +, single copy chromosomal gene; −, gene deleted; ++, multicopy episomal gene.
N-terminal end of the Glo4p shows the characteristics of a mitochondrial targeting sequence (40), namely a complete lack of negative charges and the presence of several positive charges, particularly arginine residues. The program PSORT predicts a possible cleavage site for the matrix protease at sequence position 21. However, the full-length polypeptide expressed in *E. coli* is hardly soluble and enzymatically inactive, whereas an N-terminal truncated polypeptide lacking the first 10 amino acids is soluble and enzymatically active. Taken together, the observations strongly indicate that Glo4p is indeed located in the mitochondrial matrix and an N-terminal targeting sequence must be cleaved off to produce an active enzyme. Also for rat liver it has been shown (16) that glyoxalase II is present in the cytoplasm and the mitochondria. It is not known whether the mammalian isoenzymes are encoded by different genes. But what is the significance of a mitochondrial glyoxalase II? We could not detect any glyoxalase I activity in mitochondria (not shown). As a result, there should be no S-2-lactoylglutathione as substrate in this compartment for the glyoxalase II enzyme and therefore no demand for the enzyme in mitochondria. Maybe the Glo4p accepts other thiol esters of glutathione that are formed spontaneously or by other enzymes in the mitochondria (16).

Although we could not detect a phenotype of the strains deficient for one, two, or three glyoxalase enzymes in normal vegetative growth on standard media, the growth of these strains was inhibited on media containing different amounts of methylglyoxal. When grown on glucose, strains deficient for glyoxalase I or deficient for both glyoxalase II enzymes are extremely sensitive to exogenous methylglyoxal. However, during growth on glycerol the presence of the glyoxalase I gene alone results in some resistance, presumably because of the enhanced expression of GLO1 on this carbon source. The implication of this finding is discussed below. Our experiments with growth inhibition of single deletion strains show that both the cytoplasmic (Glo2p) and the mitochondrial (Glo4p) enzyme must be present to achieve optimal protection from exogenous methylglyoxal (Fig. 6). Even the higher total amount of active glyoxalase II of the strains with multiple episomal copies of one glyoxalase II gene as compared with the wild type does not compensate for a complete lack of the other isoenzyme. Therefore, in yeast all three proteins involved in the glyoxalase system are needed for the detoxification of exogenous methylglyoxal *in vivo*. However, up to now the biological significance of the resistance to exogenous methylglyoxal is not known.

The amino acid sequences of Glo2p and the Glo4p of yeast are 59.1% identical to each other and 41.2 and 34.2% identical to the human glyoxalase II sequence (15), respectively. Searching the protein sequence data bases for protein homologues to Glo2p, Glo4p, and the human Hgrp1p revealed 16 additional homologous proteins of yet unknown function (Fig. 3). Protein sequences from widely divergent organisms are represented (archaeabacteria, eubacteria, fungi, plants, and animal), and most of them were found by systematic sequencing of the respective genomes. Six regions of significant high similarity (boxes I-VI) could be involved in the function of the proteins. These regions are distributed along the total protein and taken together comprise more than 20% of the total protein length. There is one sequence from *M. jannaschii* that lacks box 6. Interestingly, beside yeast there are three other organisms represented up to now, *E. coli*, *Haemophilus influenzae*, and *Synechocystis sp.* (all eubacteria) with two homologous proteins. But in contrast to yeast, the protein sequences of each pair of the procaryotes are only around 30% identical, and the proteins belong to different subgroups of extended similarity. The future will show if all these sequences are in fact glyoxalase II proteins.

From experiments on specific chemical modification resulting in inactivation of the glyoxalase II enzyme, it was proposed that histidine, arginine (or lysine), and aspartic acid (or glutamic acid) residues are required for the function of glyoxalase II (1, 41, 42). Each of these residues can be found in one or more of the conserved boxes, especially three histidine residues are totally conserved in box II (TH-H-DH).

However, the exact biological role of the ubiquitous glyoxalase system remains in the dark. The notion that the system could be involved in the regulation of cell proliferation (1, 8, 9) is not supported by our data as none of the deletion mutants showed a change in growth rate on either glucose or glycerol as sole carbon source. Maybe the system is important for survival under some still unknown physiological condition which leads to a much higher methylglyoxal level in the cell than present under “normal” growth condition. It has been shown (43) that the glutathione content in *S. cerevisiae* varies between different growth conditions. Therefore, another alternative role of the glyoxalase system could be the recycling of glutathione that has been “trapped” spontaneously by methylglyoxal resulting in the formation of hemithioacetal (1, 44). This hypothesis is supported by the fact that even in the presence of active glyoxalase I but in the absence of any glyoxalase II enzyme the growth in methylglyoxal-containing medium is inhibited. Another possible explanation of this growth inhibition toxicity of high SLG levels has to be assumed. The deletion mutants generated in this work will be very helpful to test these possibilities to some extent.

Acknowledgments—We thank Dr. R. Schricker for the suggestions on the preparation of mitochondria and Dr. G. Schatz (Basel) for providing the antibodies used. We are grateful to Dr. P. Briza and R. Nestelbacher for the help with the data base searches and analysis. We thank the rotation students A. Eiböck, D. Almer, R. Regl, K. Onder, and E. Döppler, who participated in some of the experiments presented here.

Note Added in Proof—Recently a cDNA sequence for a glyoxalase II enzyme from *Arabidopsis thaliana* was characterized by Riddrstrom and Mannervik (46) (GenBank/EMBL accession no. Y08357). The deduced protein sequence is different from the sequence of the putative glyoxalase II from *A. thaliana* shown in Fig. 3 in the present article. The sequence in Ref. 46 shares all six homology boxes shown in Fig. 3 and is more closely related to the sequences of the two yeast glyoxalase II proteins (37.3% and 36.0% identities compared to the Glo2p and Glo4p sequences, respectively) than the sequence shown in Fig. 3.

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