Identification of the Structural Gene for Glyoxalase I from Saccharomyces cerevisiae*

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The structural gene for glyoxalase I (GLO1) of Saccharomyces cerevisiae was identified. The GLO1 gene contained an open reading frame with 326 amino acids, and the molecular weight of the gene product (Glo1p) deduced from the DNA sequence was calculated to be 37,207.06. Glyoxalase I activity increased approximately 95-fold when the GLO1 gene was introduced into the yeast cell with a multicopy plasmid, and the resultant transformant showed the increased resistance against methylglyoxal. Since the knockout mutant of the GLO1 gene of haploid strain of S. cerevisiae was still viable, the GLO1 gene was thought to be unnecessary for growth of the yeast. The GLO1 gene was overexpressed in two kinds of glutathione-deficient mutants, γ-glutamylcysteine synthetase-deficient (gsh1−) and glutathione synthetase-deficient (gsh2−), respectively, and the sensitivities to methylglyoxal were compared. The gsh1-deficient mutant, which could not produce glutathione at all, was hypersensitive to methylglyoxal, and overproduction of the Glo1p did not restore the growth arrest caused by exogenously added methylglyoxal. The gsh2-deficient mutant, which accumulates γ-glutamylcysteine (an intermediate of glutathione biosynthesis), was also sensitive to methylglyoxal compared with the isogenic wild type strain, although the growth arrest caused by methylglyoxal was partially restored by overexpression of the GLO1 gene. Purified glyoxalase I from yeast could use γ-glutamylcysteine as a substrate (*kcat/Km* = 1.89 × 107 M⁻¹ s⁻¹, glutathione: 3.47 × 10⁴ M⁻¹ s⁻¹, γ-glutamylcysteine).

Methylglyoxal is a typical 2-oxoaldehyde in living cells. Methylglyoxal is synthesized both enzymatically and nonenzymatically in the cells, although the aldehyde is mainly formed from dihydroxyacetone phosphate by methylglyoxal synthase in microorganisms (1–7). Methylglyoxal was, therefore, once thought to be an intermediate of glycolysis. However, methylglyoxal was found to inhibit the growth of various kinds of cells from microorganisms to mammals (8–10). We have been systematically studying the metabolic fate of methylglyoxal in several microorganisms and found that methylglyoxal is metabolized to lactic acid by two different routes (11). One route is a glyoxalase system in which glyoxalase I and glyoxalase II are involved. In this route, methylglyoxal is condensed with glutathione to give S-3-lactoylgluthathione by the action of glyoxalase I, and the glutathione thiolester is then hydrolyzed to lactic acid and glutathione by glyoxalase II. Another route is a reduction/oxidation system consisting of methylglyoxal reductase (12) and lactaldehyde dehydrogenase (13). Methylglyoxal is reduced to lactaldehyde by NADPH-dependent methylglyoxal reductase, and lactaldehyde is further oxidized to lactic acid by NAD⁺-dependent lactaldehyde dehydrogenase whose activity is linked with NADPH dehydrogenase (14). We named these two routes the “glycolytic methylglyoxal pathway” (11). In some microorganisms, methylglyoxal is directly oxidized to pyruvic acid by 2-oxaloaldehyde dehydrogenase (methylglyoxal dehydrogenase) (15–18). Methylglyoxal was also reduced to acetol by aldehyde reductase, and the enzyme was partially purified from a yeast, Hansenula mrakii IFO 0895 (19).

Glyoxalase I activity was found in almost all organisms, thus the enzyme has been thought to be ubiquitous (for review, see Ref. 11). Nevertheless, the molecular weight and subunit structure were different, depending on its enzyme source. Mammalian enzymes are dimer, with two identical subunits, and a molecular weight of one subunit = 20,000–24,000. On the other hand, microbial enzymes so far reported are monomer. The molecular weight of bacterial glyoxalase I (Mw = 19,000) (20) was somewhat smaller compared with those of eukaryotic microorganisms (yeast, 32,000–38,000; mold, 36,000) (21–23). The glyoxalase I gene was first cloned and sequenced from Pseudomonas putida IFO 3738 by our group (24), and later Lu et al. (25) also cloned the gene from P. putida. Ranganathan et al. (26) and Kim et al. (27) cloned and sequenced the cDNA for human glyoxalase I. Human glyoxalase I was found to show 57% identity with bacterial enzyme at the C-terminal two-thirds at the amino acid level (27). We have cloned a part of the structural gene for glyoxalase I from Saccharomyces cerevisiae and also determined amino acid sequences of some tryptic peptides of it (28).

A genome sequence project of S. cerevisiae has been completed, and the DNA sequences are available in data base (Saccharomyces Genome Database). Homology search of such tryptic peptides of yeast glyoxalase I revealed that a hypothetical protein, showing the homology with human glyoxalase I and also containing the same sequence of tryptic peptides that we had reported, was mapped on chromosome XIII. In this study, we identify this hypothetical protein as glyoxalase I and also try to analyze the in vivo function of glyoxalase I by using glutathione-deficient mutants.

MATERIALS AND METHODS

Microorganisms and Cultures—Strains of S. cerevisiae and plasmids used in this study are listed in Table I. Cells of S. cerevisiae were cultured in SD minimum medium (2% glucose, 0.67% yeast nitrogen base, pH 5.5) with appropriate amino acids and bases at 28 °C and reciprocal shaking.

Polymerase Chain Reaction (PCR)—PCR primers corresponding to peak 5 (sense primer, primer 5S) and peak 16 (reverse primer, primer

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1 The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s).
16R) were as follows: primer 5S, 5'-TTTACACGGAACACTTGGT-3'; and primer 16R, 5'-CTCAATCCGATCTACATGACG-3'. PCR primers for cloning the full-length gene of the GLO1 were: primer 1S, 5'-TATAGCTGAGCCTCAATCCGATCTACATGACG-3'; and primer 1R, 5'-GGGCTGCAGTAAATGGTGAATCACAATATCT-3'. These primers were designed to contain the SpeI site (shown by italic letters). PCR conditions were: denaturation at 94 °C, 1 min; annealing at 60 °C, 2 min; extension at 72 °C, 2 min; 30 cycles. After PCR, the amplified DNA fragments (approximately 370-bp fragment by primers 5S and 16R, and the fragment was purified as described above. The resultant plasmid (pIGLKOa, see Fig. 2) was digested with BamHI and HindIII, and a 740-bp fragment was cloned into the HindIII site of YEp5 (URA3). The resultant plasmid (pIGLKOa, see Fig. 2) was digested with XbaI, and then introduced into the ura3-32 strains of S. cerevisiae by electroporation (Gene Pulser II, Bio-Rad) under the conditions of 1.5 kV, 200 ohms, and 16 μF. The URA3+ strains were selected on SD agar plate without ura3. Integrative disruption of the GLO1 gene by pIGLKOa (YEp5 + glo1::URA3) was confirmed by Southern analysis (32).

Genomic Southern Analysis—Chromosomal DNAs of S. cerevisiae wild type and the glo1::URA3 mutant were digested with BamHI, separated by agarose gel electrophoresis, and transferred onto the nylon membrane (High-bond N, Amersham Corp., respectively). The HindIII-BamHI fragment (740 bp), which is the same fragment in pIGLKOa, was labeled using an ECL DNA labeling kit (Amersham Corp.) and was used as a probe. The wild type gave an approximately 840-bp fragment, while the mutant gave an approximately 5,900-bp fragment which was due to the insertion of pIGLKOa into the GLO1 locus.

Growth Experiments—Cells were cultured in SD medium at 28 °C with reciprocal shaking for 16 h. A small portion of the culture was transferred into a test tube (0.9 × 16 cm) containing 5 ml of the fresh SD medium containing various concentrations of methylglyoxal, and the optical density of the culture at 610 nm (A610) was monitored periodically. The initial A610 was adjusted between 0.05 and 0.08. When the growth in glycerol medium was examined, 2% glycerol was used instead of glucose in the SD medium.

Preparation of Cell Extracts—Cells of S. cerevisiae were cultured in a 2-liter Sakaguchi flask containing 1 liter of SD medium with appropriate amino acids and bases at 28 °C with reciprocal shaking. Cells were collected at the log phase, washed once with 0.85% NaCl solution, and suspended in 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM phenylmethlysulfonyl fluoride. Cells were disrupted by glass beads in a Braun homogenizer at 0 °C for 2 min. Cell homogenates were centrifuged at 25,000 × g for 20 min at 4 °C, and resultant supernatants were used as cell extracts.

Enzyme Assay—Glyoxalase I and methylglyoxal reductase activities were assayed as described previously (23, 33). To investigate the substrate specificity of purified glyoxalase I, the concentrations of thiol compounds (glutathione and γ-glutamylcysteine) were varied in the reaction mixture in which the concentration of methylglyoxal was fixed at 2 mM. One unit of glyoxalase I and methylglyoxal reductase was defined as the amount of enzyme forming 1 μmol of S-α-lactoylglutathione/min at 25 °C (23), and as the amount of enzyme oxidizing 1 μmol of NADPH/min at 25 °C (30), respectively. Protein was measured according to the method of Lowry et al. (34).

Purification of Glyoxalase I—S. cerevisiae YPH250 carrying pE24GLO1 was cultured in a 2-liter Sakaguchi flask containing 1 liter of SD medium with 25 μg/ml t-tryptophan, t-histidine, t-leucine, and t-lysine, and 30 μg/ml adenine at 28 °C with reciprocal shaking for 16 h. After cultivation, the cells were collected by centrifugation, washed once with 0.85% NaCl solution, and suspended in 10 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM phenylmethlysulfonyl fluoride. Cells (30 g wet weight from a 6-liter culture) were disrupted by glass beads in a Dyno-Mill at 0 °C for 10 min. Homogenates were centrifuged at 25,000 × g for 30 min at 4 °C, and the resultant supernatants were used for the purification of glyoxalase I. Purification procedures for glyoxalase I were described in our previous report (22). Final preparation gave a single band on SDS-polyacrylamide gel electrophoresis (35).

Chemicals—Methylglyoxal was purchased from Sigma. Glutathione and γ-glutamylcysteine was obtained from Kojhin, Tokyo, Japan. Restriction enzymes and other DNA modification enzymes were purchased from Takara Shuzo, Co., Ltd., Kyoto, Japan.

RESULTS

Molecular Cloning of the GLO1 Gene—Previously we cloned a part of the structural gene for glyoxalase I (GLO1) from S. cerevisiae using a Agt11 expression library with anti-glyoxalase-I-IgG as a probe (28). We also determined the amino acid sequences of the trypptic peptides (peaks 5, 13, and 16) of glyoxalase I of S. cerevisiae (28). Later, Ranganathan et al. (26) and Kim et al. (27) independently cloned and sequenced the cDNA for human glyoxalase I. Similar sequences of peaks 5 and 16 were found in the amino acid sequence of human glyoxalase I deduced from the cDNA sequence. We designed the PCR primers corresponding to peaks 5 and 16, respectively, and then performed the PCR. An approximately 370-bp fragment was amplified. The nucleotide sequence of the PCR fragment showed high homology with that of human glyoxalase I cDNA. We then made a homology search against the Saccharomyces Genome Database, and found that a putative open reading frame sharing homology with human glyoxalase I was mapped on chromosome XIII (SWISS-PROT accession no. S55115; hypothetical protein YM9571.15c). The amino acid sequence of

### Table I

| Strain       | Genotypea | Reference or sourceb |
|--------------|-----------|----------------------|
| S288C        | MATa SUC2 mal6 gal2 CUP1 | YGSC                 |
| YPH250       | MATa trp1-1Δ his3Δ200 leu2-1Δ lys2-101 ade2-101 ura3-32 | YGSC                 |
| YNN27        | MATa trp1-1Δ ura3-32 gal2 u2a101 | Y. Ohtake (40)       |
| Y1H          | MATa trp1-1Δ ura3-32 gal2 gsh1 | Ohtake et al. (40)   |
| Y250GKO1     | MATa trp1-1Δ ura3-32 gal2 gsh2 | This study           |
| YNGKO1       | MATa trp1-1Δ ura3-32 gal2 gsh1 | This study           |
| YHGKO1       | MATa trp1-1Δ ura3-32 gal2 gsh1 | This study           |
| YLGKO1       | MATa trp1-1Δ ura3-32 gal2 gsh1 | This study           |
| Plasmid      |           |                      |
| YeEp24      | URA3, ampC | Bostein et al. (46)  |
| YEp5        | URA3, ampC | Stuhl et al. (47)    |
| pUC19        | ampC      | Yanisch-Perron et al. (48) |
| pE24GLO1     | YEp24 + GLO1 | This study           |
| pIGLKOa      | YIp5 + glo1::URA3 | This study           |

a ampC, ampicillin-resistant.
b YGSC, Yeast Genetic Stock Center, University of California at Berkeley.
this hypothetical protein contained the tryptic peptides of peaks 5, 13, and 16 that we had reported previously.

To identify whether or not this hypothetical protein codes the yeast glyoxalase I, we tried to clone the full-length gene containing both 5'- and 3'-flanking regions by PCR. We designed the sense-primer (primer 1S) at the position of approximately 750 bp upstream from the putative initiation codon, and the reverse-primer (primer 1R) approximately 750 bp downstream from the putative stop codon of the YM9571.15c protein, respectively. Theoretically, a 2,500-bp fragment must be amplified by PCR, and we actually obtained the 2,500-bp PCR fragment using the chromosomal DNA of \textit{S. cerevisiae} S288C (data not shown). Both PCR primers (1S and 1R) were designed to contain the SpI site, which was not reported to occur in this region according to the genome sequence of chromosome XIII deposited in the data base (EMBL accession no. Z49810). The nucleotide sequence of the fragment completely coincided with that found in the data base (Fig. 1). The gene contained an open reading frame with 326 amino acids, and the molecular weight of the gene product was calculated to be 37,207.06. The same sequences of peaks 5, 13, and 16 were found in the coding region of this gene (Fig. 1).

A multicopy plasmid (YEp24) carrying the 2,500-bp PCR fragment (pE24GLO1) was transformed into \textit{S. cerevisiae}, and the glyoxalase I activity of the transformants was assayed. As shown in Table II, glyoxalase I activity in the transformant increased approximately 95-fold compared with that of the control. We then disrupted the corresponding gene on the chromosome by using the integrate type plasmid (pIGLKOa) (Fig. 2). As shown in Table II, the glyoxalase I activity could not be detected in the cell extracts of the glo1::URA3 strain Y250GKO1. From these results, we identified the hypothetical protein as the structural gene for glyoxalase I of yeast.
protein YM9571.15c on chromosome XIII as the structural gene for glyoxalase I, GLO1, of S. cerevisiae.

Comparison of Amino Acid Sequences of Glyoxalase Is—Fig. 3 shows the comparison of the amino acid sequence of glyoxalase Is so far identified (P. putida, human, tomato (EMBL accession no. Z48183), and S. cerevisiae), and the putative glyoxalase I homologue of Schizosaccharomyces pombe (SWISS-PROT accession no. Q69751). Glyoxalase I of S. cerevisiae and S. pombe shared 50% homology with each other at the amino acid level, although they showed relatively low similarities (approximately 30%) with glyoxalase Is from other species. Bacterial glyoxalase I showed high homology (52–53%) with enzymes from higher eukaryotes at amino acid level (Fig. 4).

Effect of GLO1 Gene Dosage on Growth—The effect of the GLO1 gene dosage on the sensitivity to methylglyoxal was tested. As shown in Fig. 5, the growth rate of S. cerevisiae strain YPH250 and that carrying YE24 decreased by increasing concentrations of methylglyoxal added into the medium, and it was inhibited by 6 mM methylglyoxal. On the other hand, YPH250 carrying pE24GLO1 showed a higher resistance against methylglyoxal. The glo1::URA3 strain Y250GKO1 was hypersensitive to methylglyoxal, and 4 mM methylglyoxal completely inhibited growth.

Growth of YPH250 with or without plasmids (YE24 and pE24GLO1) and Y250GKO1 in glycerol medium was also investigated. As shown in Fig. 6, no difference was found in the growth rate of these strains in glycerol medium.

**TABLE II**

| Strain/plasmid       | Specific activitya Glyoxalase | Methylglyoxal | reductase |
|----------------------|-------------------------------|---------------|-----------|
|                      | unit/mg protein               |               |           |
| YPH250               | 0.134 ± 0.023                 | 0.036 ± 0.005 |
| YPH250/YE24          | 0.136 ± 0.025                 | 0.032 ± 0.006 |
| YPH250/pE24GLO1      | 12.7 ± 2.13                   | 0.034 ± 0.006 |
| Y250GKO1             | NDb                          | 0.033 ± 0.005 |

a Values are a summary of five independent experiments (mean ± S.D.).
b Not determined.

Effect of GLO1 Gene Dosage in Glutathione-deficient Mutants—Glutathione is one of the substrates for glyoxalase I. Glutathione is synthesized by two sequential reactions catalyzed by γ-glutamylcysteine synthetase (GSH1 gene product) and glutathione synthetase (GSH2 gene product). The pE24GLO1 was introduced into S. cerevisiae YNN27 (ura3, YH1 (ura3 gsh1), and YL1 (ura3 gsh2), respectively. These strains are isogenic, except for the mutations of glutathione biosynthesis. The GLO1 gene on the chromosome of each strain was also disrupted by pIGLKOa. Glyoxalase I activities of each strain were as follows: YNN27, 0.136 ± 0.027 unit/mg of protein; YH1, 0.133 ± 0.025; YL1, 0.141 ± 0.026; YH1/YE24, 0.134 ± 0.031; YH1/YE24, 0.135 ± 0.028; YL1/YE24, 0.134 ± 0.025; YNN27/pE24GLO1, 10.5 ± 2.66; YH1/pE24GLO1, 11.3 ± 2.88; YL1/pE24GLO1, 10.9 ± 2.53; YNGKO1, not detected; YHGKO1, not detected; YLGKO1, not detected; values are shown as mean ± S.D. (n = 3).

As shown in Fig. 7, the gsh1-deficient mutant (YH1) was hypersensitive to methylglyoxal. The growth of YH1 was completely inhibited by 1 mM methylglyoxal, and overexpression of the GLO1 gene could not restore the growth arrest caused by 1 mM methylglyoxal in YH1. Growth of YH1 was poor under aerobic conditions, even though methylglyoxal was not added in the medium (Fig. 7). This was presumably due to the increased sensitivity to oxidative stress in YH1 caused by the lack of glutathione. Glutathione plays a crucial role in scavenging the reactive oxygen species in S. cerevisiae (36). Since the mutant deficient in both GSH1 and GLO1 genes simultaneously (YHGO1) still showed comparable growth with its counterpart (YH1), the reduced growth rate exhibited by YH1 compared with the isogenic wild type strain (YNN27) was not thought to be the the result of the defect of glyoxalase I.

YLI (gsh2) was also sensitive to methylglyoxal compared with the wild type (YNN27), although the growth arrest of YL1 in the medium containing 1 mM and 2 mM methylglyoxal was partially restored by overexpression of the GLO1 gene; however, this was not the case for 4 mM methylglyoxal.

Substrate Specificity of Glyoxalase I—Glyoxalase I was purified from S. cerevisiae YPH250 overexpressing the GLO1 gene to the homogenous state on SDS-polyacrylamide gel electrophoresis. Specific activity of the purified enzyme was 1.197 μmol/min/mg of protein, which was also consistent with the value (1.130 μmol/min/mg of protein) reported by Marmstal et
Activity was plotted against the concentrations of glutathione and γ-glutamylcysteine, an intermediate of glutathione biosynthesis. As shown in Fig. 8, both of thiols gave a Michaelis-Menten type curve at the fixed concentration of methylglyoxal (2 mM). The apparent $K_m$ values for glutathione and γ-glutamylcysteine were estimated to be 0.41 mM and 1.2 mM, respectively. The $k_{cat}/K_m$ values were calculated to be $1.89 \times 10^3$ M$^{-1}$ s$^{-1}$ and $3.47 \times 10^4$ M$^{-1}$ s$^{-1}$ when glutathione and γ-glutamylcysteine were used, respectively.

**DISCUSSION**

Identification of the GLO1 Gene—The molecular weight of the GLO1 gene product (Glo1p) of *S. cerevisiae* was calculated to be 37,207.06 from the amino acid sequence deduced from the DNA sequence. According to the amino acid sequence of Glo1p, three potential N-glycosylation sites (Asn-X-Thr/Ser; X, any amino acid except for Pro) were found (Asn-24, Asn-126, and Asn-184). Douglas et al. (37) suggested that yeast glyoxalase I contained 0.75% carbohydrate, thus N-linked oligosaccharide(s) may attach to these asparagine residues. Ranganathan et al. (26) predicted potential phosphorylation sites in human and bacterial glyoxalase Is, and two of them, Thr-96 and Ser-144, were conserved in Glo1p of *S. cerevisiae*, and an additional potential phosphorylation site, Thr-249, was found in Glo1p. Previously we reported that glyoxalase I of yeast could be phosphorylated (38), thus these amino acid residues may be a target for some Ser/Thr protein kinase(s).
Glyoxalase I so far purified were reported to be inhibited by EDTA, except for the \emph{P. putida} enzyme, and to contain one zinc atom per subunit. Ranganathan et al. (26) predicted a possible \(\text{Zn}^{2+}\)-binding site of human and \emph{P. putida} glyoxalase Is. The amino acid sequence of the \(\text{Zn}^{2+}\)-binding motif of glyoxalase Is is highly conserved in the glyoxalase Is listed in Fig. 3. In the case of Glo1p of \emph{S. cerevisiae}, Glu-89 and His-92 are in the correct configuration when aligned with one of the Glu-110, His-117, Cys-129, or Glu-133 sites. Interestingly, the Glo1p of \emph{S. cerevisiae} had an additional \(\text{Zn}^{2+}\)-binding motif at the C-terminal region; i.e. Glu-242 and His-249 showed the correct configuration if aligned with one of the Glu-263, His-269, Cys-281, or Glu-285 sites. However, it has been reported that yeast glyoxalase I contained one zinc per one molecule (21).

According to the alignment of glyoxalase Is, it seemed to consist of five conserved regions, which we designated regions I, II, III, IV, and V (Fig. 3). Glyoxalase Is do not share so high a homology with each other in their N-terminal regions compared with other regions. Yeast (\emph{S. cerevisiae} and \emph{S. pombe}) glyoxalase IIs seemed to delete some amino acid residues in the N-terminal region when compared with other species. Interestingly, yeast glyoxalase Is have a long C-terminal stretch with approximately 150 amino acid residues, and similar sequences in regions I, II, III, and V were found in this stretch (regions I’, II’, III’, and V’, respectively) (Fig. 3). Ranganathan et al. (26) indicated that the sequence Ser-Leu-Tyr-Phe-Leu-Ala-Tyr (residues 69–75) of human glyoxalase I showed limited homology with plasma glutathione peroxidases from the rat and human. The sequence is a core motif of region II in glyoxalase I and is conserved among these species; therefore, region II might be concerned with the binding of glutathione.

As we described above, mammalian glyoxalase I is a dimer with two identical subunits. The molecular weight of one subunit of mammalian enzyme is similar to that of bacterial enzyme, and the amino acid sequence of the human enzyme shared higher similarity with the bacterial enzyme rather than yeast enzymes (Fig. 3). On the other hand, yeast glyoxalase Is had a C-terminal stretch that contained similar sequences of regions I–V, except for region IV. It may be possible that the bacterial enzyme is a prototype of the mammalian enzyme, and yeast enzymes have evolved from bacterial enzymes by repeating the same gene cassette tandemly, although region IV was deleted or mutated, except for the \(\text{Zn}^{2+}\)-binding motif. Mammalian glyoxalase I then consisted of two identical subunits that did not have a C-terminal stretch. However, further experiments, such as truncation of the C-terminal stretch of yeast glyoxalase I, are necessary to prove this hypothesis.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig5.png}
\caption{Effect of the \emph{GLO1} gene dosage on growth in methylglyoxal-containing medium. Cells were cultured in a test tube containing 5 ml SD minimum medium with appropriate amino acids, adenine, and various concentrations of methylglyoxal (○, 0 mM; ●, 2 mM; □, 4 mM; ■, 6 mM; and ○, 8 mM) at 28 °C with reciprocal shaking. In the case of YPH250, 30 \(\mu\)g/ml uracil was also added. □, YPH250; ■, YPH250/YPE24; ○, YPH250/pE24GLO1; ■, Y250GKO1.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig6.png}
\caption{Effect of the \emph{GLO1} gene dosage on growth in glycerol medium. Cells were cultured in a test tube containing 5 ml SD minimum medium, in which 2% glycerol was used instead of 2% glucose, with appropriate amino acids and adenine without methylglyoxal at 28 °C with reciprocal shaking. In the case of YPH250, 30 \(\mu\)g/ml uracil was also added. □, YPH250; ■, YPH250/YPE24; ○, YPH250/pE24GLO1; ■, Y250GKO1.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Fig7.png}
\caption{Effect of the \emph{GLO1} gene dosage in the glutathione-deficient mutants on growth in methylglyoxal-containing medium. Cells were cultured in a test tube containing 5 ml SD minimum medium with various concentrations of methylglyoxal (○, 0 mM; ●, 1 mM; □, 2 mM; and ■, 4 mM) at 28 °C with reciprocal shaking. In the case of YNN27, YH1, and YL1, 30 \(\mu\)g/ml uracil was also added.}
\end{figure}
GLO1 gene is not necessary for the growth of the yeast cell, although the glo1::URA3 mutant was hypersensitive to methylglyoxal. Penninckx et al. (39) reported that glo1-deficient mutant could not grow in the glycerol medium, which was presumably due to the accumulation of toxic level of methylglyoxal in the cells from the metabolism of glycerol. However, as shown in Fig. 6, the growth rates of wild type and the glo1::URA3 strain Y250GKO1 in glycerol medium were the same. However, it should be noted that the generation time of each strain was extremely prolonged in glycerol medium, and the maximum growth was lower compared with that in glucose medium. The mutant that we constructed in this study specifically deleted the GLO1 gene by integrative disruption, and the mutant completely lost the glyoxalase I activity. Methylglyoxal can be metabolized to lactic acid by an alternative route other than the glyoxalase system; i.e., a reduction/oxidation system consisting of methylglyoxal reductase and lactaldehyde dehydrogenase. Methylglyoxal reductase activity in the glo1::URA3 strain Y250GKO1 was, however, at the same level as that of the wild type cell (Table II). Further genetic analysis must await for an explanation of the differences of the behavior of the mutants.

Glyoxalase I requires glutathione for scavenging methylglyoxal. The biosynthesis of glutathione is catalyzed by two sequential reactions by γ-glutamlycysteine synthetase and glutathione synthetase. The former is a rate-limiting enzyme for glutathione biosynthesis. The GLO1 gene could be overexpressed in each glutathione-deficient mutant, although overexpression of the GLO1 gene could not confer the resistance to the gsh1-deficient mutant against 1 mM methylglyoxal (Fig. 7). On the other hand, in the case of the gsh2-deficient mutant, growth arrest caused by 1–2 mM methylglyoxal was partially restored by the overexpression of the GLO1 gene. These results confirmed that glutathione was necessary for Glo1p to scavenge methylglyoxal in yeast cell. The results also suggested another possibility, i.e., γ-glutamlycysteine may partially serve as a substrate for glyoxalase I. Ohtake et al. (40) reported that the gsh2-deficient mutant accumulated a large amount of γ-glutamlycysteine in the cell (YNN27, 0.050%, dry weight cell; YL1, 0.518%, dry weight cell). We then purified glyoxalase I from the wild type strain (YNN27, 0.050%, dry weight cell; YL1, 0.005%, dry weight cell). We then purified glyoxalase I from the wild type strain (YNN27, 0.050%, dry weight cell; YL1, 0.005%, dry weight cell). The kcat/Km value was approximately 1,000-fold lower if γ-glutamlycysteine was used as a substrate (glutathione, 1.89 × 105 m−1 s−1; γ-glutamlycysteine, 3.47 × 104 m−1 s−1), although γ-glutamlycysteine was found to be a substrate for glyoxalase I from yeast, despite the negative result reported by Woodward and Reinhardt (41).

The double mutants, YHGK01 (glo1::URA3 gsh1) and YLGK01 (glo1::URA3 gsh2), were expected to be extremely sensitive to methylglyoxal. However, the resultant mutants were still viable and showed normal growth in SD minimum medium without methylglyoxal (Fig. 7). These results suggested that the intracellular concentration of methylglyoxal being synthesized in normal metabolism was estimated to be fairly low in the yeast cell. Although yeast has an alternative route to metabolize methylglyoxal to lactic acid (methylglyoxal reductase and lactaldehyde dehydrogenase), the Km value of methylglyoxal reductase for methylglyoxal was reported to be 5.88 mM (12), which is presumably far removed from the physiological concentration of methylglyoxal in vivo. Vander Jagt et al. (42) reported that methylglyoxal is reduced to acetal (hydroxyacetone) by aldose reductase (Km = 8 μM, kcat/Km = 2.96 × 105 M−1 s−1), although the enzyme catalyzing methylglyoxal to acetal was not found in S. cerevisiae (43).

It has been more than 80 years since methylglyoxal was discovered (44, 45), but the physiological significance of methylglyoxal and its metabolic pathway remain to be determined. The genome projects are going to be completed in various organisms, and glyoxalase I or its homologue are found in various species of organisms, suggesting that the enzyme may be ubiquitous in living systems. Since S. cerevisiae has advantages in genetic manipulation and biochemical analysis, identification of the GLO1 gene may contribute to the elucidation of the intrinsic meaning of the glyoxalase system in organisms.

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