13

Methylglyoxal and Lipid Hydroxperoxide as Endogenous Cytotoxic Molecular Species: Detoxification and Regulation of Gene Expression in Yeasts

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Introduction

In 1978, Hinnen, Hicks and Fink first reported the transformation of protoplast cells of *Saccharomyces cerevisiae* using polyethylene glycol and $CaCl_2$. Harashima, Takagi and Oshima (1984) indicated that transformants obtained by the protoplast method were sometimes diploids or polyploids, because cell fusion occurred during the transformation. On the other hand, Ito *et al.* (1983) reported that intact yeast cells of *S. cerevisiae* could be transformed by treating the cells with alkaline cations. The transformants obtained showed the same ploidy as that of the recipient (Harashima, Takagi and Oshima, 1984). Through several modifications, the alkaline cation method (lithium acetate method) was established as a convenient method for the transformation of yeasts, not only *S. cerevisiae* but also other yeast strains such as *Hansenula polymorpha* (Roggenkamp *et al.*, 1986) and *Candida biodinni* (Sakai, Kazarimoto and Tani, 1991).

As well as the method for transformation, studies of the construction of vectors have also been developed. In the transformation of yeast, the genes complementing the auxotrophic markers, such as *HIS3, LEU2* and *URA3* are used as marker genes. Several genes for resistance to drugs such as G418 (Webster and Dickson, 1983) and tunicamycin (Rine *et al.*, 1983), are also used as dominant selectable marker genes

Abbreviations: BHP, butyl hydroperoxide; BSO, buthionine sulphoximine; CDNB, 1-chloro-2,4-dinitrobenzene; CHAPS, 3-cholamidopropyldimethylammonio-1-propane sulphonate; EDTA, ethylenediaminetetraacetic acid; GPx, glutathione peroxidase; GSH-I, γ-glutamylcysteine synthetase; GSH-II, glutathione synthetase; GST, glutathione *S*-transferase; HPLC, high-pressure liquid chromatography; HSE, heat-shock element; HSF, heat-shock factor; HSP, heat-shock protein; IPTG, isopropyl-β-*D*-thiogalactopyranoside; MCO, metal-catalysed oxidation; mRNA, messenger RNA; PHGPx, phospholipid hydroperoxide glutathione peroxidase; Pi, inorganic phosphate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SOD, superoxide dismutase; UAS, upstream activating sequence.

Biotechnology and Genetic Engineering Reviews — Vol. 12, December 1994
0264–8725/94/12/467–508 $20.00 + $0.00

and can be used to select transformants of prototropic and industrial strains of yeast. The author has also investigated the possibility of using some stress-resistance genes as selectable markers for the transformation of yeasts.

Several environmental stresses are known to trigger intracellular alterations in micro-organisms, e.g. cellular responses against extracellular signals or the induction of some genes. On the other hand, intracellular stresses, such as waste materials in the cells or some metabolites possessing cytotoxicity, would also affect the growth or metabolism of micro-organisms. Against those extracellular and/or intracellular stresses, micro-organisms have some mechanisms for adaptation or resistance. In this chapter, I have chosen two kinds of stresses, i.e. methylglyoxal and lipid hydroperoxide as the endogenous cytotoxic molecular species.

Methylglyoxal is a typical cellular 2-oxoaldehyde. Formation of this compound is performed both enzymatically and non-enzymatically, and the major precursors of methylglyoxal are dihydroxyacetone phosphate or glyceraldehyde 3-phosphate, which are derived from glycolysis, an ubiquitous energy-generating system in organisms. Though methylglyoxal is a normal metabolite, it arrests the growth of various organisms at millimolar concentrations. Because the compound is toxic, its synthesis and degradative pathway must be strictly controlled in cells to avoid its overaccumulation.

On the other hand, lipid hydroperoxide is also one of the endogenous cytotoxic molecular species, and it affects the growth or proliferation of cells. Lipid hydroperoxide is synthesized both enzymatically and non-enzymatically from unsaturated fatty acid or lipid containing unsaturated fatty acid moieties. Phospholipids included in the cytoplasmic or mitochondrial membrane are also peroxidized. Lipid hydroperoxide is degraded in the presence of transition metal ions to yield radicals such as the alcoxy radical and the peroxy radical. These radicals are reactive oxygen species and they cause radical chain reactions together with other lipids, lipid hydroperoxide and oxygen. Thus, lipid hydroperoxide would be one of the oxidative stresses in living cells.

In this chapter, the biochemical aspects of metabolism of methylglyoxal and lipid hydroperoxide in yeast cells are described, as are the genes corresponding to the metabolism or detoxification of methylglyoxal and lipid hydroperoxide in yeast cells, and the possibility of the use of such genes as selectable marker genes for the transformation of yeast is discussed.

Glyoxalase systems in yeasts

METABOLISM OF METHYLGLYOXAL IN MICRO-ORGANISMS

Formation of methylglyoxal

The major route for formation of methylglyoxal in microbial cells is catalysed by methylglyoxal synthase (EC 4.2.99.11) (*Figure 1*). The enzyme catalyses the conversion of dihydroxyacetone phosphate to methylglyoxal without formation of Schiff base (Hopper and Cooper, 1972). Methylglyoxal synthases were purified from several micro-organisms, such as *Escherichia coli* (Hopper and Cooper, 1972), *Pseudomonas saccharophilia* (Cooper, 1974), *Proteus vulgaris* (Tsai and Gracy,

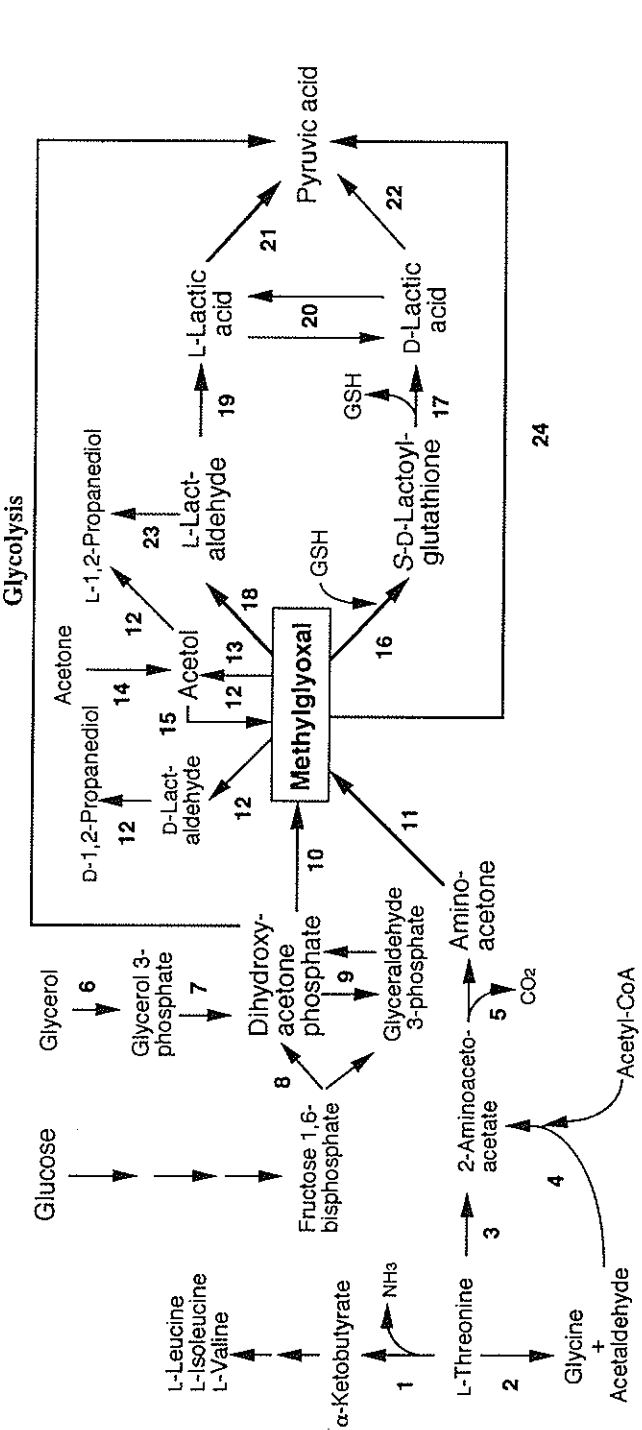

Figure 1. Metabolism of methylglyoxal. The enzymes indicated by the numbers are: 1, threonine deaminase; 2, threonine aldolase; 3, threonine dehydrogenase; 4, aminoacetone synthase; 5, spontaneous decarboxylation; 6, glycerol kinase; 7, glycerol 3-phosphate dehydrogenase; 8, fructose 1,6-bisphosphate aldoiase; 9, triosephosphate isomerase; 10, methylglyoxal synthase; 11, monoamine oxidase; 12, aldolase reductase; 13, aldehyde reductase; 14, acetone mono-oxygenase; 15, acetol dehydrogenase; 16, glyoxalase I; 17, glyoxalase II; 18, methylglyoxal reductase; 19, iactaldehyde dehydrogenase; 20, lactate racemase; 21, L-lactate dehydrogenase; 22, D-lactate dehydrogenase; 23, propanediol oxidoreductase; 24, 2-oxoaldehyde dehydrogenase.

1976) and *S. cerevisiae* (Murata *et al.*, 1985b). The same enzyme activity was also reported in goat liver (Ray and Ray, 1981). The properties of these enzymes have been well documented and are similar in terms of substrate specificity and susceptibility to inorganic phosphate (Pi). The molecular weight (M_r) of the enzyme purified from *E. coli* and *P. saccharophilia* is 67 kDa. The enzyme from *P. vulgaris* has an M_r of 135 kDa and is composed of two identical subunits. The M_r of *S. cerevisiae* enzyme is 26 kDa, significantly lower than the bacterial enzymes. The M_r of the goat liver enzyme has not been reported.

Methylglyoxal is also synthesized in the catabolism of L-threonine. *Saccharomyces cerevisiae* cells degrade L-threonine added to the medium as a sole source of nitrogen (Murata *et al.*, 1986c). In accordance with the disappearance of L-threonine in the medium, a large amount of aminoacetone is accumulated. Aminoacetone is converted to methylglyoxal by monoamine oxidase (EC 1.4.3.4).

Non-enzymatic formation of methylglyoxal from dihydroxyacetone phosphate, glyceraldehyde 3-phosphase, dihydroxyacetone and glyceraldehyde during the metabolism of acetone and glycerol has been reported in microbial cells as well as those of mammals. Details are described in a recent paper (Inoue and Kimura, in press).

Degradation of methylglyoxal

Methylglyoxal is metabolized to lactic acid by two different routes (*Figure 1*). One route is the glyoxalase system consisting of glyoxalase I and glyoxalase II. Detailed descriptions of the enzymes in this route are given below.

Another route is the reduction–oxidation system consisting of NADPH-dependent methylglyoxal reductase and NAD-dependent lactaldehyde dehydrogenase. In this route, methylglyoxal is first reduced to lactaldehyde and then oxidized to lactic acid. Methylglyoxal reductases were purified from several micro-organisms, such as *S. cerevisiae* (Murata *et al.*, 1985a), *Hansenula mrakii* (Inoue *et al.*, 1991b), *Aspergillus niger* (Inoue *et al.*, 1988) and *E. coli* (Saikusa *et al.*, 1987). The molecular structures of methylglyoxal reductases from microbial cells are similar to each other. They are monomers with molecular weights varying from 36 to 43 kDa. Methylglyoxal reductase of *S. cerevisiae* contains 5% (w/w) carbohydrate (Murata *et al.*, 1985a). *Aspergillus niger* has two kinds of methylglyoxal reductases, MGR-I and MGR-II, which can be separated by hydrophobic column chromatography (Inoue *et al.*, 1988). The M_r of MGR-I and MGR-II were estimated to be 36 kDa and 38 kDa, respectively.

NADP, one of the reaction products of methylglyoxal reductase, inhibited the activities of methylglyoxal reductases from eukaryotic micro-organisms. The type of inhibition of MGR-I by NADP was competitive, and the K_i value for NADP was estimated to be 490 μM. On the other hand, the inhibition of MGR-II and the enzyme from *H. mrakii* was of mixed type and the K_i values for NADP were 45 μM and 250 μM, respectively (Inoue *et al.*, 1988, 1991b). The K_i value for NADP of the enzyme from *S. cerevisiae* was 70 μM (Murata *et al.*, 1985a).

Methylglyoxal reductases purified from yeasts (*S. cerevisiae* and *H. mrakii*) and mould (MGR-I and MGR-II from *A. niger*) were inactivated by a brief incubation with substrates such as glyoxal, methylglyoxal and phenylglyoxal in the absence of NADPH (Murata *et al.*, 1985a; Inoue *et al.*, 1988, 1991b). The inactivation was not observed when the enzyme was preincubated with NADPH prior to exposure to 2-

oxoaldehydes. Since methylglyoxal and phenylglyoxal are known to modify the arginine residue in proteins (Takahashi, 1968), methylglyoxal reductases from these sources may contain the arginine residue in the NADPH-binding site.

Lactaldehyde thus formed is further metabolized to lactic acid by lactaldehyde dehydrogenase (*Figure 1*). Purification and characterization of lactaldehyde dehydrogenase from microbial cell has been reported for *E. coli* (Sridhara and Wu, 1969) and *S. cerevisiae* (Inoue *et al.*, 1985). The M_r of the *E. coli* enzyme was estimated to be 100 kDa (Sridhara and Wu, 1969), whereas Baldoma and Aguilar (1987) reported that the M_r of the enzyme, measured by SDS-PAGE, was 55 kDa. On the other hand, the enzyme from *S. cerevisiae* was a monomer with an M_r of 40 kDa. The *E. coli* enzyme catalyses the oxidation of L-lactaldehyde to L-lactic acid in the presence of NAD, and is almost specific for L-lactaldehyde; but other aldehydes, such as D-lactaldehyde (relative activity, 5%), pyruvaldehyde (relative activity, 3%), D,L-glyceroaldehyde (relative activity, 3%) and propionaldehyde (relative activity, 12%), are also slightly oxidized by the enzyme. On the other hand, lactaldehyde dehydrogenase from *S. cerevisiae* was almost completely specific to L-lactaldehyde, i.e. utilization of D-lactaldehyde by the enzyme was only 0.2% compared with that of L-lactaldehyde. The enzyme was specific to NAD, and NADP could not substitute for NAD. Recently, Inoue and Kimura (1994) found that NADH formed by the reaction of lactaldehyde dehydrogenase is reoxidized by NAD(P)H dehydrogenase, which is closely linked with lactaldehyde dehydrogenase.

Other routes

Methylglyoxal is reduced to acetol in the presence of NADH by aldehyde reductase. The enzyme was partially purified from *H. mrakii* (Inoue *et al.*, 1992a). This enzyme activity has not been reported in *S. cerevisiae* (Murata *et al.*, 1986a). Some bacteria, such as *Bacillus subtilis* and *Pseudomonas putida* could directly oxidize methylglyoxal to pyruvic acid by the action of 2-oxoaldehyde dehydrogenase (Willetts and Turner, 1970; Rhee *et al.*, 1987). Details are described by Inoue and Kimura (in press).

GLYOXALASE SYSTEM

Glyoxalase I

Conversion of methylglyoxal to D-lactic acid *via* *S*-D-lactoylglutathione is catalysed by the glyoxalase system, consisting of glyoxalase I and glyoxalase II in the presence of glutathione. Methylglyoxal is non-enzymatically condensed with glutathione to give an adduct, hemi-mercapal (hemi-thioacetal), which is the intrinsic substrate for glyoxalase I (*Figure 1*). The dissociation constant (K_d) for hemi-mercaptal is 3×10^{-3} M (Vander Jagt, 1993).

Since methylglyoxal is cytotoxic, bacterial cells such as *E. coli* or *Pseudomonas putida* cannot grow in media containing 1.0–1.2 mM methylglyoxal (Egyud and Szent-Gyorgyi, 1966; Rhee, Murata and Kimura, 1987). The author and his co-workers found that the yeast *Hansenula mrakii* IFO 0895 was highly resistant to methylglyoxal and was able to grow in a medium containing up to 25 mM methylglyoxal (Inoue *et al.*, 1991a). The specific activity of glyoxalase I in cell extracts of

H. mrakii (1.48–2.11 units mg^{-1} protein) (Inoue *et al.*, 1991a) was relatively higher than those of *S. cerevisiae* (0.238 units mg^{-1} protein) (Kosugi *et al.*, 1988), *A. niger* (0.026 units mg^{-1} protein) (Inoue *et al.*, 1987), *P. putida* (0.145 units mg^{-1} protein) (Rhee, Murata and Kimura, 1986) and *E. coli* (0.012 units mg^{-1} protein) (Rhee, Murata and Kimura, 1987).

Glyoxalase I is widely distributed in organisms and has been purified from various sources, from mammals to micro-organisms. Mammalian glyoxalase Is are dimers, with molecular weights around 43–48 kDa. On the other hand, microbial glyoxalase Is are monomers and molecular weights vary from 19 kDa to 38 kDa. Douglas, Seddon and Nakagawa (1986) suggested that yeast glyoxalase I contained approximately 0.75% (w/v) carbohydrate.

Each subunit of mammalian glyoxalase Is contains one atom of zinc (Aronsson, Marmstal and Mannervik, 1978; Marmstal, Aronsson and Mannervik, 1979). The zinc ion is located in the catalytic site of the enzyme and can be replaced by other bivalent metal ions, such as Mg^{2+}, Mn^{2+}, Co^{2+} and Ni^{2+} (Uotila and Koivusalo, 1975; Han *et al.*, 1977; Aronsson *et al.*, 1981; Sellin *et al.*, 1983; Sellin and Mannervik, 1984). The metal ion in the active site is suggested to be co-ordinated to two nitrogen and to four oxygen atoms (Sellin *et al.*, 1987), although the amino acid residues involved in the co-ordination were not identified (Mannervik and Ridderstorm, 1993).

The activity of glyoxalase I purified from *S. cerevisiae* was inhibited by ethylenediaminetetraacetic acid (EDTA), and the activity of EDTA-inhibited enzyme was restored partially by Mg^{2+} or Ca^{2+}, and slightly by Mn^{2+} (Murata *et al.*, 1986d). Fe^{2+}, Ni^{2+} and Co^{2+} were all without any effect on reactivation. Glyoxalase I purified from *P. putida* (Rhee, Murata and Kimura, 1986) was not inhibited by EDTA. The enzyme from a yeast, *H. mrakii*, was also insensitive to bivalent metal ion chelators such as EDTA, 1,2-cyclohexanediaminetetraacetic acid and 8-hydroxyquinoline (Inoue *et al.*, 1991a). In analogy with other microbial glyoxalase Is, the activity of the enzyme from *A. niger* was also inhibited by Zn^{2+} (Inoue *et al.*, 1987). The enzyme was protected from inhibition by Zn^{2+} by the addition of an equimolar amount of EDTA. The activity of glyoxalase I from the mould was inhibited by EDTA, and the inhibition was found to be a competitive type against hemi-mercaptal (K_i=1.3 mM). This inhibitory effect of EDTA on the reaction might be due to the structural similarity between hemi-mercaptal and EDTA. Douglas and Shinkai (1985) suggested the model for the reaction of glyoxalase I. They indicated that two oxygen atoms of hemi-mercaptal at the position of C-1 and C-2 could easily chelate Zn^{2+} in the enzyme with the subsequent isomerization of hemi-mercaptal. On the other hand, EDTA also has a similar structure of the oxygen atoms, being able to chelate bivalent metal ions. Therefore, EDTA may compete with hemi-mercaptal at the active site of the mould glyoxalase I. Mannervik, Lindsstorm and Bartfai (1972) suggested that glyoxalase I from mammals contained Zn^{2+} and that the inhibitory effect of EDTA was due to a removal of Zn^{2+}, the EDTA-inhibited enzyme being reactivated by Zn^{2+} and/or other bivalent metal ions. The reaction using the mould glyoxalase I was inhibited by EDTA; however, the mould enzyme was not found to be inactivated by either preincubation with EDTA or dialysis against the buffer containing EDTA. The mould enzyme, as well as the bacterial (*P. putida*) and the yeast (*H. mrakii*) enzymes, may not contain Zn^{2+} in its active site, or Zn^{2+} might be tightly associated with the enzyme.

Glyoxalase I activity in the haploid cells of *S. cerevisiae* changes when the cells are exposed to the opposite-type mating factor. It seems to be regulated through the phosphorylation/dephosphorylation state of the enzyme (Inoue *et al.*, 1989, 1990a; Inoue and Kimura, 1991). The enzyme activity in *S. cerevisiae* also increases when the cells are exposed to a high-cell concentration culture. In this case, cell–cell interaction affects the amount of glyoxalase I mRNA (Inoue, Yano and Kimura, 1993d). From these observations, glyoxalase I is not only a detoxification enzyme of methylglyoxal in the cells but it may also have some significant functions relating to cell growth, proliferation and differentiation.

Glyoxalase II

Glyoxalase II is an alternative enzyme in the glyoxalase system. It catalyses the hydrolysis of *S*-D-lactoylglutathione, formed by the reaction of glyoxalase I, to glutathione and D-lactic acid (*Figure 1*). Glyoxalase IIs have been purified from various sources. Mammalian enzymes are distributed in various tissues, such as brain, liver and erythrocytes. The enzymes are found in mitochondria as well as in the cytosol, although other organelles are unlikely to contain the enzyme (Jerzykowski *et al.*, 1978; Talesa *et al.*, 1988; Uotila, 1989). The molecular weighs of glyoxalase IIs vary within the range 18–30 kDa. Specific activity of the enzyme is approximately 600–900 μmol min^{-1} mg^{-1} protein, except for yeast enzymes (*S. cerevisiae*, 1.34 μmol min^{-1} mg^{-1} protein; *H. mrakii*, 31.9 μmol min^{-1} mg^{-1} protein) (Murata *et al.*, 1986b; Inoue and Kimura, 1992b). The K_m value for *S*-D-lactoylglutathione is in the range 86–450 μM, except for an extraordinarily low K_m value of the enzyme from *S. cerevisiae* (Murata *et al.*, 1986b). The values of k_{cat} of calf brain and rat erythrocyte were estimated to be 16×10^3 min^{-1} and 17×10^3 min^{-1}, respectively, with k_{cat}/K_m values of about 10^8 M^{-1} min^{-1}; suggesting that the hydrolysis of *S*-D-lactoylglutathione by glyoxalase II is diffusion limited (Guha, Vander Jagt and Creighton, 1988).

Glyoxalase II can hydrolyse a wide variety of *S*-hydroxyacylglutathione derivatives, such as *S*-acetyl, *S*-lactoyl, *S*-glycoyl, *S*-mandelyl, *S*-glyceroyl, *S*-acetoacetylglutathiones. Glyoxalase II from *S. cerevisiae* was, however, specific to *S*-D-lactoylglutathione. *Saccharomyces cerevisiae* has other enzymes that are active to *S*-D-lactoylglutathione as well as *S*-acetylglutathione, and one of the enzymes was purified (Murata *et al.*, 1987). The enzyme (P3) showed group-transfer activity and catalysed the reaction to form acetyl-coenzyme A from *S*-acetylglutathione and coenzyme A. *S*-D-Lactoylglutathione-hydrolysing activity was not divided into more than one peak during purification from the yeast, *H. mrakii* (Inoue and Kimura, 1992b; Inoue *et al.*, 1994a).

Glyoxalase II is inhibited by many derivatives of glutathione. *S*-(Substituted-carbobenzoxy)glutathione derivatives are the strongest competitive inhibitors; suggesting the occurrence of a hydrophobic substrate-binding site in glyoxalase II (Al-Timari and Douglas, 1986; Principato *et al.*, 1989). Hemi-mercaptal, a non-enzymatic condensation product between methylglyoxal and glutathione, inhibited glyoxalase II (Uotila, 1973; Oray and Norton, 1980; Murata *et al.*, 1986b; Rae *et al.*, 1990; Rae, Board and Kuchel, 1991; Inoue and Kimura, 1992b). Yeast glyoxalase IIs were also inhibited by hemi-mercaptal, although the activity of the P3-enzyme, which hydrolysed *S*-D-lactoylglutathione, was not affected by hemi-mercaptal (Murata *et al.*, 1987).

Glyoxalase I-related genes in *Saccharomyces cerevisiae*

GLYOXALASE I-DEFICIENT MUTANTS: A COMPLEMENTARY GENE

Although methylglyoxal is a normal metabolite in living cells, it arrests the growth of several organisms at millimolar concentrations. Detoxification of methylglyoxal is mainly performed by glyoxalase I. To clone the gene for glyoxalase I from *S. cerevisiae*, the mutants highly sensitive to methylglyoxal were isolated. Several kinds of mutants which showed slow growth in a medium containing methylglyoxal were obtained, some of which showed a temperature-sensitive phenotype (M26, M35, M39, M50 and M52). When such mutants were cultured at 23°C, the cells showed almost the same growth rate compared with that of wild-type cells in the presence of 0.5 mM methylglyoxal (*Figures 2a,b*). On the other hand, when the mutants were cultured at 35°C in the presence of 0.5 mM methylglyoxal, the growth of M35 and M39 was slightly inhibited (*Figure 2e*). In the presence of 1.0 mM methylglyoxal, notable growth inhibition was observed in all mutants (*Figure 2f*). Glyoxalase I activity in the mutants was assayed and was found to be lower than that of the wild-type strain in mutants M35 and M39, whereas no difference in enzyme activity was observed in other mutants. Therefore, the decrease in glyoxalase I activity was thought to be a major factor for the growth inhibition of M35 and M39 in the presence of methylglyoxal at 35°C. Penninckx, Jespers and Legrain (1983) also reported that the growth rate of a glyoxalase I-deficient yeast mutant was slightly lower than that of the parental cells.

To clone the gene corresponding to glyoxalase I, a genomic DNA library of *S. cerevisiae* was screened for complementation to the mutation of M39. Several candidates were obtained, and the mutation was phenotypically complemented in all transformants. A recombinant plasmid was recovered from one clone and was designated p39-b. It contained 3.8 kb of *S. cerevisiae* chromosomal DNA. The nucleotide sequence of the 3.8 kb fragment was determined. It contained one open reading frame of 510 bp and the molecular weight (M_r) of the polypeptide deduced from the nucleotide sequence was calculated to be 18 875 Da (170 amino acids) (*Figure 3*). The gene has four putative TATA-like sequences at 1002 bp (TATATA), 989 bp (TATA), 434 bp (TATAA) and 133 bp (TATA) upstream, respectively, from the translational initiation codon (ATG). The polyadenylation signal (AATAAA) was located 131 bp downstream of translational termination codon (TGA).

Glyoxalase I purified from *S. cerevisiae* was digested with trypsin and the N-terminal amino acid sequences of two tryptic peptides obtained by reverse-phase high-pressure liquid chromatography (HPLC) (peaks 5 and 13) were determined. The sequence was as follows: peak 5, Phe–Tyr–Thr–Glu–His–Phe–Gly; peak 13, Asp–Pro–Asp–Gly–Tyr–Ser–Ile–Glu–Val–Val–Pro–Xaa–Gly. These sequences were not found in the amino acid sequence deduced from the nucleotide sequence of the gene, herewith sequenced. Therefore, the gene cloned and sequenced was thought not to correspond to the structural gene of yeast glyoxalase I.

GLYOXALASE I GENE AND REGULATION OF ITS EXPRESSION

Glyoxalase I activation conferring (GAC) gene from S. cerevisiae

Growth of the yeast *S. cerevisiae* was inhibited in the presence of 2 mM methylglyoxal.

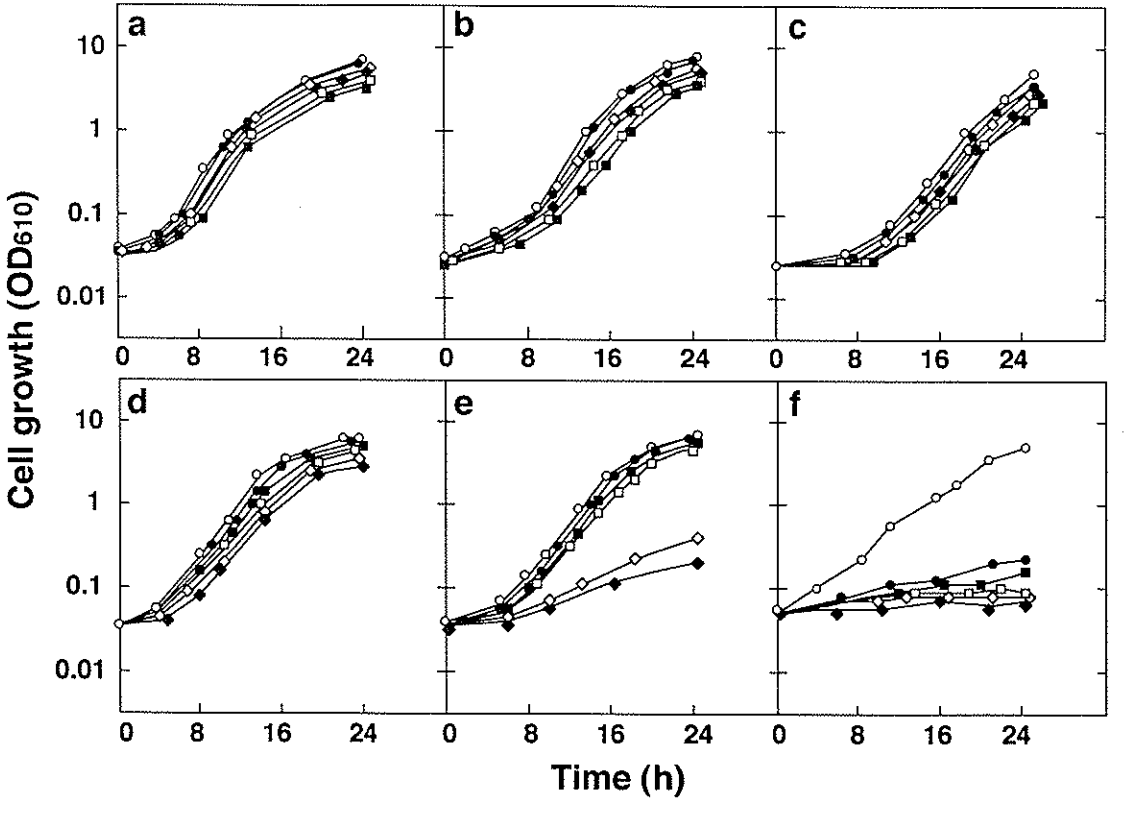

Figure 2. Growth of methylglyoxal-sensitive mutants. Cells were cultured in the synthetic dextrose (SD) minimal medium containing 0 mM (a and d), 0.5 mM (b and e), and 1.0 mM (c and f) methylglyoxal, with reciprocal shaking at 23°C (a, b and c) or 35°C (d, e and f), respectively. Cell growth was monitored by measuring the optical density of the culture at 610nm (OD_{610}). Symbols: O, wild type; ●, M26; ◊, M35; ♦, M39; □, M50; ■, M52.

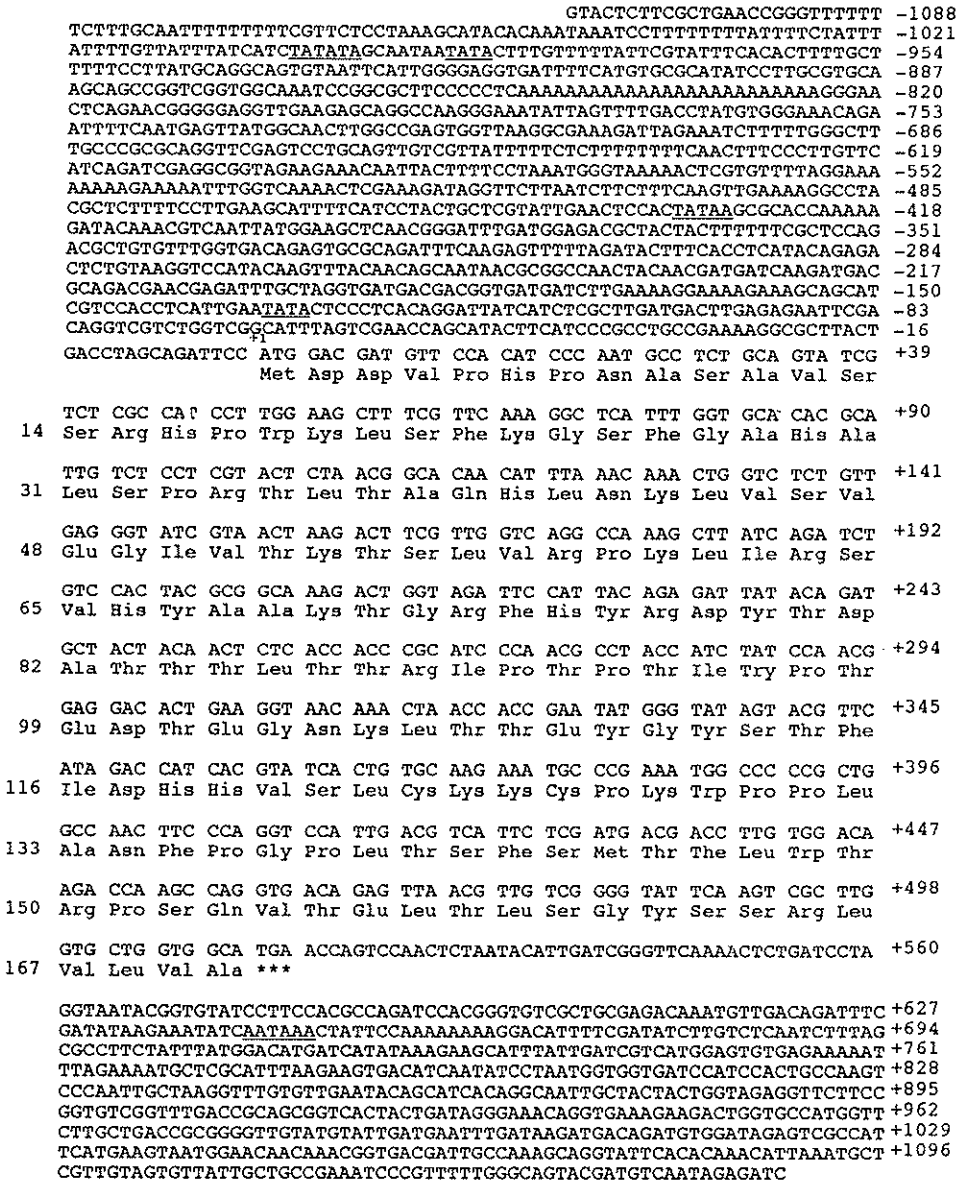

Figure 3. Nucleotide sequence of a DNA fragment in p39-b. The recombinant plasmid (p39-b) contained a 3.8 kb inserted fragment and the whole sequence of the fragment was determined. The longest open reading frame and its 5'- and 3'-flanking regions are shown. Putative TATA-like sequences and the polyadenylation signal are indicated by underlining. Numbers on the right-hand side of the figure show the nucleotide position, which started from the translational initiation codon (ATG). Numbers on the left-hand side of the figure show the amino acid position, beginning from the N-terminal methionine (Met).

The addition of an equimolar amount of glutathione or L-cysteine to the culture containing methylglyoxal eliminated the toxic effect of methylglyoxal, indicating that this effect is chemically neutralized by sulphydryl compounds. However, in the presence of a large amount of methylglyoxal (20 mM) and glutathione (20 mM), growth of yeast cells was inhibited. Since a non-enzymatic adduct (hemi-mercaptal) of methylglyoxal and glutathione is an intrinsic substrate for glyoxalase I, the yeast genomic DNA library was screened for the glyoxalase I gene by selecting transformants showing rapid growth on minimum agar plates containing 20 mM methylglyoxal and 20 mM glutathione. Three candidates were obtained and the glyoxalase I activity in these clones was appreciably higher (three- to fivefold) than that of the control strain (Murata *et al.*, 1988).

Since the phenotypic characters of these clones were identical, the nucleotide sequence of the inserted DNA in a hybrid plasmid from one of the clones was determined, and an open reading frame of 318 bp was identified. The M_r of the gene product deduced from the DNA sequence was calculated to be 14 700 Da (106 amino acids). However, yeast glyoxalase I is a monomer with M_r of 32 kDa. Thus, the open reading frame found was not thought to correspond to yeast glyoxalase I. Thus, we named the gene *GAC* (glyoxalase I activation conferring) (Inoue *et al.*, 1990c). The *GAC* gene gave two transcripts with different molecular sizes in *S. cerevisiae* cells. Cells dosed with the *GAC* gene by multicopy plasmids could grow in a medium containing methylglyoxal at concentration giving arrested growth of the control strain, suggesting that the *GAC* gene product was in fact functioning in the detoxification of methylglyoxal.

To confirm that the *GAC* gene was not the structural gene for yeast glyoxalase I, an expression system of the *GAC* gene in *E. coli* was constructed. The coding region of the *GAC* gene was ligated downstream of a *trc* promoter in an expression vector (pKK233-2) of *E. coli* and the resultant plasmid was introduced into *E. coli*. Expression of the *GAC* gene was analysed by Maxi-cell method and a protein with an M_r of approximately 15 kDa was found to be synthesized. To monitor the expression of the *GAC* gene in *E. coli* easily, the *GAC* gene was fused with a *lacZ* gene encoding the β-galactosidase of *E. coli*. The fusion gene was ligated downstream of a *tac* promoter of the expression vector pKK223–3 and the replication origin (*ori*) of the plasmid DNA was replaced with that of pUC19. Although the pKK223–3 is a multicopy vector, the *ori* of the plasmid was from pBR322. The copy number of pUC19 in *E. coli* cells is much higher than that of pBR322 because of the mutation near the *ori* region of pUC19. The resultant plasmid (pUG-Lac) was introduced into *E. coli* JM109 and expression was induced by IPTG (isopropyl-β-D-thiogalactopyranoside). The GAC–LacZ fusion protein was expressed in *E. coli*, and β-galactosidase activity could be detected. The GAC–LacZ fusion protein was purified and the N-terminal amino acid sequence was determined. The sequence coincided with that deduced from the nucleotide sequence of the *GAC* gene. A full-length gene of *GAC* was then ligated downstream of the *tac* promoter using the same system of *GAC–lacZ* fusion, and the resultant plasmid (pUGAC) was introduced into *E. coli* JM109. GAC protein produced in the *E. coli* cell reached approximately 7% of the total cellular proteins, although glyoxalase I activity did not increase (Inoue and Kimura, in press). Therefore, the *GAC* gene was confirmed as not encoding the structural gene for the yeast glyoxalase I.

Molecular cloning of the structural gene for glyoxalase I from S. cerevisiae

A structural gene for yeast glyoxalase I (*GLO1*) was cloned using anti-glyoxalase I IgG as a probe from the genomic DNA library constructed with λgt11. Approximately 2×10^5 plaques were screened, and a positive clone was obtained. The recombinant phage (λGI-10) was purified and re-transfected to *E. coli* Y1090, and the expression of the fusion protein with β-galactosidase and yeast glyoxalase I (GloI) was examined by immunoblotting analysis. A lysate of *E. coli* Y1090 transfected with λGI-10 was found to contain a fusion protein of LacZ–GloI. The λGI-10 contained approximately 3.2 kb of insert DNA in the *Eco*RI site of the vector. The nucleotide sequence of the insert DNA downstream of *Eco*RI was determined, and the amino acid sequence deduced from the nucleotide sequence coincided with that of a peak in the peptide fractionation. From the results of immunoblotting and nucleotide sequence analysis, we concluded that a part of the *GLO1* gene was cloned on to λGI-10 (Inoue *et al.*, 1991c).

Using the *Eco*RI-*Eco*RI small fragment of λGI-10 as a probe (approximately 500 bp), the *S. cerevisiae* genomic DNA library constructed using YEp13 as a vector was screened. A recombinant plasmid (p89-1) was isolated from one of the positive clones and the insert DNA was sequenced. The amino acid sequence deduced from the nucleotide sequence had the same sequence as those of peaks 5 and 13 in peptide fractionation. Recently a cDNA of human glyoxalase I was cloned and sequenced by independent groups (Kim *et al.*, 1993; Mannervik and Riddestrom, 1993; Ranganathan *et al.*, 1993). Sequence of our peaks 5 and 13 showed homology with an amino acid sequence deduced from the cDNA sequence of human glyoxalase I.

A structural gene for glyoxalase I of *Pseudomonas putida* was also cloned and its nucleotide sequence was determined (Rhee, Murata and Kimura, 1987, 1988). Human glyoxalase I showed 51% homology at the nucleotide level and 42% homology at amino acid level with *P. putida* glyoxalase I. Mammalian glyoxalase I is a dimer (M_r=43–48 kDa) composed of identical subunits, and each subunit contains one Zn^{2+} at its catalytic site. The binding frequency of zinc atoms to the proteins was thought to be as follows: His>>Glu>Asp=Cys, and these amino acids have appropriate spacing (Valee and Auld, 1990). Ranganathan *et al.* (1993) predicted that the amino acids concerned with Zn^{2+} binding are Glu100 and His103, with one of Asp121, His127, Cys139 or Glu143. They also suggested that the Zn^{2+}-binding motif of bacterial glyoxalase I was: Glu91 and His94 with one of Asp112, His118, Cys130 or Glu132. However, Rhee, Murata and Kimura (1986) reported that *P. putida* glyoxalase I was not inhibited by an excess amount (10 mM) of EDTA and suggested no contribution of metal ions in the catalytic function of the bacterial enzyme.

Effect of the GAC gene product on the expression of the GLO1 gene in S. cerevisiae

Although the expression of the *GAC* gene in *E. coli* did not increase the glyoxalase I activity, a yeast dosed with the gene showed increased activity of glyoxalase I and then showed resistance to methylglyoxal. Therefore, the biological function of the *GAC* gene product in *S. cerevisiae* cells should be a subject of considerable interest. The mRNA level of the *GLO1* gene in *S. cerevisiae* dosed with the *GAC* gene

increased in yeast cells compared with that of control cells (*Figure 4*) (Inoue *et al.*, 1991c). The result suggested that the *GAC* gene product affected the activity of yeast glyoxalase I at the mRNA level of the *GLO1* gene; i.e. transcriptional activation or enhancing the stability of *GLO1* mRNA.

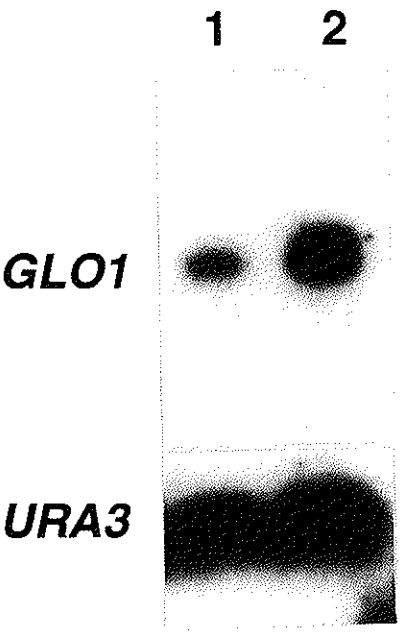

Figure 4. Effect of the *GAC* gene on the expression of the *GLO1* gene in *S. cerevisiae*. Total RNA prepared from the *S. cerevisiae* cell transformed with YEp13 (lane 1, vector alone as control) and YEp13 + *GAC* (lane 2) was subjected to electrophoresis in an agarose gel containing formaldehyde and transferred on to a nylon membrane. The level of *URA3* mRNA was used as an internal control by re-probing the membrane as indicated at the bottom.

It is known that the 5'-flanking regions of several yeast genes contain a specific DNA sequence for the binding of the *trans*-activator for transcription, designated UAS (upstream activating sequence) (Guarente, 1984, 1988; Struhl, 1987). Several proteins that bind to the UAS and activate the transcription of yeast genes have been identified (Verdier, 1990). DNA binding proteins often contain the Zn^{2+} ion in their molecule through tetrahedral co-ordination of cysteine residues, the protein being termed a 'zinc finger' (Johnston, 1987a). In this model, the DNA binding protein binds to the specific site of the 5'-flanking region of the gene and accelerates the transcription of DNA through interaction with RNA polymerase (Allison and Ingles, 1989). The Zn^{2+} co-ordinated by cysteine residues is essential for the protein to bind to DNA (Johnston, 1987b). According to amino acid sequences deduced from the DNA sequence of *GAC* gene, the gene product is expected to contain five cysteine residues near the carboxyl terminus. However, the spacing of the cysteine residues is not similar to that of the C_6 zinc fingers of GAL4 and other yeast activator proteins (Inoue *et al.*, 1990c). It bears a similarity to the C_5 zinc finger, which is characteristic of the hormone receptor superfamily.

Recent findings suggested that the GAC protein produced in *E. coli* had the capability for DNA binding; the results of a gel retardation assay indicated that GAC

protein produced in *E. coli* could bind to the chromosomal DNA of *S. cerevisiae* (H. Ginya, 1991). This suggested that the *GAC* gene product may function in the yeast cell to activate the transcription of the *GLO1* gene through interaction with its 5'-flanking region.

Oxidative stress response in *Hansenula mrakii*

All aerobic organisms use molecular oxygen for respiration or the oxidation of nutrients to acquire the energy to live. The molecular oxygen is reduced to water through the acceptance of four electrons. During the reduction of molecular oxygen, several reactive oxygen species are formed; i.e. acceptance of one, two and three electrons leads to the formation respectively, of the suproxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (HO•) (*Figure 5*). Such reactive oxygen species have been reported as causative agents in several degenerative diseases (Ames, 1983; Cerutti, 1985). They attack almost all cell components, DNA, protein and lipid membranes, and sometimes cause lethal damage to the cells. Both prokaryotic and eukaryotic cells have defensive methanisms against such oxidative damage. *Escherichia coli* and *Salmonella typhimurium* cells have the *oxyR*-controlled regulon of hydrogen peroxide-inducible genes (Christman, Storz and Ames, 1989; Storz, Tartaglia and Ames, 1990). *Escherichia coli* cells also have a *soxRS* regulon, which is induced by the superoxide anion radical (Greenberg *et al.*, 1990; Tsaneva and Weiss, 1990; Wu and Weiss, 1991; Nunoshiba *et al.*, 1992). The yeast *S. cerevisiae* has cytochrome *c* peroxidase (Yonetani, 1970) as well as a superoxide dismutase-catalase system.

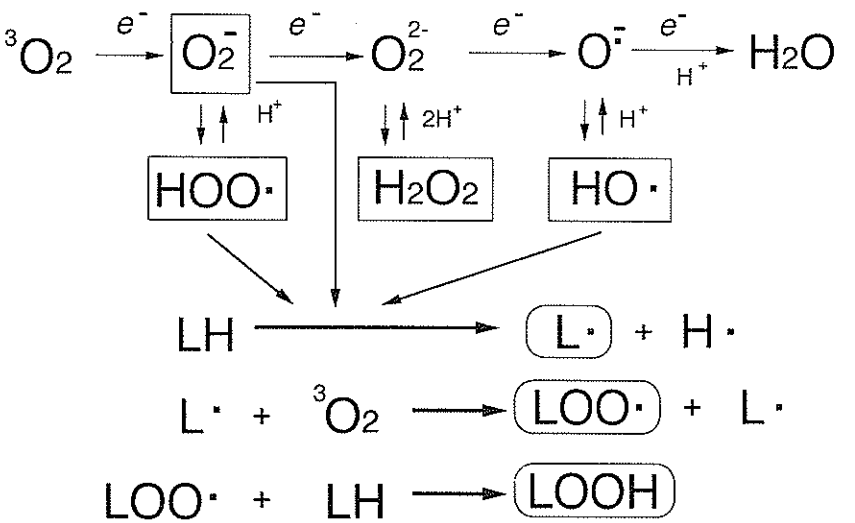

Figure 5. Reduction of molecular oxygen to water and generation of reactive oxygen species and lipid peroxidation.

Lipid hydroperoxides are also a reactive oxygen species. Radiation, halocarbons, some drugs and herbicides have been known to cause oxidative stress, and are able to peroxidize the biological membrane *in vivo*. Among the reactive oxygens, HO•, as well as the perhydroxyl radical (HOO•) can extract *bis*-allylic hydrogen atoms of

unsaturated fatty acids (LH) to form lipid alkyl radicals (L•). The L• is oxidized by molecular oxygen to generate a lipid peroxy radical (LOO•), and the LOO• thus formed reacts with LH to give lipid hydroperoxide (LOOH) and L•. The radical chain reaction is then propagated (*Figure 5*). Occurrence of the lipid hydroperoxides in the biological membrane may be one of the major reasons for oxidative damage of the cells.

Contrasting with the voluminous studies of the defensive mechanism against reactive oxygen such as O_2^- and H_2O_2 in bacterial cells (Demple and Amabile Cuevas, 1991; Farr and Kogoma, 1991; Storz and Tartaglia, 1992), studies of adaptation or resistance to oxidative stress in yeast have been fewer (Collinson and Dawes, 1992; Jamieson, 1992). Furthermore, the defensive mechanism for oxidative stress caused by lipid hydroperoxide has not been studied in detail in micro-organisms (Inoue *et al.*, 1990b, 1992b). As the first step in this study, the author and his co-workers screened several yeast strains for resistance against lipid hydroperoxide, and found that *Hansenula mrakii* IFO 0895 could grow in a medium containing 4 mM linoleic acid hydroperoxide in which all other yeast strains tested could not grow (Inoue *et al.*, 1990b). The resistance was proved to be due to a membrane-bound glutathione peroxidase which was induced when the yeast was exposed to oxidative environments.

INDUCTION OF GLUTATHIONE PEROXIDASE BY LIPID HYDROPEROXIDE IN *H. MRAKII*

Screening of yeast resistant to linoleic acid hydroperoxide

Several yeast strains in laboratory collections were screened for resistance against 1 mM linoleic acid hydroperoxide. Among the yeast strains tested, *Hansenula* yeast (*H. anomala* IFO 0149, *H. californica* IFO 0800, *H. canadensis* IFO 0973, *H. canadensis* IFO 0976, *H. jadinii* IFO 0987, *H. saturnus* IFO 0117 and *H. mrakii* IFO 0895) could grow in the linoleic acid hydroperoxide-containing medium. *Hansenula mrakii* IFO 0895 could grow in the minimal medium containing 4 mM linoleic acid hydroperoxide (*Figure 6a*). Other strains, except for *Rhodotorula minuta* IFO 0387, *Zygo-saccharomyces rouxii* IFO 0487, *Metschnikowia zobellii* IFO 1680, *Candida maltosa* IFO 1975 and *Pichia kluveri* IFO 1165, could not grow in the medium containing 1 mM linoleic acid hydroperoxide. *Metschnikowia zobellii* belongs to the Spermophthoraceae and several strains in this family are plant parasites. Synthesis of lipid hydroperoxide catalysed by lipoxygenase is known as one of the defensive responses of plants against microparasites. Thus, *M. zobellii* IFO 1680 may have a resistance mechanism to lipid hydroperoxide.

Linoleic acid hydroperoxide is degraded radically in the presence of metal ions to yield many secondary degraded products, such as aldehydes, ketones and carboxylic acids. To investigate whether or not the tolerance of *H. mrakii* to the oxidative stress of linoleic acid hydroperoxide resulted from resistance to the secondary degraded products of linoleic acid hydroperoxide, the compound was incubated with $CuSO_4$ to promote the radical chain reaction, and the mixture was then added to the medium. As shown in *Figure 6b*, the cells of *H. mrakii* could grow in a medium containing 1 mM linoleic acid hydroperoxide or 5 μM $CuSO_4$ alone, but cell growth was completely inhibited in a medium containing 1 mM linoleic acid hydroperoxide together with

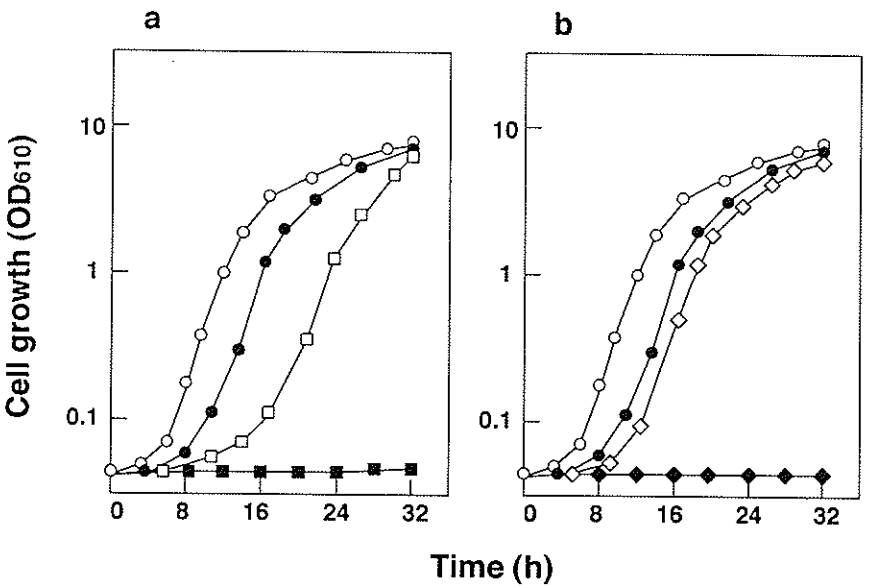

Figure 6. Effect of lipid hydroperoxide on the growth of *H. mrakii*. (a) Cells of *H. mrakii* IFO 0895 were cultured in the SD minimal medium containing O mM (O), 1 mM (●), 4 mM (□) and 10 mM (■ linoleic acid hydroperoxide at 28°C, respectively. Cell growth was monitored by measuring the OD_{610}. (b) Cells of *H. mrakii* were cultured in the absence of chemicals (O) or the presence of 1 mM linoleic acid hydroperoxide (●), 5 μM $CuSO_4$ (◊) or a mixture of 1 mM linoleic acid hydroperoxide and 5 μM $CuSO_4$ (♦). In the latter case, the mixture was preincubated at 28°C for 12 h before it was added to the medium.

5μM $CuSO_4$. This suggested that *H. mrakii* was resistant to linoleic acid hydroperoxide itself but not to the secondary degraded products of lipid hydroperoxide.

Induction of glutathione peroxidase by linoleic acid hydroperoxide

Residual linoleic acid hydroperoxide in the culture of *H. mrakii* was analysed by thin layer chromatography. The hydroperoxide moiety of the compound was found to be reduced to an alcohol moiety. Thus, the tolerance of *H. mrakii* to linoleic acid hydroperoxide may result from the reduction of the hydroperoxide moiety of the linoleic acid hydroperoxide to an alcohol moiety (Inoue *et al.*, 1990b). Reduction of the hydroperoxide moiety to alcohol is expected to be catalysed by several peroxidases. The activity of potential candidates, such as ascorbate peroxidase (Boveris *et al.*, 1980), chloride peroxidase (Hager, 1970), cytochrome *c* peroxidase (Yonetani, 1970), NAD-peroxidase (Dolin, 1957), peroxidase (Kenten and Mann, 1954) and glutathione peroxidase (Awasthi, Beutler and Srivastava, 1975) was assayed in the soluble and insoluble fractions of cell homogenates of *H. mrakii*, which was grown in the medium with or without 1 mM linoleic acid hydroperoxide. Among the enzymes assayed, only glutathione peroxidase was found to be dramatically induced when the cells of *H. mrakii* were cultued with 1 mM linoleic acid hydroperoxide (Inoue *et al.*, 1990b). Almost all of the glutathione peroxidase was recovered from the insoluble fractions using several detergents such as Triton X-100, lubrol PX (polyethylene-glycol (9) lauryl ether) and CHAPS (3-cholamidopropyldimethylammonio-1-propane sulphonate). No activity was detected in the soluble fractions obtained by ultra-

centrifugation of cell homogenates at 105 000 *g* for 60 min (Tran, Inoue and Kimura, 1993a). Thus, glutathione peroxidase of *H. mrakii* was induced by linoleic acid hydroperoxide and it seemed to be bound to the cell membrane.

INDUCTION OF GLUTATHIONE PEROXIDASE BY REACTIVE OXYGEN IN *H. MRAKII*

Hansenula mrakii could grow in the medium containing 4 mM linoleic acid hydroperoxide, in which no other yeast strain tested could grow. Resistance against lipid hydroperoxide was due to a glutathione peroxidase which was induced when the yeast was exposed to exogenous lipid hydroperoxide (Inoue *et al.*, 1990b). Among the rective oxygen species, the hydroxy radical (HO•) as well as the perhydroxy radical (HOO•), which is generated by protonation of the superoxide anion radical, can initiate the peroxidation of unsaturated fatty acid. Thus, *H. mrakii* cells were incubated in a mixture in which O_2^- or HO• was generated to investigate whether glutathione peroxidase was induced.

Intact cells of yeast (possessing cell walls) were first incubated in a HO•- and O_2^--generating system; however, glutathione peroxidase was not induced. The reaction rate constant (k) of HO• for abstraction of the *bis*-allylic hydrogen atom of unsaturated fatty acid is estimated to be $10^8\ M^{-1}\ s^{-1}$ (Barber and Thomas, 1978), although the radical is generally thought not to be involved in the peroxidation process of membrane lipid *in vivo*, because the HO• in the cytosol would react non-specifically with other compounds surrounding the radical before it reached the membrane (Pryor, 1986; Aikens and Dix, 1991). Therefore, when intact cells are used, the cell wall might prevent the radical from reaching or interacting with the cell membrane. Thus, protoplasts of *H. mrakii* were exposed to HO• and O_2^-, respectively.

When the protoplasts of *H. mrakii* were incubated with H_2O_2 alone, or Fe^{2+} + ADP, glutathione peroxidase activity was not detected in any of the cases (*Figures 7a,b,c*). However, when the protoplasts were incubated in the HO•-generating mixture ($H_2O_2 + Fe^{2+} \rightarrow HO\bullet + OH^- + Fe^{3+}$; Fenton reaction) for 1 h, the cells regained growth after a 20 h lag time, and at this time glutathione peroxidase activity was detected (*Figure 7d*).

To examine whether or not other reactive oxygen radicals, such as O_2^- can induce the synthesis of glutathione peroxidase in *H. mrakii*, the protoplasts were treated in an O_2^--generating system. When protoplasts of *H. mrakii* cells were pre-incubated with xanthine or xanthine oxidase alone (*Figures 7e,f*), glutathione peroxidase activity was not detected. However, when the protoplasts were pretreated for 1 h in a mixture generating O_2^- (xanthine + xanthine oxidase), glutathione peroxidase was induced after a 10 h time lag (*Figure 7g*). The superoxide anion radical itself is thought not to be able to withdraw the *bis*-allylic hydrogen atom of unsaturated fatty acid (Gebicki and Bielski, 1981; Halliwell and Gutteridge, 1990), although it serves as proton acceptor to yield the perhydroxyl radical ($O_2^- + H^+ \rightarrow HOO\bullet$) (Bielski, Arudi and Sutherland, 1983; Sawyer, McDowell and Yamaguchi, 1988). It has been reported that HOO• could initiate lipid peroxidation (Gebicki and Bielski, 1981; Bielski, Arudi and Sutherland, 1983; Aikens and Dix, 1991). At physiological pH (i.e. pH 6.8), only 1% of O_2^- exists as HOO• (pKa=4.88), though HOO• may be formed more abundantly near the surface of the membrane where the pH might be lower owing to the negative surface charge of the membrane (Bielski, 1978; Barber, 1980). Therefore,

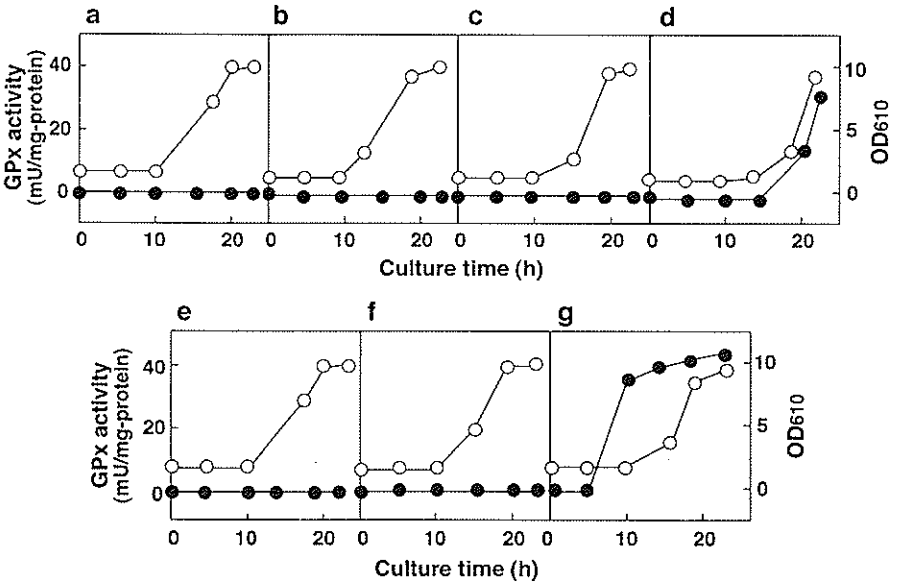

Figure 7. Induction of glutathione peroxidase by reactive oxygen. Upper panel: induction of glutathione peroxidase by HO•; protoplasts of *H. mrakii* were pretreated with (a) no chemicals, (b) 30 mM H_2O_2, (c) 0.1 mM $FeSO_4$ + 0.25 mM ADP, and (d) 0.1 mM $FeSO_4$ + 0.25 mM ADP + 30 mM H_2O_2. Lower panel: induction of glutathione peroxidase by O_2^-; protoplasts of *H. mrakii* were pretreated with (e) 0.5 mM xanthine, (f) 2 mU ml^{-1} xanthine oxidase, and (g) 0.5 mM xanthine + 2 mU ml^{-1} xanthine oxidase. Symbols: O, OD_{610}; ●, glutathione peroxidase (GPx) activity.

induction of glutathione peroxidase by the xanthine + xanthine oxidase system may be due to HOO• rather than O_2^-.

The following putative model for adaptation to oxidative stress in *H. mrakii* is suggested. Reactive oxygen species such as HO• and HOO• attack the membrane of protoplasts, and initiate the peroxidation of membrane lipid. Peroxidation of the membrane lipid constitutes damage to the cell, and it may serve as a signal for the yeast to trigger the synthesis of some apparatus for adaptation to oxidative stress.

ROLE OF GLUTATHIONE PEROXIDASE AGAINST OXIDATIVE STRESS IN *H. MRAKII*

Wild-type cells of *H. mrakii* were highly resistant to the oxidative stres caused by lipid hydroperoxide. The resistance was due to a glutathione peroxidase which was induced when the yeast was exposed to the oxidative environments (Inoue *et al.*, 1990b; Tran, Inoue and Kimura, 1993b). To investigate the role of glutathione peroxidase, the mutants sensitive to lipid hydroperoxide were isolated.

Two mutants (M1 and M9) showing small colonies on the minimal agar plate containing 2 mM *tert*-butyl hydroperoxide at 30°C were obtained. Wild-type cells of *H. mrakii* could make large colonies under the same conditions. To confirm the phenotypes of M1 and M9, each cell was cultured in a liquid medium containing *tert*-butyl hydroperoxide, linoleic acid hydroperoxide and linolenic acid hydroperoxide, respectively, with various concentrations, at 28°C and 35°C. Wild-type cells as well as M1 and M9 cells showed almost the same growth rates at 28°C, and they could

grow in a 2 mM *tert*-butyl hydroperoxide-, linoleic acid hydroperoxide-, and linolenic acid hydroperoxide-containing medium (*Figure 8*). At 35°C, wild-type cells showed the same growth rate at 28°C in the medium containing each lipid hydroperoxide. M1 could not grow in the medium containing 1 mM *tert*-butyl hydroperoxide, 2 mM linoleic acid hydroperoxide, and 1 mM linolenic acid hydroperoxide at 35°C. On the other hand, M9 could grow in the medium containing 1 mM lipid hydroperoxide after a 1–2 day lag time, although the growth of M9 was also completely inhibited by 2 mM lipid hydroperoxide at 35°C (*Figure 8*).

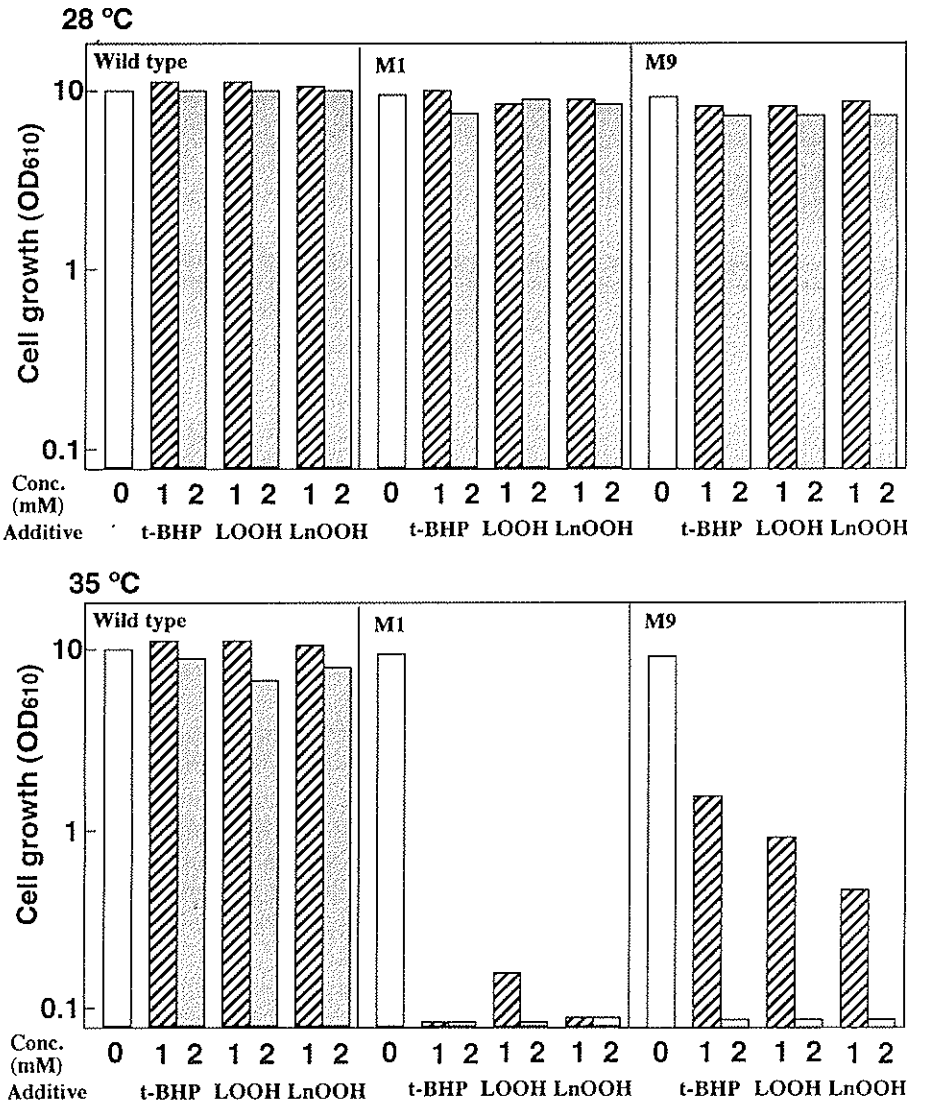

Figure 8. Effect of temperature on growth of lipid hydroperoxide-sensitive mutants. Cells (wild type, M1 and M9) were cultured in the SD medium containing 0 mM (▭), 1 mM (▩) and 2 mM (▩) lipid hydroperoxides, at 28°C (upper panel) and 35°C (lower panel), respectively. Cell growth was monitored by measuring the OD_{610}. Each bar indicates the OD_{610} of the 3 day culture. t-BHP, *tert*-butyl hydroperoxide; LOOH, linoleic acid hydroperoxide; LnOOH, linolenic acid hydroperoxide.

The resistance against lipid hydroperoxide of the wild-type cells of *H. mrakii* was due to glutathione peroxidase, which was induced when the cells were cultured in the presence of lipid hydroperoxide (Inoue *et al.*, 1990b). Hence, the activities of glutathione peroxidase in M1 and M9 were examined. Cells of the wild type, M1 and M9 were cultured in the minimal medium at 28°C and 35°C, respectively. When the OD_{610} of the medium reached approximately 1.0, *tert*-butyl hydroperoxide was added to each medium, and the cultivation was continued until the OD_{610} reached 8.0. At 35°C, M1 and M9 could not grow after the additions of 1 mM and 2 mM *tert*-butyl hydroperoxide, respectively, so the cells were harvested at the same time as the wild-type cells. The activity of glutathione peroxidase was assayed. The wild type, as well as M1 and M9, induced glutathione peroxidase at 28°C, and specific activity increased in association with the amount of *tert*-butyl hydroperoxide added (*Table 1*). Both wild type and M9 induced the enzyme when 1 mM *tert*-butyl hydroperoxide was added at 35°C, while M1 failed to induce glutathione peroxidase. When 2 mM *tert*-butyl hydroperoxide was added, neither M1 nor M9 induced glutathione peroxidase.

Table 1. Glutathione peroxidase activities in lipid hydroperoxide-sensitive mutants

Strain	GPx activity (mU mg^{-1} protein)					
	tert-BHP added into medium					
	0 mM		1 mM		2 mM	
	28°C	35°C	28°C	35°C	28°C	35°C
Wild type	ND[a]	ND	31	26	180	140
M1	ND	ND	25	ND[b]	130	ND[b]
M9	ND	ND	28	26	110	ND[b]

Cells (wild type, M1 and M9) were cultured in SD medium at 28°C and 35°C, respectively. When the OD_{610} reached approximately 1.0, *tert*-BHP was added and cultivation was carried out until OD_{610}=8.0.
[a] Not detected.
[b] Cells did not grow after the addition of *tert*-BHP, so the cells were collected at the same time as the wild-type cells.

To investigate whether or not the temperature-sensitive phenotypes of M1 and M9 were due to the instability of glutathione peroxidase at higher temperature, the glutathione peroxidase induced at 28°C and prepared from each strain was incubated at 28°C, 35°C and 37°C, respectively. Glutathione peroxidase prepared from each strain was stable after at least 1 h incubation at 37°C. Several possibilities could be speculated, e.g. mutation(s) might occur on the gene(s) encoding some positive regulators involved in the biosynthesis of glutathione peroxidase, or mutation(s) might occur to decrease the thermal stability of the mRNA of glutathione peroxidase or to decrease the translational efficiency of flutathione peroxidase mRNA at higher temperature (Inoue, Tran and Kimura, 1993).

As described above, the glutathione peroxidase was thus proved to be essential for *H. mrakii* to survive under the oxidative stress caused by lipid hydroperoxide.

PURIFICATION OF MEMBRANE-BOUND GLUTATHIONE PEROXIDASE FROM *H. MRAKII*

Purification of glutathione peroxidase from H. mrakii

As described above, the glutathione peroxidase was recovered from the insoluble fractions of the cell homogenates of *H. mrakii* (Inoue *et al.*, 1990b). Therefore, the

glutathione peroxidase of *H. mrakii* appeared to occur in a membrane-bound form. Since CHAPS has a high critical micellar concentration value and small micellar size, preferable properties for the purification of an enzyme, this detergent was chosen to solubilize the glutathione peroxidase from total membrane fractions of *H. mrakii*. As shown in *Table 2*, almost all glutathione peroxidase activity remained in the precipitates after KCl-treatment; suggesting that the glutathione peroxidase was bound strongly to the biological membrane. After treatment of the precipitates with 0.5% CHAPS, the enzyme was completely solubilized. Recovery of glutathione peroxidase in the CHAPS extracts was approximately 82%. Therefore, almost all the glutathione peroxidase was believed to be bound to the biological membrane of *H. mrakii* cells.

Table 2. Recovery of glutathione peroxidase activity in membrane fractions

Step	Total activity (unit)	Recovery (%)
Homogenates	3.52	100[a]
200 000 × *g*		
Supernatant	0.22	6.30
Pellet	3.30	93.7
2 M KCl, 200 000 × *g*		
Supernatant	0.40	11.4
Pellet	2.90	82.4
0.5% CHAPS, 200 000 × *g*		
Supernatant	2.90	82.4
Pellet	ND[b]	–

[a] Total activity of homogenates was taken as 100%.
[b] Not detected.

Glutathione peroxidase was purified to the homogenous state as judged by SDS-PAGE from the total membrane fractions of *H. mrakii* (Tran, Inoue and Kimura, 1993a), and the molecular weight of the purified enzyme was estimated to be 28 kDa.

Glutathione peroxidases purified and characterized so far can be divided into two groups: a selenium-dependent enzyme (glutathione peroxidase) and a selenium-independent enzyme (glutathione *S*-transferase). The glutathione peroxidase purified from *H. mrakii* (*Hansenula*-GPx) was different from the glutathione peroxidases so far purified in substrate specificity. As shown in *Table 3*, various lipid hydroperoxides and their methyl esters were well used by *Hansenula*-GPx. Some properties of the enzyme are summarized in *Table 4* in comparison with glutathione peroxidases from other sources. Specific activity of purified *Hansenula*-GPx for *tert*-butyl hydroperoxide (625 μmol min^{-1} mg^{-1} protein) was higher than those of other glutathione peroxidases as listed in *Table 4* (human erythrocyte GPx-I, 103.3 μmol min^{-1} mg^{-1} protein (Awasthi, Beutler and Srivastava, 1975); pig heart PHGPx, 15.5 μmol min^{-1} mg^{-1} protein (Ursini, Maiorino and Gregolin, 1985); rat liver GPx-II, 3.2 μmol min^{-1} mg^{-1} protein (Prohaska and Ganther, 1977); rat liver microsome GST, 1.4 μmol min^{-1} mg^{-1} protein for cumene hydroperoxide (Morgensten and DePrierre, 1983); *Mucor hiemalis* GPx, 187.2 μmol min^{-1} mg^{-1} protein (Aisaka, Uwajima and Terada, 1983); *Euglena gracilis* GPx, 42 μmol min^{-1} mg^{-1} protein (Overbaugh and Fall, 1985)). Hydroperoxide of phosphatidylcholine, a representative of membrane phospholipid, was preferably reduced by the *Hansenula*-GPx. Usually glutathione peroxidase and glutathione *S*-transferase were not active on hydroperoxides of phosphatidylcholine and cholesterol,

except for the phospholipid hydroperoxide glutathione peroxidase (PHGPx) reported by Ursini, Maiorino and Gregolin (1985) (*Table 4*). Cholesterol-5α-hydroperoxide was also reduced by the *Hansenula*-GPx.

Table 3. Substrate specificity of glutathione peroxidase from *H. mrakii*

Substrate	Relative activity (%)
Hydrogen peroxide	0
tert-Butyl hydroperoxide	100
Cumene hydroperoxide	144
Methyl ethyl ketone hydroperoxide	101
Benzoyl peroxide	59
Di-*tert* butyl peroxide	0
Dicumene peroxide	0
cis-Vaccenic acid hydroperoxide	162
Linoleic acid hydroperoxide	142
Linolenic acid hydroperoxide	109
Oleic acid hydroperoxide	98
Methyl linolate hydroperoxide	150
Methyl linolenate hydroperoxide	124
Methyl oleate hydroperoxide	123
Cholesterol 5α-hydroperoxide	120
Phosphatidylcholine hydroperoxide	120
Substrate for glutathione *S*-transferase:	
o-Dinitrobenzene	0
1-Chloro-2,4-dinitrobenzene	0
1,2-Dichloro-4-nitrobenzene	0
p-Nitrobenzyl chloride	0
p-Nitrophenethyl bromide	0
4-Nitropyridine-*N*-oxide	0
1,2-Epoxy-3-(*p*-nitrophenoxy) propane	0

Activity on *tert*-butyl hydroperoxide was taken as 100%.

The *Hansenula*-GPx could not reduce hydrogen peroxide, while the other glutathione peroxidases, except for glutathione peroxidase from *Mucor hiemalis* (Aisaka, Uwajima and Terada, 1983), can use it as a substrate. Since the glutathione *S*-transferase could not reduce hydrogen peroxide, *Hansenula*-GPx was thought to be a kind of glutathione *S*-transferase. However, as shown in *Table 3*, the *Hansenula*-GPx did not catalyse the conjugation of glutathione with electrophilic compounds such as 1-chloro-2,4-dinitrobenzene and *o*-dinitrobenzene, which are used as substrates of glutathione *S*-transferase in yeast (Habig, Pabst and Jakoby, 1974; Kumagai *et al.*, 1988).

One of the characteristics of the *Hansenula*-GPx is that the enzyme is strongly bound to the biological membrane. Morgenstern and co-workers (Morgenstern *et al.*, 1980; Morgenstern, Guthenberg and DePrierre, 1982; Morgenstern and DePrierre, 1983) purified the glutathione *S*-transferase from the membrane fractions of rat liver microsome, although the enzyme was not active on phospholipid hydroperoxide. PHGPx with activity on phosphatidylcholine hydroperoxide was purified from cytosolic fractions of pig heart (Ursini, Maiorino and Gregolin, 1985). The *Hansenula*-GPx has not been subjected to a direct analysis of selenium as yet because of insufficient availability of the purified enzyme. However, whether the *Hansenula*-GPx contains selenium or not, the enzyme previously purified can be distinguished

Table 4. Properties of glutathione peroxidases from different sources

Enzyme source	Subcellular localization	Molecular weight	No. of subunits	Substrate			
				H_2O_2	*tert*-BHP	PLHP	CDNB
Human erythrocyte (GPx-I)[a]	Cytosol	95 000	4	Yes	Yes	No	No
Pig heart (PHGPx)[b]	Cytosol	20 000	1	Yes	Yes	Yes	No
Rat liver (GPx-II, GST)[c]	Cytosol	47 000	2	No	Yes	No	Yes
Rat liver microsome (GST)[d]	Membrane	14 000	1	No	?[g]	No	Yes
Mucor hiemalis[e]	Cytosol	45 000	2	No	Yes	No	No
Euglena gracilis[f]	Cytosol	130 000	4	Yes	Yes	No	No
Hansenula mrakii	Membrane	28 000	?	No	Yes	Yes	No

[a] Data shown for the enzyme from human erythrocyte (Awasthi, Beutler and Srivastava, 1975).
[b] Data shown for pig heart (Ursini, Maiorino and Gregolin, 1985).
[c] Data shown for rat liver (Prohaska and Ganther, 1977).
[d] Data shown for rat liver microsome (Morgenstern, Guthenberg and DePrierre, 1982; Morgenstern and DePrierre, 1983).
[e] Data shown for *M. hiemalis* (Aisaka, Uwajima and Terada, 1983).
[f] Data shown for *E. gracilis* (Overbaugh and Fall, 1985).
[g] Cumene hydroperoxide was used instead of *tert*-butyl hydroperoxide.
Abbreviations: *tert*-BHP, *tert*-butyl hydroperoxide; PLHP, phospholipid hydroperoxide; CDNB, 1-chloro-2,4-dinitrobenzene; GPx, glutathione peroxidase; PHGPx, phospholipd hydroperoxide glutathione peroxidase; GST, glutathione *S*-transferase.

from classical types of glutathione peroxidase and glutathione *S*-transferase so far purified because of its substrate specificity and intracellular localization.

Glutathione peroxidase is thought to play an important role in the protection of cells against oxidative stress, such as peroxidation of membrane lipids. Indeed, the *Hansenula*-GPx was shown to be essential for *H. mrakii* to protect the cells against oxidative stress caused by lipid hydroperoxide by using mutants (Inoue, Tran and Kimura, 1993). Since classical glutathione peroxidases have been purified from cytosolic fractions of cell homogenates, the membrane-protective function of the enzyme was supposed to be associated with a preceding action of phospholipase (Grosmann and Wendel, 1983). Glutathione *S*-transferases have been purified from the cytoplasm as well as from membrane fractions; however, the direct reduction of peroxidized membrane lipids by glutathione *S*-transferase has been disputed (Gibson, Hornbrook and McCay, 1980; Tan *et al.*, 1984). On the other hand, PHGPx was shown to be able to reduce peroxidized membrane lipids, thus suggesting an alternative mechanism for protection of the membrane from peroxidation through direct interaction of the enzyme with peroxidized membrane lipids. Although PHGPx activity was also reported to be detected from the cell membranes, the PHGPx purified so far has been from the cytosolic fractions (Ursini, Maiorino and Gregolin, 1985; Roveri *et al.*, 1992). From this point of view, the *Hansenula*-GPx turns out to be of physiological interest. Because the enzyme was purified from the membrane fractions and was active toward hydroperoxide of phosphatidylcholine, a representative of membrane phospholipid, it can serve as evidence for the physiological function of glutathione peroxidase in the direct reduction of peroxidized membrane lipids (Inoue and Kimura, 1992c) (*Figure 9*).

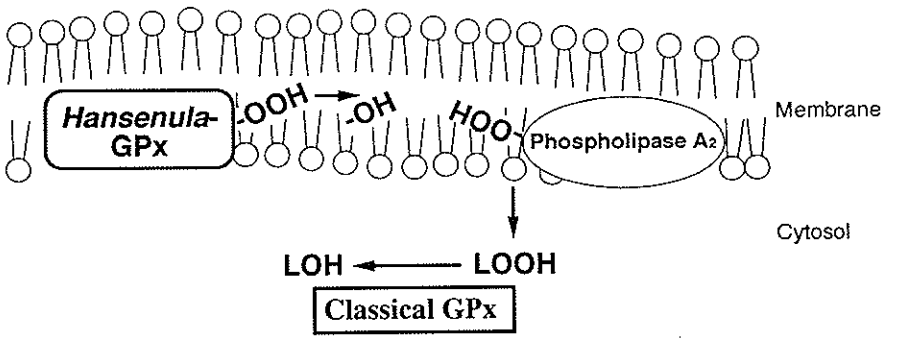

Figure 9. Possible model for protection of membrane lipid by glutathione peroxidase of *H. mrakii*.

Oxidative stress response in *Saccharomyces cerevisiae*

Several environmental stresses are known to trigger intracellular alterations in organisms. Organisms of all types show the synthesis of stress-inducible proteins, and the most advanced undertanding of the stress-inducible proteins has been obtained from the study of heat-shock protein (HSP). A sudden increase in temperature of the environment in which cells are growing induces increased synthesis of a set of heat-shock mRNAs and proteins in the cells. When *E. coli* cells are shifted from 30°C to 42°C, the intracellular concentration of σ^{32} increases 15- to 20-fold. The σ factor is one of the components of RNA polymerase of *E. coli* and substitution of a vegetative σ factor (σ^{70}) to σ^{32} changes the RNA polymerase so as specifically to recognize the HSP-encoding genes. When the yeast cells are exposed to the heat-shock stress, the heat-shock factor (HSF), which constitutively binds to the specific sequence (HSE, heat-shock element) of the genes encoding HSP, is trimerized and phosphorylated by heat shock, and the phosphorylated HSF then activates the transcription of *HSP* genes.

Intracellular stresses would also affect the expression of *hsp* genes. For example, reactive oxygen species, which are generated during respiration in aerobic organisms, are known to induce the synthesis of some stress shock proteins. *Escherichia coli* and *S. typhimurium* have *oxyR*-controlled hydrogen peroxide-inducible genes. When the bacterial cells are treated with low doses of hydrogen peroxide, synthesis of at least 30 proteins is induced. The synthesis of nine of these is positively controlled by OxyR protein. OxyR binds to the promoter regions of several genes such as *katG* (catalase), *ahpC* and *ahpF* (alkylhydroperoxide reductase) and *gorA* (glutathione reductase), whose products are concerned with the protection of the cells against oxidative stress, to activate their expression. OxyR belongs to the LysR family and it binds to its own promoter region of the *oxyR* gene to control its transcription negatively (Storz *et al.*, 1993). Several oxidative stress proteins are known to be induced by multiple types of stress such as heat shock and carbon starvation; however, the molecular mechanisms of the overlapping regulation have not been well elucidated.

Recently the author and his co-workers have started to study the mechanism for adaptation and resistance to oxidative stress caused by lipid hydroperoxide in yeasts (Inoue *et al.*, 1990b, 1992a, 1993; Inoue, Kobayashi and Kimura, 1993; Inoue, Tran and Kimura, 1993; Tran, Inoue and Kimura, 1993a,b). As described in the previous

section, most yeast strains tested, except for a few strains of the *Hansenula* genus, could not grow in medium containing lipid hydroperoxide (Inoue *et al.*, 1990b). Among them *H. mrakii* has a membrane-bound glutathione peroxidase that is induced by lipid hydroperoxide as well as reactive oxygen species, such as the superoxide anion radical and the hydroxyl radical, to protect the membrane phospholipid from peroxidation (Inoue *et al.*, 1990b; Inoue, Tran and Kimura, 1993; Tran, Inoue and Kimura, 1993a,b).

MOLECULAR CLONING AND PHENOTYPIC CHARACTER OF OXIDATIVE STRESS RESISTANT GENE FROM *S. CEREVISIAE*

Cloning

A genomic DNA library of *S. cerevisiae* was screened for the gene(s) that confer resistance against lipid hydroperoxide in yeast. Several candidates were obtained by screening the transformants showing rapid growth on lipid hydroperoxide-containing medium. Finally two independent clones (designated No. 2 and No. 10) were obtained (*Figure 10a*). Growth of the control strain carrying YEp13 in the medium containing 0.75 mM *tert*-butyl hydroperoxide and 0.5 mM linoleic acid hydroperoxide was inhibited for approximately 2 days, and then the cells started to grow (*Figure 10b*). On the other hand, the transformants No. 2 and No. 10 could grow at almost same growth rate compared with growth without chemicals. By the addition of 2.0 mM *tert*-butyl hydroperoxide and 1.0 mM linoleic acid hydroperoxide, the growth of the control strain was completely inhibited. Whereas both transformants (No. 2 and No. 10) could grow in the medium containing *tert*-butyl hydroperoxide and linoleic acid hydroperoxide up to 2.0 mM and 1.0 mM, respectively (*Figure 10b*).

Plasmids isolated from strains No. 2 and No. 10 were named pYHP2 and pYHP10, respectively. The size of the inserted fragment in pYHP2 and pYHP10 was 5.9 kb and 4.0 kb, respectively. The restriction map of the inserted fragment was different in the two plasmids (Inoue *et al.*, 1993). Several kinds of genes corresponding to the resistance to oxidative stress caused by lipid hydroperoxide may exist on the genome of *S. cerevisiae*. Collinson and Dawes (1992) reported that *S. cerevisiae* cells have at least four polypeptides involved in the peroxide-stress response. Jamieson (1992) and Flattery-O'Brien, Collinson and Dawes (1993) also reported that *S. cerevisiae* has two distinct adaptive responses to hydrogen peroxide and a superoxide-generating reagent (menadione). Therefore, amplification of such a gene would allow the transformant cell to resist oxidative stress.

The transformant carrying pYHP10 could grow in the medium containing *tert*-butyl hydroperoxide, linoleic acid hydroperoxide and hydrogen peroxide up to 2.0 mM, 1.0 mM and 5.0 mM, respectively, in which the control strain could not grow. The gene was designated as *OSR*, oxidative stress resistant gene (Inoue, Kobayashi and Kimura, 1993).

Glutathione-dependent phenotype

Glutathione is synthesized by two sequential reactions catalysed by γ-glutamylcysteine synthetase (GSH-I, EC 6.3.2.2) and glutathione synthetase (GSH-II, EC 6.3.2.3) in

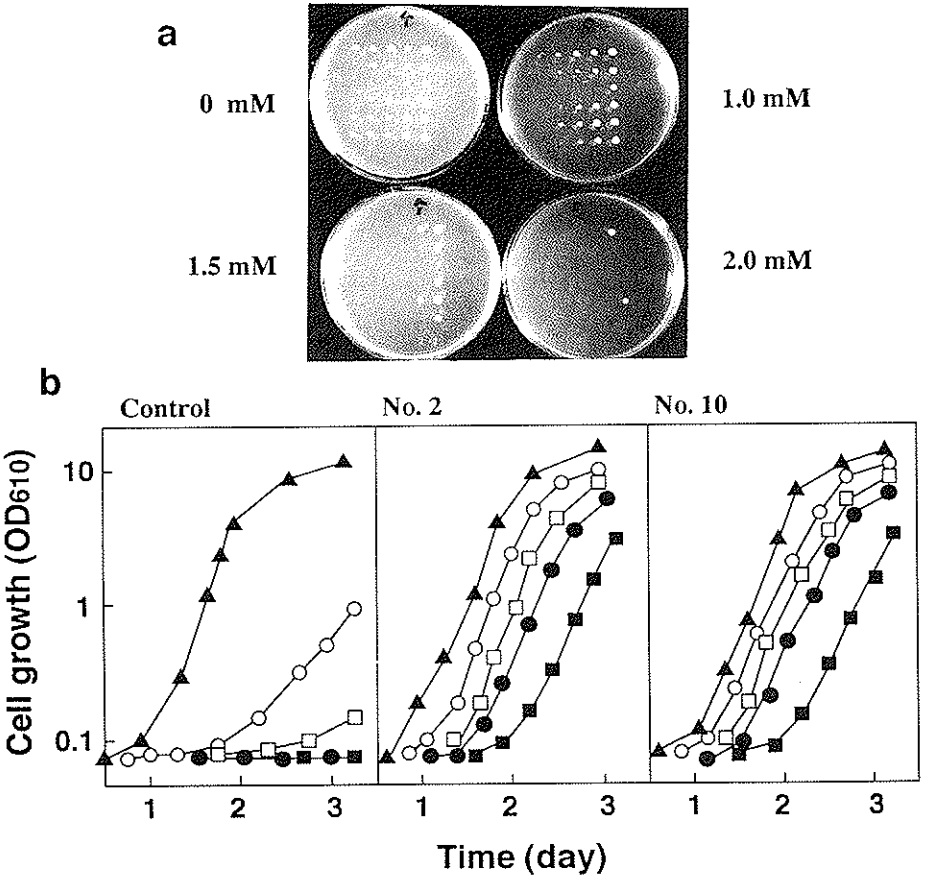

Figure 10. Growth of *S. cerevisiae* cells dosed with the *OSR* gene. (a) Screening of lipid hydroperoxide resistant clones from a genomic DNA library. Several candidates obtained by primary screening were replica plated on the SD minimal agar plate containing *tert*-butyl hydroperoxide with various concentrations as indicated. (b) Growth of yeast transformants in lipid hydroperoxide-containing medium. Symbols: ▲, without chemicals; O, 0.75 mM *tert*-butyl hydroperoxide; ●, 2.0 mM *tert*-butyl hydroperoxide; □, 0.5 mM linoleic acid hydroperoxide; ■, 1.0 mM linoleic acid hydroperoxide.

the presence of ATP. Glutathione is distributed in almost all aerobic organisms and the compound has many biological functions in the cells, e.g. detoxification of various cytotoxic compounds, maintenance of redox potential in the cells, cofactor for various enzymes, protection of SH group of various proteins, transportation of amino acids (constituting γ-amino cycle), and so on. Both prokaryotic and eukaryotic cells have a glutathione-dependent detoxification system for xenobiotics (Inoue and Kimura, in press). In order to investigate whether or not resistance of the yeast transformant carrying pYHP10 was glutathione-dependent, buthionine sulphoximine (BSO), a potent inhibitor for GSH-I, was added to the medium containing *tert*-butyl hydroperoxide. BSO alone did not affect the growth of the transformants carrying pYHP10 and YEp13, whereas when BSO and *tert*-butyl hydroperoxide were added simultaneously, the growth of both transformant was completely arrested (*Figure 11*).

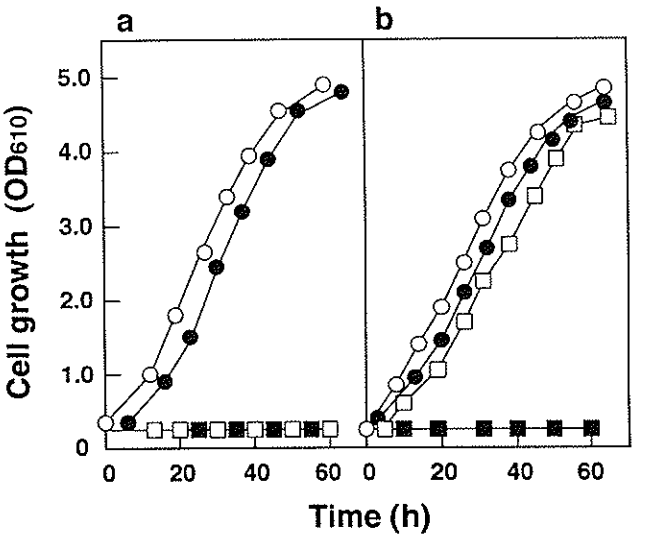

Figure 11. Effect of BSO on growth of yeast transformants. *Saccharomyces cerevisiae* carrying YEp13 (a) or pYHP10 (b) was cultured in the SD medium containing BSO and/or *tert*-butyl hydroperoxide. Symbols: O, without chemicals; ●, 0.1 mM BSO; □, 1.5 mM *tert*-butyl hydroperoxide; ■, 0.1 mM BSO + 1.5 mM *tert*-butyl hydroperoxide.

Saccharomyces cerevisiae has superoxide dismutases (Cu,Zn-SOD (Goscin and Fridovich, 1972; Bermingham-McDonogh, Gralla and Valentine, 1988), Mn-SOD (Ravindranath and Fridovich, 1975; Marres *et al.*, 1985)) for disproportion of the superoxide anion radical, and cytochrome *c* peroxidase (*CCP1* (Yonetani and Ray, 1965; Kaput, Golz and Blobel, 1982)) as well as catalases (*CTT1* (Seah, Bhatti and Kaplan, 1973; Hartig and Ruis, 1986) and *CTA1* (Seah and Kaplan, 1973; Cohen, Rapat and Ruis, 1988)) for the reduction or breakdown of hydrogen peroxide; however, glutathione-dependent enzymes for scavenging of reactive oxygen species in *S. cerevisiae* have not been identified at the genetic level. The author and his co-workers proved that a yeast, *H. mrakii*, has a membrane-bound glutathione peroxidase to protect the cell against peroxidation of membrane lipid (Inoue *et al.*, 1990b; Inoue, Tran and Kimura, 1993; Tran, Inoue and Kimura, 1993a,b). In mammalian cells, glutathione peroxidase is one of the major enzymes that scavenge hydrogen peroxide and lipid hydroperoxide. Jacobson *et al.* (1989) have proposed that alkyl hydroperoxide reductase serves as a prokaryotic equivalent to the glutathione reductase/glutathione peroxidase system in mammalian cells. The intracellular glutathione concentration of *S. cerevisiae* is comparable with that of the mammalian cell, and yeast also has glutathione reductase (Racker, 1955); thus the occurrence of some glutathione-dependent enzymatic systems for scavenging the reactive oxygen species in *S. cerevisiae* would be expected.

NUCLEOTIDE SEQUENCE AND FUNCTION OF THE *OSR* GENE IN *S. CEREVISIAE*

Nucleotide sequence of the OSR gene

The nucleotide sequence of the *OSR* gene was determined. *Figure 12* shows the 2297

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                                                                GATCGGTGCAGGTCCCATGAGATAAACTTTTGCGTTGAGTCTGGACTCTTGATGTATCTCGGA -547

    CTAAAGCATTCATTCTGCTAAGGGAAGCGTCTGGCTAAGTTCAGCCAGACTTCCATTCTACAACCCTTTTTACTAGCTGAGCCTTCTTCCC -456

    AGGAAGCAAAAAAAAAAATCTTGCTACTTTCTCTTCATGACGCAGCGTGGTGAGTGCATTGCTCTTTCCCTACCTACGCCGAACATATATGT -365

    CATATAACGGAAGTCATAAACGGTGGCTCCTTTTTGTTTTTGCTGGTCAACCTTAAAGAAACAAAAACTCTAATATGATTTCTTGGCATAG -274

    ACCCACACACCCTCGGAAAACCTCATTTTTCAAAAGAAAAAAAAATGCGTTTTTGTTTGCGAAAAATAACGTCCCTCCCTCTGTTTTCCTTC -183

    TTCAGATGGCCTTGAAGGTGAGGAATGAGGTGGTTGCTGCATCAGCTAAAACTTTTCATTGCAAAGTCATCAGAGCTCAAAGTTTCATAAA -92

    AGAATATAACATTCAAGATACTTTAAAAGGCTTGGCGCAGTAATTTTTCCCTATCGATAACCGTCAGGAATACCAGGAAACTAATAGAGTC -1

    ATG ATC ACC GGT AAA GAA TTG AGA ATC ATC TCT CTT TTG ACC TTA GAC ACG GTT TTT TTC CTA TTG GAA +69
  1 Met Ile Thr Gly Lys Glu Leu Arg Ile Ile Ser Leu Leu Thr Leu Asp Thr Val Phe Phe Leu Leu Glu

    ATT ACC ATA GGT TAT ATG TCA CAT TCA TTG GCC TTG ATT GCC GAT TCA TTT CAC ATG TTG AAT GAT ATC +138
 24 Ile Thr Ile Gly Tyr Met Ser His Ser Leu Ala Leu Ile Ala Asp Ser Phe His Met Leu Asn Asp Ile

    ATC TCT CTT TTA GTG GCA CTA TGG GCT GTG GAT GTG GCC AAA AAC AGG GGT CCA GAC GCT AAA TAC ACT +207
 47 Ile Ser Leu Leu Val Ala Leu Trp Ala Val Asp Val Ala Lys Asn Arg Gly Pro Asp Ala Lys Tyr Thr

    TAT GGA TGG AAA AGA GCG GAA ATT TTG GGT GCT TTA ATC AAT GCT GTT TTT CTT ATT GCC CTG TGT TTC +276
 70 Tyr Gly Trp Lys Arg Ala Glu Ile Leu Gly Ala Leu Ile Asn Ala Val Phe Leu Ile Ala Leu Cys Phe

    TCT ATT ATG ATT GAA GCT TTA CAA AGA TTG ATT GAA CCT CAA GAA ATT CAA AAC CCA AGG TTG GTT TTA +345
 93 Ser Ile Met Ile Glu Ala Leu Gln Arg Leu Ile Glu Pro Gln Glu Ile Gln Asn Pro Arg Leu Val Leu

    TAC GTT GGT GTA GCA GGG TTA ATT TCT AAT GTC GTA GGT TTA TTT TTG TTC CAC GAT CAT GGC AGC GAT +414
116 Tyr Val Gly Val Ala Gly Leu Ile Ser Asn Val Gal Gly Leu Phe Leu Phe His Asp His Gly Ser Asp

    AGT CTG CAC TCA CAC TCT CAT GGC TCT GTG GAA AGC GGG AAT AAC GAT TTG GAC ATA GAA TCT AAT GCG +483
139 Ser Leu His Ser His Ser His Gly Ser Val Glu Ser Gly Asn Asn Asp Leu Asp Ile Glu Ser Asn Ala

    ACT CAT TCC CAC TCT CAT GCA TCT CTT CCA AAC GAT AAT TTG GCC ATC GAT GAA GAT GCT ATT TCG AGT +552
162 Thr His Ser His Ser His Ala Ser Leu Pro Asn Asp Asn Leu Ala Ile Asp Glu Asp Ala Ile Ser Ser

    CCT GGG CCC TCA GGG CAA ATT GGT GAA GTG TTG CCA CAA TCA GTA GTA AAC AGA TTA TCA AAC GAA AGC +621
185 Pro Gly Pro Ser Gly Gln Ile Gly Glu Val Leu Pro Gln Ser Val Val Asn Arg Leu Ser Asn Glu Ser

    CAA CCC TTA TTG AAC CAC GAT GAT CAT GAC CAC AGC CAT GAA TCA AAG AAA CCA GGT CAT CGT TCT TTG +690
208 Gln Pro Leu Leu Asn His Asp Asp His Asp His Ser His Glu Ser Lys Lys Pro Gly His Arg Ser Leu

    AAT ATG CAT GGT GTC TTC TTA CAT GTA CTA GGT GAT GCT CTG GGT AAT ATT GGT GTT ATT GCA GCT GCT +759
123 Asn Met His Gly Val Phe Leu His Val Leu Gly Asp Ala Leu Gly Asn Ile Gly Val Ile Ala Ala Ala

    TTG TTT ATT TGG AAA ACT GAA TAT TCT TGG AGA TAT TAC TCG GAT CCA ATC GTT TCT TTA ATC ATC ACC +828
254 Leu Phe Ile Trp Lys Thr Glu Tyr Ser Trp Arg Tyr Tyr Ser Asp Pro Ile Val Ser Leu Ile Ile Thr

    ATT ATT ATT TTC TCT TCC GCT CTG CCC TTA TCA CGT AGA GCT TCA AGA ATT TTA CTA CAG GCT ACT CCT +897
277 Ile Ile Ile Phe Ser Ser Ala Leu Pro Leu Ser Arg Arg Ala Ser Arg Ile Leu Leu Gln Ala Thr Pro

    TCT ACA ATT TCT GCT GAT CAG ATT CAA AGA GAG ATT TTG GCA GTA CCT GGC GTG ATA GCG GTC CAT GAC +966
300 Ser Thr Ile Ser Ala Asp Gln Ile Gln Arg Glu Ile Leu Ala Val Pro Gly Val Ile Ala Val His Asp

    TTC CAC GTC TGG AAC TTA ACT GAA TCA ATA TAT ATT GCA TCT ATC CAC GTT CAA ATA GAC TGT GCA CCT +1035
323 Phe His Val Trp Asn Leu Thr Glu Ser Ile Tyr Ile Ala Ser Ile His Val Gln Ile Asp Cys Ala Pro

    GAT AAA TTC ATG AGC TCC GCC AAG CTG ATA AGA AAA ATA TTC CAT CAA CAC GGT ATT CAT TCT GCA ACT +1104
346 Asp Lys Phe Met Ser Ser Ala Lys Leu Ile Arg Lys Ile Phe His Gln His Gly Ile His Ser Ala Thr

    GTT CAA CCA GAA TTT GTC TCT GGA GAT GTT AAT GAG GAT ATT CGC AGA AGA TTT TCT ATC ATA GCA GGT +1173
369 Val Gln Pro Glu Phe Val Ser Gly Asp Val Asn Glu Asp Ile Arg Arg Arg Phe Ser Ile Ile Ala Gly

    GGT TCA CCA TCT TCG TCT CAA GAA GCC TTT GAC AGC CAT GGA AAC ACT GAG CAT GGT AGA AAA AAG AGT +1242
392 Gly Ser Pro Ser Ser Ser Gln Glu Ala Phe Asp Ser His Gly Asn Thr Glu His Gly Arg Lys Lys Ser

    TCA CCT ATT GCC TAT GGT GCT ACT ACA CAT CAT CTA ATT GTA TTG TAGATGACGCTGTAAACTGCAATACTTCCAA +1318
415 Ser Pro Ile Ala Tyr Gly Ala Thr Thr His His Leu Ile Val Leu ***

    TTGCCTGTAATAAAGATATAAAGGGCTGAAGCGTCGGTGAATTTATCCAAGGTTCTACAGAGAACATAAACTATATACTATTTACATAATT +1409

    AATTCTTAGACGTATAGGAAGAAGCAGTTCTTCCTAACTCAAGAACTGCGTTATTCAATGTATGAATACGCTATCTTTTAGCTATAAATCT +1500

    CCATTAAAAATTTGTATGCAATATGTGCCACAGTCCTTCCTCATCTTTTTTTGAGACTTTTAACCCAGACATAAAATACCCCCATACATC +1591

    CAGAATAGCGTTTTTATCTCTCAAATAGTTAACTTTTTTCAGCGCGCGCAGAGCAGGAAACAAATTAAGCTCACTTCGGTGTGGTAC
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Figure 12. Nucleotide sequence of the *OSR* gene. Putative TATA-like sequences and the polyadenylation signal are indicated by underlining. Numbers on the right-hand side of the figure show the nucleotide position, which started from the translational initiation codon (ATG). Numbers on the left-hand side of the figure show the amino acid position beginning from the N-terminal methionine (Met).

bp nucleotide sequence and deduced amino acid sequence derived from the DNA sequence. It contained an open reading frame with 1287 bp (encoding 429 amino acids), and the molecular weight of the peptide was calculated to be 47 075 Da. The 5'-non-coding region of the *OSR* gene contained four putative TATA-like sequences at the position of –371 (TATAT), –362 (TATAA), –291 (TATGA) and –87 (TATAA).

A homology search revealed that the *OSR* gene was identical with *ZRC1*, the zinc-resistant conferring gene of *S. cerevisiae* (Kamizono *et al.*, 1989). The amino acid sequence near the C-terminus of OSR was different from that of ZRC1. The OSR protein consisted of 429 amino acids, whereas the ZRC1 protein contained 442 amino acids. The amino acid sequence near the C-terminus of OSR was ^{422}TTHHLIVL, whereas that of ZRC1 was ^{422}TTASSNCIVDDAVNCNTSNCL. This was due to a one-base deletion of the *OSR* gene. If we add 'G' 1270 bp downstream of ATG, the reading frame is shifted and the amino acid sequence thereafter completely coincides with that of ZRC1. However, we could not read 'G' at this position.

It was interesting that the *OSR* gene was identical with the *ZRC1* gene. The *OSR* gene was cloned independently using a different probe (lipid hydroperoxide resistance). Reactive oxygen species in the cells are formed by various routes. Besides the respiration of molecular oxygen, reactive oxygen species are formed by several biochemical reactions, such as xanthine+xanthine oxidase reaction to form the superoxide anion radical. On the other hand, transition metal ions also produce reactive oxygen species by metal-catalysed oxidation (MCO) systems. One possible explanation why the same gene was cloned by different methods is that both probes (zinc ion and oxidative stress) are closely related to the reactive oxygen species. Furthermore, the *ZRC1* gene was recently cloned in a study of ageing of *S. cerevisiae* by Guarente and his co-workers (M. Nishizawa, personal communication). Oxidative stress is known to be one of the factors that determine ageing (Ames and Shigenaga, 1992). Therefore, zinc ions, which may trigger the generation of reactive oxygen species, and oxidative stress, which is caused by reactive oxygen species, may cause similar effects in the cell, and may also be concerned with ageing.

Gene disruption

When the *OSR* gene was introduced into *S. cerevisiae* with the multicopy plasmid, the yeast showed higher resistance against oxidative stress caused by several hydroperoxides, such as *tert*-butyl hydroperoxide and linoleic acid hydroperoxide. To investigate the function of the *OSR* gene product in *S. cerevisiae* cells, the corresponding gene on the chromosomal DNA was disrupted by replacing with the *URA3* gene.

Growth of a knockout mutant in the presence of *tert*-butyl hydroperoxide was compared with that of wild-type cell. First of all, since we could obtain the disruptant using a haploid cell, *OSR* gene seemed not to be necessary for viability of the yeast. As shown in *Figure 13*, the knockout mutant could grow in the *tert*-butyl hydroperoxide-containing medium up to 0.5mM, although the growth rate was slightly reduced. In the presence of 0.7 mM *tert*-butyl hydroperoxide the mutant could not grow at all, whereas the wild-type cell could grow. These results suggested that the *OSR* gene product is necessary for scavenging the excess amount of oxidants produced during the normal metabolism of the cell.

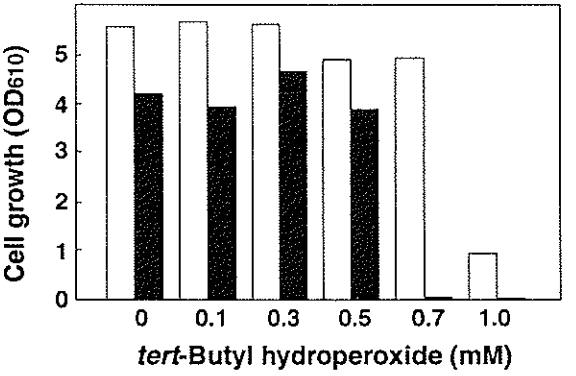

Figure 13. Growth of an *OSR* gene disruptant. *Saccharomyces cerevisiae* whose *OSR* gene was disrupted (*osr::URA3*) was cultured in the SD minimal medium containing *tert*-butyl hydroperoxide. White bars indicate the growth of wild type, and black bars indicate the growth of disruptant. Each bar indicates the OD_{610} of 3 days' culture.

Glutathione content

Previously the author proposed that the function of the *OSR* gene product seemed to be expressed in a glutathione-dependent manner (Inoue, Kobayashi and Kimura, 1993). Intracellular glutathione content was then measured in wild type, knockout mutant and transformant cells carrying YEp24 + *OSR* and YCp50 + *OSR*. The glutathione content in knockout mutants was lowered approximately 44% compared with that in wild-type cells, whereas the cells dosed with the *OSR* gene had approximately a three-fold higher glutathione content (*Figure 14a*).

In the biosynthesis of glutathione, γ-glutamylcysteine synthetase (GSH-I) is a key enzyme. The effect of the *OSR* gene on the expression of the *GSH-I* gene in *S. cerevisiae* was then investigated by quantitative slot blot hybridization (*Figure 14b*). The amount of mRNA of the *GSH-I* gene in the knockout mutant cell was lower compared with that of the wild-type cell. On the other hand, the mRNA level of the *GSH-I* gene was slightly increased in the cells harbouring YEp24 + *OSR* and YCp50 + *OSR*. Biosynthesis of glutathione is believed to be controlled at the substrate level, i.e. GSH-I is a rate-limiting enzyme and the GSH-I activity is negatively controlled by glutathione (feedback inhibition). It implies that the increase in the mRNA level of the *GSH-I* gene may not directly reflect the amount of intracellular glutathione. The results also suggested a possibility that the *OSR* gene product may stabilize the *GSH-I* mRNA, or inhibit the degradation of mRNA of the *GSH-I* gene.

Stress response of OSR gene expression

The promoter activity of the 5'-flanking region (612 bp) of the *OSR* gene was examined by constructing an *OSR'–'lacZ* fusion. The *Sau*3AI–*Eco*RV fragment (*Figure 15a*; 744 bp) was cloned into the *Sma*I site of pMC1871 (Shapira *et al.*, 1983) which contained the *lacZ* gene of *E. coli* without its original promoter region and N-terminal eight amino acids. The resultant fusion gene contained the 5'-non-coding region of the *OSR* gene, 45 amino acids beginning from N-terminal methionine and *lacZ* structural gene (*Figure 15a*). The fusion gene was cloned into YCp50 and

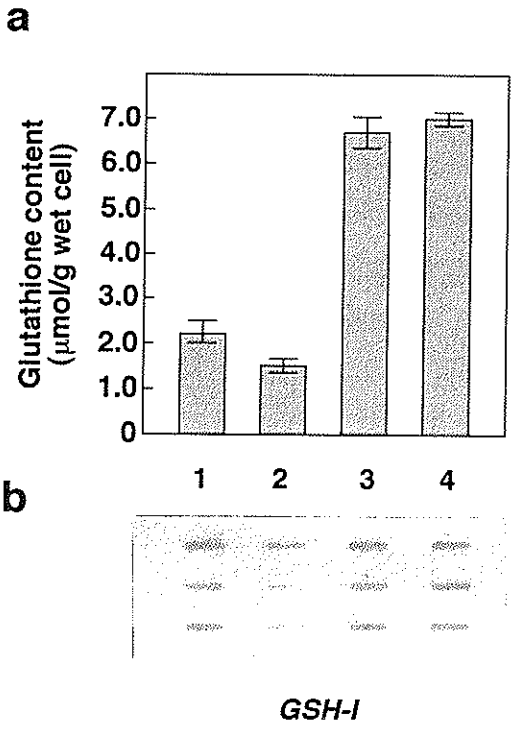

Figure 14. Effect of the *OSR* gene on the biosynthesis of glutathione. (a) Intracellular glutathione content. Lane 1, wild type; lane 2, knockout mutant; lane 3, wild type carrying YEp24 + *OSR*; lane 4, wild type carrying YCp50 + *OSR*. (b) Effect of the *OSR* gene on the mRNA level of the *GSH-I* gene in *S. cerevisiae*. Each slot corresponds to the lanes in (a). Total RNA was prepared from each cell and the amount of *GSH-I* mRNA was monitored by slot hybridization. Results of three independent experiments are shown.

YEp24, respectively, and each recombinant plasmid was transformed into *S. cerevisiae*. β-Galactosidase activity could be detected from both transformants carrying YCp50 + *OSR'–'lacZ* and YEp24 + *OSR'–'lacZ*; suggesting that the 5'-non-coding region of *OSR* gene has the promoter activity in *S. cerevisiae*. These observations show that the *OSR* gene is constitutively expressed in *S. cerevisiae* cells, which was also confirmed by Northern blotting analysis (Inoue, Kobayashi and Kimura, 1993). Expression of the *OSR* gene under oxidative conditions was then investigated using the *OSR'–'lacZ* fusion gene. The activity increased in accordance with the increased concentrations of *tert*-butyl hydroperoxide and cumene hydroperoxide added to the medium (*Figure 15b*).

The expression of many yeast genes is known to be regulated at the transcriptional level by DNA-binding proteins. To investigate whether some proteins bind to the promoter regions of the *OSR* gene, a gel retardation assay was conducted (*Figure 16a*). Band shift was observed; however, unexpectedly, the shift was also observed even when the cell extracts prepared from those cells not exposed to oxidative stress were used. Band shift was suppressed by the addition of an excess of competitor, thus some proteins specifically recognizing the 5'-flanking region of *OSR* gene might constitutively exist and bind to this region.

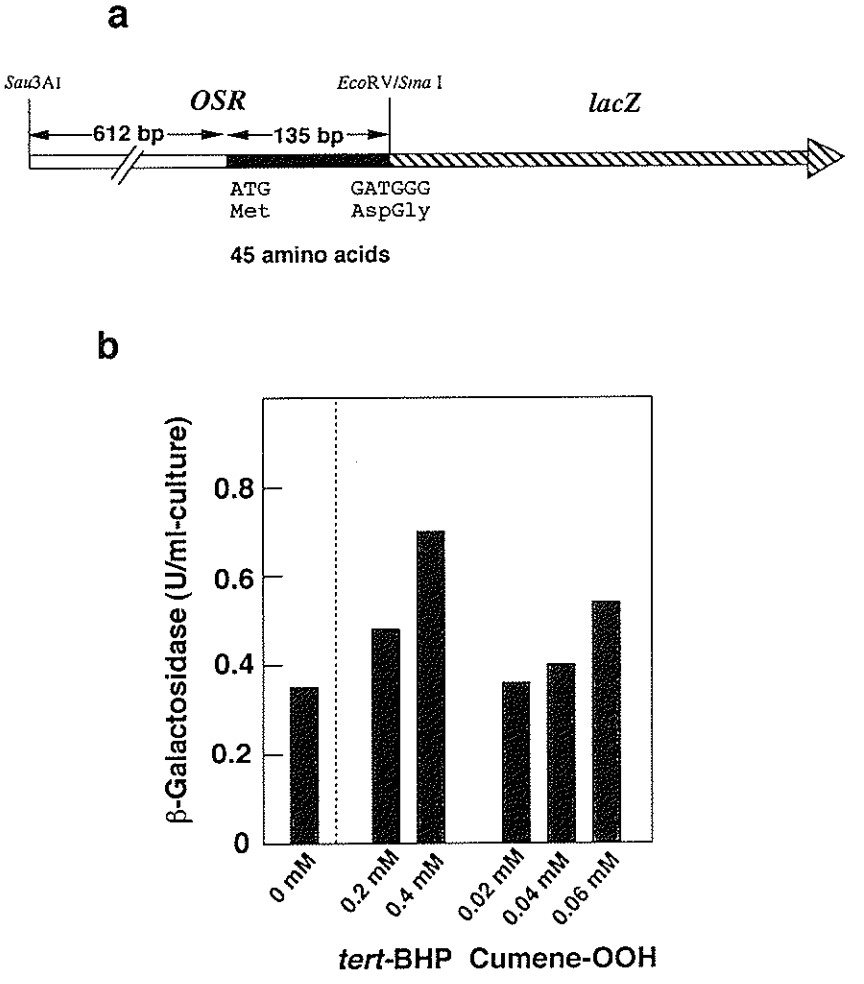

Figure 15. Effect of oxidative stress on translational efficiency. (a) Structure of *OSR'–'lacZ* fusion. ▭, 5'-flanking region of *OSR* gene; ▬, a part of the open rearing frame of *OSR* gene (45 amino acids); ▭, *lacZ* gene. (b) *S. cerevisiae* carrying the *OSR'–'lacZ* fusion gene with single copy vector (YCp50) was cultured in the SD minimal medium containing low concentrations of lipid hydroperoxide as indicated, and β-galactosidase activity was assayed. *tert*-BHP, *tert*-butyl hydroperoxide; Cumene-OOH, cumene hydroperoxide.

The amount of mRNA of the *OSR* gene after the treatment of cells with sublethal concentrations of *tert*-butyl hydroperoxide and cumene hydroperoxide was examined (*Figure 16b*). No significant changes were observed before and after the treatment. Therefore, the transcription of the *OSR* gene was not affected by the oxidative stress. On the other hand, the translational level seemed to be positively regulated under the oxidative conditions (*Figure 15b*); the region involved might be within the first 45 amino acids (within 135 bases from the initiation codon, AUG) (*Figure 15a*). The secondary structure of mRNA of this region is shown in *Figure 17*. Total secondary structure energy was calculated to be –41.2 kcal mol^{-1}. This structure is, therefore, expected to be possible. Translational efficiency of this mRNA may be

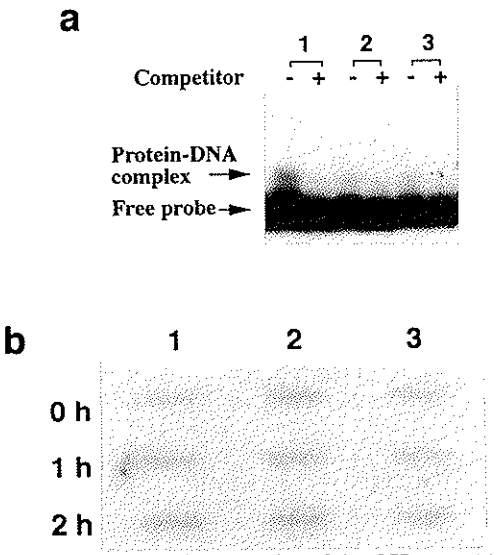

Figure 16. Effect of oxidative stress on the expression of the *OSR* gene. (a) Gel retardation assay. *Saccharomyces cerevisiae* was cultured in SD minimal medium without chemicals, 0.3 mM *tert*-butyl hydroperoxide and 0.1 mM cumene hydroperoxide, respectively, and cell extracts were prepared from each cell. The 5'-flanking region of the *OSR* gene (612 bp as indicated in *Figure 15a*) was labelled with ^{32}P and was used as a probe. Cell extracts used in each lane were prepared from the cells cultured with: lane 1, without chemicals; lane 2, 0.3 mM *tert*-butyl hydroperoxide; land 3, 0.1 mM cumene hydroperoxide, respectively. (b) Slot hybridation of the *OSR* gene. Cells of *S. cerevisiae* cultured for 16 h without oxidative stress were washed and resuspended in the fresh SD medium containing no chemicals (slot 1), 0.3 mM *tert*-butyl hydroperoxide (slot 2) or 0.1 mM cumene hydroperoxide (slot 3), and incubated at 30°C with reciprocal shaking. Total RNA was prepared after 1 h and 2 h, respectively, and slot hybridization was carried out using the *OSR* gene as a probe.

lower in the yeast cell because of its high secondary structure energy. The effect of heat shock on the translational efficiency was examined using the *OSR*'–'*lacZ* fusion, although no change of β-galactosidase activity was observed. Alteration of the translational efficiency under the oxidative conditions appears to be of interest.

UTILIZATION OF THE *OSR* GENE AS A SELECTABLE MARKER GENE FOR YEAST TRANSFORMATION

In the transformation of *S. cerevisiae*, complemental genes for the auxotrophic markers of host strains are commonly used for the selection of transformant. Several drug-resistant genes are also used if the host strains do not have appropriate auxotrophic markers. Since the amplification of the *OSR* gene has proved to make the yeast transformant resistant against oxidative stress caused by lipid hydroperoxide, the plasmid carrying the *OSR* gene (pYHP10) was introduced into *S. cerevisiae* S288C (*MAT*α *SUC2 mal mel gal2 CUP1*), LB1-10B (*MAT*α *mnn1 SUC2 mal gal2 CUP1*) and LB1-16A (*MAT*α *mnn2 SUC2 mal gal2 CUP1*), which were commonly used as laboratory strains. These yeast strains have no appropriate markers such as *leu2* and *ura3* for the detection of transformants, although transformants could be screend for resistance against 2.0 mM *tert*-butyl hydroperoxide on the minimal agar plate. By

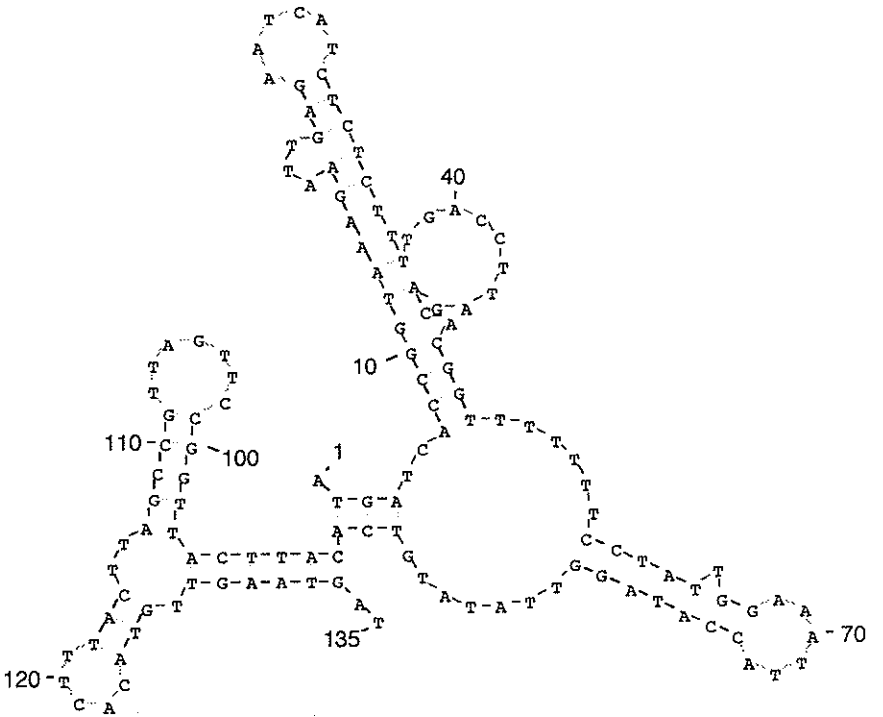

Figure 17. Secondary structure of mRNA of the *OSR* gene; the first 45 amino acid region.

Southern blotting analysis using a part of the *bla* gene in the vector (YEp13), pYHP10 was maintained in the *tert*-butyl hydroperoxide-resistant transformants. Therefore, the *OSR* gene was proved to be useful as the selection marker gene in the transformation of *S. cerevisiae* having no appropriate markers (Inoue *et al.*, 1993).

Most industrial users of yeast probably will not want to grow the transformants continuously in the presence of lipid hydroperoxide, because the chemical would cause the oxidation of the product. Therefore, the previously cloned *OSR* gene must be modified for use in conjunction with integration of transforming DNA into the genome of the host strain, with multicopy, and must confer on the transformants stable resistance against lipid hydroperoxide.

The gene product of *OSR* has not been identified yet; however, the gene could be used as a selection marker for the transformation of *S. cerevisiae* strains that do not have an appropriate auxotrophic marker. Promoter replacement study has also been started, to make it more suitable as the selectable marker gene.

Conclusions

Methylglyoxal and lipid hydroperoxide are endogenous cytotoxic molecular species. Micro-organisms as well as mammals have several defensive mechanisms against these stresses. By amplification of the genes corresponding to resistance against these stresses, breeding of useful organisms would be expected. Indeed, the author and his co-workers succeeded in producing *S*-D-lactoylglutathione, which is a reaction

product of glyoxalase I and has several physiological activities, by using genetically engineered *E. coli* cells (Inoue and Kimura, 1992a). The *OSR* gene was found to be useful as a selectable marker gene for the transformation of *S. cerevisiae* strains that do not have appropriate auxotrophic markers.

It has been believed that micro-organisms do not have peroxidases that use glutathione as the electron donor. Micro-organisms are believed to use cytochrome *c* as an electron donor for the peroxidase reaction (cytochrome *c* peroxidase). In plants, ascorbate is an electron donor for ascorbate peroxidase. In mammalian systems, glutathione is used as an electron donor. However, the author and his co-workers have discovered a peroxidase that uses glutathione as an electron donor in the yeast, *H. mrakii*. Furthermore, the glutathione peroxidase is tightly bound to the biological membrane. Very recently, we found that the enzyme is bound to the cell membrane as well as both the inner and outer membranes of mitochondria, an organelle in which large amounts of reactive oxygen species are formed by respiration. Molecular characterization of glutathione peroxidase in *H. mrakii* would give us a hint not only for the elucidation of the mechanisms to protect membrane phospholipid but also for the evolution of mechanisms against oxidative stress in organisms.

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