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Dissertação de candidatura ao grau de Doutor em Ciências Biomédicas, especialidade de Bioquímica, apresentada no Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto.

Orientador: Professor Doutor Pedro Moradas Ferreira (Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Portugal)

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Aos meus Pais

Á Isabel, ao Miguel, e à Catarina

De acordo com o nº 2 do Artigo 8º do Decreto-Lei nº 388/70, parte dos resultados apresentados nesta dissertação encontram-se publicados, como a seguir se descrimina:

Costa V, Reis E, Quintanilha A, and Moradas-Ferreira P (1993). Aquisition of ethanol tolerance in *Saccharomyces cerevisiae*: the key role of the mitochondrial superoxide dismutase. *Arch Biochem Biophys,* **300:** 608-614.

Costa V, Amorim MA, Reis E, Quintanilha A, and Moradas-Ferreira P (1997). Mitochondrial superoxide dismutase is essential for ethanol tolerance of *Saccharomyces cerevisiae* in the post-diauxic phase. *Microbiol,* **143:** 1649-1656.

No cumprimento do Decreto-Lei acima mencionado, foi decidido apresentar esta dissertação na forma de artigos. Declara-se ainda serem da nossa responsabilidade o planeamento e execução das experiências, assim como a interpretação, discussão e redacção dos resultados.

Agradecimentos

O trabalho apresentado nesta dissertação teve o apoio de várias pessoas e Instituições, às quais gostaria de deixar os meus sinceros agradecimentos.

Em primeiro lugar, não poderia deixar de agradecer ao Professor Doutor Pedro Moradas Ferreira (ICBAS e IBMC), pelas capacidades cientificas demonstradas na orientação desta tese, assim como pela sua atitude sempre positiva, que ajudou a ultrapassar os momentos mais difíceis deste trabalho. Gostaria ainda de expressar o meu apreço pelo sua amizade.

Desejo igualmente agradecer ao Professor Doutor Alexandre Quintanilha (ICBAS e IBMC), pelo constante interesse e apoio ao projecto, pelas sugestões e pelo estusiasmo transmitido.

À Professora Doutora Claudina Rodrigues Pousada (ICBAS e IGC), gostaria de agradecer o empenho e incentivos ao trabalho realizado sobre a função dos factores de transcrição Yap1p e Yap2p.

Ao Professor Doutor Cláudio Sunkel (ICBAS e IBMC), e à Professora Doutora Maria João Saraiva (ICBAS e IBMC), queria agradecer o equipamento de laboratório compartilhado e as revistas científicas colocadas à disposição.

Não podia deixar de agradecer ao Professor Doutor H. Sies e ao Doutor T.P.M. Akerboom (Universidade de Dusseldorf), pela forma amigável com que me receberam no seu laboratório, assim como pelo apoio prestado no doseamento de glutationa. Ao João Santos (IBMC), queria expressar os meus agradecimentos pela disponibilidade e sugestões para a realização de "Northern blots". Ao Professor Doutor R. Levine (NIH), por ter partilhado connosco a sua experiência na análise da oxidação de proteínas, o meu muito obrigado.

A realização deste projecto contou com a colaboração de várias pessoas que gentilmente cederam estirpes de *S. cerevisiae* ou plasmídeos. Ao Professor Doutor T. Bilinski (Universidade de Lublin), ao Doutor A.P.G.M. van Loon (Hoffman-La Roche, Suiça), ao Doutor Y. Ohtake (Asahi Breweries Ltd), e à Professora Doutora Claudina Rodrigues Pousada (ICBAS e IGC), pelas células *sod1*Δ, DL1/*sod2*Δ, YNN27/*gsh1*Δ, e W303/*yap1*Δ/*yap2*Δ/*yap1*Δ*yap2*Δ, respectivamente, e à Doutora E. Gralla (Universidade da Califórnia, Los Angeles), ao Doutor L. Grivel (Universidade de Amsterdam), e ao Professor Doutor H. Ruis (Universidade de Viena), pelos plasmídeos contendo os genes *SOD1*, *SOD2*, e *CTT1*, respectivamente, os meus agradecimentos.

A todos os elementos da Unidade de Stress em Microrganismos (IBMC), não podia deixar de agradecer toda a ajuda e o excelente ambiente proporcionado para a realização deste trabalho; à Amélia Amorim, ao Eduardo Reis, e à Alexandra Teixeira, pelo empenho neste projecto, e ao Paulo Fernandes, pelas sugestões, deixo um agradecimento especial. À Ana Maria (laboratório de

Bioquímica, ICBAS), queria expressar a minha gratidão pela forma como se desdobrou para prestar rapidamente todo o apoio solicitado.

Ao Centro de Citologia Experimental/Instituto de Biologia Molecular e Celular, pelas infraestruturas e outros meios colocados à disposição para a realização deste trabalho, os meus agradecimentos.

Finalmente, gostaria de deixar um obrigado muito especial à Isabel, pelo seu apoio incondicional, pelas sugestões e revisões que fez a esta dissertação, mas principalmente pela sua paciência e pelas palavras amigas importantes em momentos mais difíceis. E ainda ao Miguel e à Catarina, pelos sorrisos que fazem esquecer tudo...

O trabalho apresentado nesta tese foi realizado no âmbito do projecto "Microrganismos em ambientes extremos: mecanismos de adaptação" (PRAXIS XXI 2/2.1/BIO/20/94), e foi financiado pela JNICT (Bolsa de Doutoramento BD/50/90-IF) e pelo PRODEP.

Abbreviations

ACT1	Actin
APN1	AP endonuclease
ARE	AP1 recognition element
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BSA	bovine serum albumin
CCO	cytochrome c oxidase
CCP1	cytochrome c peroxidase
CDNB	1-chloro-2,4-dinitrobenzene
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]1-propanesulfonate
COQ3	ubiquinol
CPH1	cyclophylin 1
CTA1	catalase A
CTT1	catalase T
CUP1	metallothionein
DNA	deoxyribonucleic acid
DNP	dinitrophenyl
DS	diauxic shift
DTT	dithiothreitol
GLR	glutathione reductase
GRX	glutaredoxin
GSH	reduced glutathione
GSSH	oxidised glutathione
GSH1	γ- glutamyl-cysteine synthetase
HAP	heme activator protein
8-OH-Gua	8-hydroxyguanine
HOG	high osmolarity glycerol response
HS	heat shock
HSP	heat shock protein
IPG	immobilised pH gradient
MALDI	mass assisted laser desorption
MAP	mitogen activated protein
MCO	metal ion catalysed oxidation
MDA	malondialdehyde
MOPS	4-morpholine propane sulfonic acid
mRNA	messenger RNA
MSN	multicopy suppressor of *snf1*Δ
MSRA	methionine sulfoxide reductase
NAD	nicotinamide adenine dinucleotide
NADH	NAD-reduced
NEM	N-ethyl-maleimide
NTG	endonuclease three-like glycosylase
OGG	8-oxoguanine glycosylase

PAGE	polyacrylamide gel electrophoresis
PD	post-diauxic
PKA	protein kinase A
PLA2	phospholypase A2
PMA	plasma membrane ATPase
PMG1	phosphoglycerate mutase
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
SKN	suppressor of *kre9Δ*
SOD	superoxide dismutase
SPE2	S-adenosylmethionine decarboxylase
STRE	stress response element
TCA	trichloroacetic acid
TPI1	triosephosphate isomerase
TRX2	thioredoxin 2
TPX=TSA=TPP	thioredoxin peroxidase
TRR1	thioredoxin reductase
UBI4	polyubiquitin
YAP	yeast activator protein
YKL026c	putative glutathione peroxidase
ZWF	glucose-6-phosphate dehydrogenase

Contents

Summary

Ethanol, one of the major end products of yeast fermentation, is toxic to yeast cells above a critical concentration, affecting their growth, viability and fermentation rate. The characterisation of the molecular mechanisms involved in the acquisition of ethanol tolerance is, therefore, of biotechnological importance. A correlation between the induction of the mitochondrial superoxide dismutase (SOD) activity and the acquisition of ethanol tolerance in exponential phase *Saccharomyces cerevisiae* cells led to the search for the role of antioxidant defences in ethanol resistance, and to the identification of oxidative damages that might contribute to ethanol-induced cell death.

Our results indicate that MnSOD, unlike CuZnSOD, is essential for the acquisition of ethanol tolerance of yeast cells at the exponential phase, as well as during growth to the diauxic shift and post-diauxic shift phase, in which cells have a high, constitutive MnSOD activity. The analysis of *SOD* expression during growth from the exponential to the post-diauxic shift phase indicate that two regulatory mechanisms are involved in the induction of MnSOD and CuZnSOD activity: synthesis *de novo* of Sod proteins, until the diauxic shift phase, and post-transcriptional or post-translational regulation, during the post-diauxic phase. Ethanol does not alter the activities of either enzyme in cells from the diauxic shift or post-diauxic phases. Using respiration deficient mutants, we have shown that reactive oxygen species produced in the mitochondria mediate ethanol toxicity in yeast cells, and may regulate Sod activity during stress.

The effect of ethanol on *SOD* expression was compared with that of an oxidant, namely hydrogen peroxide. Unlike ethanol, hydrogen peroxide enhances synthesis *de novo* of MnSOD. The analysis of the role of Yap1p and Yap2p transcription factors in the regulation of SOD during exposure to hydrogen peroxide showed that transcriptional activation of *SOD1* gene is Yap1p-dependent, while the induction of *SOD2* gene expression is partially Yap1p-dependent. Moreover, Yap2p seems to have a role in sustaining the transcriptional activation of *SOD2*, and both Yap1p and Yap2p seem to be

indirectly involved in the post-translational activation of MnSOD apoprotein. Consistent with an important function of Yap2p in the adaptive response, disruption of *YAP2* reduces the capacity of *yap1*Δ cells to enhance H_2O_2 resistance by a sublethal stress pre-treatment.

This study also aimed to identify and correlate oxidative modifications in lipids and proteins with ethanol and hydrogen peroxide toxicity. Ethanol does not induce lipid peroxidation, even in ethanol sensitive *sod2*Δ cells. Ethanol causes, however, a mild oxidation of a subset of proteins, suggesting that protein oxidation may contribute to ethanol induced cell death in *S. cerevisiae*. The analysis of oxidative damages induced by hydrogen peroxide showed that lipid peroxidation is induced by this oxidant; however, it is not a major factor for the increased sensitivity of *yap1*Δ cells to hydrogen peroxide. In contrast, lipid peroxidation may contribute to the even higher sensitivity of *yap1*Δ*yap2*Δ cells to hydrogen peroxide, suggesting that antioxidant defences regulated by both Yap1p and Yap2p factors are important for protection from lipid peroxidation. Hydrogen peroxide also induces protein oxidation; however, the accumulation of oxidised proteins is not higher in cells hypersensitive to peroxide (*yap1*Δ*yap2*Δ).

These results indicate that oxidative stress mediates ethanol toxicity in *Saccharomyces cerevisiae*, the mitochondrial superoxide dismutase protecting yeast cells from the increased production of reactive oxygen species induced by ethanol.

Resumo

O etanol, um dos principais produtos da fermentação de leveduras, é, acima de uma concentração crítica, tóxico para estas células, afectando o seu crescimento, viabilidade e taxa de fermentação. A caracterização dos mecanismos moleculares envolvidos na aquisição de tolerância ao etanol assume, portanto, uma grande importância biotecnológica. A correlação encontrada entre a indução da actividade da superóxido dismutase (SOD) mitocondrial e a aquisição de tolerância ao etanol em células de *Saccharomyces cerevisiae* levou à pesquisa do papel das defesas antioxidantes na resistência ao etanol, assim como à identificação dos danos oxidativos que podem contribuir para a morte celular induzida pelo etanol.

Os resultados obtidos indicam que a MnSOD, ao contrário da CuZnSOD, é essencial para a aquisição de tolerância ao etanol em leveduras, não só em fase exponencial, como também durante o crescimento para as fases diauxica e pós-diauxica, nas quais as células apresentam uma elevada actividade constitutiva da MnSOD. A análise da expressão da SOD durante o crescimento da fase exponencial para as fases diauxica e pós-diauxica indica que dois mecanismos reguladores estão envolvidos na indução da actividade das MnSOD e CuZnSOD durante o crescimento: síntese *de novo* de proteínas Sod, até à fase diauxica, e regulação pós-transcripcional ou pós-traducional, durante a fase pós-diauxica. O etanol não altera as actividades de nenhuma das enzimas em células nas fases diauxica ou pós-diauxica. Usando mutantes deficientes respiratórios, foi demonstrado que as espécies reactivas de oxigénio produzidas na mitocôndria contribuem para a toxicidade do etanol em leveduras, e podem regular a actividade de Sod durante o stress.

O efeito do etanol na expressão de *SOD* foi comparado com o efeito de um oxidante, neste caso, peróxido de hidrogénio. Ao contrário do etanol, o peróxido de hidrogénio aumenta a síntese *de novo* de MnSOD. A análise do papel dos factores de transcrição Yap1p e Yap2p na regulação dos genes *SOD* durante a exposição ao peróxido de hidrogénio revelou que a activação

transcripcional do gene *SOD1* é dependente do Yap1p, ao passo que a indução da expressão do gene *SOD2* é parcialmente dependente de factor. Mais ainda, o Yap2p parece ter um papel na manutenção da activação transcripcional do gene *SOD2*, e ambos Yap1p e Yap2p parecem estar indirectamente envolvidos na activação pós-traducional da apoproteina da MnSOD. Consistente com uma importante função do Yap2p na resposta adaptativa, a disrupção de *YAP2* reduz a capacidade de células *yap1*Δ aumentarem a resistência ao H_2O_2 por um pré-tratamento sub-letal.

Este estudo teve também como objectivo pesquisar e correlacionar modificações oxidativas em lípidos e proteínas com a toxicidade do etanol e peróxido de hidrogénio. O etanol não induz peroxidação lipídica, mesmo em células *sod2*Δ sensíveis este stress. O etanol causa, no entanto, uma oxidação suave de um sub-grupo de proteínas, o que sugere que a oxidação de proteínas pode contribuir para a morte celular induzida pelo etanol em *S. cerevisiae*. A análise dos danos oxidativos induzidos pelo peróxido de hidrogénio revelou que a peroxidação de lípidos é induzida por este oxidante; no entanto, não é o principal factor a contribuir para o aumento da sensibilidade de *yap1*Δ ao peróxido de hidrogénio. Em contraste, a peroxidação de lípidos pode contribuir para a ainda maior sensibilidade das células *yap1*Δ*yap2*Δ ao peróxido de hidrogénio, o que sugere que as defesas antioxidantes reguladas pelos factores Yap1p e Yap2p são importantes para a protecção contra a peroxidação de lípidos. O peróxido de hidrogénio induz, também, oxidação de proteínas; no entanto, a acumulação de proteínas oxidadas em células sensiveis a este stress (*yap1*Δ*yap2*Δ) é semelhante à observada na estirpe selvagem.

Os resultados obtidos indicam que a toxicidade do etanol está associada a um stress oxidativo em *Saccharomyces cerevisiae*, e a superóxido dismutase mitocondrial protege as células de levedura do aumento da produção de espécies reactivas de oxigénio induzida pelo etanol.

Resumé

L'ethanol est le produit final de la fermentation de la levure, mais quand sa concentration atteind des niveaux critiques l'alcool devient toxique, et comme conséquence la croissance, la viabilité et le taux de fermentation sont affectées negativement. Du point de vue biotechnologique, c'est important de connaitre les mechanismes moleculaires qui sont associés à l'acquisition de la tolerance des levures à l'ethanol. La corrélation entre l'induction de l' activité de l'enzyme superoxide dismutase (SOD) mitochondriel et l'acquisition de la tolerance à l'ethanol chez *Saccharomyces cerevisiae* a conduit à la recherche du rôle des défenses antioxydantes dans la resistance à l'ethanol, et à l'identification des dommages oxydatifs qui peuvent contribuer à la morte par l'ethanol.

Nos résultats montrent que la MnSOD, au contraire de la CuZnSOD, est essentiel pour l'acquisition de la tolerance à l'ethanol dans les cellules qui sont en phase exponentiel, et aussi pendant la croissance pour la phase diauxique et post-diauxique, une phase dans laquelle les cellules ont un haut niveau d'activité de MnSOD constitutive.

L'analyse de l'expression de SOD pendant la croissance de la phase exponentiel pour la phase post-diauxique montre qu'il y a deux mechanismes responsables par l'induction de MnSOD et CuZnSOD: synthèse *de novo* des proteines Sod jusqu'à la phase diauxique, et régulation post-transcription ou post-traduction pendant la phase post-diauxique. Mais l'ethanol ne change pas l'activité des enzymes des cellules dans les phases diauxique et post-diauxique. En utilisant des cellules que ne respirent pas, on a montré que les espèces d'oxygène reactives produites dans la mitochondrie contribuent à la toxicité de l'ethanol et elles peuvent réguler l'activité des SOD pendant le stress.

L'effet de l'ethanol sur l'expression des SOD a été comparé à l'effet d'un oxydant comme le peroxide d'hydrogène. Au contraire de l'ethanol, le peroxide d'hydrogène augmente la syntèse *de novo* de MnSOD. L'analyse du rôle des facteurs de transcription Yap1p et Yap2p dans la régulation des SOD pendant le stress avec le peroxide, a montré que l'expression de *SOD1* est très

dépendant de Yap1p, et que l'expression de *SOD2* a une faible dépendence. En plus ça, le Yap2p semble avoir un rôle dans le maintien de l'activation transcriptionel de *SOD2*, et les deux facteurs sont indirectement engagés dans l'activation post-traduction de l'apoproteine MnSOD. En accord avec la function de Yap2p dans l'acquisition de la tolerance au peroxide, la disruption du gène *YAP2* réduit la capacité des cellules *yap1*Δ d'acquerir la resistance par un pre-traitement avec une concentration sous-letal de peroxide.

Un des objectifs de ce travail a été d'étudier la corrélation des changements oxydatifs dans les lipides et dans les proteines avec la toxicité de l'ethanol et du peroxide d'hydrogène. Le peroxide induit la peroxidation des lipides, mais ça n'est pas un facteur qui soit lié à la sensibilité des cellules *yap1*Δ. Au contraire, la peroxidation des lipides dans les cellules *yap1*Δ*yap2*Δ peut contribuer à l'haute sensibilité vers le peroxide, ce qui sugère que les defenses antioxydantes regulées par les deux facteurs sont importantes pour la protection des lipides. Le peroxide d'hydrogène oxyde aussi les proteines, mais l'oxydation est semblabe dans les cellules hipersensibles au peroxide. L'ethanol ne produit pas de peroxidation dans les lipides, même dans les cellules très sensibles à l'ethanol (*sod2*Δ). Pourtant, l'ethanol induit une faible oxydation des proteines.

Les résultats presentés dans ce travail indiquent que la toxicité de l'ethanol est associé au stress oxydatif in *Saccharomyces cerevisiae*, et l'enzyme mitochondriel SOD protège les cellules de levure des espèces reactives de l'oxygène induites par l'ethanol.

General Introduction

The molecular defences against reactive oxygen species in yeast

1- Yeast cells, an ideal system to study oxidative stress responses

Yeasts have been used in traditional biotechnologies, such as backing, brewing, distiller's fermentations, and wine making, for centuries. During these industrial applications, yeasts are exposed to numerous environmental stress conditions, including supraoptimal temperatures, oxidation, hyperosmolarity, ionic stress, organic acids, alcohols and nutrient limitation and starvation, which can occur in concert and sequentially (Attfield, 1997). The adaptation of *Saccharomyces cerevisiae* cells to stress conditions has been extensively studied in recent years (see Hohmann and Mager, 1997). Yeasts exhibit a complex array of both specific and general stress responses, which involve cell sensing, signal transduction, transcriptional and posttranslational control, protein targeting to organelles, accumulation of protectants, and repair of damaged molecules. The efficiency of these events determines the ability of yeasts to withstand stressful environments and, to a large extent, whether they meet the necessary commercial standards in industry.

Understanding how yeast cells adapt to changing environmental and physiological conditions has implications, not only in biotechnology but also in medicine. In fact, yeasts have remarkable similarities to human cells at the macromolecular and organelle level, and a number of yeast proteins, such as Ras (Morishita *et al.*, 1995), catalase A (de Hoop *et al.*, 1993) and superoxide dismutase (Rabizadeh *et al.*, 1995), have been shown to be functionally interchangeable with the highly homologous human proteins. Thus, much of what we learn using yeasts as model systems to study stress responses will help us understand the molecular mechanisms underlying stress resistance in mammals and other eukaryotes. A particular interest into the protective systems against reactive oxygen species (ROS) increased in recent years, since oxidative stress has been linked to various disease states (e.g. neurodegenerative diseases, cancer and atherosclerosis) and ageing.

Saccharomyces cerevisiae cells can be easily manipulated, either genetically, through construction of defined mutants and reporter genes, or

physiologically, through manipulation of growth and environmental conditions. These features make *S. cerevisiae* an ideal system to study oxidative stress responses. A number of primary antioxidant defences, systems of metal ion homeostasis or detoxification, and repair mechanisms, have now been demonstrated to be involved in oxidative stress protection in yeast. This chapter summarises current understanding of the effects of pro-oxidants, the oxidative stress response and the acquisition of increased resistance to reactive oxygen species (ROS) in the yeast *S. cerevisiae*.

2- *Generation of reactive oxygen species (ROS)*

Yeasts, as aerobic cells, have to face the toxic side-effects of molecular oxygen, namely the production of ROS, such as superoxide ($O_2^{\cdot-}$) and hydroxyl ($OH^{\cdot}$) radicals, hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), which are generated during normal cellular metabolism: e.g., superoxide radicals are produced via one electron reduction of molecular oxygen during mitochondrial respiration, and H_2O_2 in reactions catalysed by oxidases. In addition, the production of ROS is induced under different stress conditions: a) during exposure to pro-oxidants in the medium, such as menadione or paraquat, which easily cross membranes and undergo auto-oxidation in the presence of oxygen, generating superoxide radicals; b) when the oxygen pressure increases (hyperoxia or re-oxygenation of hypoxic cells); c) during exposure to ionising radiations, which causes homolytic fission of O-H bonds in water, generating hydroxyl radicals; and d) by the metal-dependent decomposition of hydrogen peroxide, via the Fenton reaction:

$$Metal^{n+} + H_2O_2 \rightarrow Metal^{(n+1)+} + HO^{\cdot} + OH^{-}$$

The oxidised form of the metal ion can be reduced by superoxide radicals, via the Haber-Weiss reaction:

$$Metal^{(n+1)+} + O_2^{\cdot-} \rightarrow Metal^{n+} + O_2$$

From these reactions, it is clear that any increase in the levels of redox active metal ions (e.g., copper and iron), superoxide radicals or hydrogen peroxide, are likely to promote the production of the highly reactive hydroxyl radicals. These ROS, in combination with other atoms or larger molecules, can generate other oxygen radicals, including alkoxyl ($RO^{\cdot}$) or peroxyl ($ROO^{\cdot}$) radicals, e.g., in lipids (see Fig. 1).

ROS have different half-lives (Table 1): hydroxyl radicals have the highest rate constant for the reaction with target molecules, and its reactions occur at the site of generation; in contrast, relatively stable ROS, such as superoxide radicals, hydrogen peroxide or peroxyl radicals, can diffuse from their site of generation, producing oxidative damages in other target sites.

Table 1- Half-life of reactive oxygen species.

Reactive oxygen species	$t_{1/2}$ (s)
Superoxide radical ($O_2^{\cdot-}$)	(enzymic)
Hydrogen peroxide (H_2O_2)	(enzymic)
Hydroxyl radical ($OH^{\cdot}$)	10^{-9}
Singlet oxygen ($^1O_2$)	10^{-5}
Alkoxyl radical ($RO^{\cdot}$)	10^{-6}
Peroxyl radical ($ROO^{\cdot}$)	7

Adapted from Sies (1993)

3- *Molecular damages induced by ROS*

ROS damage all cellular components by oxidising lipids, proteins and nucleic acids (Halliwell and Gutteridge, 1989). Under normal physiological conditions, antioxidant defence mechanisms are almost certainly adequate to maintain ROS at basal, unharmful levels and to repair cellular damages. These defences operate at different levels. Primary defences prevent the formation of ROS or neutralise them, while secondary defences repair or remove oxidatively damaged molecules. Cells face an oxidative stress when ROS levels exceed the antioxidant capacity of the cells.

Although other ROS can produce cellular damages, the hydroxyl radicals are the key causative agents in oxidative stress. The molecular mechanisms underlying oxidative modification of macromolecules are complex. The development of methods for identifying and measuring some products of macromolecule oxidation allowed the characterisation of the types of damages associated with ageing and numerous diseases. It is known that the oxidation of nucleic acids leads to base and sugar damages, single strand breaks, abasic sites and DNA-protein cross-links (Halliwell and Aruoma, 1993). In recent years, investigators started to analyse oxidative modification of DNA in *S. cerevisiae* cells. It has been shown that H_2O_2 and $O_2^{\cdot}$ generating compounds induce the production of 8-hydroxyguanine (8-OH-Gua), one of the major products of base damage (Woodford *et al.*, 1995; Lee and Park, 1988). $O_2^{\cdot}$ generating compounds also generate strand breaks (Lee and Park, 1988). In addition, DNA damage induced by hydroxyl radicals, generated by ionising radiation or by the Fenton reaction, increase the frequency of intrachromosomal recombination (Frankenberg *et al.*, 1993; Brennan *et al.*, 1994).

ROS-induced damages to lipids involves the oxidation of polyunsaturated fatty acids by an auto-catalytic process, leading to the production of fatty acids hydroperoxides and other secondary products, such as epoxides, aldehydes and alkanes (Fig. 1). Some of these products are highly reactive (able to subsequently damage DNA and proteins) and may be considered as second toxic messengers which disseminate and increase initial free radical events (Halliwell and Gutteridge, 1989). Lipid peroxidation impairs the structural integrity of membranes by generating shorter fatty acyl chains and increasing membrane fluidity. In mammalian cells, the formation of malondialdehyde is frequently used as a measure of ROS-mediated lipid peroxidation. Although malondialdehyde can also be generated by oxidative

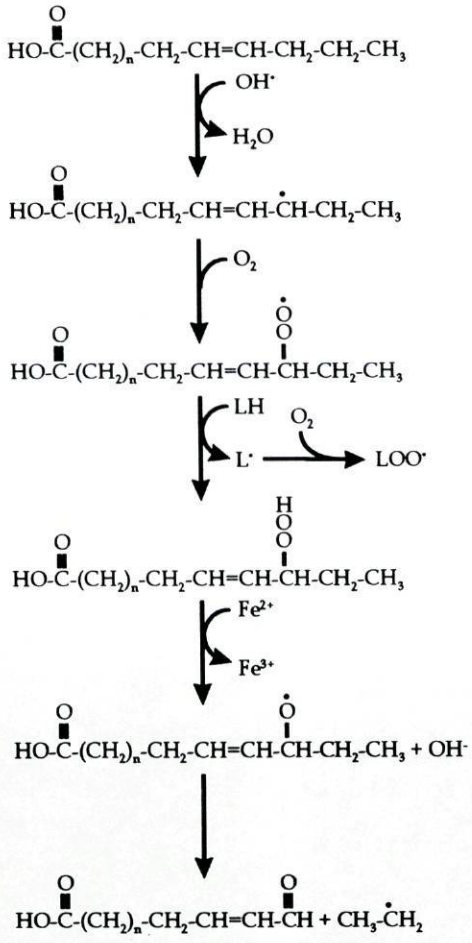

Fig. 1- Peroxidation of polyunsaturated fatty acids.

attacks on deoxyribose and amino acid residues in proteins (Esterbauer *et al.*, 1991), lipid peroxidation is the predominant source. Yeast cells are unable to synthesise polyunsaturated fatty acids; however, exogenous fatty acids are preferentially internalised and incorporated into membranes when present in the growth media (Bossie and Martin, 1989). The concentration of polyunsaturated fatty acids in the growth media is, therefore, a major determinant of the amount of malondialdehyde originated from lipid peroxidation. Studies on lipid peroxidation in *S. cerevisiae* showed that high concentrations of hydrogen peroxide increase the production of malondialdehyde (Steels *et al.*, 1994). It was recently shown that a sublethal heat shock also increases malondialdehyde levels in nonthermotolerant yeast

cells (Guerzoni *et al.*, 1997). These strains are unable to increase the unsaturation level of the cellular fatty acids at supraoptimal temperatures. Unsaturation is catalysed by oxygen consuming desaturases, which may contribute to the prevention of oxidative damages by counteracting the increased partitioning of oxygen into membranes as the temperature rises (Smotkin *et al.*, 1991).

ROS-induced damages to proteins involves oxidation of amino acid residues side chains to hydroxy or carbonyl derivatives, scission of the polypeptide chain (at proline, glutamate or aspartate residues), and protein cross-linking, leading to increased proteolytic susceptibility and decreased biological activity (Stadtman, 1993; Levine *et al.*, 1994). Most of the studies on protein oxidation have been performed using ionising radiation or metal-catalysed reaction to generate hydroxyl radicals. All amino acid residues are targets for reaction with $OH^{\cdot}$ produced by ionising radiation; however, sulphur containing and aromatic amino acid residues are particularly sensitive to ionising radiation (Table 2). The oxidation of amino acid residues by radiolysis involves α-hydrogen abstraction by hydroxyl radicals (Fig. 2A). The carbon-centered alkyl radical ($R^{\cdot}$) formed reacts with oxygen, generating a peroxyl intermediate ($ROO^{\cdot}$), which is converted to a peroxide (ROOH) and subsequently to an alkoxyl radical ($RO^{\cdot}$). The alkyl, alkylperoxyl, and alkoxyl radicals may react with other amino acid residues, generating new alkyl radicals and, therefore, amplifying the oxidation of the protein. Alkoxyl radicals undergo decomposition, leading to peptide bond cleavage by either the diamide or α-amidation mechanism (Fig. 2B). In contrast, the metal ion catalysed oxidation (MCO) of proteins (involving the formation of hydroxyl radicals by the Fenton reaction) is mainly a site-specific process in which only one or a few amino acids at metal-binding sites on the protein are preferentially oxidised. Histidine, proline, arginine, lysine, methionine and cysteine have been identified as major targets for oxidation by MCO systems (Table 2).

Table 2- Oxidation of amino acid residues in proteins.

Amino acid	Oxidation products
Arginine	glutamic semialdehyde
Cysteine	disulphide cross links, cysteic acid
Glutamic acid	oxalic acid, pyruvic acid
Histidine	aspartate, asparagine, 2-oxohistidine
Lysine	2-amino-adipic semialdehyde
Methionine	methionine sulfoxide, methionine sulfone
Proline	glutamate, pyroglutamate, cis/trans-4-hydroxyproline, 2-pyrrolidone, glutamic semialdehyde, γ-aminobutyric acid
Threonine	2-amino-3-ketobutyric acid
Tryptophan	2-, 4-, 5-, 6-, and 7-hydroxytryptophan, nitrotryptophan, kynurenine, 3-hydroxykynurinine, formylkynurinine
Tyrosine	Tyr-Tyr cross-links, 3,4-dihydroxyphenylalanine, Tyr-O-Tyr, cross-linked nitrotyrosine

Adapted from Berlett and Stadtman (1997)

Among the various oxidative modifications of amino acid residues in proteins, carbonyl formation may be an early marker for protein oxidation. The most likely amino acid residues to form carbonyl derivatives are arginine, proline, lysine and histidine. The oxidative cleavage of the peptide chain also leads to the generation of carbonyl derivatives. However, carbonyl groups may also be introduced into proteins by mechanisms that do not involve oxidation of amino acid residues. α,β-unsaturated alkenals, produced during lipid peroxidation, may react with sulphydryl groups of proteins to form stable covalent thioether adducts carrying carbonyl groups. Similarly, Schiff bases obtained by reaction of reducing sugars (e.g. glucose) with the ε-amino group of lysyl residues in proteins may, on Amadori rearrangements, yield ketoamine protein conjugates. At present, only one study on protein oxidation in *S. cerevisiae* was reported. Fortuniak *et al.* (1996) showed that the content of protein carbonyls and glutathione-protein mixed disulphides increase as yeast cells grow from the exponential to the stationary phase; however, no additional

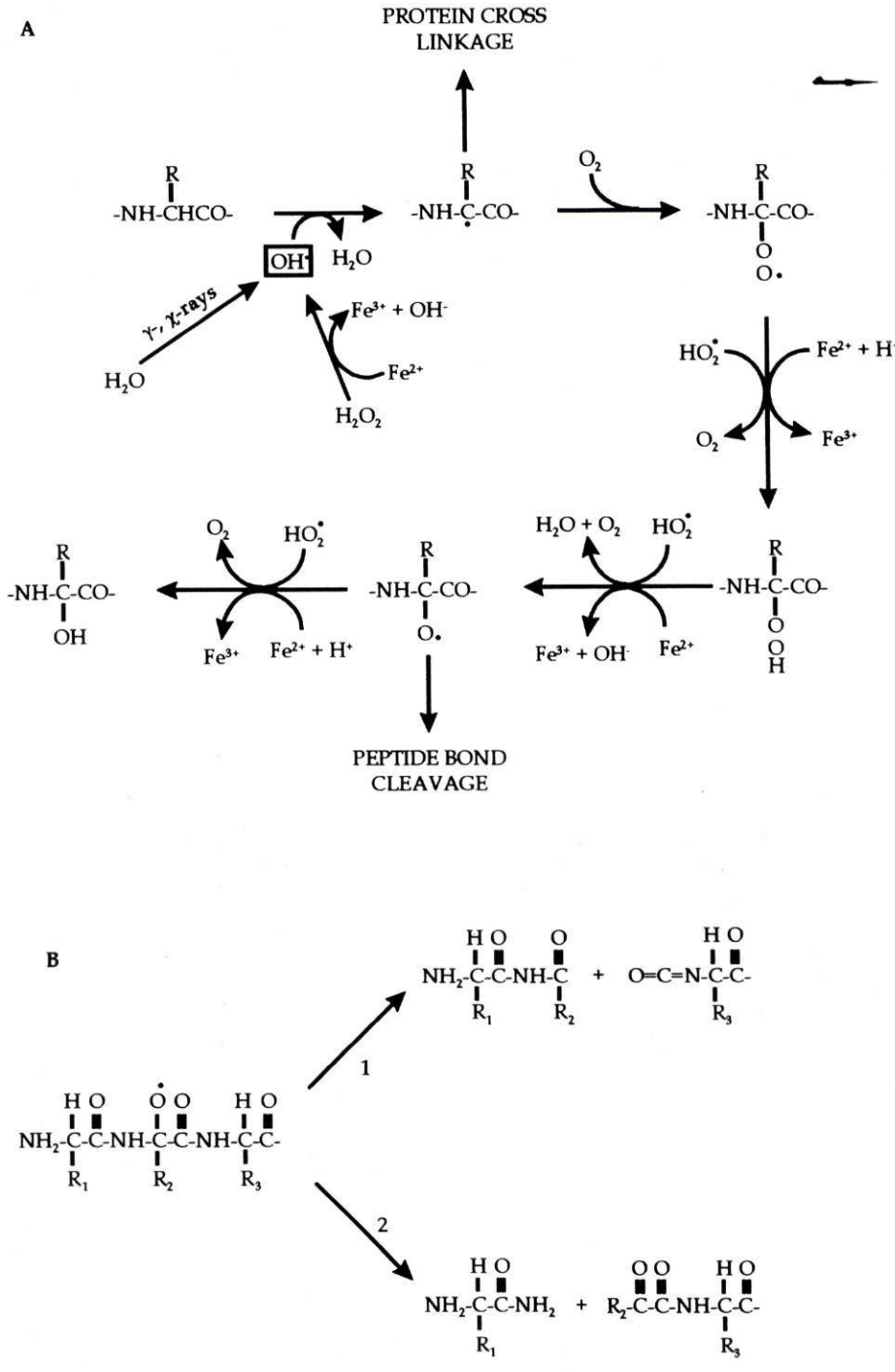

Fig. 2- A) Protein oxidation mediated by reactive oxygen species; B) Peptide bond cleavage by the (1) diamide and (2) α-amidation pathways. Adapted from Berlett and Stadtman (1997).

effect is observed in cells deficient in superoxide dismutase or catalase, or in cells with decreased glutathione levels, suggesting the existence of compensatory antioxidant mechanisms.

4- Antioxidant defence mechanisms in yeast

Halliwell and Gutteridge (1989) have defined an antioxidant as "any substance that, when present at low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate". All aerobic organisms contain a great diversity of antioxidant defences, which are organised to act at three levels: prevention, interception and repair.

4.1- Systems of metal ion homeostasis

As described above, the highly reactive hydroxyl radical is generated by the metal ion-catalysed univalent reduction of H_2O_2 by the Fenton reaction (Halliwell and Gutteridge, 1989). It is not surprising, therefore, that systems of metal ion detoxification or metal ion homeostasis constitute a first line of defence against ROS, by preventing their formation. Copper and iron are the two metal ions that can catalyse the Fenton reaction, and both increase the synthesis of antioxidant defences, namely superoxide dismutase (Sod1p) and metallothionein (Cup1p) by copper, and thioredoxin peroxidase (Tpx) by iron (see Table 7) (Lee and Hassan, 1985; Furst *et al.*, 1988; Kim *et al.*, 1989). Moreover, disruption of genes encoding antioxidant defences increase the sensitivity of yeast cells to metal ions (see Table 6B): *sod1*Δ cells are sensitive to copper and iron (Culotta *et al.*, 1995; Wisnicka *et al.*, 1998), and *cup1*Δ cells are sensitive to copper (Fogel and Welch, 1982). It is known that these metals participate in a variety of enzymatic reactions, being essential trace elements for normal physiological functions of the cells. This intrinsically cytotoxic yet essential nature of both copper and iron implies that cells must possess fine-

tuned mechanisms to regulate metal ion reduction, uptake, distribution, sequestration and removal for normal homeostatic control.

The first line of regulation in metal ion homeostasis occurs at the level of transport, and involves several proteins. Prior to transport, Cu(II) and Fe(III) have to be reduced by the plasma membrane Cu(II)/Fe(III) reductase, encoded by the *FRE1* and *FRE2* genes (Dancis *et al.*, 1990; Georgatsou and Alexandraki, 1994; Georgatsou *et al.*, 1997). High affinity transport of Fe(II) is mediated by Fet3p, a multicopper ferroxidase that oxidises Fe(II) to Fe(III), and Ftr1p (Askwith *et al.*, 1994; Dancis *et al.*, 1994; De Silva *et al.*, 1995; Stearman *et al.*, 1996). Besides this high affinity iron transporter system, yeast cells possess a low affinity transporter, Fet4p, which also mediates the uptake of cadmium and cobalt (Dix *et al.*, 1994). High affinity transport of Cu(I) into yeast cells involves two integral membrane proteins, Ctr1p and Ctr3p (Dancis *et al.*, 1994; Knight *et al.*, 1996). The levels of copper and iron reductases and transporters are inversely proportional to metal ion availability. Yeast cells maintain this ratio by regulating gene expression and protein turnover: a) (*FET3* and *FTR1*) / (*CTR1* and *CTR3*) expression is induced by iron/copper starvation and repressed by iron/copper repletion, respectively (Askwith *et al.*, 1996; Dancis *et al.*, 1994; Knight *et al.*, 1996); b) *FRE1* gene expression is induced by either iron or copper starvation and repressed by either iron or copper repletion (Hasset and Kosman, 1995), while *FRE2* gene expression is not upregulated by copper depletion (Georgatsou *et al.*, 1997); c) Ctr1p and Mac1p, the transcription factor that strongly activates *FRE1*, *CTR1* and *CTR3* gene expression under copper starvation, are both rapidly degraded under conditions of copper excess (Ooi *et al.*, 1996; Zhu *et al.*, 1998).

Once inside the cells, copper has to be transported from Ctr1p to specific proteins, such as cytochrome c oxidase (CCO) in the mitochondria, Sod1p in the cytosol, and Fet3p in the plasma membrane (Fig. 3). This is performed by several copper chaperones, including Cox17p, Atx1p, and lys7p, which contain a highly conserved metal-binding motif MTCXXC. Cox17p transports copper to

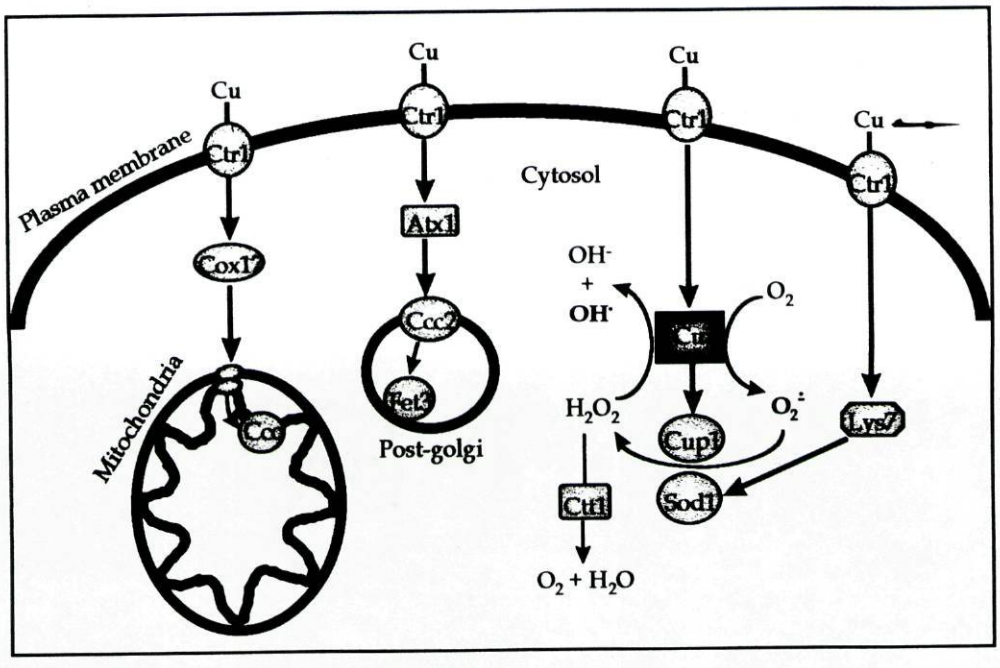

Fig. 3- Copper homeostasis *vs* toxicity in yeast.

the mitochondria for insertion into cytochrome c oxidase (Glerum *et al.*, 1996). Atx1p carries copper to Ccc2p, a copper translocating ATPase (Lin *et al.*, 1997). Atx1p directly interacts with the Atx1-like cytosolic domains of Ccc2p and this association is stabilised by copper (Pufahl *et al.*, 1997). Ccc2p then delivers copper to Fet3p in a post-Golgi compartment diverted from the secretory pathway, before Fet3p completes its transit to the plasma membrane (Yuan *et al.*, 1997). Lys7p transports copper specifically to Sod1p (Culotta *et al.*, 1997; Gamonet and Lauquin, 1998). Consistent with Lys7p function, *lys7Δ* mutants have no Sod1p activity, being lysine and methionine auxotrophs, and displaying an increased sensitivity to $O_2^{\cdot-}$, similarly to the observed with *sod1Δ* cells (Gamonet and Lauquin, 1998; see 4.2); nevertheless, they contain normal levels of the Sod1p apoprotein. The Sod1p activity is restored by addition of copper to the growth medium or to cellular extracts, suggesting that copper loading of Sod1p may represent a mechanism of controlling enzyme activity at the post-translational level (Gamonet and Lauquin, 1998). In fact, it is known that approximately one-third of the total cellular Sod1p exists as apoprotein, under normal physiological conditions (Petrovic *et al.*, 1996).

To prevent the formation of ROS under conditions of copper or iron excess, these metal ions are accumulated in the vacuoles (Eide *et al.*, 1993; Szczypka *et al.*, 1997) or sequestered in the cytosol. The major molecules involved in intracellular sequestration of copper in *S. cerevisiae* are metallothioneins, Cup1p and Crs5p, and the cytosolic superoxide dismutase, Sod1p (Tamai *et al.*, 1993; Culotta *et al.*, 1994; Culotta *et al.*, 1995). Metallothioneins are a ubiquitous family of low molecular weight proteins, which bind copper through thiolate bonds to cysteine residues (Kagi, 1991). The role of Sod1p in copper buffering seems to be functionally related with the role of metallothioneins. Indeed, the overexpression of *CUP1* suppresses copper sensitivity of *sod1*Δ cells (Tamai *et al.*, 1993) and *vice versa* (Culotta *et al.*, 1995). Copper binds the imidazolium group of four histidine residues of Sod1p, participating directly in the dismutation of superoxide radicals; however, the toxicity of copper can also be unrelated to the induction of oxidative damages. This hypothesis is supported by the following data: a) copper sensitivity of *sod1*Δ mutants is independent of oxygen; b) the deletion of *BSD1* or *BSD2* genes, or the overexpression of *ATX1*, which suppress the oxygen related defects associated with the loss of Sod1p (see 4.2; Liu and Culotta, 1994; Lapinskas *et al.*, 1995; Lin and Culotta, 1995), fail to suppress the copper toxicity of *sod1*Δ mutants (Culotta *et al.*, 1995); and c) Ace1p transcription factor, which activates *SOD1* and *CUP1* gene expression under copper excess, and the Ace1p-binding sites in *SOD1* gene promoter are essential for the protection against copper toxicity, but not aerobic defects, afforded by Sod1p (see 4.2; Culotta *et al.*, 1995). In fact, copper is known to bind to nitrogen, oxygen or sulphur ligands in biomolecules, thereby inactivating enzymes and disrupting cellular functions (Karlstrom and Levine, 1991).

Cadmium, in contrast with copper and iron, is a non essential transition metal and does not participate in the Fenton reaction; however, cadmium is detoxified conjugated to glutathione (Singhal *et al.*, 1987). Bis(glutathionato)-cadmium complexes are transported to the vacuole by Ycf1p (Li *et al.*, 1997), an

MgATP-energised glutathione S-conjugate transporter. It is not, therefore, surprising that *gsh1*Δ and *ycf1*Δ mutants are sensitive to cadmium, and Ycf1p synthesis is induced by this metal ion (see Tables 6B and 7B; Brennan and Schiestl, 1996; Wemmie *et al.*, 1994b). Ycf1p also confers resistance to 1-chloro-2,4-dinitrobenzene (Li *et al.*, 1996) and diamide (Wemmie and Moye-Rowley, 1997), indicating that Ycf1p can act as a multidrug resistance determinant in *S. cerevisiae*. In fact, the amino-terminal of Ycf1p is essential for its function and shares extensive homology to the human multidrug resistance-associated protein, Mrp1p (Wemmie and Moye-Rowley, 1997). The depletion of glutathione by cadmium increases ROS steady-state levels. The association of cadmium with oxidative stress is supported by several evidences, such as: a) cells grown in the absence of oxygen are more resistant to cadmium; b) intracellular free radical-sensitive reporter compounds are activated by cadmium; c) the recombinagenic activity of cadmium is suppressed by the free radical scavenger N-acetylcysteine; d) cadmium induces the expression of *UBI4* gene, which encodes polyubiquitin, known to target oxidised proteins for degradation by the proteasome; and e) yeast cells deficient in the proteasome or in the mitochondrial superoxide dismutase are sensitive to cadmium (see Table 6B; Jungmann *et al.*, 1993; Culotta *et al.*, 1995).

4.2- *Primary antioxidant defences*

In order to maintain ROS at physiological, unharmful levels, yeast cells possess both enzymic and non-enzymic primary antioxidant defences (Table 3). A number of enzymes function cooperatively to assure the complete reduction of superoxide radicals to water, thus preventing the production of hydroxyl radicals: superoxide dismutases catalyse the dismutation of superoxide radicals to hydrogen peroxide; catalases, cytochrome c peroxidase and thioredoxin peroxidase decompose hydrogen peroxide. Small molecules, such as glutathione and metallothioneins, act as free radical scavengers. Antioxidant

Table 3 - Yeast primary antioxidant defences.

Gene (Product)	Function
SOD1 (Sod1p; cytoplasmic) *SOD2* (Sod2p; mitochondrial)	Dismutation of superoxide radicals
CTA1 (Catalase A; peroxisomal) *CTT1* (Catalase T; cytoplasmic)	Decomposition of hydrogen peroxide
CCP1 (Cytochrome c peroxidase)	Reduction of hydrogen peroxide
GSH1[a] (Glutathione)	Scavenging of free radicals; Conjugation with electrophilic substrates; Binding of cadmium
YKL026c (putative glutathione peroxidase)	Reduction of hydrogen peroxide
CUP1 (Metallothionein)	Binding of copper, preventing the Fenton reaction; Scavenging of superoxide and hydroxyl radicals
TRX2 (Thioredoxin) *TPX=TSA=TPP* (Thioredoxin peroxidase)	Reduction of hydrogen peroxide and alkyl hydroperoxides; Scavenging of sulphur containing radicals
COQ3 (Ubiquinol)	Scavenging of perferryl, lipid and lipid peroxyl radicals; Reduction of α-tocopheroxyl radical
SPE2[b] (Polyamines)	Binding of copper, preventing the Fenton reaction; Protection of lipids from oxidation

[a] *GSH1* encodes γ-glutamyl-cysteine synthetase, which catalyses the first and rate-limiting step in glutathione biosynthesis; [b] *SPE2* encodes S-adenosylmethionine decarboxylase, which is involved in polyamine biosynthesis; SOD = superoxide dismutase.

defences, such as Sod2p and Ccp1p, are present in mitochondria, which is not surprising, attending to the fact that most ROS are generated from the incomplete reduction of molecular oxygen during oxidative phosphorylation. Mitochondria also possess ubiquinol (Coq3p), which is able to scavenge lipid and lipid peroxyl radicals, as well as regenerate the lipophilic antioxidant α-tocopherol. In agreement with Coq3p function, *coq3Δ* cells are sensitive to polyunsaturated fatty acids, which generate lipid peroxides and peroxyl radicals by autoxidation reactions (Do *et al.*, 1996). These defences immediately eliminate ROS, preventing oxidative modification of mitochondrial components and, therefore, the induction of respiratory incompetence. Indeed, the

protection of the mitochondrial function is important for oxidative stress resistance, as put in evidence by the high sensitivity of respiration deficient mutants (petites) to hydrogen peroxide and superoxide radicals (Collinson and Dawes, 1992; Flattery-O'Brien *et al.*, 1993).

A subset of the yeast antioxidant defences are constitutively expressed in unstressed cells, while others are induced by pro-oxidants. Moreover, the expression in unstressed cells is carbon source- and growth phase-dependent. A number of genes encoding antioxidant defences are repressed by glucose, being derepressed with the respiratory adaptation that follows glucose exhaustion during aerobic batch fermentation. It is presumably to tolerate the much higher levels of endogenous production of ROS during respiratory, as compared to fermentative, growth that yeast cells growing by respiration of non-fermentable substrates (e.g. ethanol or glycerol), or at the post-diauxic shift phase, express higher levels of these antioxidant activities (see Table 7A) and display higher oxidative stress tolerances (Jamieson, 1992; Jamieson *et al.*, 1994; Steels *et al.*, 1994). The key protective role of superoxide dismutases during respiratory growth conditions is illustrated by the high induction of both superoxide dismutases activities in cells grown on non-fermentable carbon sources or grown on glucose to the post-diauxic shift phase, (Westerbeek-Marres *et al.*, 1988; Lee *et al.*, 1996; Costa *et al.*, 1997). Yeast cells growing on fatty acids also express higher levels of the peroxisomal catalase A, which is required to decompose the high amounts of H_2O_2 produced by peroxisomal fatty acid oxidation (Ruis and Hamilton, 1992). This induction of antioxidant defences during respiratory adaptation of yeast can, therefore, be considered as a "stress" response, counteracting the increased production of ROS that respiratory maintenance will entail.

Consistent with an important role in oxidative stress protection, yeast cells deficient in antioxidant defences display oxygen-dependent growth defects: *sod1*Δ and *sod2*Δ cells grow slower on non-fermentable carbon sources; *sod1*Δ*sod2*Δ, *gsh1*Δ, *coq3*Δ and *spe2*Δ mutants are unable to grow under these

conditions (Table 4; van Loon *et al.*, 1986; Ohtake and Yabuuchi, 1991; Tzagoloff and Dieckmann, 1990; Balasundaram *et al.*, 1993; Tamai *et al.*, 1993; Longo *et al.*, 1996); *sod1Δsod2Δ* die within a few days at the stationary phase (Longo *et al.*, 1996). The cytosolic superoxide dismutase (Sod1p) seems to be one of the key primary antioxidant defences. Indeed, *sod1Δ* mutants are sensitive even to atmospheric levels of oxygen (cellular growth is slower due to an increase in the G1 phase), display lysine, cysteine and methionine auxotrophy, and undergo elevated spontaneous mutagenesis, (Bilinski *et al.*, 1985; Gralla and Valentine, 1991; Liu *et al.*, 1992; Tamai *et al.*, 1993; Lee *et al.*, 1996). *sod1Δ* mutants share with *zwf1Δ* cells the auxotrophic requirement for methionine and poor aerobic growth, and both deficiencies are due to reduced levels of

Table 4 - Oxygen-dependent growth defects of yeast cells deficient in antioxidant defences (-, unable to grow; <, slow growth; =, normal growth).

Mutation	Fermentative growth	Respiratory growth	References
sod1Δ[a, b]	<	<	1-4
sod2Δ	<	<	4-6
gsh1Δ	<	-	7-8
trx2Δ[c]	<		9
glr1Δ		=	10
tpxΔ	<		11
zwf1Δ[c]	<		12
spe2Δ		-	13
coq3Δ		-	14
apn1Δ[b]	<		15
ogg2Δ[b]	<		16

[a] Lys/Met/Cys auxotrophs; [b] undergo high spontaneous mutagenesis; [c] *trx1Δtrx2Δ* and *zwf1Δ* cells are Met/Cys auxotrophs

References: 1- Bilinski *et al.*, 1995; 2- Gralla and Valentine, 1991; 3- Tamai *et al.*, 1993; 4- Longo *et al.*, 1996; 5- van Loon *et al.*, 1986; 6- Westerbeek-Marres *et al.*, 1988; 7- Ohtake and Yabuuchi, 1991; 8- Stephen and Jamieson, 1997; 9- Muller, 1991; 10- Grant *et al.*, 1996b; 11- Chae *et al.*, 1993; 12- Slekar *et al.*, 1996; 13- Balasundaram *et al.*, 1993; 14- Tzagoloff and Dieckmann, 1990; 15- Ramotar *et al.*, 1991; 16- Brunner *et al.*, 1998

NADPH (59-67% of wild type levels), which is necessary for the thioredoxin-dependent PAPS reductase and sulfite reductase steps of methionine biosynthesis (Slekar *et al.*, 1996). In agreement with this hypothesis, both phenotypes are suppressed in *sod1*Δ cells by overexpression of *TKL1*, which increases the production of NADPH (Slekar *et al.*, 1996). *TKL1* encodes transketolase, the rate-limiting enzyme in the non-oxidative reactions of the pentose phosphate pathway.

The aerobic-dependent methionine and lysine auxotrophy of *sod1*Δ cells, as well as the higher sensitivity of these mutants to hyperoxia and superoxide radicals (see 5.2), is reversed when cells are treated with 2mM manganese (Chang and Kosman, 1989). This protective role of manganese seems to be due to its ROS-scavenging activity (Archibald and Fridovich, 1982). Consistent with these results, disruption of *BSD1* gene (Liu *et al.*, 1992; Lapinskas *et al.*, 1995), or overexpression of *ATX2* gene (Lin and Culotta, 1996), suppress the auxotrophic requirements and the superoxide radicals sensitivity of *sod1*Δ cells, by increasing the levels of manganese. Bsd1p (=Pmr1p) is a P-type ATPase localised in the Golgi, and seems to function in the transport of both calcium and manganese ions into the secretory pathway (Lapinskas *et al.*, 1995). Atx2p is also a membrane protein localised in the Golgi, and its function is mediated through the high affinity manganese transporter, Smf1p (Lin and Culotta, 1996).

Methionine, but not lysine, auxotrophy of *sod1*Δ mutants, as well as its respiratory growth defect, is reversed when cells are treated with 250μM copper (only in cells overexpressing *CUP1* gene; Tamai *et al.*, 1993). This seems to be in contradiction with the pro-oxidant effect of copper (discussed in 4.1). Nevertheless, other observations indicate that higher levels of copper can, under some circumstances, be beneficial to yeast cells. Indeed, disruption of *BSD2* gene (Liu *et al.*, 1992; Liu and Culotta, 1994), and overexpression of *ATX1* (Lin and Culotta, 1995), suppress methionine and lysine auxotrophy of *sod1*Δ cells, by increasing the levels of copper. Bsd2p is an endoplasmic reticulum

protein, and prevents copper (and cadmium) hyperaccumulation by exerting negative control over the Smf1p metal transporter (Liu *et al.*, 1997). The molecular mechanism by which copper reverses the oxidative damages in *sod1*Δ cells disrupted in *BSD2* or overexpressing *ATX1* is Cup1p-independent, and was attributed to the ROS-scavenging activity of copper (free copper ions or a redox-active copper complex other than Cup1p-metallothionein or SOD). The function of *BSD* and *ATX* gene products provides further evidence for an important role of metal ion homeostasis in oxygen toxicity.

4.3- Secondary antioxidant defences

Secondary antioxidant defences repair or remove the products of oxidation damage to DNA, proteins and lipids. Studies reported by several authors started to unravel yeasts antioxidant defences involved in repair of oxidised molecules (Table 5). Amongst oxidative damages, base modification is an important class of lesions due to its lethal or mutagenic effect. Indeed, failure to maintain the genomic integrity has been associated with ageing and degenerative diseases, including cancer (Halliwell and Aruoma, 1993). Oxidised bases have to be replaced by the following enzymatic system: DNA glycosylases remove the damaged bases, by cutting the glycosylic bond; AP endonucleases subsequently cut out the sugar-phosphate remnant, at the apurinic or apyrimidinic site; repair is completed by DNA polymerase and ligase. In *S. cerevisiae*, the excision repair of oxidised bases involves three DNA glycosylases, Ogg1p, Ogg2p (=Ntg1p) and Ntg2p, and a 3'-diesterase/AP nuclease, Apn1p (Ramotar *et al.*, 1991; Girard and Boiteux, 1997; Bruner *et al.*,1998). Ogg1p and Ogg2p catalyse the excision of 8-hydroxyguanine (8-OH-Gua) and 2,6-diamino-4-hydroxy-5-formamido pyrimidine (FapyGua) generated by oxidation of guanine nucleotides (Eide *et al.*, 1996; Karahalil *et al.*, 1998), while Apn1p acts on the abasic sites (Ramotar *et al.*, 1991). Consistent with their function in DNA repair, *ogg2*Δ and *apn1*Δ mutants undergo high spontaneous mutagenesis (see Table 4; Bruner *et al.*, 1998; Ramotar *et al.*, 1991).

Table 5- Yeast secondary antioxidant defences.

Gene (Product)	Function
OGG1 (8-oxoguanine glycosylase/lyase) *OGG2* (8-oxoguanine glycosylase/lyase) *NTG2* (endonuclease three-like glycosylase)	Excision of oxidised DNA bases
APN1 (AP endonuclease)	Cleavage of apurinic/apyrimidinic (AP) sites; Generation of 3'-hydroxyl groups at AP sites
PLA2 (phospholypase A2)	Hydrolysis of oxidised fatty acids esters
YKL026c (putative glutathione peroxidase)	Reduction of alkyl hydroperoxides
MSRA (methionine sulfoxide reductase)	Reduction of methionine sulfoxide
GSH1[a] (glutathione)	Reduction of protein disulphides
TRX2 (thioredoxin)	Reduction of protein disulphides; Reduction of oxidised glutathione
GLR1 (glutathione reductase)	Reduction of oxidised glutathione
TRR1 (thioredoxin reductase)	Reduction of oxidised thioredoxin
ZWF (glucose-6-phosphate dehydrogenase)	Reduction of $NADP^+$ to NADPH
GRX1 / *GRX2* (glutaredoxin)	Reduction of protein disulphides
UBI4 (polyubiquitin)	Marking oxidised proteins for degradation by the 26S proteasome

As discussed in 3), lipid peroxidation impairs membrane integrity. The repair of oxidised lipids is, therefore, critical for cell survival. Several enzymes are known to be involved in the excision repair of oxidised lipids in mammalian cells (Halliwell and Guteridge, 1989). A membrane-bound phospholipase A_2 is activated by oxidation and hydrolyses the oxidised fatty acids esters in phospholipid membranes. A new acyl-CoA is then reacylated by an acyltransferase. The lipid hydroperoxide (LOOH) released is reduced to the correspondent alcohol (LOH) by glutathione peroxidase, at the expense of glutathione. Yeast cells contain phospholypases A2, and the mitochondrial enzyme seems to be important for the adaptation and maintenance of membranes under respiratory growth conditions (Yost *et al.*, 1991). A putative glutathione peroxidase (*YKL026c*) was recently identified during genome sequencing (*Saccharomyces* Genome Database). Both phospholypases A2 and

glutathione peroxidase are likely to have an important function in oxidative stress protection, however, it remains to be demonstrated. It is predictable that these or other unidentified components involved in repair of oxidised lipids can be induced. Indeed, uncharacterised yeast genes, when overexpressed, confer resistance against lipid hydroperoxides (Inoue *et al.*, 1993). When lipid hydroperoxides are not reduced to alcohols, they undergo fragmentation, yielding highly toxic products that have to be eliminated. In this respect, glutathione seems to display an important role, as 4-hydroxy-2-nonenal (a major product of lipid peroxidation) depletes glutathione and cells respond by increasing glutathione synthesis (Wonisch *et al.*, 1997). In addition, glutathione and glutathione reductase are essential for resistance to malondialdehyde (Turton *et al.*, 1997).

Oxidised proteins can be reactivated by reduction of the oxidised amino acid residues. For example, methionine sulfoxide is reduced to methionine by methionine sulfoxide reductase, encoded by the *MSRA* gene (Gibson and Large, 1985). Disulphide bonds, generated by oxidation of cysteine residues, can be reduced by glutathione, glutaredoxins and thioredoxins (Holmgren, 1989; Meister, 1995), which have been shown to contribute to the protection of yeast cells against oxidative aggressions (Stephen and Jamieson, 1996; Luikenhuis *et al.*, 1998; Kuge and Jones, 1994; Chae *et al.*, 1994). The expression of both glutaredoxin genes (*GRX1* and *GRX2*) increases in cells grown on glucose to the stationary phase, probably in response to the respiratory metabolism of post-diauxic-shift phase (Luikenhuis *et al.*, 1998). A subset of enzymes function in backup by catalysing the reduction of oxidised glutathione or thioredoxin (glutathione reductase and thioredoxin reductase, respectively) formed during inactivation of ROS and reduction of protein disulphide bonds, or by providing NADPH for these reactions (glucose-6-phosphate dehydrogenase).

Oxidised proteins exhibit enhanced rates of proteolytic degradation, which may be related to an increased hydrophobicity and denaturation of the

proteins, as put in evidence in red blood cells (Pacifici and Davies, 1990). In *S. cerevisiae*, different observations suggest that oxidatively modified proteins are substrates for the ubiquitination system for intracellular protein turnover: lowered ubiquitin levels, caused by inactivation of the polyubiquitin gene (*UBI4*), can increase the sensitivity of yeast to H_2O_2 inactivation (Cheng *et al.*, 1994); *UBI4* expression is strongly induced in yeast cells adapting from fermentative to respiratory metabolism (Watt and Piper, 1997), and by exposure of *sod1*Δ cells to 100% oxygen (Lee *et al.*, 1996). The ubiquitination system functions to mark proteins for degradation by the multicatalytic protease, the 26S proteasome (Smith *et al.*, 1996); however, a direct involvement of the ubiquitin system / 26S proteasome on the turnover of oxidised proteins has not yet been reported.

5- *Responses to oxidative stress*

A number of antioxidant defences, including superoxide dismutases and glutathione, are already present in yeast cells under normal physiological conditions; however, they confer a limited capacity to resist a sudden oxidative aggression. The induction of antioxidant defences is, thus, essential for cells survival. This is clearly illustrated by the data summarised in tables 6 and 7. Yeast cells deficient in antioxidant defences display an increased stress sensitivity (Table 6). An oxidative stress response is triggered when cells are treated with low concentrations of either H_2O_2 or $O_2^{\cdot-}$ generating compounds (Table 7), leading to the acquisition of resistance to a subsequent challenge with lethal concentrations of these oxidants (Jamieson, 1992; Collinson and Dawes, 1992; Steels *et al.*, 1994). The adaptation to stress conditions involves early responses, which provide an almost immediate protection against sublethal stress conditions, and late responses, which provide an efficient protection against a severe stress, and allow cells to return to non stress conditions, in a rapid and ordered way. Early responses result in the post-transcriptional activation of pre-existing defences, as well as signal transduction pathways that initiate late

Table 6 - Increased stress sensitivity of yeast cells deficient in antioxidant defences.

A- Sensitivity to oxidants (+, sensitive; -, not sensitive).

Mutation	Hyperoxia	$O_2^{\cdot}$	H_2O_2	Diamide	References
sod1Δ	+	+	+		1, 2
sod2Δ	+[b]	+			3-5
ctt1Δ	-	-	+[c-e]		1, 5-7
cta1Δ	-	-	+[c-e]		1, 7
gsh1Δ		+[d, e]	+[d, e]		8-9
glr1Δ		+[f]	+[d, e]	+	10-14
ycf1Δ				+	15
trx2Δ			+		16
tpxΔ		+	+		17- 18
zwf1Δ		-	+[d, g]		13, 19-21
cup1Δ		+			22
spe2Δ	+				23
grx1Δ		+	-		24
grx2Δ		-	+		24
msraΔ			+		25
ubi4Δ			+[f]		26
coq3Δ	-	-	+		27-28
apn1Δ[a]			+		29
ogg2Δ[a]		+	+		30

B- Sensitivity to metal ions and other stress conditions (+, sensitive; -, not sensitive).

Mutation	Cu	Fe	Cd	Heat shock	Ethanol stress	Osmotic stress	References
*sod1*Δ	+[h]	+	-	+	-		31-35
*sod2*Δ	-		+	-	+[d, e]		31, 33-35
*ctt1*Δ		-		+	-[i]	+	6, 33, 36
*cta1*Δ				+	-[i]		33
*ccp1*Δ		-		+	-[i]		32-33
*gsh1*Δ			+				37
*ycf1*Δ			+				38
*cup1*Δ	+						39-40
*ubi4*Δ				+[b]			26

[a] high spontaneous mutagenesis; [b] enhanced under respiratory growth conditions; [c] *cta1*Δ*ctt1*Δ double mutants; [d] impaired acquisition of tolerance by a sublethal stress pre-treatment; [e] impaired acquisition of tolerance by growth to post-diauxic phase; [f] only under respiratory growth conditions; [g] sensitivity also observed in other mutants of the pentose phosphate pathway (*rpe1*Δ, *tkl1*Δ*tkl2*Δ, *tal1*Δ or *gnd1*Δ);[h] O_2-independent; [i] V. Costa and P. Moradas-Ferreira, unpublished

References: 1- Bilinski *et al.*, 1995; 2- Bermingham-McDonogh *et al.*, 1988; 3- van Loon *et al.*, 1986; 4- Westerbeek-Marres *et al.*, 1988; 5- Zhu and Scandalios, 1992; 6- Wieser *et al.*, 1991; 7- Izawa *et al.*, 1996; 8- Stephen and Jamieson, 1996; 9- Izawa *et al.*, 1995; 10- Grant *et al.*, 1996b; 11- Lee and Hassan, 1985; 12- Grant *et al.*, 1996a; 13- Izawa *et al.*, 1988; 14- Muller, 1996; 15- Wemmie and Moye-Rowley, 1997; 16- Kuge and Jones, 1994; 17- Chae *et al.*, 1993; 18- Lee and Park, 1998; 19- Slekar *et al.*, 1996; 20- Nogae and Johnston, 1990; 21- Juhnke *et al.*, 1996; 22- Tamai *et al.*, 1993; 23- Balasundaram *et al.*, 1993; 24- Luikenhuis *et al.*, 1998; 25- Moskovitz *et al.*, 1997; 26- Cheng *et al.*, 1994; 27- Do *et al.*, 1996; 28- Grant *et al.*, 1997; 29- Ramotar *et al.*, 1991; 30- Eide *et al.*, 1996; 31- Culotta *et al.*, 1995; 32- Wisnicka *et al.*, 1998; 33- Davidson *et al.*, 1996; 34- Costa *et al.*, 1993; 35- Costa *et al.*, 1997; 36- Schuller *et al.*, 1994; 37- Brennan and Schiestl, 1996; 38- Szczypka *et al.*, 1994; 39- Fogel and Welch, 1982; 40- Hamer *et al.*, 1985

Table 7- Expression of yeast antioxidant defences.

A- Effect of oxidants and respiratory growth conditions
(+, induction; =, no effect; -, repression)

Gene	O_2	Respiratory growth	Growth to PDS	Hyperoxia	$O_2^{\cdot-}$	H_2O_2	Diamide	References
SOD1	+	+	+	+	+	+		1-6
SOD2	+	+	+	+[a]	+	+		3, 4, 7-12
CTT1		+	+	+[a]	=	+		1, 11, 13-15
CTA1	+	+		+[a]	=	=		11-12, 16
CCP1	+[b]		+[b]			+[b]		17-18
GSH1			=[c]		+	+		19-22
GLR1		=	+[d]			+	+	19, 23-24
YKL026c[e]			+					25
TRX2					=	+	+	20, 26-27
TRR1						+	+	27
TPX				+				28
ZWF1			-			+[f]		18, 23
CUP1	-	+		+	+[a]	=		29-31
GRX1			+		+	+		32
GRX2			+		+	+		32
UBI4		+		+[a]	+	+		11, 33-34
OGG2					+			35

B- Effect of metal ions and other stress conditions (+, induction; =, no effect).

Gene	Fe	Cu	Cd	Heat shock	Ethanol stress	Osmotic stress	References
SOD1		+		+	=		2, 4-5
SOD2				+	+[g]		4, 36
CTT1		+		+	+	+	14, 37-39
CCP1					=		40
GSH1				=[c]	=[c]		36
GLR1				=	=		36
YCF1			+				41
TRX2					=		40
TPX	+			=			27
CUP1		+		+			30, 42-43
GRX1				+		+	32
GRX2				+		+	32
UBI4			+	+	+		44-46

[a] enhanced in *sod1*Δ cells; [b] Ccp1 activity; [c] assessed by glutathione content; [d] mitochondrial function-dependent; [e] putative glutathione peroxidase (*Saccharomyces* Genome Database: genome-www.stanford.edu); [f] only G6PDH activity (not mRNA-*ZWF1*); [g] only Sod2p activity (not mRNA-*SOD2* levels)

References: 1- Gregory *et al.*, 1974; 2- Galiazzo *et al.*, 1991; 3- Galiazzo and Labbe-Bois, 1993; 4- Costa *et al.*, 1997; 5- Lee and Hassan, 1985; 6- Schnell *et al.*, 1992; 7- Autor, 1982; 8- Lowry and Zitomer, 1984; 9- Westerbeek-Marres *et al.*, 1988; 10- Flattery-O'Brien *et al.*, 1997; 11- Lee *et al.*, 1996; 12- Jamieson *et al.*, 1994; 13- Ruis and Hamilton, 1992; 14- Belazzi *et al.*, 1991; 15- Marchler *et al.*, 1993; 16- Zimniak *et al.*, 1976; 17- Djavadi-Ohaniance *et al.*, 1978; 18- Izawa *et al.*, 1996; 19- Grant *et al.*, 1996a; 20- Stephen *et al.*, 1995; 21- Stephen and Jamieson, 1996; 22- Stephen and Jamieson, 1997; 23- Izawa *et al.*, 1995; 24- Grant *et al.*, 1996b; 25- DeRisi *et al.*, 1997; 26- Kuge and Jones, 1994; 27- Morgan *et al.*, 1997; 28- Kim *et al.*, 1989; 29- Strain and Culotta, 1996; 30- Tamai *et al.*, 1993; 31- Liu and Thiele, 1996; 32- Luikenhuis *et al.*, 1998; 33- Cheng *et al.*, 1994; 34- Watt and Piper, 1997; 35- Eide *et al.*, 1996; 36- Costa *et al.*, 1993; 37- Lapinskas *et al.*, 1993; 38- Wieser *et al.*, 1991; 39- Schuller *et al.*, 1994; 40- V. Costa and P. Moradas-Ferreira, unpublished; 41- Wemmie *et al.*, 1994b; 42- Furst *et al.*, 1988; 43- Thiele, 1988; 44- Jungmann *et al.*, 1993; 45- Finley *et al.*, 1987; 46- Piper *et al.*, 1994

responses, including the synthesis *de novo* of stress proteins and antioxidant defences. A number of studies has provided evidence that stress conditions trigger both specific and general responses. For ex., both $O_2^{\cdot}$ and H_2O_2 induce the synthesis of stress proteins, including some heat shock proteins, which can be divided into 3 groups: proteins induced by both H_2O_2 and $O_2^{\cdot}$, proteins induced by H_2O_2 alone and proteins induced by $O_2^{\cdot}$ alone (Flattery-O'Brien *et al.*, 1993; Jamieson *et al.*, 1994). Specific effects triggered by H_2O_2 and $O_2^{\cdot}$ are also observed at the level of cell cycle arrest: H_2O_2 causes *RAD9*-dependent arrest in G_2, while $O_2^{\cdot}$ causes *RAD9*-independent arrest in G_1 (Flattery-O'Brien and Dawes, 1998). The differences in the stress response induced by H_2O_2 and $O_2^{\cdot}$ may, therefore, be related to the specific phase in which cells are arrested. In fact, G_2 arrest caused by methyl-2-benzimidazil carbamate increase resistance to H_2O_2 but not to $O_2^{\cdot}$, whereas G_1 arrest mediated by α-factor enhance resistance to $O_2^{\cdot}$ but not to H_2O_2 (Flattery-O'Brien and Dawes, 1998).

5.1- Biochemical and metabolic alterations

The adaptive responses to oxidative stress have been studied in cells exposed to hyperoxia, superoxide generating compounds, hydrogen peroxide or lipid hydroperoxides, and thiol oxidants, such as diamide. In general, when an antioxidant defence is induced by an oxidant, it is essential at least for the acquisition of stress tolerance by a sublethal stress pretreatment.

5.1.1- Hyperoxia

Superoxide dismutases and thioredoxin peroxidase are the most important antioxidant defences conferring protection to hyperoxia, as indicated by the increased expression of *SOD1*, *SOD2*, and *TPX* genes (Westerbeek-Marres *et al.*, 1988; Kim *et al.*, 1989). The cooperative function of Sod1p, Sod2p, and Tpx assure the reduction of $O_2^{\cdot}$ and H_2O_2 to H_2O and O_2, decreasing the formation of $OH^{\cdot}$ by the Fenton reaction. In agreement with the role played by

superoxide dismutases, *sod1*Δ or *sod2*Δ mutant cells are highly sensitive to hyperoxia (Bilinski *et al.*, 1985; van Loon *et al.*, 1986; Bermingham-McDonogh *et al.*, 1988). The hypersensitivity of *sod2*Δ mutants to hyperoxia is further enhanced in cells growing by respiration and is reversed when the mitochondrial respiratory function is eliminated (the rho° state; Guidot *et al.*, 1993), supporting the key role of Sod2p in the detoxification of $O_2^{\cdot}$ generated by the respiratory chain. Consistent with this model, the induction of *SOD2* gene expression is enhanced in *sod1*Δ cells (Lee *et al.*, 1996), in order to increase the capacity of these mutants to eliminate $O_2^{\cdot}$ within the mitochondria. As a consequence, the flux of $O_2^{\cdot}$ to the cytosol decreases, which is particularly beneficial when cells are deficient in Sod1p. The enhanced stress response in *sod1*Δ cells is also observed at the level of *CTA1*, *CTT1*, and *UBI4* gene expression (Lee *et al.*, 1996). In wild type cells, transcriptional activation of *CTA1* and *CTT1* genes by hyperoxia is weak (Gregory *et al.*, 1974), and the resistance of *cta1*Δ*ctt1*Δ cells is similar to that of wild type cells (Bilinski *et al.*, 1985). The increased expression of *CTA1* and *CTT1* may help *sod1*Δ cells to decompose H_2O_2 originated from $O_2^{\cdot}$, and the induction of *UBI4* promotes the targeting of oxidised proteins for turnover. In spite of this compensatory induction, *sod1*Δ cells are permanently arrested at G1 phase, due to the inhibition of autoregulated G1 cyclins, Cln1p and Cln2p (Lee *et al.*, 1996). Hyperoxia resistance has also been linked to polyamine production, since *spe2*Δ mutants have a low resistance to 95% O_2, and this sensitivity can be reversed by *SOD1* overexpression (Balasundaram *et al.*, 1993). The importance of polyamines in the prevention of oxygen toxicity is also highlighted by the loss of respiratory competence when polyamine-depleted *spe2*Δ cells are grown in air (Balasundaram *et al.*, 1993). In both cases, the higher sensitivity of *spe2*Δ cells is probably due to the accumulation of oxidative damages caused by hydroxyl radicals, as polyamines are known to quelate iron metal ions, preventing the Fenton reaction, and decrease lipid peroxidation (Lovaas and Carlin, 1991).

5.1.2- *Superoxide radicals*

Data has been accumulated indicating that superoxide dismutases and glutathione have critical roles in the protection of yeast cells against $O_2^{\cdot-}$ generating compounds: *SOD1*, *SOD2* and *GSH1* gene expression is induced by $O_2^{\cdot-}$; *sod1*Δ and *sod2*Δ cells are hypersensitive to $O_2^{\cdot-}$; and the acquisition of $O_2^{\cdot-}$ resistance by a sublethal stress pretreatment or by growth to the post-diauxic phase is lower in *gsh1*Δ cells (Lee and Hassan, 1985; Bilinski *et al.*, 1985; Zhu and Scandalios, 1992; Galiazzo and Labbe-Bois, 1993; Stephen *et al.*, 1995; Stephen and Jamieson, 1996). The role of glutathione in the protection of yeast cells from the redox cycling compound menadione is not restricted to the scavenging of $O_2^{\cdot-}$; indeed, glutathione is involved in the elimination of menadione, which is exported from the cells as a glutathione S-conjugate. *GSH1* expression is, thus, induced both to increase $O_2^{\cdot-}$ scavenging and to counterbalance the depletion of glutathione during menadione detoxification (Zadzinski *et al.*, 1998). Disruption of *GLR1* gene also decreases $O_2^{\cdot-}$ resistance in cells growing by respiration, but not by fermentation, further supporting the key role of glutathione in $O_2^{\cdot-}$ scavenging (Grant *et al.*, 1996b). Cup1p metallothionein, in addition to the above mentioned role in metal ion homeostasis (see 4.1), plays an important role in $O_2^{\cdot-}$ detoxification. In fact, *CUP1* overexpression increases yeast resistance to $O_2^{\cdot-}$, and *CUP1* expression is enhanced in *sod1*Δ cells exposed to $O_2^{\cdot-}$ (Liu and Thiele, 1996). The protection afforded by Cup1p has been attributed to its capacity to scanvenge $O_2^{\cdot-}$ and $OH^{\cdot}$ (Felix *et al.*, 1993); however, Cup1p is not functionally equivalent to Sod1p, as *CUP1* overexpression does not completely reverse the sensitivity of *sod1*Δ cells to $O_2^{\cdot-}$, and does not restore the growth of *sod1*Δ cells under hyperoxia. Thioredoxin peroxidase (Tpx) is also important for $O_2^{\cdot-}$ resistance, since *tpx*Δ cells display an increased sensitivity of to $O_2^{\cdot-}$, and *TPX* gene expression is induced by $O_2^{\cdot-}$. Thioredoxin peroxidase removes thiyl radicals formed during the reaction of $O_2^{\cdot-}$ with thiol groups (e.g., in glutathione, thioredoxin, metallothioneins, or even proteins), and reduces H_2O_2 (produced by SODs) and

alkyl peroxides (generated by reaction of $O_2^{\cdot}$ with biomolecules, such as fatty acids) (Kim *et al.*, 1989; Chae *et al.*, 1993 and 1994; Lee and Park, 1998).

The induction of secondary antioxidant defences by $O_2^{\cdot}$ suggests that primary antioxidant defences are not sufficient to avoid oxidative modification of biomolecules. Indeed, the oxidation of guanine bases in DNA, as well as the formation of strands breaks, was detected in cell exposed to $O_2^{\cdot}$. At present, only the Ogg2p glycosylase was shown to be important for repair of $O_2^{\cdot}$-induced DNA damages: *OGG2* gene expression is induced by $O_2^{\cdot}$, and *ogg2*Δ mutants are sensitive to $O_2^{\cdot}$ (Eide *et al.*, 1996; You *et al.*, 1998). So far, there are no evidences demonstrating that $O_2^{\cdot}$ causes protein oxidation in yeasts; however, they are likely to occur, as $O_2^{\cdot}$ increase glutathione and glutaredoxin levels, and both are known to reduce disulphide bonds in proteins. The higher sensitivity of *gsh1*Δ and *grx1*Δ mutants to $O_2^{\cdot}$ suggests that glutathione and glutaredoxin might have an important role in repair of oxidised proteins (Stephen and Jamieson, 1996; Luikenhuis *et al.*, 1998).

5.1.3- *Hydrogen peroxide*

Enzymes able to decompose H_2O_2, such as catalases, cytochrome c peroxidase, and thioredoxin peroxidase, play important roles in H_2O_2 resistance: a) *CTT1* gene expression is induced by H_2O_2, and the acquisition of H_2O_2 tolerance, either by a mild stress pre-treatment or during growth to post-diauxic phase, is impaired in catalase (*cta1*Δ*ctt1*Δ) mutants (Bilinski *et al.*, 1985; Wieser *et al.*, 1991; Marchler *et al.*, 1993; Izawa *et al.*, 1996); b) Ccp1p activity increase in cells exposed to H_2O_2 (Izawa *et al.*, 1996); and c) *tpx*Δ mutants have an increased sensitivity to H_2O_2, and expression of yeast *TPX* enhances the resistance of *E. coli* to H_2O_2 (Chae *et al.*, 1993; Ahn *et al.*, 1996). Thioredoxin peroxidase reduces H_2O_2 at the expense of thioredoxin, which is subsequently reduced by thioredoxin reductase. Therefore, it is not surprising that *TRX2* and *TRR1* gene expression is also induced by H_2O_2, and *trx2*Δ mutants have an

increased sensitivity to H_2O_2 (Kuge and Jones, 1994; Morgan *et al.*, 1997). The putative glutathione peroxidase (*YKL026c*) may also have ~~an~~ important function in H_2O_2 detoxification. Indeed, glutathione peroxidases use glutathione to reduce H_2O_2, and the expression of *GSH1* and *GLR1* genes increases in cells exposed to H_2O_2 (Izawa *et al.*, 1995; Grant *et al.*, 1996b; Stephen and Jamieson, 1997). The reduction of glutathione and thioredoxin requires NADPH, which is produced in the pentose phosphate pathway. A role of NADPH synthesis in H_2O_2 detoxification is supported by different data: glucose-6-phosphate dehydrogenase activity is induced by H_2O_2, and mutants deficient in enzymes of the pentose phosphate pathway (*zwf1*Δ, *rpe1*Δ, *tkl1*Δ*tkl2*Δ, *tal1*Δ, and *gnd1*Δ) have a higher sensitivity to H_2O_2 (Nogae and Johnston, 1990; Juhnke *et al.*, 1996).

Yeast cells exposed to H_2O_2 increase the expression of genes encoding other antioxidant defences which are not involved in H_2O_2 decomposition, such as superoxide dismutases and secondary defences. The induction of SOD activities increase the capacity of the cells to eliminate $O_2^{\cdot}$ (Schnell *et al.*, 1992; Jamieson *et al.*, 1994), which can be produced due to the hyperoxia-like situation generated by H_2O_2 decomposition to H_2O and O_2. The induction of secondary antioxidant defences by H_2O_2, as well as the higher sensitivity of mutants deficient in these defences, indicate that the repair of molecules oxidatively modified by H_2O_2 is essential for H_2O_2 resistance. Data obtained with *grx2*Δ (glutaredoxin), *gsh1*Δ (glutathione synthesis), *trx2*Δ (thioredoxin), and *msra*Δ (methionine sulfoxide reductase) mutants show that the reduction of cysteine and methionine residues oxidised by H_2O_2 is important for cellular protection (Kuge and Jones, 1994; Izawa *et al.*, 1995; Stephen and Jamieson, 1996; Moskovitz *et al.*, 1997; Luikenhuis *et al.*, 1998). *msrA*Δ mutants exposed to H_2O_2 accumulate high amounts of both free and protein-bound methionine sulfoxide (Moskovitz *et al.*, 1997). The H_2O_2-induced increase in free methionine sulfoxide is likely due to the oxidation of free methionine and release of methionine sulfoxide from oxidised proteins as a consequence of proteolytic

degradation. The degradation of damaged proteins mediated by the ubiquitin / proteasome system also seems to be important for cellular protection, as *ubi4Δ* cells grown by respiration are sensitive to H_2O_2 (Cheng *et al.*, 1994). Regarding DNA damage, the Ogg2p glycosylase and the Apn1p AP endonuclease/3'-diesterase are essential for repair of oxidised bases (Ramotar *et al.*, 1991; Eide *et al.*, 1996). The observation that *S. cerevisiae APN1* overexpression renders Chinese hamster cells more resistant to H_2O_2 (Tomicic *et al.*, 1997), and suppresses the sensitivity to oxidants of *E. coli* mutants lacking exonuclease III and endonuclease IV (Ramotar *et al.*, 1991; Wilson *et al.*, 1995), further supports a role of Apn1p in the repair of oxidised bases. Regarding lipid peroxidation, it was recently shown that Coq3p (ubiquinol) has an important role in cellular protection (Grant *et al*, 1997), probably by scavenging perferryl, lipid and lipid peroxyl radicals.

5.1.4- Diamide

Diamide oxidises thiol groups, e.g., of glutathione. Oxidised glutathione can be reduced by glutathione reductase and by thioredoxin. Therefore, yeast cells adapt to diamide by increasing *GLR1*, *TRX2*, and *TRR1* gene expression (Grant *et al.*, 1996b; Morgan *et al.*, 1997). Consistent with glutathione reductase function, *glr1Δ* mutants are hypersensitive to diamide (Muller, 1996). *YCF1* disruption also increases diamide sensitivity, suggesting that vacuolar sequestration of diamide conjugated with glutathione may be involved in detoxification, as shown for cadmium and CDNB (Wemmie and Moye-Rowley, 1997).

5.1.5- Heat shock, ethanol, and osmostress

A number of unrelated stress conditions, including heat shock, ethanol and osmostress, seem to have pro-oxidant effects. Indeed, all these stresses induce antioxidant defences (Table 7B; Jamieson, 1992; Flattery-O'Brien *et al.*,

1993; Mager and Moradas-Ferreira, 1993; Costa *et al.*, 1993; Marchler *et al.*, 1993; Steels *et al.*, 1994). Ctt1p is an example of an antioxidant activity induced not only by sublethal concentrations of hydrogen peroxide, but also by a mild heat shock, ethanol stress and osmostress, and contributing to survival of cells exposed to all these stress conditions, except ethanol (Bilinski *et al.*, 1985; Wieser *et al.*, 1991; Marchler *et al.*, 1993; Schuller *et al.*, 1994). Heat shock also increases *SOD1*, *SOD2*, *GRX1*, *GRX2*, and *UBI4* gene expression, *sod1*Δ, *ccp1*Δ, and *ubi4*Δ cells bearing a lower thermotolerance (Finley *et al.*, 1987; Cheng *et al.*, 1994; Davidson *et al.*, 1996; Costa *et al.*, 1997; Luikenhuis *et al.*, 1998). Several evidences further support that heat shock induce an oxidative stress: heat shock impairs coupling of oxidative phosphorylation and causes a sustained stress response in cell growing by respiration (heat shock response in fermenting cells is transient); growth under anaerobic conditions and *CTT1* or *SOD1* overexpression increases thermotolerance; and *CTT1* overexpression quenches the activation of an intracellular free radical-sensitive molecular probe by heat shock (Lindquist, 1986; Patriarca and Maresca, 1990; Davidson *et al.*, 1996). Ethanol enhances *UBI4* expression and Sod2p activity, and *sod2*Δ mutants are very sensitive to ethanol, even when pre-treated by a mild heat or ethanol stress, or grown to the post-diauxic phase (Costa *et al.*, 1993 and 1997; Watt and Piper, 1997). Several oxidative stress-sensitive yeast mutants are also osmostress sensitive even under anaerobic conditions, and/or display an increased heat sensitivity (Krems *et al.*, 1995). A stress cross-tolerance analysis in fourteen yeast strains showed a correlation between H_2O_2 tolerance and resistance to heat shock and osmostress (Lewis *et al.*, 1997). It is, therefore, clear that heat, ethanol, osmotic and oxidative stresses induce responses in yeast that share certain identical molecular targets in the building of resistance.

5.2- *Cell signalling and control of gene transcription*

As stated above, the adaptive responses involve transcriptional activation of genes that participate in cellular defence against oxidative stress

conditions. Understanding how information is transmitted to targets in the nucleus has been driving research in recent years. In *S. cerevisiae*, a stress regulator, Skn7p (=Pos9p), harbours domains with high homology to response regulators present on prokaryotic two-component systems, as well as to the yeast Hsf1p DNA-binding domain (Krems *et al.*, 1995 and 1996). Skn7p regulates the induction of *TRX2* and *TRR1* by H_2O_2, t-butyl hydroperoxide and diamide (Table 8), *skn7*Δ strains being sensitive to peroxides, hyperoxia, menadione and cadmium (Krems *et al.*, 1996; Morgan *et al.*, 1997). Skn7p also regulates cell wall and G1 cyclin genes, and the transcriptional activation of these genes requires phosphorylation of the Skn7p Asp427 residue (Brown *et al.*, 1993 and 1994; Morgan *et al.*, 1995). Phosphorylation of response regulator proteins is mediated by membrane bound histidine kinases (Bourret *et al.*, 1991). Sln1p is the only known histidine kinase in *S. cerevisiae*, and, together with the response regulator protein Ssk1p, forms the two component system that controls the HOG MAP kinase pathway, which activates an osmotic stress response (Ota and Varshavsky, 1993; Maeda *et al.*, 1994). Whether Sln1p mediates Skn7p phosphorylation remains to be demonstrated. Gene regulation by Skn7p requires post-translational activation of the protein, as *SKN7* expression is not induced by H_2O_2 (Krems *et al.*, 1996); however, the transcriptional activation of *TRX2* and *TRR1*, as well as binding of Skn7p to the *TRX2* promoter, does not require phosphorylation of Asp427, in contrast to the transcriptional activation of cell wall and G1 cyclin genes (Morgan *et al.*, 1997). Nevertheless, the receiver domain is essential for H_2O_2 resistance, suggesting that it may play a structural function necessary for the oxidative stress response. These results suggest that Skn7p might function in a system altering gene expression in response to intracellular redox balance, similar to the redox-sensing control of mammalian AP1 and the redox-controlled response regulators of prokaryotic cells (Demple and Amabile-Cuevas, 1991).

Skn7p is epistatic to Yap1p, as *SKN7* disruption does not enhance the sensitivity of *yap1*Δ cells to oxidants (Krems *et al.*, 1996). Yap1p (=Pdr4p

Table 8 - Yeast genes regulated by oxidant-responsive transcription factors.

Gene	Principal inducer(s)	Transcription factor	References
SOD2	Post-diauxic phase growth	HAP2/3/4/5	1, 2
		Msn2p/Msn4p	
CTT1	H_2O_2	Msn2p/Msn4p ?	3
GSH1 [a]	H_2O_2	Yap1p	4
	$O_2^{\cdot-}$	Unknown $O_2^{\cdot-}$ activated factor	5
GLR1	Post-diauxic phase growth	Yap1p	6
		HAP2/3/4/5?	
YCF1	Cd	Yap1p	7
TRX2	H_2O_2 (not $O_2^{\cdot-}$); diamide	Yap1p	5, 8, 9
	H_2O_2; tBOOH; diamide	Skn7p	9, 10
TRR1	H_2O_2; tBOOH; diamide	Skn7p	9, 10
	H_2O_2; diamide	Yap1p	9
CUP1	$O_2^{\cdot-}$	Hsf1p	11
TPS1	?	Yap1p (probably through indirect action on STRE)	12
SSA1	H_2O_2	Yap1p	5

[a] *GSH1* encodes γ-glutamyl-cysteine synthetase, which catalyses the first and rate-limiting step in glutathione biosynthesis

References: 1- Flattery-O'Bryen *et al.*, 1997; 2- Pinkham *et al.*, 1997; 3- Marchler *et al.*, 1993; 4- Wu and Moye-Rowley, 1994; 5- Stephen *et al.*, 1995; 6- Grant *et al.*, 1996a; 7- Wemmie *et al.*, 1994b; 8- Kuge and Jones, 1994; 9- Morgan *et al.*, 1997; 10- Krems *et al.*, 1996; 11- Liu and Thiele, 1996; 12- Gounalaki and Thiereos, 1994

=Snq3p=Par1p) belongs to a family of eight bZIP proteins with overlapping but distinct biological functions (Fernandes *et al.*, 1997). Yap1p and Yap2p show strong homology with Gcn4p, the *S. cerevisiae* AP1 factor that activates the transcription of genes in response to amino acid starvation and other environmental stresses (Hinnebusch, 1988; Hope and Struhl, 1985; Engleberg *et al.*, 1994). Yap proteins have, thus, been referred to as homologues of the mammalian c-jun oncogene product, which, as homodimer or as heterodimer with c-fos, forms the AP1 transcription factor, known to be activated by H_2O_2 and UV light (Pahl and Baeuerle, 1994); however, it was recently shown that Yap proteins are clearly distinct from AP1 factors (Fernandes *et al.*, 1997). Yap

proteins bind most efficiently to TTACTAA, a sequence that differs at position ±2 from the optimal AP1 site (ARE; TGACTCA). Therefore, the recognition of Gcn4-binding site and Yap-binding site by Yap1p and Gcn4p, respectively, is inefficient (Bossier *et al.*, 1993; Wu *et al.*, 1993; Hirata *et al*, 1994; Fernandes *et al.*, 1997). Moreover, Yap-dependent transcription is inhibited by high levels of protein kinase A (PKA), unlike Gcn4p transcriptional activity, which is stimulated by protein kinase A (Fernandes *et al.*, 1997). PKA inhibits Yap-dependent transcriptional activity by decreasing Yap protein levels. Furthermore, PKA inhibits DNA-binding of at least Yap1p.

YAP1 and *YAP2* were isolated as genes whose overexpression overcomes growth arrest by diverse drugs (e.g., cycloheximide, 1,10-phenanthroline or 4-nitroquinoline-1-oxide) or which lead to the capacity to tolerate normally toxic levels of ion chelators and zinc (Schnell *et al.*, 1992; Bossier *et al.*, 1993; Wu *et al.*, 1993). It was recently shown that *YAP1* overexpression increase more than three-fold the mRNA-levels for seventeen genes (deRisi *et al.*, 1997). One of these genes, *ATR1*, encodes a protein that confers resistance to drugs, such as aminotriazole and 4-nitroquinoline-1-oxide (Kanazawa *et al.*, 1988; Gompel-Klein and Brendel, 1990). Interestingly, *ATR1* gene expression is regulated by both Yap1p and Gcn4p through a common ARE; however, Yap1p has a higher affinity to *ATR1* ARE and may have a more important function in the activation of *ATR1* gene expression (Coleman *et al.*, 1997). A putative glutathione transferase gene is induced more than 7-fold, and may be involved in drug detoxification, as shown in mammalian cells (Hayes and Strange, 1995).

The role of Yap1p in oxidative stress protection was first suggested when *yap1*Δ null mutants were found to display an increased sensitivity to H_2O_2, t-butyl hydroperoxide, menadione and elevated oxygen pressure (Schnell *et al.*, 1992; Kuge and Jones, 1994). Notably, these cells contain lower activities of enzymes involved in oxygen detoxification (superoxide dismutase and glucose-6-phosphate dehydrogenase) and maintenance of reduced

glutathione levels (glutathione reductase), although overexpression / disruption of *YAP1* only affects cellular glutathione +/- 30% of normal levels (Schnell *et al.*, 1992). The adaptive response to H_2O_2, but not to menadione, either by stress pretreatment or by growth to the stationary phase, is also highly affected in *yap1*Δ cells (Stephen *et al.*, 1995). In addition, loss of *YAP1* renders yeast cells hypersensitive to cadmium (shown above to act as pro-oxidant) and malondialdehyde (a product of molecular oxidations), and *YAP1* overexpression enhance cadmium resistance (Wu *et al.*, 1993; Hirata *et al.*, 1994; Wemmie *et al.*, 1994a; Turton *et al.*, 1997).

In agreement with its function in heavy metal ions and oxidative stress protection, Yap1p directly regulates a number of genes encoding antioxidant defences (Table 8). One of the Yap1p-regulated genes, *YCF1*, encodes a member of the ATP-binding cassette (ABC) family of membrane transporter proteins. *YCF1* overexpression results in cadmium tolerance, whereas *ycf1*Δ mutant strains are hypersensitive to this metal (Wemmie *et al.*, 1994b). The Yap1p binding sites in the *YCF1* promoter display only partial homology to that found in the *GSH1*, *TRX2* and *GLR1* promoters (Wemmie *et al.*, 1994b). *TRX2* overexpression confers *YAP1*-dependent resistance to H_2O_2 and tBOOH, and the Yap1p-binding sites in the *TRX2* promoter are essential for both basal and stress induced activity (Kuge and Jones, 1994). This H_2O_2 and tBOOH resistance mediated by *TRX2* shows the importance of thioredoxin for oxidative stress protection, through its actions in either reducing ROS or aiding protein repair by means of its protein oxidoreductase activity (Tables 3 and 5). The activation of *GLR1* expression in cells exposed to H_2O_2, and during growth to post-diauxic phase, is Yap1p-dependent and, in both cases, the increase of glutathione reductase activity is essential for the acquisition of H_2O_2 tolerance (Grant *et al.*, 1996a, and 1996b; Izawa *et al.*, 1998).

Of the seventeen genes identified in a DNA microarray screen as being induced by *YAP1* overexpression, five bear homology to aryl-alcohol oxidoreductases; however, their function in *S. cerevisiae* has not been

characterised (in ligninolytic fungi they participate in coupled redox reactions, oxidising aromatic and aliphatic unsaturated alcohols to aldehydes, with the production of H_2O_2). Other four genes belonging to the general class of dehydrogenases/oxidoreductases are induced, and might play important functions in oxidative stress protection (deRisi *et al.*, 1997). It has to be emphasised that only two thirds of these genes contain Yap1p binding sites (TTACTAA or TGACTAA) in their promoter region, suggesting that they are directly regulated by Yap1p. Moreover, some genes containing Yap1p binding sites in the promoter are not differentially regulated by *YAP1* overexpression. Transcriptional regulation of these genes may require a cooperation between Yap1p and another transcription factor, but this remains to be demonstrated.

Gene regulation by Yap1p requires post-translational activation of the protein, and the Yap1p-dependent transcription under stress conditions is activated at different levels, depending on the stress factor. Regulation in the presence of cadmium occurs at the level of the activation domain, while stimulation of Yap1p activity by H_2O_2 is regulated at the level of promoter occupancy (Fernandes *et al.*, 1997). Yap1p-mediated transcription induced by diamide and diethylmaleate was shown to be predominantly regulated at the level of nuclear import, as shown for other transcription factors (Vandromme *et al.*, 1996), although a small increase in the DNA-binding ability also contributes to the increased transcription (Kuge *et al.*, 1997). The regulated localisation of Yap1p is mediated by three conserved cysteine residues in the C-terminal cysteine-rich domain (Bossier *et al.*, 1993; Wu *et al.*, 1993; Hirata *et al.*, 1994; Kuge *et al.*, 1997). Mutant proteins lacking two of these cysteine residues are unable to complement the H_2O_2 hypersensitivity of *yap1*Δ cells (Wemmie *et al.*, 1997), suggesting that Yap1p-dependent transcription induced by H_2O_2 is also regulated at the level of its relocalisation from the cytoplasm to the nucleus. These results indicate that Yap1p activity is dependent on its redox status, as shown for the AP1 factor (Frame *et al.*, 1991), which is consistent with ARE-dependent transcriptional activation being inhibited by N-acetyl cysteine, a scavenger for reactive oxygen intermediates and precursor for glutathione

(Hirata *et al.*, 1994). Yeast cells expressing mutant Yap1 proteins that show constitutive nuclear localisation have slightly increased expression levels of *GSH1* and *GLR1*, but not *TRX2*. In addition, these cells show increased resistance to thiol oxidants but not to H_2O_2, suggesting that Yap1p is not the only factor important for *TRX2* regulation by H_2O_2 (Kuge *et al.*, 1997). Interestingly, although Yap1p transcriptional activation is enhanced by both H_2O_2 and diamide, the oxidative stress conditions elicited by these oxidants are differentially sensed by two domains of Yap1p (Wemmie *et al.*, 1997). These domains serve as a site for negative control of function during diamide-induced stress, but act as positive regulatory sites upon H_2O_2 exposure. The carboxyl terminus of Yap1p (containing the transcriptional activation and cysteine rich domains) is necessary and sufficient for response to diamide-induced oxidative stress, however it serves to inhibit the ability of the protein to transactivate in response to diamide. The carboxyl or amino terminus of the protein alone is not sufficient for response to H_2O_2, as the transcriptional activation domain is required for normal transactivation during H_2O_2-induced stress. These results are consistent with the observation that mutant proteins, lacking the transcriptional activation domain, are unable to normally complement the H_2O_2 hypersensitivity of *yap1*Δ cells, and increase diamide resistance (Wemmie *et al.*, 1997).

As shown for Yap1p, Yap2p plays a critical function in the regulation of the adaptive response to H_2O_2 (Stephen *et al.*, 1995) and mediates the response to cadmium (Wu *et al.*, 1993; Hirata *et al.*, 1994). Stimulation of Yap2p activity by cadmium is regulated, at least in part, at the level of the activation domain (Fernandes *et al.*, 1997). The use of ARE-containing reporter constructs in *yap1*Δ and *yap2*Δ disruption strains provided evidence that Yap1p is the major contributor to pro-oxidants induced transcription directed by this sequence, whereas Yap2p contributes to only a marginal extent (Hirata *et al.*, 1994). Nevertheless, overproduction of Yap2p in a *yap1*Δ background indicated that Yap2p may have some functional homology to Yap1p, and, therefore, may also

activate the transcription of genes encoding antioxidant defences, in response to oxidants. However, regarding Yap2p, no target gene has yet been identified.

Apart from being involved in direct transcriptional activation of the above mentioned genes, Yap1p has also been implicated in activation of genes controlled by the general stress response element (STRE; consensus CCCCT). Evidence was obtained that Yap1p regulates stress-induced and PKA-inhibited transcription of *TPS2* gene, encoding trehalose phosphate phosphatase, through the multiple STRE elements in the *TPS2* promoter (Gounalaki and Thireos, 1994). In addition, Yap1p is required for transcription of a STRE-controlled reporter gene and may act downstream of PKA, under stress conditions (Gounalaki and Thireos, 1994). This is in agreement with the evidence that STRE-regulated genes are also under negative regulation by cAMP (Marcher *et al.*, 1993). No indication exists that Yap1p binds directly to the STRE. This factor may, therefore, regulate transcription of some STRE-controlled genes in an indirect manner; however, Yap1p does not play a role in transcriptional activation of all STRE-regulated genes. In fact, the heat and osmostress-induced transcription of the *HSP12* gene was found to be largely unaffected in *yap1Δyap2Δ* cells, despite the presence of mutiple functional STREs in the *HSP12* promoter, and *HSP12* is only weakly induced by H_2O_2, as compared to its induction by other stress conditions (Varela *et al.*, 1995). Therefore, the molecular mechanism of the, apparently indirect, activation of the STRE by Yap1p remains to be characterised.

Two zinc finger proteins, encoded by the *MSN2* and *MSN4* genes, bind to STRE sequences and directly mediate STRE-dependent transcriptional induction (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996). These two genes were first isolated as multicopy supressors of the invertase defects of *snf1Δ* protein kinase mutants (Estruch and Carlson, 1993). Msn2p and Msn4p are rapidly and reversibly translocated from the cytoplasm to the nucleus under stress conditions (Gorner *et al.*, 1998), similarly to the observed with Yap1p in response to oxidative stress (Kuge *et al.*, 1997). Nuclear relocalisation

of Msn2p and Msn4p is inversely correlated to cAMP levels and PKA activity. STRE-mediated transcription is activated by pleiotropic stress conditions, including oxidative stress, heat shock, osmotic stress, carbon or nitrogen source starvation, low pH, weak lipophilic acids and ethanol (Schuller *et al.*, 1994; Martinez-Pastor *et al.*, 1996). Consistent with Msn2p and Msn4p function, the disruption of *MSN2* and *MSN4* genes severely reduce basal STRE-mediated transcription, as well as heat shock-, low pH-, sorbate-, and ethanol-induced transcription of a STRE-driven reporter gene (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996). The induction by oxidative stress and osmostress is not affected in this mutant, however the level of transcription of the reporter gene is very low, compared to the observed in wild type cells. In agreement with these observations, *msn2Δmsn4Δ* cells display a pleiotropic stress sensitivity. In addition, *MSN2* or *MSN4* overexpression, as well as constitutive translocation of Msn2p into the nucleus in cells with low PKA activity, increases general stress resistance and results in constitutive expression of STRE regulated genes (Martinez-Pastor *et al.*, 1996; Gorner *et al.*, 1998); however, heat shock- and osmostress-induced transcriptional activation still occurs in *msn2Δmsn4Δ* cells, suggesting that at least another transcription factor seems to be involved in STRE-mediated stress response (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996).

STREs are present in the promoter of *CTT1* and other genes encoding heat shock proteins or involved in trehalose biosynthesis (Marchler *et al.*, 1993; Schuller *et al.*, 1994; Kuge and Jones, 1994; Varela *et al.*, 1995; Mager and de Kruijff, 1995; Martinez-Pastor *et al.*, 1996; DeRisi *et al.*, 1997). *CTT1* gene expression is negatively regulated by cAMP/PKA and is induced by nutrient starvation, heat shock, ethanol, osmostress, and H_2O_2 through STREs (Marchler *et al.*, 1993; Schuller *et al.*, 1994; Table 8). *CTT1* expression is also enhanced by oxygen, via heme, through the HAP1p transcription factor. Besides, heat shock-induced *CTT1* expression requires a synergistic effect of two of the three

positive signals that regulate *CTT1* transcription: O_2 via heme, low cAMP, and heat shock (Ruis and Hamilton, 1992).

As discussed in 4.2, yeast cells growing by respiration on non-fermentable substrates, or at the post-diauxic shift phase, express higher levels of antioxidant activities (Table 7A), presumably to tolerate the much higher levels of endogenous production of ROS during respiratory growth (Jamieson, 1992; Jamieson *et al.*, 1994; Steels *et al.*, 1994). *CTT1* and *SOD2* gene expression is induced by growth on non-fermentable carbon source and at the diauxic-shift, partialy through STRE sequences (Belazzi *et al.*, 1991; Flattery-O'Brien *et al.*, 1997; Pinkham *et al.*, 1997), and the induction of *SOD2* expression at the diauxic-shift decreases in *msn2Δmsn4Δ* cells (Boy-Marcotte *et al.*, 1998); however, the induction of a STRE-driven reporter gene in cells grown by respiration (on ethanol) is not affected in *msn2Δmsn4Δ* cells (Martinez-Paster *et al.*, 1996), further supporting the above mentioned hypothesis of another transcription factor being involved in STRE-mediated stress response. It can also be speculated that this putative factor may have a key role in the activation of defence mechanisms that contribute to the high resistance of stationary phase *msn2Δmsn4Δ* cells (Martinez-Pastor *et al.*, 1996).

The induction of *SOD2* gene expression in cells grown to post-diauxic phase or adapting to respiratory growth conditions is also dependent on the heteromeric transcriptional activator complex HAP2/3/4/5 (Flattery-O'Brien *et al.*, 1997; Pinkham *et al.*, 1997). This complex binds to the consensus sequence CCAAT, and is responsible for induction of several genes important for respiration (Forsburg and Guarente, 1989; Olesen and Guarente, 1990; Rosenkrantz *et al.*, 1994; NcNabb *et al.*, 1995). *UBI4* activation in cells growing by respiration is also dependent on the HAP2/3/4/5-factor (Watt and Piper, 1997). Both *SOD2* and *UBI4* genes are also regulated by the oxygen/heme-responsive HAP1 factor, known to be involved in the regulation of genes encoding heme proteins such as *CTT1* and *CTA1* and components of the mitochondrial respiratory chain, through the CGGnnnTAnCGG consensus core

sequence (Zitomer and Lowry, 1992; Zhang and Guarente, 1994). It is known that HAP2/3/4/5-binding sites are present in the *GLR1* promoter (Collinson and Dawes, 1995), and the induction of *GLR1* gene expression at the post-diauxic phase is only partially Yap1p-dependent (Grant *et al.*, 1996a). It is, therefore, likely that the HAP2/3/4/5-complex plays a role in the regulation of *GLR1* expression at this growth phase.

It was recently shown that the heat shock factor (Hsf1p) also plays a critical role in oxidative stress protection. In fact, Hsf1p activates *CUP1* transcription in response to $O_2^{\cdot}$ (but not to H_2O_2), as well as to heat shock. Hsf1p contains two transcriptional activation domains: an amino terminal region, which is required for transient activation of genes containing heat shock elements (HSE, consensus GAAnnAAGnnnGAA), and a carboxyl terminal domain, which is required for a sustained activation in response to heat shock (Sorger, 1990; Nieto-Sotelo *et al.*, 1990). The activation of *CUP1* transcription in response to heat shock and $O_2^{\cdot}$ (and glucose starvation) requires a rapid phosphorylation of the carboxyl terminal domain; however, Hsf1p is differentially phosphorylated in response to heat shock and $O_2^{\cdot}$, which is consistent with the transient/sustained nature of both Hsf1p phosphorylation and *CUP1* activation in response to heat shock/$O_2^{\cdot}$ (Liu and Thiele, 1996).

As discussed in 4.1, oxidative stress and metal ion toxicity are intimately linked. Therefore, it is not surprising that genes of oxidative stress protection are regulated by metal ion-responsive transcription factors (Table 9). *SOD1* gene, as well as metallothioneins *CUP1* and *CRS5* genes, is transcriptionally induced by copper through the action of copper-sensing transcription factor, Ace1p (Thiele, 1988; Welch *et al.*, 1989; Culotta *et al.*, 1994; Gralla *et al.*, 1991). Ace1p has a highly basic amino terminal region containing 12 cysteine residues in five configurations of Cys-X-Cys or Cys-X-X-Cys, which can chelate copper or silver as can the metallothioneins (Dameron *et al.*, 1991; Casas-Finet *et al.*, 1992). Copper binding affects the conformation of the DNA-binding domain present in the amino terminal, allowing Ace1p to bind to the consensus

sequence 5'-TTTTGCTG-3' present in the promoter region (Furst *et al.*, 1988; Furst and Hamer, 1989). *CUP1* promoter contains four Ace1p binding sites and copper induces *CUP1* transcription 20- to 50-fold (Butt *et al.*, 1984; Thiele and Hamer, 1986). In comparation, *CRS5* promoter contains a single Ace1p binding site and the induction of *CRS5* transcription by copper is low (3- to 5-fold; Culotta *et al.*, 1994). In agreement with these observations, Cup1p plays a major role in cellular protection against copper toxicity, while the protection afforded by Crs5p is modest (Culotta *et al.*, 1995).

Table 9 - Metal ion-responsive transcription factors, whose action may influence resistance against oxidants.

Gene (Product)	Inducer	Transcription factor	Reference
SOD1 (Sod1p)	Cu	Ace1p (copper responsive)	Gralla *et al.*, 1991
CUP1 (Metallothionein)	Cu	Ace1p	Zhou *et al.*, 1992
CRS5 (Metallothionein)	Cu	Ace1p	Culotta *et al.*, 1994
CTT1 (Catalase T)	H_2O_2	Mac1p	Jungmann *et al.*, 1994

Messenger RNA-*CTT1* levels also increase in cells exposed to copper; however, the induction is not mediated by Ace1p (Lapinskas *et al.*, 1993). Interestingly, Mac1p, a transcription factor related to the copper-dependent Ace1p, is required but not sufficient for the H_2O_2-induced transcription of *CTT1* (Jungmann *et al.*, 1994). In addition, *mac1*Δ cells grow slowly, are sensitive to H_2O_2, heat shock, cadmium and zinc, and are respiration deficient (Jungmann *et al.*, 1994). These phenotypes are rescued by addition of copper, which is probably related to the role of Mac1p in copper transport mediated through *FRE1*, *CTR1* and *CTR5* gene expression (Ooi *et al.*, 1996; Zhu *et al.*, 1998).

The transcription factors discussed up to now are directly involved in the activation of genes encoding systems of metal ion homeostasis and primary antioxidant defences. Recently, Imp2p has been implicated in the regulation of genes required for repair of oxidised DNA (Masson and Ramotar, 1996). Imp2p

transcription factor lacks a DNA-binding motif; however it possesses a carboxyl terminal leucine-rich repeat and is able to activate a reporter gene via an acidic amino-terminal domain (Masson and Ramotar, 1996). Imp2p was previously shown to be required for expression of genes repressed by glucose (Lodi *et al.*, 1995). *imp2Δ* cells are hypersensitive to H_2O_2, bleomycin and and heat shock, are sporulation deficient, and show a leaky phenotype on non-fermentable carbon sources (Donnini *et al.*, 1992; Masson and Ramotar, 1996). Moreover, *imp2Δ* cells accumulate strand breaks when exposed to H_2O_2 and bleomycin; however, Imp2p-target genes, encoding antioxidant defences involved in DNA repair, remain to be identified.

6- Final remarks

The oxidative damages inherent of life in aerobic environments are counteracted by numerous antioxidant defences highly conserved from prokaryotic to eukaryotic organisms. Studies in yeasts have significantly contributed to unravelling the highly complex molecular mechanisms underlying oxidative modification of macromolecules and its reversal or prevention by antioxidants. It has been clearly shown that distinct systems for gene activation function in response to different oxidants: e.g., *GSH1* is more strongly induced by $O_2^{\cdot}$ than H_2O_2, *YAP1* deletion abolishing H_2O_2 induction but not $O_2^{\cdot}$ induction (Stephen *et al.*, 1995). This is analogous to the situation in *E. coli*, where H_2O_2 activates genes through the OxyR transcriptional activator while $O_2^{\cdot}$ controls a separate stress response regulated by the soxRS locus (Demple and Amabile-Cuevas, 1991). Under certain stress conditions, more than one system is induced: e.g., *CTT1* can be activated by H_2O_2, through the STRE (Marchler *et al.*, 1993), and also by the Mac1p transcription factor (Jungmann *et al.*, 1994). The complex nature of the regulatory mechanisms is further illustrated by the finding that the presence of a stress response element in the promoter of one gene is not sufficient for its coregulated transcriptional activation with other genes containing the same regulatory element: e.g.,

ethanol, which is known to induce the expression of *CTT1* through STREs, does not affect the transcription of the *TPS1*, in spite of the presence of multiple STREs in the promoter of this gene.

Studies in yeasts have also been revealing how systems of oxidative stress protection are induced by other types of stress. Both heat shock and ethanol lead to an increase in two major antioxidant enzymes, the cytoplasmic catalase T and the mitochondrial Sod2p (Ruis and Hamilton, 1992; Costa *et al.*, 1993), probably to allow a more efficient ROS trapping in respiratory cultures subject to these stresses. The damages due to heat and ethanol, as well as the molecular responses of yeast to these stresses, show remarkable similarity (Piper *et al.*, 1994). Both heat and ethanol stress enhance incomplete reduction of molecular oxygen by the mitochondrial respiratory chain, an important source of endogenous production of $O_2^{\cdot}$. They might also increase oxidative membrane damage by increasing partitioning of molecular oxygen from the aqueous to the lipid phases of cells (Steels *et al.*, 1994).

The challenge for the studies presented in this dissertation was to elucidate the molecular mechanisms underlying the regulation of antioxidant defences important for the acquisition of ethanol and hydrogen peroxide resistance in *Saccharomyces cerevisiae* cells. In addition, this study aimed at improving our understanding about the oxidative damages contributing to cell death induced by ethanol and hydrogen peroxide.

Experimental Research

Aims of the project

The molecular mechanisms involved in the acquisition of stress tolerance in *Saccharomyces cerevisiae* are not completely understood. The main objectives of this project were to investigate the role of superoxide dismutases and glutathione in ethanol and hydrogen peroxide resistance, and to identify oxidative damages induced by these stresses.

The work presented in chapter 1 aimed at elucidating the role of glutathione, glutathione reductase, and superoxide dismutases in the acquisition of ethanol tolerance. For this purpose, we analysed: a) glutathione levels and glutathione reductase activity during adaptation of yeast to cells to a sublethal heat shock or ethanol, and during growth to the post-diauxic phase; b) superoxide dismutase activity and mRNA-*SOD* levels during growth from the exponential to the post-diauxic phase; and c) ethanol tolerance of *gsh1*Δ, *sod1*Δ and *sod2*Δ mutants. The correlation between ethanol toxicity and the production of reactive oxygen species in the mitochondria was studied using respiration deficient mutants.

The studies described in chapter 2 aimed to investigate the role of Yap1p and Yap2p transcription factors in the regulation of *SOD* gene expression and superoxide dismutases activity during adaptation of yeast cells to hydrogen peroxide, and its correlation to hydrogen peroxide resistance.

In order to identify oxidative modifications in biomolecules induced by ethanol and hydrogen peroxide, we determined malondialdehyde concentration, as an indicator of lipid peroxidation, and analysed protein oxidation by immunodetection of carbonyls in proteins separated by two-dimensional gel electrophoresis (chapter 3).

The results obtained are described and discussed in detail in the next chapters.

Chapter 1

Mitochondrial superoxide dismutase is essential for ethanol tolerance of *Saccharomyces cerevisiae*

SUMMARY

This study reports the role of glutathione and both superoxide dismutases - CuZnSOD (encoded by *SOD1*) and MnSOD (encoded by *SOD2*) - in the build-up of ethanol tolerance of *Saccharomyces cerevisiae* cells. Three different pre-conditions were used to induce the acquisition of ethanol resistance: a sublethal heat shock or ethanol stress pretreatment, and growth from exponential to post-diauxic phase. Heat shock (37°C) and 8% ethanol stress have no effect on glutathione concentration and glutathione reductase activity, suggesting that the development of ethanol resistance is independent of these antioxidant defences. In agreement with these results, ethanol resistance of *gsh1*Δ cells is similar to wild type cells. It was previously shown that heat shock and 8% ethanol increase MnSOD activity (Costa *et al.*, 1993), which is consistent with data showing that the acquisition of ethanol tolerance is impaired in *sod2*Δ, but not *sod1*Δ, cells pre-exposed to a sublethal heat shock or ethanol stress. Strikingly, *sod1*Δ cells have an even higher ethanol tolerance, compared to the wild type strain, which was associated to a higher MnSOD activity. The activity of both CuZnSOD and MnSOD increase from the exponential to the diauxic shift and to post-diauxic phase. The levels of mRNA-*SOD1* and mRNA-*SOD2* increase from the exponential to the diauxic shift phase; however, during the post-diauxic phase mRNA-*SOD1* levels decrease while mRNA-*SOD2* levels remain identical. These data indicate the existence of two regulatory mechanisms involved in the induction of SOD activity during growth: synthesis *de novo* of the proteins (until the diauxic shift), and post-transcriptional or post-translational regulation (during the post-diauxic phase). Ethanol does not alter the activities of either enzyme in cells from the diauxic shift or post-diauxic phases. Results from experiments using *sod1*Δ and *sod2*Δ mutants show that MnSOD, but not CuZnSOD, is also essential for ethanol tolerance of diauxic shift and post-diauxic cells. Evidence that ethanol toxicity is correlated with the production of reactive oxygen species in the mitochondria is obtained from results with respiration deficient mutants. In these cells, the

induction of superoxide dismutase activity by ethanol is low; also, the respiratory deficiency restores the capacity of *sod2*Δ cells to acquire ethanol tolerance.

INTRODUCTION

The increasing ethanol concentration during batch fermentation affects growth, viability and fermentation rate of yeast cells (van Uden, 1984), and this toxicity has been associated with protein denaturation and membrane fluidity, leading to membrane leakage (Casey and Ingledew, 1986; Piper, 1995). Ethanol has also been considered to be responsible for promoting mitochondria DNA mutagenesis (Bandas and Zakharov, 1980) and mitochondria have been suggested as a target for ethanol damage (Aguilera and Benitez, 1985; Sá-Correia and van Uden, 1986). As for other stress conditions, *S. cerevisiae* cells seem to be more ethanol tolerant when they reach stationary phase (Werner-Washburne *et al.*, 1993; Piper, 1995). Exponential cells can also become tolerant to lethal ethanol concentrations if they undergo a previous sublethal heat or ethanol stress (Watson and Cavicchioli, 1983). During both "stress" adaptations, the expression of a subset of proteins is highly induced (Plesset *et al.*, 1982; Werner-Washburne *et al.*, 1993), including the stress proteins Hsp26, Hsp30 (Panaretou and Piper, 1992), Hsp70, Hsp104 (Sanchez *et al.*, 1992), and catalase T (Belazzi *et al.*, 1991; Wieser *et al.*, 1991); however, only Hsp104 was shown to be required for the acquisition of ethanol tolerance. Plasma membrane ATPase activity (Panaretou and Piper, 1990; Rosa and Sá-Correia, 1996), as well as the accumulation of trehalose (Odumeru *et al.*, 1993) and the increase in membrane concentrations of unsaturated fatty acids and ergosterol (Beaven *et al.*, 1982; Del Castillo Agudo, 1992), were also correlated with ethanol tolerance (Piper, 1995).

Heat shock and ethanol are two stress agents that have been associated with oxidative aggressions, by increasing the synthesis of oxygen free radicals (Cederbaum, 1989; Fridovich, 1981; Freeman *et al.*, 1990). The induction of antioxidant defences, such as catalase T and MnSOD, in *Saccharomyces cerevisiae*

cells exposed to ethanol (Wieser *et al.*, 1991; Costa *et al.*, 1993), also suggests a correlation between ethanol toxicity and the production of reactive oxygen species (ROS). In this study, we analysed the role of antioxidant defences, namely glutathione, glutathione reductase, and superoxide dismutases (both CuZnSOD, encoded by *SOD1*, and MnSOD, encoded by *SOD2*), in the adaptive response leading to ethanol resistance.

MATERIAL AND METHODS

Yeast strains and growth conditions

The strains of *Saccharomyces cerevisiae* used in this study are listed in Table 1. Respiration deficient mutants were prepared by prolonged exposure to ethidium bromide and selected as cells unable to form colonies on YPG plates (1% yeast extract, 2% bactopeptone, 3% glycerol). Cells were grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose) to early exponential phase (OD_{600}=0.6), diauxic shift phase (OD_{600}=3±0.1 for the aBR10 strain; OD_{600}=3.9± 0.1 for DL1, DL1*sod2*Δ and Dscd2-2C strains) or post-diauxic phase (OD_{600}=5.7± 0.1 for the aBR10 and DL1*sod2*Δ strains; OD_{600}=7 for the DL1 strain; OD_{600}=9 for the Dscd2-2C strain) (Fig. 1), in an orbital shaker, at 26°C, and 120 rpm, with a ratio of flask volume / medium volume of 5:1. Growth of aBR10p and DL1*sod2*Δp cells (respiration deficient mutants) in the exponential phase was similar to the observed in the aBR10 and DL1*sod2*Δ cells (data not shown).

Table 1. *Saccharomyces cerevisiae* **strains used in this study.**

Strain	Genotype	Reference/Source
aBR10	a *gal1 trp1 his4 ade cyc1*	Rymond *et al.* (1983)
aBR10p†	[aBR10] rho⁻	This work
Dscd2-2C	α *ura3 arg4 sod1*	Bilinski *et al.* (1985)
DL1	α *leu2,3, 2-112, his3-11,3-15 ura3-251, 3-372, 3-328*	van Loon *et al.* (1986)
DL1*sod2*	[DL1]*sod2*::*LEU2*	van Loon *et al.* (1986)
DL1*sod2*p†	[DL1] *sod2* rho⁻	This work

†respiration deficient mutants.

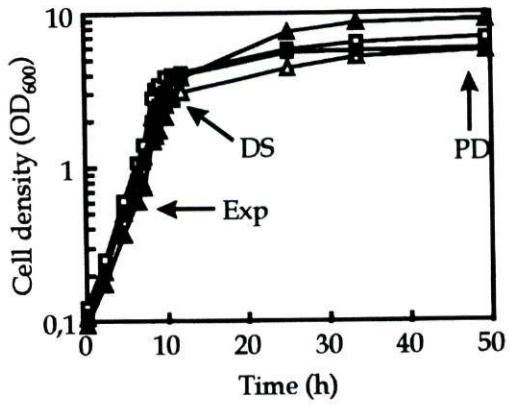

Fig. 1. Growth of *S. cerevisiae* aBR10 (Δ), DL1 (□), *sod1*Δ (▲) and *sod2*Δ (■) cells in YPD medium. Arrows indicate the growth phases at which the experiments were performed: the exponential phase (Exp), the diauxic shift phase (DS) and the post-diauxic phase (PD).

Ethanol tolerance

Yeast cells, growing in early exponential phase at 26°C, or pre-exposed to a sublethal heat shock (37°C for 30 min) or ethanol stress (8%, v/v, for 30 min), were subsequently treated with 14% (v/v) ethanol. Yeast cells growing at the diauxic shift or post-diauxic phase were treated with 14% (v/v) or 20% (v/v) ethanol during 30 min. Cell viability was determined by standard dilution plate counts on YPD medium containing 1.5% agar. Colonies were counted after growth at 26°C for 3 days. Viability was expressed as the percentage of the colony-forming units of non-stressed cells.

Glutathione determination

Sample processing for cellular glutathione assays was performed as described by Akerboom and Sies (1981). Yeast cells were lysed by combining equal volumes of 2M perchloric acid and a cell suspension in 0.1M potassium phosphate buffer (pH 7.0) containing 2mM EDTA. The mixture was vigorously shaken, in the presence of glass beads, for 3 min. Short pulses of 30 s were used, with 30 s intervals on ice. Proteins were removed by centrifugation. For glutathione disulphide determinations, acidified extracts were treated with

17mM NEM (final concentration) and neutralised to pH 7.0 with 2M KOH / 0.3M MOPS. The excess of NEM was removed from the supernatant using a Sep-Pack C18 cartridge (Water Associates, Milford, MA) (Adams *et al.*, 1983). Glutathione disulphide was eluted in 2.5 ml phosphate buffer. Reduced glutathione was assayed, following its conjugation with CDNB at 340-400 nm (ε=6.8x10^6 cm^2/mol), at room temperature (Brigelius *et al.*, 1983). Glutathione disulphide was assayed by the method of Tietze (1969). The rate of colour development was monitored at 412 nm. The concentration was determined by reference to a GSSG standard added to the assay cuvette (internal standard). Protein concentration was determined by the method of Bensadoun and Weinstein (1976), using bovine serum albumin as standard.

Enzymatic activity measurement

Yeast extracts were prepared in potassium phosphate buffer, by vigorous shaking of the cell suspension, in the presence of glass beads, for 3 min. Short pulses of 30 s were used, with 30 s intervals on ice. Proteins were assayed by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Samples for glutathione reductase (GLR; EC. 1.6.4.2.) determination were prepared in 0.1M potassium phosphate buffer (pH 7.0) containing 1mM EDTA. GLR activity was followed spectrophotometrically at 365 nm (Hg-line filter; ε=3.4x10^6 cm^2/mol), by monitoring the oxidation of NADPH, at room temperature (Goldberg and Spooner, 1983).

Samples for superoxide dismutase (SOD; EC. 1.15.1.1.) determination were prepared in 0.05M potassium phosphate buffer (pH 7.8) containing 0.1mM EDTA. One hundred µg of total proteins were used for the enzymatic assay. Total SOD activity was determined spectrophotometrically at 550 nm, in the presence of cytochrome c, using the xanthine - xanthine oxidase system (Flohé and Otting, 1984). MnSOD activity was assayed in the presence of 2mM KCN. SOD activity of extracts was determined by reference to a standard curve

prepared with known amounts of bovine SOD (Sigma), and expressed as U (mg protein)$^{-1}$.

Preparation and analysis of RNA

RNA was isolated as described by Brown (1994). Total RNA (30 μg) was denatured with glyoxal and dimethyl sulfoxide, blotted onto Hybond N membranes and probed as described by Sambrook *et al.* (1989). The following gene probes were used: a 0.5 kb *Hin*dIII fragment of *SOD1* gene (Bermingham-McDonogh *et al.*, 1988); a 2 kb *Bam*HI fragment of *SOD2* gene (Marres *et al.*, 1985); a 1.1 kb *Eco*RI fragment of *CTT1* gene (Spevak *et al.*, 1983); and a 1 kb *Hin*dIII - *Eco*RI fragment of *ACT1* gene (Gallwitz and Sures, 1980). Band intensities were evaluated using a Ultra Scan XL Enhancer Laser Densitometer.

Statistical analysis

Data are expressed as mean values ± SD of at least 3 independent experiments. Values were compared by computing standard Student's t-test. The 0.05 level was chosen as the point of statistical significance throughout.

RESULTS

Heat shock and ethanol does not affect glutathione concentration and glutathione reductase activity

The analysis of glutathione concentration of *S. cerevisiae* aBR10 cells exposed to heat shock or 8% ethanol, showed that GSH and GSSG levels did not significantly change after both stresses (Table 2). The activity of glutathione reductase also remained constant during these stress conditions (Table 2), which is consistent with the maintenance of glutathione levels. These results suggest that glutathione metabolism in *S. cerevisiae* is not affected by either heat or ethanol sublethal stress.

Table 2. Glutathione levels and glutathione reductase activity are not affected by heat shock and 8% ethanol.

Exponential phase cells of *S. cerevisiae* aBR10 growing in YPD were exposed to 8% ethanol (ES) or heat stressed at 37°C (HS). Glutathione levels (GSH, reduced form; GSSG, oxidised form) and glutathione reductase (GLR) activity were determined as described in Methods. Values are mean ± SD of five independent experiments.

Sample	nmol GSH (mg protein)$^{-1}$	pmol GSSG (mg protein)$^{-1}$	mU GLR (mg protein)$^{-1}$
Control	0.66 ± 0.08	18.9 ± 2.5	51.8 ± 2.5
HS-15 min	0.73 ± 0.06	20.7 ± 3.5	48.4 ± 1.7
HS-30 min	0.63 ± 0.08	22.1 ± 1.5	46.1 ± 2.1
HS-60 min	0.64 ± 0.02	21.8 ± 0.9	44.6 ± 2.1
ES-15 min	0.59 ± 0.04	15.9 ± 1.6	54.3 ± 4.6
ES-30 min	0.65 ± 0.06	18.6 ± 2.2	52.3 ± 3.9
ES-60 min	0.68 ± 0.06	19.5 ± 2.8	52.0 ± 2.6

MnSOD is essential for the acquisition of ethanol tolerance in cells pre-exposed to a sublethal heat shock or ethanol stress

The activity of the mitochondrial superoxide dismutase, MnSOD, is induced in exponentially growing yeast cells exposed to a sublethal heat shock and ethanol. In contrast, the activity of the cytosolic superoxide dismutase, CuZnSOD, is not affected under these stress conditions (Costa *et al.*, 1993). Both heat shock and 8% ethanol are known to increase ethanol resistance of yeast cells, ethanol pretreatment conferring a higher acquisition of ethanol tolerance (Costa *et al.*, 1993). The specific adaptive response of yeast superoxide dismutases led to the search for further evidence regarding the relative role of CuZnSOD and MnSOD in the acquisition of stress tolerance. Therefore, the effect of heat shock and 8% ethanol on ethanol tolerance and superoxide dismutase activity of *S. cerevisiae* cells, deficient in either CuZnSOD (*sod1*Δ) or MnSOD (*sod2*Δ), was assessed.

The analysis of ethanol tolerance in *sod1*Δ cells showed that this mutation did not impair the acquisition of ethanol tolerance (Fig. 2A). Cells pre-exposed to a

sublethal heat shock or ethanol stress became more tolerant to a subsequent lethal ethanol stress, as observed in wild type strains (Costa *et al.*, 1993). Strikingly, the viability of unstressed *sod1Δ* cells was higher than the observed in the *S. cerevisiae* aBR10 strain ($p<0.01$). In the *sod1Δ* mutant, 90% cell death occurred only after 3 h in the presence of 14% ethanol, whereas in the aBR10 cells the same mortality was observed after 1 h (Costa *et al.*, 1993).

In contrast, disruption of *SOD2* decreased the acquisition of ethanol tolerance (Fig. 2B). While the viability of *sod2Δ* cells, pre-exposed to a mild heat stress, seemed to increase slightly, cells pre-exposed to 8% ethanol did not develop ethanol tolerance. In both cases, the viability of these cells is much lower than that observed in pre-exposed aBR10 cells (Costa *et al.*, 1993), suggesting that MnSOD plays an important role in the acquisition of ethanol tolerance.

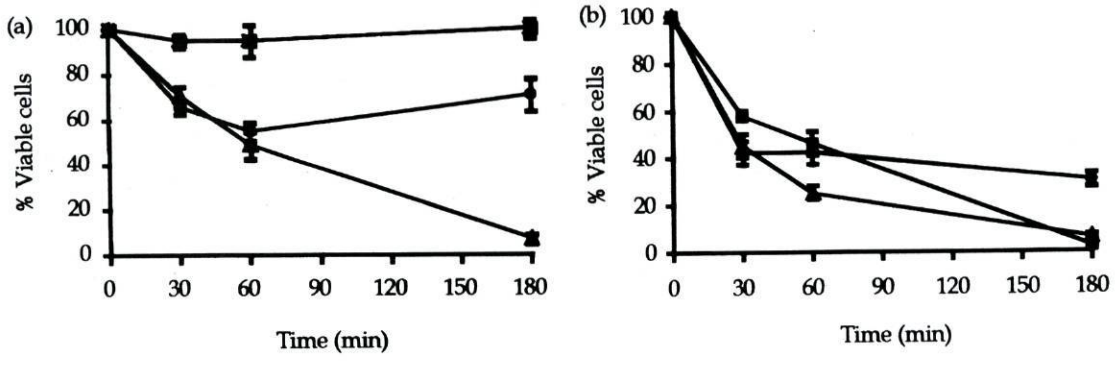

Fig. 2. Ethanol (14%, v/v) tolerance of *S. cerevisiae sod1Δ* (a), and *sod2Δ* (b) cells: unstressed cells (▲), and cells pre-treated by heat shock (37°C) for 30 min (●) or 8% (v/v) ethanol (26°C) for 30 min (■), were subsequently exposed to 14% ethanol. Data are expressed as mean values ± SD of at least five independent experiments.

CuZnSOD activity increases in sod2Δ cells exposed to ethanol

In the *sod1Δ* strain, the MnSOD activity [1.8 U (mg protein)$^{-1}$] is much higher ($p< 0.01$) than in the aBR10 strain (0.49 U (mg protein)$^{-1}$; Costa *et al.*, 1993). Interestingly enough, the MnSOD activity of *sod1Δ* cells (which was high to start with) decreased significantly after both sublethal stresses (Fig. 3a), in contrast to what was observed in the case of the *S. cerevisiae* aBR10 cells (Costa *et al.*, 1993), where the MnSOD activity increased from a low level after the

sublethal stresses. The MnSOD activity in the *sod1*Δ cells, however, was always higher than in both the unstressed and heat or ethanol stressed aBR10 cells.

In the *sod2*Δ strain, the activity of the CuZnSOD [3.9 U (mg protein)$^{-1}$] is also significantly higher ($p < 0.01$) than in the aBR10 strain (2.6 U (mg protein)$^{-1}$; Costa *et al.*, 1993). Moreover, the CuZnSOD activity of *sod2*Δ cells increased after both types of sublethal stresses (Fig. 3b) to values 2-fold higher than the observed in aBR10 cells induced under the same conditions (Costa *et al.*, 1993).

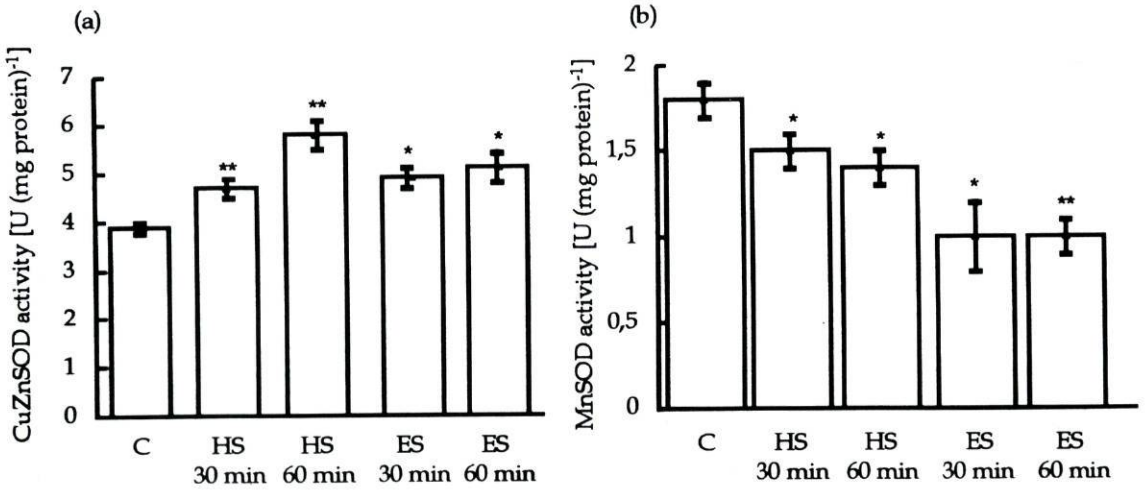

Fig. 3. (a) Analysis of CuZnSOD activity in *S. cerevisiae sod2*Δ cells. (b) Analysis of MnSOD activity in *S. cerevisiae sod1*Δ cells. Exponential phase cells growing in YPD were exposed to 8% (v/v) ethanol (ES) or heat stressed at 37°C (HS). C, constitutive levels. Values are mean values ± SD. Number of independent experiments >5. $^{*}p<0.05$; $^{**}p<0.01$.

Ethanol does not induce SODs activities in cells growing from diauxic shift to post-diauxic phase

As the post-diauxic phase yeast cells are resistant to high concentrations of ethanol (Piper, 1995), we analysed SOD activities and levels of mRNA-*SOD1* and mRNA-*SOD2* in *S. cerevisiae* aBR10 cells during the different growth phases. CuZnSOD and MnSOD activities increased 100% and 200%, respectively, from exponential to diauxic shift phase, and 35% and 170%, respectively, from diauxic shift to post-diauxic phase (Fig. 4a). Exposure of diauxic shift or post-diauxic phase cells to ethanol (14% or 20%, v/v) did not

affect either CuZnSOD or MnSOD activity (Fig. 4b). To assess if the increased superoxide dismutases activities were due to the synthesis *de novo* of the proteins, the respective mRNA levels were estimated. Our results confirmed the data previously obtained by Galiazzo and Labbe-Bois (1993): both mRNA-*SOD1* and mRNA-*SOD2* levels increased three fold during growth from the exponential to the diauxic shift phase; however, during growth to the post-diauxic phase, mRNA-*SOD1* levels decreased 40%, while mRNA-*SOD2* levels remained identical (Fig. 5). When post-diauxic cells were stressed with ethanol, both mRNA levels were further reduced 30-40%. A similar depletion of mRNA-*CTT1* and mRNA-*ACT1* was observed (Fig. 6). This effect on mRNA-*SOD* levels suggests that, besides a general decrease for mRNA levels as cells enter post-diauxic phase, ethanol stimulates mRNA degradation or inhibits transcription.

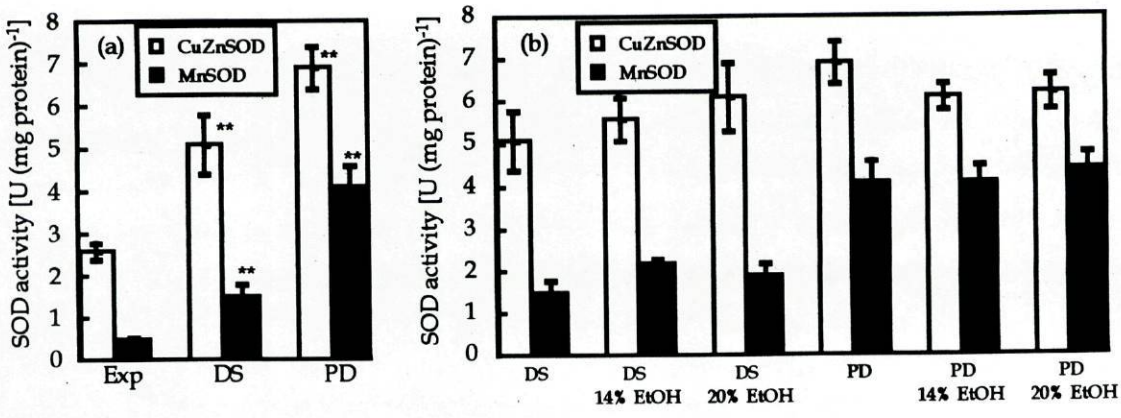

Fig. 4. Analysis of CuZnSOD and MnSOD activities in *S. cerevisiae* aBR10 cells. (a) Both CuZnSOD and MnSOD activities increase during growth from the exponential (Exp) to the diauxic shift (DS) and post-diauxic (PD) phases [results for exponential phase cells are from Costa *et al.* (1993)]. Values are mean ± SD of five independent experiments. **p<0.05 (DS compared to Exp, and PD to DS). (b) Exposure of diauxic shift (DS) and post-diauxic (PD) cells to 14% (v/v) or 20% (v/v) ethanol for 30 min does not affect CuZnSOD or MnSOD activity. Values are mean ± SD of five independent experiments.

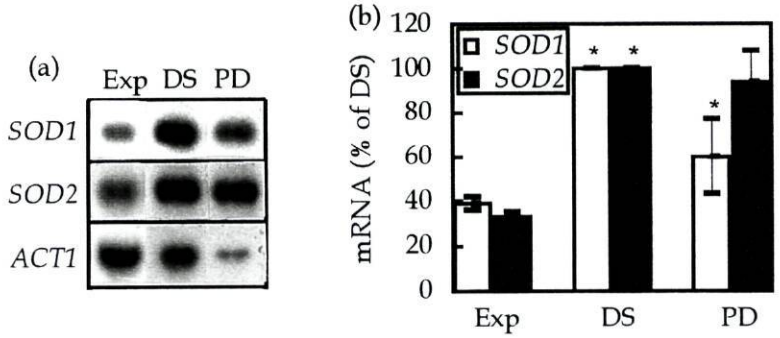

Fig. 5. (a) Northern blot analysis of mRNA-*SOD1* (CuZnSOD gene) and mRNA-*SOD2* (MnSOD gene) in *S. cerevisiae* aBR10 cells growing at the exponential (Exp), diauxic shift (DS) and post-diauxic (PD) phase. A representative experiment is shown. (b) mRNA-*SOD1* and mRNA-*SOD2* hybridization signals were quantified using a Ultra Scan XL Enhancer Laser Densitometer. As mRNA-*ACT1* levels decrease at PD phase, equal RNA loading was confirmed by ethidium bromide staining after electrophoresis. mRNA-*SOD1* and mRNA-*SOD2* levels increase three fold during growth from the exponential to the diauxic shift phase; however, mRNA-*SOD1* levels decrease in cells growing from the diauxic shift (DS) to the post-diauxic (PD) phase, while mRNA-*SOD2* levels remain constant. Values are mean ± SD of three independent experiments. *p<0.01 (DS compared to Exp cells, and PD to DS).

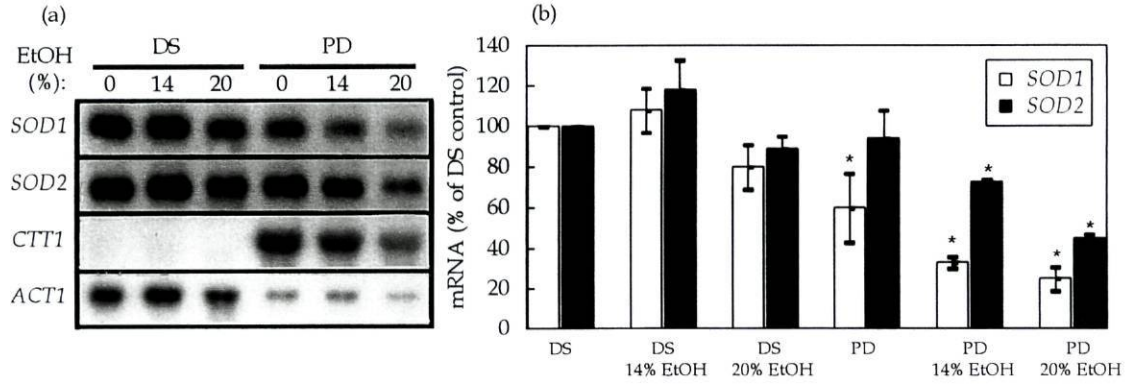

Fig. 6. (a) Northern blot analysis of mRNA-*SOD1* (CuZnSOD gene), mRNA-*SOD2* (MnSOD gene) and mRNA-*CTT1* (catalase T gene) in *S. cerevisiae* aBR10 cells. Diauxic shift (DS) and post-diauxic (PD) phase cells growing in YPD were exposed to 14% (v/v) or 20% (v/v) ethanol for 30 min. A representative experiment is shown. (b) mRNA-*SOD1* and mRNA-*SOD2* hybridization signals were quantified using a Ultra Scan XL Enhancer Laser Densitometer. As mRNA-*ACT1* levels decrease at PD phase, equal RNA loading was confirmed by ethidium bromide staining after electrophoresis. Both mRNA-*SOD* levels decrease in PD cells exposed to 14% (v/v) or 20% (v/v) ethanol for 30 min. Values are mean ± SD of three independent experiments. *p<0.01 (treated cells compared to control cells, and PD to DS).

MnSOD deficiency renders post-diauxic phase yeast cells hypersensitive to ethanol

The results in Fig. 4 suggested that ethanol tolerance at the post-diauxic phase is associated with a higher activity of CuZnSOD and MnSOD. Considering the role of superoxide dismutases in ethanol tolerance, it was decided to assess the relevance of each enzyme during growth, using *sod1*Δ and *sod2*Δ null mutants. In *S. cerevisiae* aBR10 cells, 80-90% of diauxic shift and post-diauxic phase cells exposed to 14% (v/v) ethanol stress remained viable (Table 3). During the transition from the diauxic shift to the post-diauxic phase, cells became tolerant to higher ethanol concentrations up to 20% (v/v): 55% of cells remained viable at diauxic shift phase, while more than 80% of post-diauxic phase cells survived. The analysis of ethanol tolerance in *sod1*Δ cells showed that this mutation did not impair the acquisition of ethanol tolerance during the transition to diauxic shift and post-diauxic phases (Table 3). In contrast, *sod2*Δ mutation rendered yeast cells very sensitive to ethanol (Table 3). In this mutant, 100 / 90% of diauxic shift / post-diauxic phase cells became unviable after 30 min in the presence of 20% ethanol, whereas in aBR10 cells, as well as in DL1 cells (the isogenic, wild type strain of the *sod2*Δ mutants; data not shown) only 45 / 20% of cell death occurred, respectively. Despite the high sensitivity of

Table 3. Cells deficient in MnSOD are sensitive to ethanol.

S. cerevisiae aBR10, *sod1*Δ and *sod2*Δ cells, growing in YPD at the diauxic shift or post-diauxic phase, were treated with 14% (v/v) or 20% (v/v) ethanol for 30 min. Appropriate dilutions were plated on YPD. Viable cells were assayed after growth at 26°C for three days and expressed relative to control cells (not treated with ethanol). Values are mean ± SD of five independent experiments.

S. cerevisiae strain	Viability (%)			
	Diauxic shift		Post-diauxic	
	14% EtOH	20% EtOH	14% EtOH	20% EtOH
aBR10	90 ± 3	56 ± 8	79 ± 7	82 ± 2
*sod1*Δ	83 ± 10	44 ± 15	87 ± 11	79 ± 8
*sod2*Δ	72 ± 3	0	76 ± 3	9 ± 3

sod2Δ mutants to ethanol, their tolerance slightly increased in the post-diauxic phase, compared to the diauxic shift phase.

Ethanol induces CuZnSOD activity of sod2Δ cells in the diauxic shift and post-diauxic phase

As *sod2Δ* cells are very sensitive to ethanol, but can still acquire a low degree of tolerance when reaching the post-diauxic phase, we analysed the contribution of CuZnSOD activity towards the observed tolerance. CuZnSOD activity of *sod2Δ* cells increased 40% during growth from log to diauxic shift phase (data not shown), and 55% from diauxic shift to post-diauxic phase (Table 4). Similar changes were observed in aBR10 cells (Fig. 4a). Interestingly, CuZnSOD activity in the post-diauxic phase was higher in *sod2Δ* cells [8.5 U (mg protein)$^{-1}$] than in the aBR10 strain [6.9 U (mg protein)$^{-1}$], and further increased 50-90% when *sod2Δ* cells where exposed to 14% or 20% ethanol (Table 4), in contrast to the lack of effect observed in aBR10 cells (Fig. 4b).

Table 4. CuZnSOD activity increases in post-diauxic *sod2Δ* cells exposed to ethanol.

S. cerevisiae sod2Δ cells growing in YPD at the diauxic shift or at the post-diauxic phase were treated with 14% (v/v) or 20% (v/v) ethanol for 30 min, and the activity of CuZnSOD was determined as described in Methods. Values are mean ± SD of five independent experiments. *p<0.01; **p<0.05 (treated cells compared to control cells, and PD to DS).

Ethanol (%)	CuZnSOD activity [U (mg protein)$^{-1}$]	
	Diauxic shift	Post-diauxic
0	5.5 ± 0.3	8.5 ± 0.7**
14	5.6 ± 0.3	12.9 ± 1.4*
20	6.5 ± 0.2*	16.1 ± 1.5**

Ethanol induction of MnSOD activity is low in respiration deficient mutants

The high sensitivity of *sod2*Δ cells to ethanol supports the correlation between ethanol toxicity and ROS production in the mitochondria. We addressed, therefore, the question of whether a respiration deficiency would increase ethanol tolerance of *sod2*Δ cells, since the generation of reactive species in the mitochondria is impaired. Indeed, the tolerance to 14% ethanol of respiration deficient *sod2*Δ cells (*S. cerevisiae sod2*Δp) growing exponentially was significantly enhanced when cells were pre-exposed to 8% ethanol for 30 min: 15% of exponential cells were able to form colonies after exposure to 14% ethanol for 60 min, while 80% of ethanol pre-treated cells remained viable. This acquisition of ethanol tolerance by *sod2*Δp cells was similar to that observed in the wild type DL1 strain.

To investigate if ROS produced during mitochondrial respiration are involved in the induction of MnSOD, the activity of this enzyme was analysed in respiration deficient mutants of aBR10 strain (*S. cerevisiae* aBR10p). The constitutive SOD activities of aBR10p mutants (Fig. 7a) were identical to that of wild type cells; however, when cells were exposed to 8% ethanol, only a small increase of MnSOD activity was observed (Fig. 7a). A comparative analysis with heat shock showed no effect on both enzyme activities (Fig. 7a). The analysis of mRNA levels showed that ethanol decreased mRNA-*SOD1* levels in the aBR10p strain, similar to the observed in wild type cells, but increased mRNA-*SOD2* levels (90%, after 60 min; Fig. 7b). Heat shock did not affect mRNA-*SOD1* levels and increased the levels of mRNA-*SOD2* (45% and 95% after 30 and 60 min, respectively), however the induction was lower than the determined in aBR10 cells (150 and 130%, respectively; Costa *et al.*, 1993).

DISCUSSION

When *S. cerevisiae* cells growing exponentially on glucose are exposed to a sublethal thermal or ethanol stress, antioxidant defences, such as MnSOD and catalase T, are induced and ethanol tolerance increases (Watson and

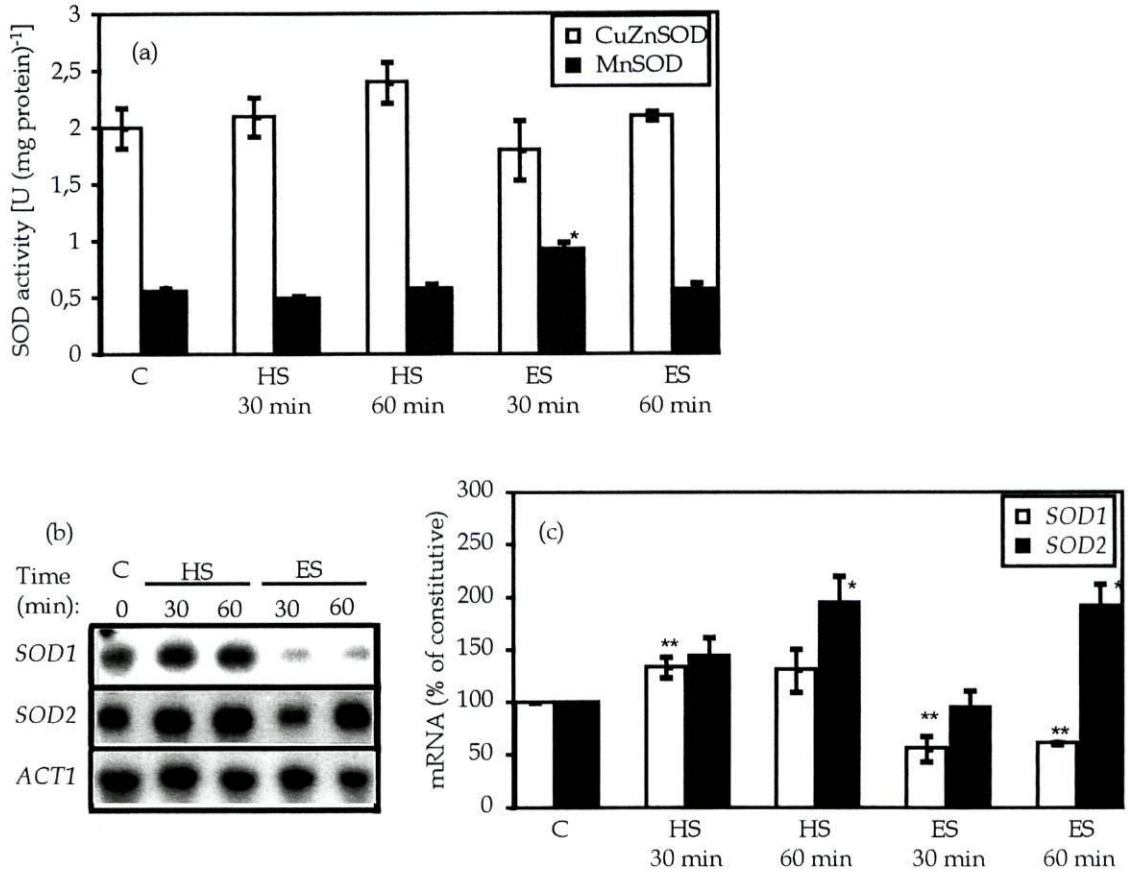

Fig. 7. Analysis of superoxide dismutases in exponential *S. cerevisiae* aBR10p cells exposed to a sub lethal heat shock (HS; 37°C) and 8% (v/v) ethanol stress (ES). C, constitutive levels. (a) CuZnSOD activity is not affected by either a heat shock or ethanol stress, while MnSOD activity is transiently induced by ethanol; Values are mean ± SD of five independent experiments. *p<0.01. (b) Northern blot analysis of mRNA-*SOD1* (CuZnSOD gene) and mRNA-*SOD2* (MnSOD gene) levels (a representative experiment is shown). (c) mRNA-*SOD1* and mRNA-*SOD2* band intensities were quantified and corrected for *ACT1* (RNA loading control). mRNA-*SOD1* levels are not induced by heat shock and even decrease during ethanol treatment. mRNA-*SOD2* levels increase upon heat shock and ethanol stress. Values are mean ± SD of three independent experiments. *p<0.01; **p<0.05.

Cavicchioli, 1983; Wieser *et al.*, 1991; Costa *et al.*, 1993; Schuller *et al.*, 1994). The yeast cells can also become more tolerant to ethanol and other stress agents when they shift from a fermentative to a respiratory growth (Piper, 1995). Indeed, when fermentation comes to an end, a number of genes down regulated by glucose are activated, including genes encoding for antioxidant defences (Werner-Washburne *et al.*, 1993; Moradas-Ferreira *et al.*, 1996). In the present work, we have analysed the correlation between glutathione levels or

superoxide dismutase activity and the acquisition of ethanol tolerance during different phases of growth.

Glutathione and ethanol tolerance

Glutathione plays an important role in the acquisition of oxidative stress resistance. Indeed, yeast cells enhance the biosynthesis of glutathione in response to oxidants (superoxide radicals and hydrogen peroxide), and *gsh1*Δ mutants are unable to increase resistance to these oxidants (Stephen *et al.*, 1995; Izawa *et al.*, 1995; Stephen and Jamieson, 1996, 1997). Our data indicate that glutathione levels (both reduced and oxidised forms) and glutathione reductase activity do not suffer significant changes during sublethal heat or ethanol stress (Table 2), suggesting that changes in the glutathione system are not necessary for the acquisition of ethanol tolerance in *S. cerevisiae*. In addition, glutathione levels do not increase when yeast cells grow to the post-diauxic phase, and ethanol resistance of post-diauxic phase *gsh1*Δ cells is high, similarly to the observed in wild type cells (data not shown), further supporting the lack of correlation between glutathione and ethanol tolerance.

Superoxide dismutases and ethanol tolerance in the exponential phase

Superoxide dismutases are essential for protection of yeast cells from a number of oxidative stress conditions, including exposure to hyperoxia, superoxide radicals, and hydrogen peroxide (Bilinski *et al.*, 1985; van Loon *et al.*, 1986; Zhu and Scandalios, 1992). The induction of *SOD1* and *SOD2* gene expression in response to these aggressions further supports a key protective function of these enzymes (Gregory *et al.*, 1974; Lee and Hassan, 1985; Westerbeek-Marres *et al.*, 1988; Schnell *et al.*, 1992; Galiazzo and Labbe-Bois, 1993; Jamieson *et al.*, 1994). Our results suggest that the cytosolic superoxide dismutase plays only a modest role in ethanol resistance of *S. cerevisiae* cells. Indeed, the deficiency in CuZnSOD did not impair the acquisition of ethanol tolerance by a sublethal heat shock of 8% ethanol pretreatment, and, despite the higher levels of CuZnSOD activity found in unstressed and pretreated *sod2*Δ mutants [3.9-4.9 U

(mg protein)$^{-1}$], compared to the aBR10 strain [2.1-2.6 U (mg protein)$^{-1}$], these cells were highly sensitive to ethanol (Fig. 8a).

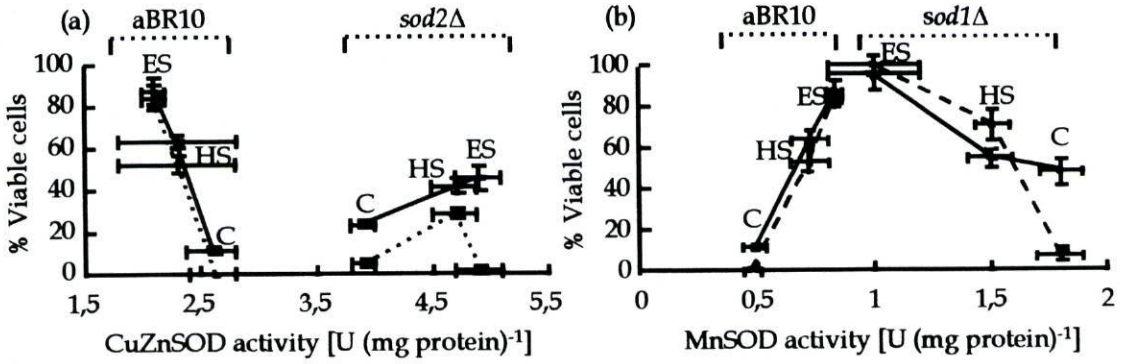

Fig. 8. Ethanol (14%, v/v) tolerance as a function of MnSOD (a) and CuZnSOD (b) activity. The viability values of aBR10 cells are from Costa *et al.*, 1993, and of *sod1*Δ and *sod2*Δ cells are from Fig. 2 (for 60 min (—) and 3 h (....) exposure to 14% ethanol). The superoxide dismutase activities of aBR10 cells are from Costa *et al.*, 1993, and of *sod1*Δ and *sod2*Δ cells are from Fig. 3: the MnSOD values were estimated in the *S. cerevisiae* aBR10 and *sod1*Δ strains; the CuZnSOD values were estimated in the *S. cerevisiae* aBR10 and *sod2*Δ strains. (C) control, (HS) heat shocked, and (ES) 8% ethanol stressed cells.

It was interesting to find that the deficiency in CuZnSOD in *S. cerevisiae* cells was overcome by an almost 4 fold increase in MnSOD activity (Fig. 3a), when compared to the aBR10 cells. However, the increase of MnSOD activity is not sufficient to compensate the activity lost by CuZnSOD deficiency. This observation suggests that compartmentalisation of MnSOD in mitochondria, where most of the superoxide radicals are produced, increases the efficiency of superoxide radicals trapping, despite total SOD activity being lower.

In contrast, the acquisition of ethanol resistance is very dependent on MnSOD, as indicated by the highest ethanol sensitivity found in *sod2*Δ cells (Fig. 2b). The correlation between MnSOD and ethanol resistance supports previous results showing that ethanol pre-exposure leads to a higher ethanol resistance and MnSOD activity, compared to the heat stress pretreatment (Costa *et al.*, 1993). In unstressed wild type cells, MnSOD activity is not sufficient to prevent the lethal effects of superoxide radicals induced by high ethanol concentrations. The

importance of mitochondrial dismutase activity is further evidenced by the results obtained with *sod1*Δ cells. These cells have a very high constitutive MnSOD activity (Fig. 3a) and a higher ethanol tolerance; however, while the aBR10 cells are able to acquire resistance to 14% ethanol by increasing the MnSOD activity from 0.49 to 0.84 U (mg protein)$^{-1}$ (Costa *et al.*, 1993), *sod1*Δ cells become more ethanol tolerant by decreasing the MnSOD activity from 1.8 to 1.0 U (mg protein)$^{-1}$. More than 90% of the *sod1*Δ yeast cells survive, even after 3 h in 14% ethanol, when the MnSOD activity is 1.0 U (mg protein)$^{-1}$.

The decrease of MnSOD activity associated with the acquisition of ethanol tolerance (Fig. 3a) might be explained as follows: a) the high MnSOD activity observed in unstressed *sod1*Δ cells might lead to an overproduction of H_2O_2 during the exposure to lethal ethanol concentrations. This increase of the ratio H_2O_2 production (by SOD) / H_2O_2 degradation (by catalase or peroxidases) might lead to H_2O_2 induced oxidative damages and, thus, to the high sensitivity of these cells to 14% ethanol; b) the decrease of MnSOD activity (to values which remain nevertheless higher than those observed in induced aBR10 cells), during pre-exposure to 8% ethanol or heat shock, prevents H_2O_2 induced damages. The overall results show that a higher ethanol tolerance is achieved when MnSOD activity is close to 1.0 U (mg protein)$^{-1}$ (Fig. 8b). For both higher or lower levels of MnSOD activity, the cells have a higher ethanol sensitivity. It is noteworthy that the ratio between superoxide and hydrogen peroxide within the cell seems to be critical for cell viability, as suggested before by Bowler *et al.* (1991).

In *E. coli* , it was shown that the overproduction of MnSOD, achieved by a multicopy plasmid bearing the *SODA* gene, increases the sensitivity of the cells to paraquat, a redox cycling agent known to generate superoxide radicals. This higher sensitivity, however, was not associated to the overproduction of H_2O_2 but to the suppression of the induction of glucose-6-phosphate dehydrogenase, leading to a low NADPH and GSH concentrations (Liochev and Fridovich, 1991). As glutathione levels of *S. cerevisiae* cells are not changed by heat or ethanol stress (Table 2), this hypothesis cannot be considered.

It is interesting to note an increase of CuZnSOD in *sod2*Δ cells. Nevertheless, cells bearing a high CuZnSOD activity (e.g. 4.9 U (mg protein)$^{-1}$ in the *sod2*Δ strain) have a much lower ethanol tolerance than those with a low MnSOD activity (e.g. 1.0 U (mg protein)$^{-1}$ in the *sod1*Δ strain): 2 and 100% survive after 3h in 14% ethanol, respectively. These results support the hypothesis put forward that trapping superoxide radicals within mitochondria by MnSOD is more efficient and prevents its flux to the cytosol, avoiding oxidative damages in cellular proteins, nucleic acids and membrane lipids (Halliwell and Gutteridge, 1989). Furtermore, the data obtained is consistent with previous observations, indicating that mitochondria might be a target in ethanol-induced death (Bandas and Zakharov, 1980; Aguilera and Benítez, 1985; Sá-Correia and van Uden, 1986).

Superoxide dismutases and ethanol tolerance in the post-diauxic phase

When cells enter the diauxic shift phase, the activity of both superoxide dismutases increased (Fig. 4a), which was correlated to higher levels of mRNA (Fig. 5), and thus to an increased translation of the apoproteins; however, the estimation of superoxide dismutase activity in cells at the post-diauxic phase revealed that the activity of CuZnSOD was only moderately induced while the activity of MnSOD was much more significantly induced, and this occurred while mRNA-*SOD1* levels decreased and mRNA-*SOD2* remained constant, compared to diauxic shift cells (Figs. 4 and 5). These results suggest that the induction of CuZnSOD and MnSOD activity during growth from log to diauxic shift phase is due to a *de novo* synthesis of the proteins. In contrast, their induction during growth to post-diauxic phase involves post-transcriptional regulatory mechanisms or post-translational activation of the apoproteins. The same seems to occur during the induction of MnSOD activity in exponential cells exposed to 8% ethanol (Costa *et al.*, 1997). These post-transcriptional regulatory mechanisms have been shown to occur in yeast cells. The CuZnSOD apoprotein is post-translationaly activated by copper during aeration of hypoxic cells both in yeast and mammalian cells (Galiazzo *et al.*, 1991; Rossi *et*

al., 1994). An increased translatability of mRNA-*RAS2*, mRNA-*ENO1*, mRNA-*RPB4* and mRNA-*BCY1*, and post-translational modifications of Bcy1p was observed as cells grow to post-diauxic phase (Brevario *et al.*, 1988; Jigami *et al.*, 1986; Werner-Washburne *et al.*, 1991).

When post-diauxic phase cells were stressed with ethanol, the superoxide dismutases activity did not change (Fig. 4b); however, the levels of both mRNA-*SOD*s decreased (Fig. 6). A similar depletion of mRNA-*CTT1* and mRNA-*ACT1* indicates that ethanol either stimulates mRNA degradation in general or represses gene transcription.

The higher superoxide dismutases activity and ethanol tolerance observed in post-diauxic cells also suggests a possible correlation between these two phenotypes. The role of MnSOD in ethanol tolerance is supported by the high sensitivity of *sod2*Δ mutant cells to ethanol during growth (Table 3). However, *sod2*Δ cells are still more tolerant at the post-diauxic phase than in the diauxic shift phase, adding support to the idea that others factors might be involved that are induced at the post-diauxic phase, such as hsp26, hsp104 and catalase T, together with changes in membrane lipids (Piper, 1995).

It is clear that ethanol tolerance is independent of CuZnSOD activity, as *sod1*Δ mutant cells display a similar tolerance as wild type cells during all growth phases (Table 3). Furthermore, despite the higher CuZnSOD activity found in *sod2*Δ mutants in the post-diauxic phase [8.5 *vs* 6.9 U (mg protein)$^{-1}$], these cells are highly sensitive to ethanol (Tables 3 and 4); however, CuZnSOD may play a minor role in ethanol tolerance of cells deficient in MnSOD. In fact, CuZnSOD activity increased in post-diauxic *sod2*Δ cells exposed to ethanol, and these cells have a higher ethanol tolerance than diauxic shift cells. These results, however, cannot rule out an important role of CuZnSOD in other stress tolerance associated to post-diauxic phase cells (Werner-Washburne *et al.*, 1993).

The H_2O_2 produced by dismutation of superoxide radicals catalysed by MnSOD can be decomposed by catalase T. Therefore, this or other antioxidant defences able to decompose H_2O_2, acting in concert with MnSOD, might have an important role in ethanol resistance. It has been shown that the *CTT1* gene is

derepressed in the post-diauxic phase, as well as upon heat or ethanol stress of log cells (Wieser *et al.*, 1991; Schuller *et al.*, 1994). In fact, the highest ethanol tolerance is achieved by the coordinated action of MnSOD and catalase T. The induction of *CTT1* gene expression is higher in exponential phase cells exposed to ethanol than in those exposed to heat shock, and sublethal ethanol pretreatment confers higher ethanol tolerance than a sublethal thermal stress (Costa *et al.*, 1993). In addition, *CTT1* is only derepressed in the post-diauxic phase, and these cells are more tolerant to ethanol than diauxic shift cells (Piper, 1995).

The overall results suggest that ethanol induces the generation of superoxide radicals in mitochondria. Therefore, it would be expected that a respiration deficient strain would be more tolerant to ethanol. Indeed, in contrast to *sod2*Δ cells, exponentially growing *sod2*Δp cells are able to acquire ethanol tolerance. Besides, the data also indicate that superoxide radicals may regulate *SOD* expression upon thermal or ethanol stress. In fact, the levels of mRNA-*SOD1* and mRNA-*SOD2* increased upon heat shock and mRNA-*SOD2* increased upon ethanol stress; however, the induction was lower than that observed in wild type cells (Fig. 4 and Table 4). The induction of MnSOD activity by ethanol was rather low in petite (respiration deficient) cells and no longer occurred upon heat shock, compared with wild type cells, giving further evidence of a post-translational regulation of MnSOD, which may involve superoxide radicals under these stress conditions. The hypothesis that the increase of MnSOD activity may be directly or indirectly regulated by mitochondrial superoxide radicals flux *per se* was previously suggested by several experimental evidences (Hassan and Fridovich, 1977; Freeman and Crapo, 1981; Moody and Hassan, 1984; Westerbeek-Marres *et al.*, 1988).

These results led to establish the link between ethanol stress and the oxidative stress response. Therefore, a further analysis of the yeast response to oxidants was performed, namely the expression of *SOD* genes and superoxide dismutases activity in the presence of hydrogen peroxide. As *SOD1* gene

promoter harbours a Yap1p-binding site, the regulation of *SOD* expression by two members of the Yap family was analysed (chapter 2).

Chapter 2

The role of Yap1p and Yap2p in the activation of superoxide dismutases induced by hydrogen peroxide in *Saccharomyces cerevisiae*

SUMMARY

In *S. cerevisiae* cells, the Yap1p bZIP transcription factor plays a ~~critic~~al role in oxidative stress protection, which is correlated with a direct function of Yap1p in the regulation of a number of genes encoding antioxidant defences. Yap2p, a Yap1p homologue, is also important for adaptive responses to hydrogen peroxide. *SOD1* gene, encoding Cu,Zn-superoxide dismutase, contains a Yap1p binding element in the promoter region. In this report, we show that the increase of mRNA-*SOD1* levels in cells exposed to hydrogen peroxide is Yap1p-dependent and Yap2p-independent. *SOD2* gene, encoding Mn-superoxide dismutase, does not contain a Yap1p binding element; however, the induction of *SOD2* gene expression by hydrogen peroxide is partially dependent on Yap1p. Moreover, Yap2p seems to have a function in sustaining the activation of *SOD2* gene expression, as the increase of mRNA-*SOD2* levels shown for wild type and *yap1Δ* cells after 60 min of stress is not observed in *yap2Δ* and *yap1Δyap2Δ* cells. Interestingly, both Yap1p and Yap2p seem to be indirectly involved in posttranslational activation of Sod2p, since the induction of MnSOD activity is completely suppressed in *yap1Δyap2Δ* double mutant. Consistent with an important function of Yap2p in the adaptive response, disruption of *YAP2* reduces the capacity of *yap1Δ* cells to enhance hydrogen peroxide resistance by a sublethal stress pre-treatment.

INTRODUCTION

In the yeast *Saccharomyces cerevisiae*, the Yap1p transcription factor plays an important function in oxidative stress protection, as indicated by the high sensitivity of *yap1Δ* null mutants to H_2O_2, t-butyl hydroperoxide, superoxide radicals and hyperoxia (Schnell *et al.*, 1992; Kuge and Jones, 1994). In addition, disruption of *YAP1* gene renders yeast cells hypersensitive to Cd (known to act as pro-oxidant) and malondialdehyde (a product of molecular oxidations), and *YAP1* overexpression enhance Cd resistance (Wu *et al.*, 1993; Hirata *et al.*, 1994; Wemmie *et al.*, 1994a; Turton *et al.*, 1997). In agreement with its function in heavy metal and oxidative stress protection, Yap1p directly regulates a number

of genes encoding antioxidant defences (see general introduction), through direct binding to a promoter element with a core consensus TTACTAA (Fernandes *et al.*, 1997).

Yap1p belongs to a family of eight bZIP proteins with overlapping but distinct biological functions (Fernandes *et al.*, 1997). Yap2p shows strong homology to Yap1p and also plays an important function in the regulation of the adaptive response to H_2O_2 (Stephen *et al.*, 1995) and Cd (Wu *et al.*, 1993; Hirata *et al.*, 1994). However, genes encoding antioxidant defences have not yet been identified as targets for Yap2p.

Superoxide dismutases are key antioxidant defences in the protection of yeast cells against oxidative damages induced by hyperoxia, superoxide radicals, and hydrogen peroxide (Bilinski *et al.*, 1985; van Loon *et al.*, 1986; Zhu and Scandalios, 1992). Yeast cells contain two superoxide dismutases: a cytosolic CuZnSOD, encoded by *SOD1*, and a mitochondrial MnSOD, encoded by *SOD2*. *SOD1* gene harbours a Yap1p-binding element in the promoter region, and CuZnSOD activity is lower in *yap1Δ* mutants (Schnell *et al.*, 1992). *SOD2* gene promoter contains a general stress response element (STRE) and a HAP2/3/4/5-binding site, which are required for induction of *SOD2* gene expression during growth on non-fermentable carbon sources and at the diauxic-shift (Flattery-O'Brien *et al.*, 1997; Pinkham *et al.*, 1997). In this study, we have investigated the role of Yap1p and Yap2p in the activation of superoxide dismutases by H_2O_2, and their contribution to H_2O_2 resistance.

MATERIAL AND METHODS

Yeast strains and growth conditions

The strains of *Saccharomyces cerevisiae* used in this study are listed in Table 1. Cells were grown in minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with appropriate amino acids (40 mg/l tryptophan, 40 mg/l histidine, 80 mg/l leucine) and nucleotides (40 mg/l uracil, 40 mg/l adenine), to early exponential phase (OD_{600}=0.6), in an orbital

shaker, at 26°C, and 120 rpm, with a ratio of flask volume / medium volume of 5:1.

Table 1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Reference/Source
W303	Matα, *ade2-1, can1-100, trp1-1, ura3-1, his3-11,15, leu2-3,112*	Wallis *et al.*, 1989
W303 *yap1Δ*	[W303] *yap1::HIS*	C. Rodrigues-Pousada
W303 *yap2Δ*	[W303] *yap2::ADE*	Bossier *et al.*, 1993
W303 *yap1Δ yap2Δ*	[W303] *yap1::HIS yap2::ADE*	C. Rodrigues-Pousada

Hydrogen peroxide tolerance

Yeast cells, growing in early exponential phase at 26°C, or pre-exposed to 0.1mM H_2O_2 for 30 min, were subsequently treated with 1.5mM H_2O_2. Cell viability was determined by standard dilution plate counts on YPD medium containing 1.5% agar. Colonies were counted after growth at 26°C for 3 days. Viability was expressed as the percentage of the colony-forming units of non-stressed cells.

Superoxide dismutase (EC 1.15.1.1.) activity

Superoxide dismutase activity was estimated using the xanthine - xanthine oxidase system (Flohé and Otting, 1984), as described in chapter 2.

Preparation and analysis of RNA

Total cellular RNA was isolated and analysed as described in chapter 2. The following gene probes were used: a 0.5 kb *Hin*dIII fragment of *SOD1* gene (Bermingham-McDonogh *et al.*, 1988); a 2 kb *Bam*HI fragment of *SOD2* gene (Marres *et al.*, 1985); and a 1 kb *Hin*dIII - *Eco*RI fragment of *ACT1* gene (Gallwitz and Sures, 1980).

Statistical analysis

Data are expressed as mean values ± SD of at least 3 independent experiments. Values were compared by computing standard Student's t-test. The 0.05 level was chosen as the point of statistical significance throughout.

RESULTS

The role of Yap1p and Yap2p in the regulation of SOD1 and SOD2 gene transcription induced by hydrogen peroxide

Yeast cells exposed to hydrogen peroxide induce the synthesis of catalase T (Marchler *et al.*, 1993), which decomposes hydrogen peroxide into water and molecular oxygen leading to an increase of intracellular oxygen concentration that favours the generation of superoxide radicals. As a response to superoxide formation, *SOD1* and *SOD2* gene expression increase upon hydrogen peroxide treatment (Schnell *et al.*, 1992; Jamieson *et al.*, 1994). To evaluate the role of Yap1p and Yap2p transcriptions factors on the regulation of *SOD1* and *SOD2* genes, we determined the respective mRNA levels in exponential phase cells exposed to 0.1mM H_2O_2 (Fig. 1). The constitutive mRNA levels were similar in wild type and in *yap1Δ*, *yap2Δ*, or *yap1Δyap2Δ* cells. In the wild type strain, the mRNA levels increased upon H_2O_2 treatment, being maximal at 30 min (60% for *SOD1*, 120% for *SOD2*) and slightly decreasing at 60 min. The induction of mRNA-*SOD1* levels was completely suppressed in *yap1Δ* cells, however it was not affected in *yap2Δ* cells (Figs. 1a and 1b). The results obtained with *yap1Δyap2Δ* double mutants were similar to those observed in *yap1Δ* cells. Unlike *SOD1* gene expression, the induction of mRNA-*SOD2* levels by H_2O_2 was only partially dependent on Yap1p (Figs. 1a and 1c). Notably, the increase of mRNA-*SOD2* levels in *yap2Δ* cells was maximal at 30 min, similarly to the observed in wild type cells; however, this stress response was highly transient, as the mRNA-*SOD2* levels decreased to control levels at 60 min, while a 70% increase was still observed in the wild type strain. In the *yap1Δyap2Δ* double mutant, the pattern of mRNA-*SOD2* levels is the sum of the observed in the

single mutants, *i.e.*, the induction was smaller (as in *yap1Δ* cells) and transient (as in *yap2Δ* cells). These results suggest that Yap2p participates in oxidative stress response by sustaining the activation of gene transcription.

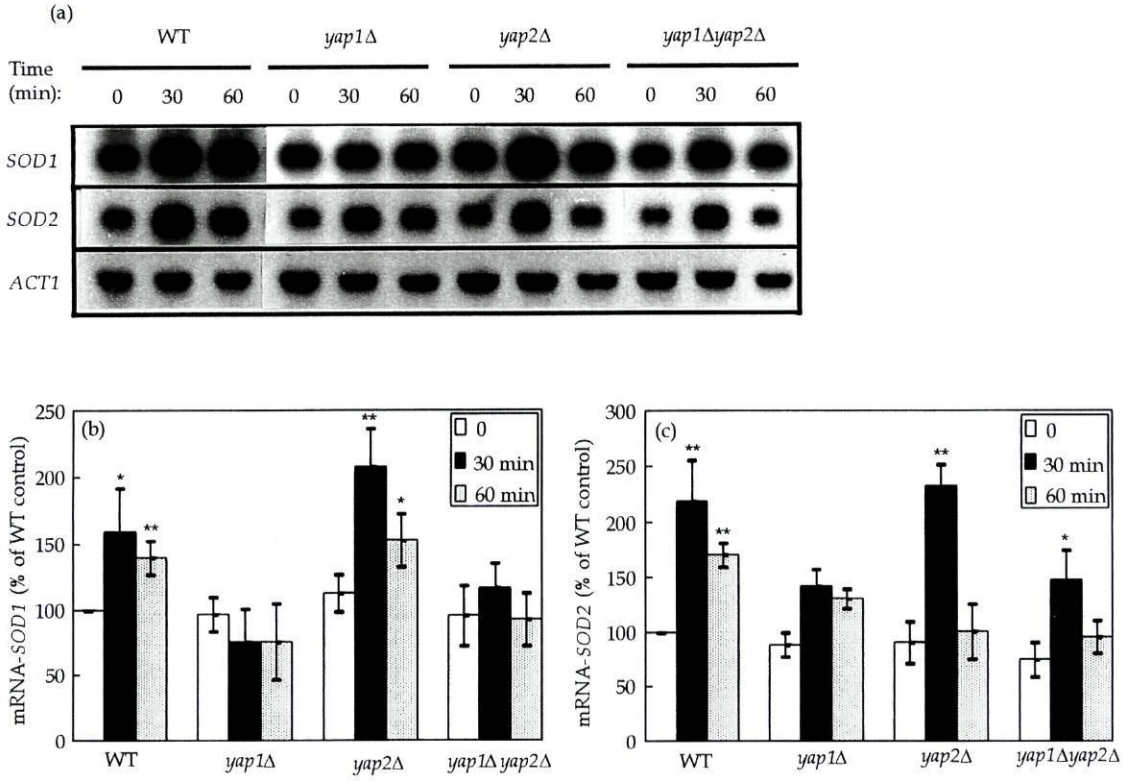

Fig. 1. (a) Northern blot analysis of mRNA-*SOD1*, and mRNA-*SOD2* in *S. cerevisiae* W303 (WT), *yap1Δ*, *yap2Δ* and *yap1Δyap2Δ* cells exposed to hydrogen peroxide. Exponential phase cells were exposed to 0.1mM H_2O_2 for 30 min and 60 min (a representative experiment is shown). mRNA-*SOD1* (b), and mRNA-*SOD2* (c) hybridization signals were quantified using a Ultra Scan XL Enhancer Laser Densitometer. Band intensities were corrected for *ACT1* (RNA loading control). Values are mean ± SD of three independent experiments. *p<0.05, **p<0.01 (treated cells compared to control cells).

Yap1p and Yap2p are indirectly involved in post-transcriptional activation of Sod2p

We have also analysed the role of Yap1p and Yap2p on CuZnSOD and MnSOD activity of yeast cells exposed to hydrogen peroxide. In agreement with the results of Schnell *et al.* (1992), CuZnSOD basal activity in *yap1Δ* cells was 65% of

wild type levels; however, it was not affected by *YAP2* disruption (Fig. 2a). In addition, CuZnSOD activity was not significantly induced in any of the strains exposed to 0.1mM H_2O_2. Unlike CuZnSOD, MnSOD activity increased 40% in the wild type cells exposed to 0.1mM H_2O_2 (Fig. 2b). *YAP1* or *YAP2* disruption did not affect basal or enhanced MnSOD activity. However, the induction of MnSOD activity is completely suppressed in the *yap1Δyap2Δ* double mutant.

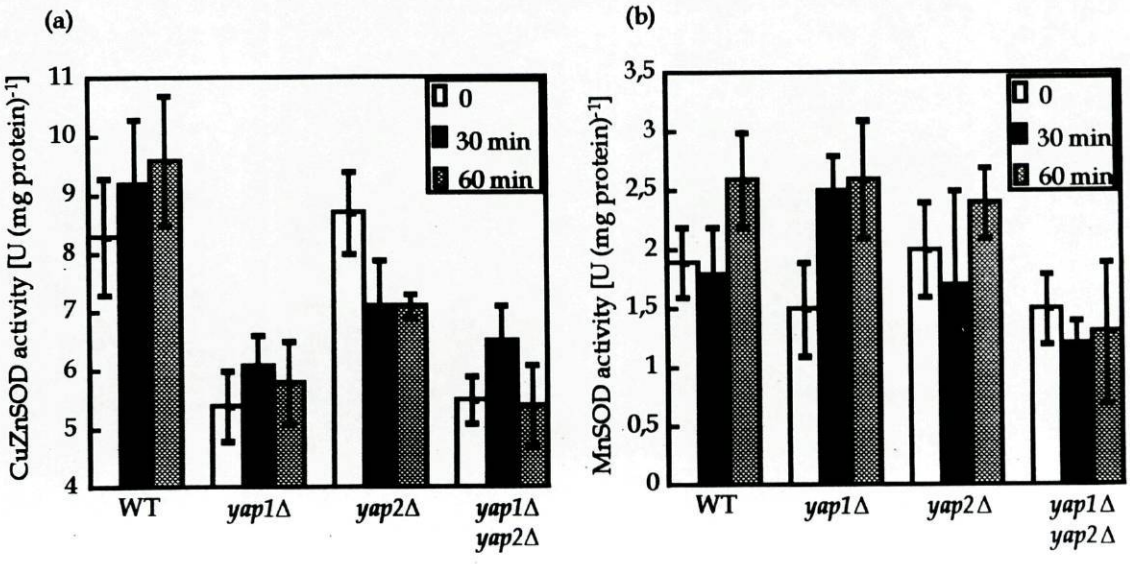

Fig. 2. Analysis of CuZnSOD (a) and MnSOD (b) activities in *S. cerevisiae* W303 (WT), *yap1Δ*, *yap2Δ* and *yap1Δyap2Δ* cells exposed to hydrogen peroxide. Exponential phase cells were treated with 0.1mM H_2O_2 for 30 min and 60 min. Values are mean ± SD of five independent experiments.

yap1Δyap2Δ cells are hypersensitive to hydrogen peroxide

Aiming at assessing if the incapacity of *yap1Δyap2Δ* cells to increase MnSOD activity was correlated with a lower stress resistance, we compared H_2O_2 tolerance of *yap1Δyap2Δ* with that of single mutants and wild type cells. Our results show that the adaptive response induced by 0.1mM H_2O_2 increased the resistance of wild type cells to a subsequent exposure to 1.5mM H_2O_2 (Fig. 3). *YAP1* disruption significantly decreased the constitutive H_2O_2 tolerance: 45% of wild type cells survived a 30 min exposure to 1.5mM H_2O_2, while only 20% of *yap1Δ* cells remained; however, the acquisition of H_2O_2 tolerance was not

impaired in *yap1Δ* cells (Fig. 3). Unlike *YAP1*, *YAP2* disruption did not affect either the constitutive or the 0.1mM H_2O_2-induced wild type resistance; however, it significantly increased the sensitivity of the *yap1Δ* cells: only 5% of *yap1Δyap2Δ* cells survived a 30 min exposure to 1.5mM H_2O_2, and, despite the acquisition of H_2O_2 resistance by a 0.1mM H_2O_2 pretreatment, *yap1Δyap2Δ* resistance was severely reduced.

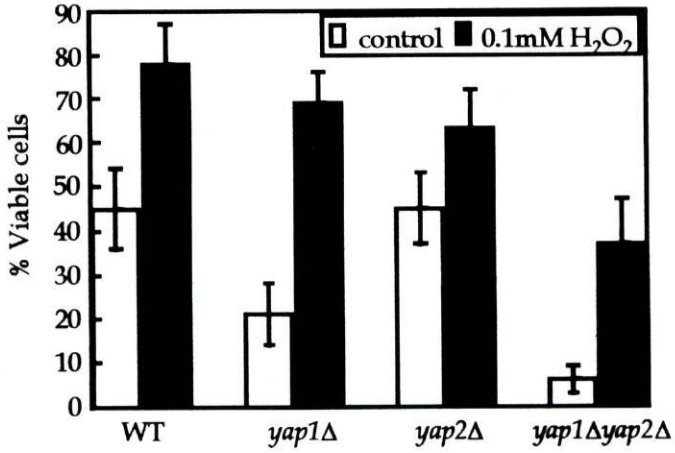

Fig. 3. H_2O_2 (1.5mM) tolerance of *S. cerevisiae* W303 (WT), *yap1Δ*, *yap2Δ* and *yap1Δyap2Δ* cells: exponential phase unstressed cells, and cells pretreated with 0.1mM H_2O_2 for 30 min, were subsequently exposed to 1.5mM H_2O_2 for 30 min. Appropriate dilutions were plated on YPD. Viable cells were assayed after growth at 26°C for three days and expressed relative to control cells (untreated). Values are mean ± SD of five independent experiments.

Cells grown to the post-diauxic phase display an increased resistance to oxidative stress (Jamieson, 1992; Jamieson *et al.*, 1994; Steels *et al.*, 1994). Aiming at investigate the role of Yap1p and Yap2p in this adaptive response, we analysed H_2O_2 resistance of the cells in the post-diauxic phase. In contrast to the observed in the exponential phase, H_2O_2 tolerance of *yap1Δyap2Δ* and wild type cells was similar: in both strains, more than 90% of post-diauxic cells survived a 60 min treatment with H_2O_2 up to 5mM.

DISCUSSION

A number of antioxidant defences and systems of metal ion homeostasis are known to contribute to oxidative-stress protection in yeast (Jamieson, 1995; Moradas-Ferreira *et al.*, 1996; Santoro and Thiele, 1997). Specific transcription factors regulate both the basal and the oxidative-stress-induced transcription of genes responsible for protection. Previous studies have shown that Yap1p, a transcription factor that belongs to a family of eight bZIP proteins (Fernandes *et al.*, 1997), plays a central role in the protection against oxidative stress conditions, including peroxides, superoxide radicals, hyperoxia and thiol oxidants (Schnell *et al.*, 1992; Hirata *et al.*, 1994; Kuge and Jones, 1994; Wemmie *et al.*, 1997). A second member of the Yap proteins, Yap2p, shows extensive homology to Yap1p (Bossier et al., 1993), and has an important function in the adaptive response to H_2O_2 (Stephen *et al.*, 1995); however, at present, the role of Yap2p is still unclear.

In this work, we have analysed the role of Yap1p and Yap2p in the expression of superoxide dismutases. A Yap-binding site is present in *SOD1* gene promoter; however, mRNA-*SOD1* basal levels are Yap1p- and Yap2p-independent. In spite of the mRNA-*SOD1* levels being similar in wild type and *yap1Δ* cells, the constitutive CuZnSOD activity seems to be partially dependent on Yap1p: CuZnSOD activity decreased 35% in the *yap1Δ* cells (similarly to the observed by Schnell *et al.*, 1992). These results suggest that Yap1p may be indirectly involved in the post-translational activation of Sod1p. It is known that, under normal physiological conditions, one-third of Sod1p exists as apoprotein (Petrovic *et al.*, 1996). Under specific conditions, the apoprotein is activated by copper, which is transported in the cytosol by the copper chaperone Lys7p to Sod1p (Galiazzo *et al.*, 1991; Culotta *et al.*, 1997). A limiting amount of Cu or Lys7p available for the activation of the Sod1 apoprotein would, therefore, result in a lower CuZnSOD activity. Although there is no evidence that Yap1p controls *LYS7* transcription, it may occur, since a Yap1p-binding site (-169TTACTAA-163) is present in *LYS7* promoter (Horecka *et al.*, 1995).

The expression of *SOD1* gene is induced by H_2O_2 (Schnell *et al.*, 1992; Fig. 1a), and *sod1*Δ mutants are more sensitive to H_2O_2 than wild type cells (Bilinski *et al.*, 1985), indicating that CuZnSOD plays an important function in the protection of yeast cells exposed to H_2O_2. We have shown that H_2O_2-induced transcriptional activation of *SOD1* is mediated by Yap1p, which is consistent with the Yap1p-binding site present in *SOD1* gene promoter. Indeed, the increase of mRNA-*SOD1* levels was impaired by disruption of *YAP1* gene, but not of *YAP2* (Fig. 1a). Therefore, *SOD1* was identified as a Yap1p target gene, increasing the number of genes encoding antioxidant defences known to be directly regulated by Yap1p: *TRX2* (thioredoxin 2), *TRR1* (thioredoxin reductase), *GSH1* (γ-glutamylcysteine synthetase) and *GLR1* (glutathione reductase) (Kuge and Jones, 1994; Wu and Moye-Rowley, 1994; Grant *et al.*, 1996b; Morgan *et al.*, 1997).

Despite the increase of mRNA-*SOD1* levels in wild type cells, CuZnSOD activity was not significantly induced (Fig. 2a). As metalloproteins are highly sensitive to oxidative inactivation (Stadtman, 1993), the induction of *SOD1* gene expression may represent a cellular adaptation in order to compensate the CuZnSOD activity lost by protein damage.

SOD2 gene expression is also induced by H_2O_2 (Jamieson *et al.*, 1994; Fig. 1b). Strikingly, the transcriptional activation of *SOD2* gene induced by H_2O_2 is partially dependent on Yap1p, in spite of the lack of a Yap1p-binding site in the promoter region. Moreover, Yap2p seems to have an important role by sustaining the induction of *SOD2* gene expression. In fact, in *yap2*Δ and *yap1*Δ*yap2*Δ mutants, mRNA-*SOD2* levels decay to control levels after 60 min of stress, while a 70% increase was still observed in the wild type strain. These results suggest that Yap1p has an important role in early responses, while Yap2p is involved in late responses.

It was previously suggested that Yap1p plays a role in the STRE-mediated response, although in an indirect manner (Gounalaki and Thireos, 1994). As *SOD2* gene promoter contains one STRE, Yap1p and Yap2p may have an indirect function in the transcriptional activation of *SOD2* in response to H_2O_2,

mediated through the STRE. A significant increase of mRNA-*SOD2* levels was still observed in *yap1Δ* and *yap1Δyap2Δ* cells exposed to H_2O_2. This induction may be directly mediated by Msn2p, Msn4p, or other unidentified transcription factor(s) that bind the STRE (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996).

Our results also indicate an important, overlapping role of Yap1p and Yap2p in the induction of MnSOD activity by H_2O_2. The increase of MnSOD activity during H_2O_2 treatment was similar in wild type and *yap1Δ* or *yap2Δ* single mutants; however, it was not observed in *yap1Δyap2Δ* cells (Fig. 2b), despite the transient induction of mRNA-*SOD2* (Fig. 2b), suggesting that Yap1p and Yap2p may have an indirect role in the post-transcriptional activation of MnSOD. A similar cooperative function of Yap1p and Yap2p was described in the transcriptional activation of the multidrug resistance genes, *PDR5* and *SNQ2*, in response to heat shock (Miyahara *et al.*, 1996).

The impaired induction of MnSOD activity in *yap1Δyap2Δ* cells was correlated with a higher sensitivity of these mutants to 1.5mM H_2O_2 (Fig. 3). In fact, the lower H_2O_2 tolerance of *yap1Δ* cells was further decreased by *YAP2* disruption. Moreover, despite the acquisition of H_2O_2 resistance by 0.1mM H_2O_2 pretreatment, the viability of *yap1Δyap2Δ* cells was severely reduced. These results indicate that Yap2p contributes to H_2O_2 resistance of *yap1Δ* cells. This role of Yap1p and Yap2p was only observed in exponential phase cells. Indeed, the high, constitutive H_2O_2 tolerance of post-diauxic phase cells was not impaired in *yap1Δyap2Δ* double mutants.

The accumulation of oxidatively modified molecules may contribute to cell death under stress conditions. Aiming at studying the correlation between oxidative damages and stress sensitivity, we analysed lipid peroxidation and protein oxidation in cells exposed to hydrogen peroxide and ethanol (chapter 3).

Chapter 3

Oxidative damages induced by hydrogen peroxide and ethanol in *Saccharomyces cerevisiae*

SUMMARY

Oxidative damages are major factors leading to cell death under specific stress conditions. In this work, we analysed oxidative modifications in lipids and proteins of *S. cerevisiae* cells exposed to hydrogen peroxide and ethanol, and investigated its correlation with the high sensitivity of *yap1Δ* and *yap1Δyap2Δ* mutants to hydrogen peroxide, and of *sod2Δ* cells to ethanol. Our results show that hydrogen peroxide increases the concentration of malondialdehyde, which is mainly produced during fragmentation of lipid hydroperoxides. *YAP1* disruption does not further increases MDA production during hydrogen peroxide stress, indicating that the higher sensitivity of *yap1Δ* cells is not associated with lipid peroxidation; however, MDA levels of *yap1Δyap2Δ* cells exposed to hydrogen peroxide are significantly higher than those observed in *yap1Δ* cells, thus the higher sensitivity of *yap1Δyap2Δ* cells to hydrogen peroxide may be related to lipid peroxidation. The analysis of protein carbonyls shows that the accumulation of oxidised proteins induced by hydrogen peroxide is not a major factor in stress sensitivity of *yap1Δyap2Δ* cells. Indeed, hydrogen peroxide induces a severe, non specific protein oxidation, which was not further increased in *yap1Δyap2Δ* cells. Protein oxidation is reduced by a sublethal hydrogen peroxide pretreatment that increases cellular resistance, suggesting a partial correlation between the accumulation of oxidised proteins and hydrogen peroxide toxicity. Some of the proteins most sensitive to oxidative modifications were identified as being cyclophilin, and the glycolytic enzymes triosephosphate isomerase and phosphoglycerate mutase. Previous results suggested that oxidative stress contributes to ethanol toxicity. Our results show that ethanol does not increase malondialdehyde levels, even in ethanol sensitive *sod2Δ* cells. Ethanol causes, however, a mild protein oxidation, suggesting a correlation between the accumulation of oxidised proteins and ethanol-induced cell death.

INTRODUCTION

Reactive oxygen species (ROS) are able to oxidise nucleic acids, proteins, lipids or carbohydrates, affecting the integrity of cell membranes and inactivating cellular functions essential for survival (Halliwell and Gutteridge, 1989). Amongst lipids, polyunsaturated fatty acids are major targets for oxidation, giving rise to fatty acyl hydroperoxides, which undergo fragmentation, generating highly reactive products, such as epoxides, aldehydes and alkanes (Halliwell and Gutteridge, 1989). One of the aldehydes (malondialdehyde, MDA) is frequently assayed as an index of lipid peroxidation. Studies in *S. cerevisiae* cells have shown that hydrogen peroxide increases the production of MDA (Steels *et al.*, 1994). Oxidative damage to proteins involves oxidation of amino acid residue side chains to hydroxy or carbonyl derivatives, scission of the polypeptide chain, and protein cross-linking, leading to increased proteolytic susceptibility and decreased biological activity (Stadtman, 1993; Levine *et al.*, 1994). In *S. cerevisiae*, it was recently shown that hydrogen peroxide oxidises methionine residues (Moskovitz *et al.*, 1997); however, the identification of the proteins modified by oxidants has not been reported.

Under physiological conditions, oxidative damages are minimised by antioxidant defences, which function at three distinct levels: preventing the generation of ROS, by fine-tuned mechanisms which regulate metal ion homeostasis; intercepting the ROS; and repairing oxidatively modified molecules (reviewed by Jamieson, 1995; Moradas-Ferreira *et al.*, 1996; Santoro and Thiele, 1997). Specific transcription factors activate the expression of genes encoding antioxidant defences under respiratory growth conditions, and in response to oxidative aggressions. In this respect, Yap1p has a major role in oxidative stress response, *yap1Δ* mutants having a high sensitivity to oxidants (see general introduction).

Aiming at studying the correlation between H_2O_2 toxicity and the induction of oxidative damages, we analysed lipid peroxidation and protein oxidation in wild type cells and in mutants highly sensitive to H_2O_2 (*yap1Δ* and

yap1Δyap2Δ). Cellular damages were also analysed in cells exposed to ethanol, in order to identify pro-oxidant effects induced by this stress agent. Lipid peroxidation was analysed by assaying malondialdehyde concentration, while protein oxidation was evaluated by immunodetection of carbonyls in proteins. Some proteins highly sensitive to oxidation were identified by mass spectrometry, giving insights into the cellular functions affected.

METHODS

Yeast strains and growth conditions

The strains of *Saccharomyces cerevisiae* used in this study are listed in Table 1. Cells were grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose) or in minimal medium (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with appropriate amino acids (40 mg/l tryptophan, 40 mg/l histidine, 80 mg/l leucine) and nucleotides (40 mg/l adenine, 40 mg/l uracil), to early exponential phase (OD_{600}=0.6), in an orbital shaker, at 26ºC, and 120 rpm, with a ratio of flask volume / medium volume of 5:1.

Table 1. *Saccharomyces cerevisiae* strains used in this study.

Strain	Genotype	Reference/Source
W303	Matα, *ade2-1, can1-100, trp1-1, ura3-1, his3-11,15, leu2-3,112*	Wallis *et al.*, 1989
W303 *yap1Δ*	[W303] *yap1::HIS*	C. Rodrigues Pousada
W303 *yap2Δ*	[W303] *yap2::ADE*	Bossier *et al.*, 1993
W303 *yap1Δ yap2Δ*	[W303] *yap1::HIS yap2::ADE*	C. Rodrigues Pousada
DL1	α *leu2,3, 2-112, his3-11,3-15 ura3-251, 3-372, 3-328*	van Loon *et al.* (1986)
DL1*sod2Δ*	[DL1]*sod2::LEU2*	van Loon *et al.* (1986)

Thiobarbituric acid reactive substances assay

Yeast extracts were prepared in 20mM sodium phosphate buffer (pH 7.2) 10% TCA, by vigorous shaking of the cell suspension, in the presence of glass beads,

for 3 min. Short pulses of 30 s were used, with 30 s intervals on ice. The homogenate was centrifuged at 5000 rpm for 15 min. Proteins were assayed by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. Thiobarbituric acid reactive substances were assayed as described by Steels *et al.* (1994). An aliquote of the sample was mixed with one volume of 0.1M EDTA and six volumes of 1% thiobarbituric acid 0.025% butylated hydroxytoluene 0.05M sodium hydroxide, and boiled 15 min. The absorvance was read at 532 nm. The results were expressed as nmol MDA (mg protein)$^{-1}$ (ε=1.4 x 10^5 M^{-1} cm^2).

Electrophoretic analysis

Yeast extracts were prepared in 50mM potassium phosphate buffer (pH 7.0) containing a mixture of protease inhibitors (30μg/ml pepstatin, 30μg/ml leupeptin, 6μg/ml antipain, 6mM EDTA), and proteins were assayed, as described above. Proteins were derivatised with 2,4-dinitrophenylhydrazine, as described by Levine *et al.* (1994). For SDS-PAGE, an aliquote of the sample was mixed with one volume of 12% SDS and 2 volumes of 20mM 2,4-dinitrophenylhydrazine in 10% (v/v) trifluoroacetic acid (a blank control was treated with 2 volumes of 10% trifluoroacetic acid alone). The mixture was incubated 30 min at room temperature, in the dark, and subsequently neutralised with 1.5 volumes of 2M Tris containing 30% glycerol and 19% 2-mercaptoethanol. Proteins (15 μg) were loaded in a polyacrylamide gel (12.5%), as described by Laemmli (1970). For 2D-analysis, an aliquote of the sample was mixed with 1ml of 10 mM 2,4-dinitrophenylhydrazine in 2M HCl, and incubated 60 min at room temperature, in the dark. Proteins were precipitated with trichloroacetic acid (10% v/v, final concentration), 10 min on ice. The protein pellets, obtained after centrifugation at 14000 rpm for 5 min, were washed twice with 1 ml of ethanol:ethyl acetate (1:1), and once with acetone, and solubilised in 9M Urea, 2% CHAPS, 2% β-mercaptoethanol, 0.8% Pharmalytes pH 3-10. Proteins (100 μg) were mixed with 3 volumes of a gel slurry (30 mg Sephadex IEF / ml solubilisation solution) and loaded on a 13 cm

immobilised pH 3-10 linear gradient (IPG) dry strip (Pharmacia), previously re-hydrated in 8M Urea, 0.5% CHAPS, 0.2% DTT, 0.25% Pharmalytes pH 3-10, 0.5% Triton X-100 (first dimension). Isoelectric focusing was performed in four phases: 1- 30 min at 150V; 2- 60 min at 300V; 3- 90 min at 3500V, 2mA, 7W, 2850V/h; 4- 245 min at 3500 V, 2mA, 7 W, 14150V/h. In the second dimension (SDS-PAGE), IPG-strips were incubated in equilibration buffer (6M Urea, 2% SDS, 0.1mM EDTA, 30% glycerol, 0.01% bromophenol blue, 50mM Tris pH 6.8) containing 10 mg/ml DTT (10 min) and 10 mg/ml DTT, 48.1 mg/ml Iodoacetamide (10 min), and loaded on a 12.5% polyacrylamide. Following 1D- or 2D-gel electrophoresis, proteins were visualised by Coomassie blue R-250 or silver staining, or blotted.

Immunoblotting

After electrophoresis, proteins were transferred to nitrocellulose, in a semi-dry system, using 39mM glycine, 48mM Tris, 0.0375% SDS, 20% methanol as the transfer buffer, during 1h, at 0.8 mA/cm^2. Nitrocellulose was probed with rabbit IgG anti-DNP (Dako) at a 1:5000 dilution, as the first antibody, and goat anti-rabbit IgG linked to horseradish peroxidase (Sigma) at a 1:5000 dilution, as the second antibody. Immunodetection was performed by chemiluminescence, using a kit from Amersham (RPN 2109).

Protein identification

Proteins were excised from polyacrylamide gels stained with 0.05% Coomassie blue, 0.5% acetic acid, 20% methanol, and destained with 30% methanol, or silver stained (O'Connell and Stults, 1997). The peptides obtained by digestion with trypsin or Lys C were analysed by MALDI-mass spectrometry, and the proteins were identified by search in a yeast peptide database (SWISS-PROT).

RESULTS

yap1Δyap2Δ deletion enhances malondialdehyde formation induced by hydrogen peroxide

It was previously shown that H_2O_2 increases the production of MDA in *S. cerevisiae*, suggesting that H_2O_2 toxicity is associated with lipid peroxidation (Steels *et al.*, 1994). Aiming at investigating if the higher sensitivity of *yap1Δ* and *yap1Δyap2Δ* mutants to H_2O_2 is correlated with a higher induction of lipid peroxidation, we determined MDA production in *yap1Δ* or *yap2Δ* single mutants, and in *yap1Δyap2Δ* double mutants exposed to 1.5mM H_2O_2 (Fig. 1). In the wild type strain, the levels of MDA increased 50% after exposure of exponential cells to 1.5mM H_2O_2 for 60 min. Similar results were obtained with *yap1Δ* or *yap2Δ* mutants; however, in the *yap1Δyap2Δ* double mutants, the increase of MDA levels was significantly higher (110%; p=0.0006).

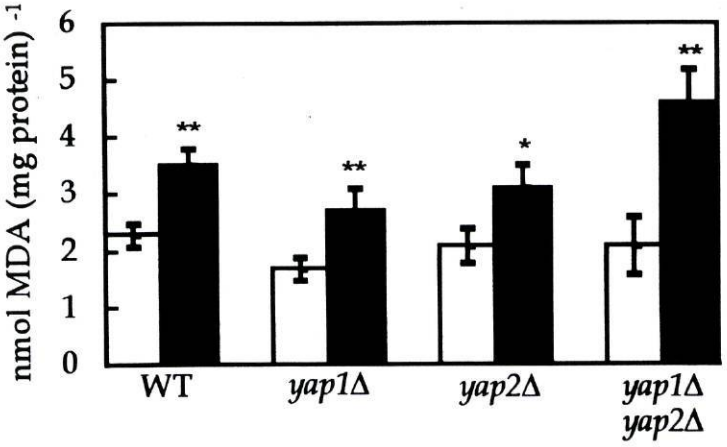

Fig. 1. Analysis of malondialdehyde (MDA) concentration in *S. cerevisiae* cells exposed to hydrogen peroxide. Exponential phase cells growing in minimal medium (□) were exposed to 1.5mM H_2O_2 for 60 min (■). Data are expressed as mean values ± SD of at least five independent experiments. $^{*}p<0.05$; $^{**}p<0.01$.

Hydrogen peroxide induces a severe, non-specific protein oxidation

To assess if the higher sensitivity of *yap1Δyap2Δ* mutants to H_2O_2 is correlated with a higher induction of protein oxidation, carbonyls in proteins separated by SDS-PAGE were analysed. Hydrogen peroxide (1.5mM) induced a high, non specific protein oxidation, which increased linearly in time, from 10 min to 2 h of exposure (Fig. 2A and B). Pretreatment of yeast cells with sublethal H_2O_2 concentrations is known to increase the resistance to a subsequent lethal H_2O_2 stress (Collinson and Dawes, 1992); pre-exposure to 0.4mM H_2O_2 attenuated but did not prevent carbonyls formation induced by 1.5mM H_2O_2 (Fig. 2C and D). When the hypersensitive *yap1Δyap2Δ* cells were stressed with H_2O_2, both the pattern and the degree of protein oxidation were similar to the observed in the wild type cells (Fig. 3).

To identify the proteins more susceptible to oxidation by H_2O_2, we performed the immunodetection of carbonyls in proteins separated by two-dimensional gel electrophoresis (Fig. 4). In addition to an overall induction of protein oxidation, we observed a high oxidation of some proteins, which were labelled P37A-B, P29A-C, P27A-E, P20A-D and P17 (Fig. 4D). Among these proteins, P29A-B, P27A-C,E, and P20A-D seem to be the most extensively oxidised, since they could not be detected even by the highly sensitive silver staining. P29C, P27D and P17 were identified by mass spectrometry as being phosphoglycerate mutase (Pmg1p), triosephosphate isomerase (Tpi1p), and cyclophilin (Cph1p).

We have also analysed the carbonyl content of proteins from *yap1Δyap2Δ* cells separated by the two-dimensional system, since it has a much higher resolution. These results confirmed that hydrogen peroxide-induced protein oxidation in *yap1Δyap2Δ* mutants was similar to the observed in wild type cells (Fig. 5).

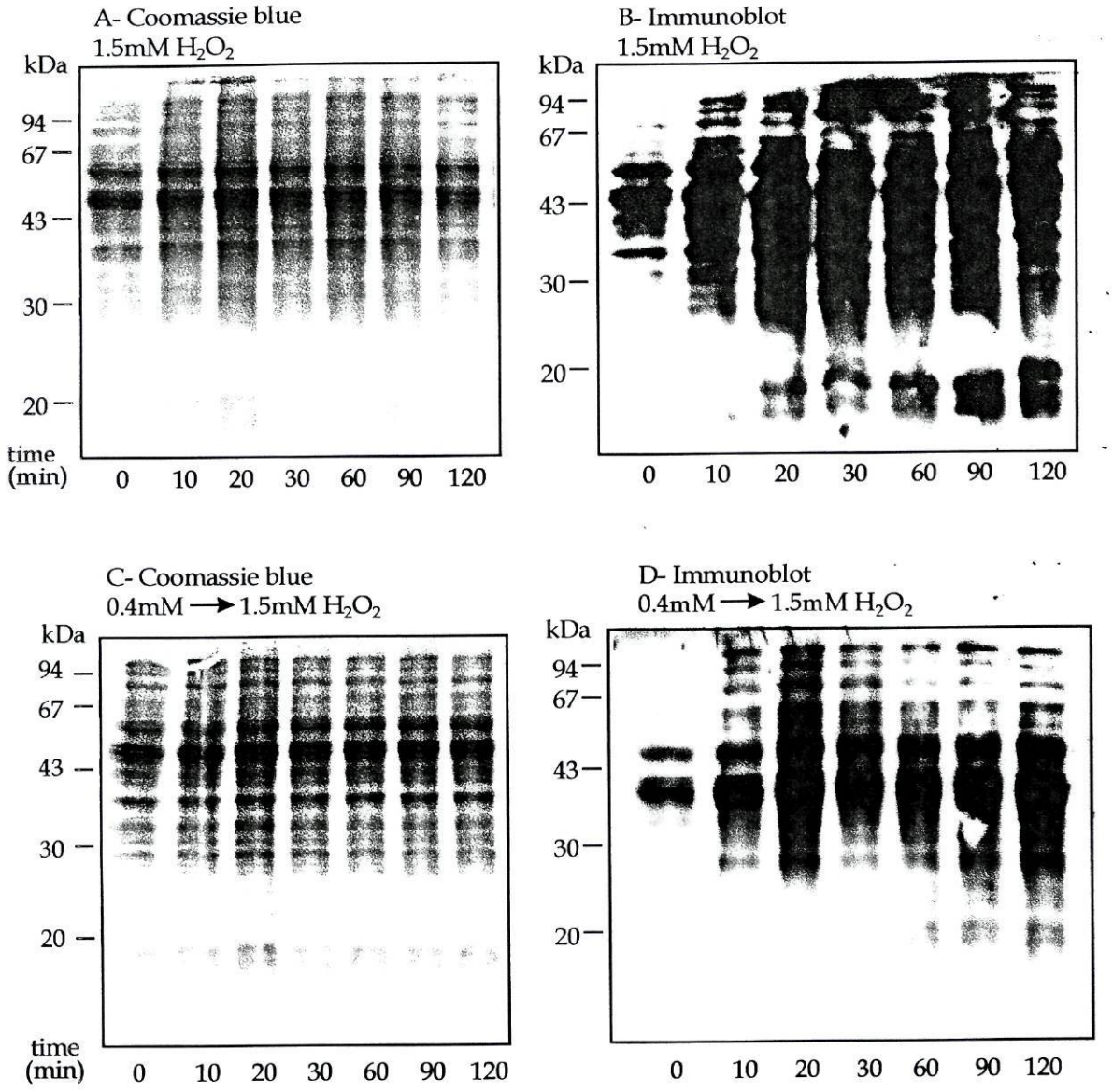

Fig. 2. Analysis by SDS-PAGE of proteins from *S. cerevisiae* W303 cells growing in minimal medium. Exponential phase cells (A and B) and cells pre-exposed to 0.4mM H_2O_2 for 30 min (C and D) were exposed to 1.5mM H_2O_2. A, C- Coomassie blue staining. B, D- Immunodetection of protein carbonyls. Proteins were isolated, derivatised, and separated by SDS-PAGE, as described in Methods. A replica gel was used to blot the proteins into nitrocellulose. Immunodetection was performed using anti-DNP antibodies, as described in Methods. The experiment was reproduced three times, using independent samples. A representative gel/blot is shown.

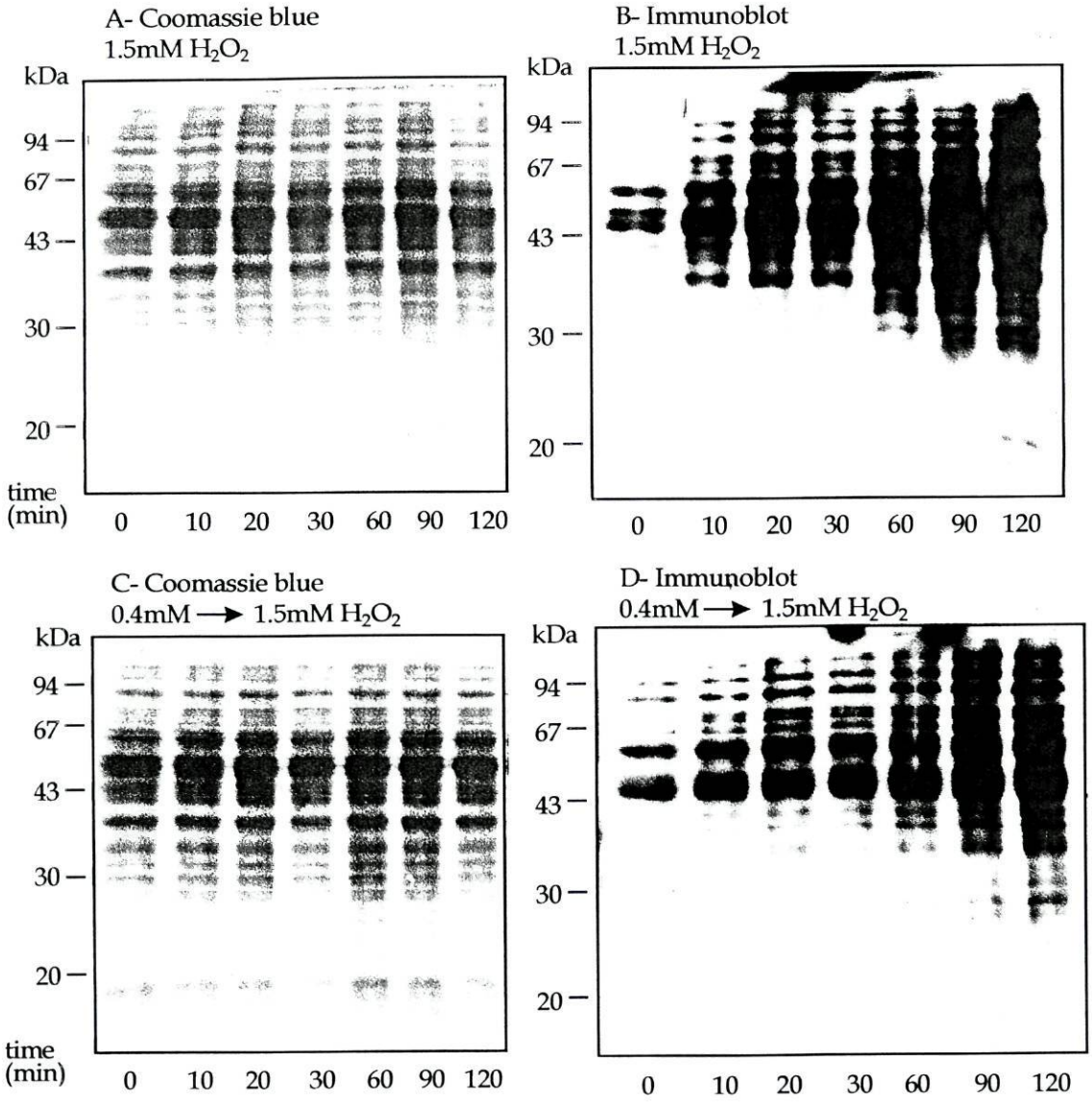

Fig. 3. Analysis by SDS-PAGE of proteins from *S. cerevisiae yap1Δyap2Δ* cells growing in minimal medium. Exponential phase cells (A and B) and cells pre-exposed to 0.4mM H_2O_2 for 30 min (C and D) were exposed to 1.5mM H_2O_2. A, C- Coomassie blue staining. B, D- Immunodetection of protein carbonyls. Samples were processed as described in Fig. 2. The experiment was reproduced three times, using independent samples. A representative gel/blot is shown.

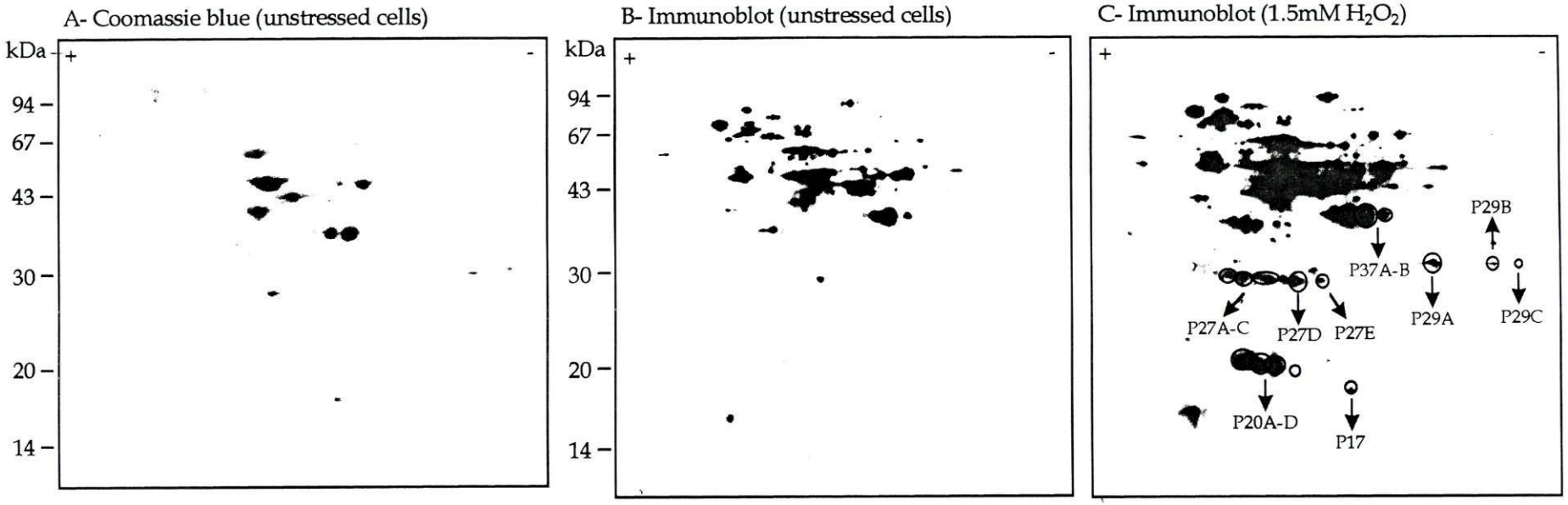

Fig. 4. Two-dimensional analysis of proteins from exponential phase S. cerevisiae W303 cells growing in minimal medium. A- Coomassie blue staining of proteins from unstressed cells. A similar pattern was obtained for proteins from cells exposed to 1.5mM H_2O_2. B, C- Immunodetection of carbonyls in proteins from unstressed cells (B) or from cells exposed to 1.5mM H_2O_2 for 60 min (C). Proteins were isolated, derivatised, separated by two-dimensional gel electrophoresis, and blotted into nitrocellulose. Immunodetection was performed using anti-DNP antibodies, as described in Methods. The experiment was reproduced three times, using independent samples. A representative gel/blot is shown.

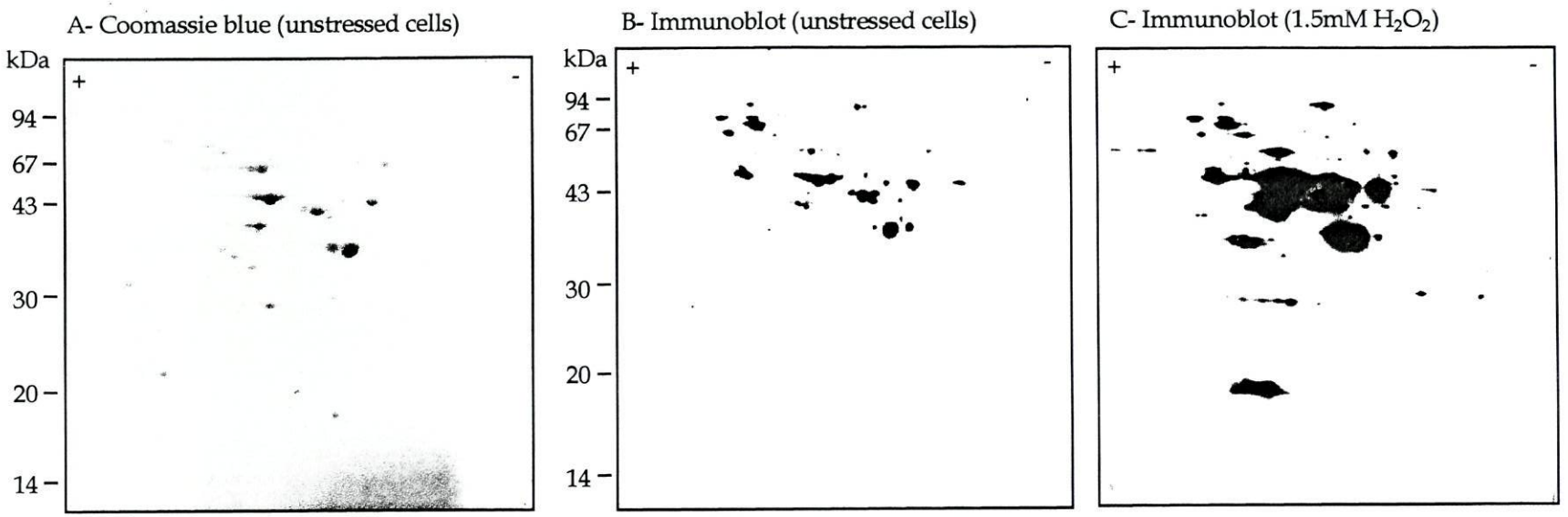

Fig. 5. Two-dimensional analysis of proteins from exponential phase *S. cerevisiae* W303 *yap1*Δ*yap2*Δ cells growing in minimal medium. A- Coomassie blue staining of proteins from unstressed cells. A similar pattern was obtained for proteins from cells exposed to 1.5mM H_2O_2. B, C- Immunodetection of carbonyls in proteins from unstressed cells (B) or from cells exposed to 1.5mM H_2O_2 for 60 min (C). Proteins were isolated, derivatised, separated by two-dimensional gel electrophoresis, and blotted into nitrocellulose. Immunodetection was performed using anti-DNP antibodies, as described in Methods. The experiment was reproduced three times, using independent samples. A representative gel/blot is shown.

Ethanol does not increase malondialdehyde formation

We have previously suggested that ethanol induces the production of ROS in *S. cerevisiae* (Costa *et al.*, 1993, 1997). It was conceivable, therefore, that ethanol might induce oxidative damages, and to evaluate lipid peroxidation, MDA concentration of exponential cells exposed to 14% ethanol was determined. Our results show that ethanol did not increase MDA formation (Fig. 6). To confirm the lack of correlation between ethanol toxicity and the production of MDA, we analysed MDA levels in cells deficient in Mn-superoxide dismutase (*sod2*Δ), which are hypersensitive to ethanol (Costa *et al.*, 1993, 1997). As observed in wild type cells, ethanol did no affect MDA concentration of *sod2*Δ cells (Fig. 6).

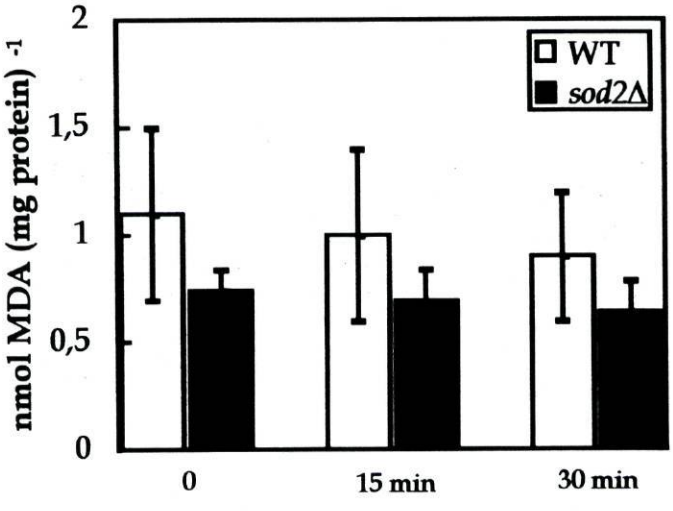

Fig. 6. Analysis of malondialdehyde (MDA) concentration in *S. cerevisiae* cells exposed to ethanol. Exponential phase cells growing in YPD were exposed to 14% ethanol for 60 min. Data are expressed as mean values ± SD of at least five independent experiments.

Ethanol induces a mild oxidation of a subset of proteins

Aiming to investigate if protein oxidation contributes to ethanol toxicity in *S. cerevisiae*, we analysed carbonyl levels in cells exposed to 14% ethanol. We did not find any major differences in carbonyls of proteins separated by SDS-PAGE, even in *sod2*Δ cells (Fig. 7B).

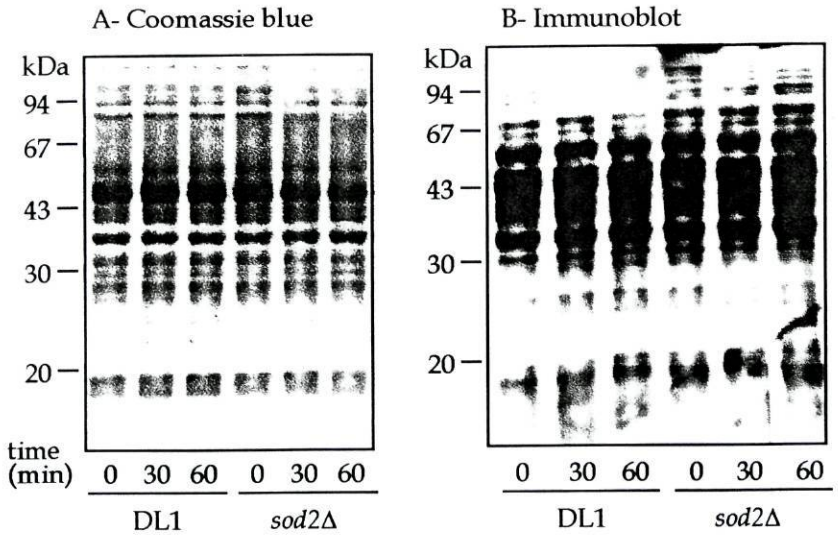

Fig. 7. Analysis by SDS-PAGE of proteins from *S. cerevisiae* DL1 and *sod2Δ* cells exposed to ethanol. Exponential phase cells growing in YPD were exposed to 14% ethanol. A- Coomassie blue staining. B- Immunodetection of protein carbonyls. Samples were processed as described in Fig. 2. 0-, negative control (sample 0 treated only with 10% trifluoroacetic acid). The experiment was reproduced three times, using independent samples. A representative gel/blot is shown.

The analysis of carbonyls after two-dimensional separation of the proteins revealed, however, an increase in the intensity of 9 spots induced by ethanol, corresponding to proteins with a molecular mass between 40 and 90 kDa (Fig. 8B and C). The low intensity of these 9 spots, compared to the intensity of the protein spots already present in control, untreated cells, might explain the similar patterns obtained by SDS-PAGE.

DISCUSSION

The identification of the molecular targets affected by oxidative stress and, thus, the cellular functions impaired, as well as the characterisation of the cellular defences protecting yeast cells from oxidative damages, is of critical importance in biotechnology, since the production of ROS has been associated with stress conditions that yeast must face during industrial processes (Attfield, 1997).

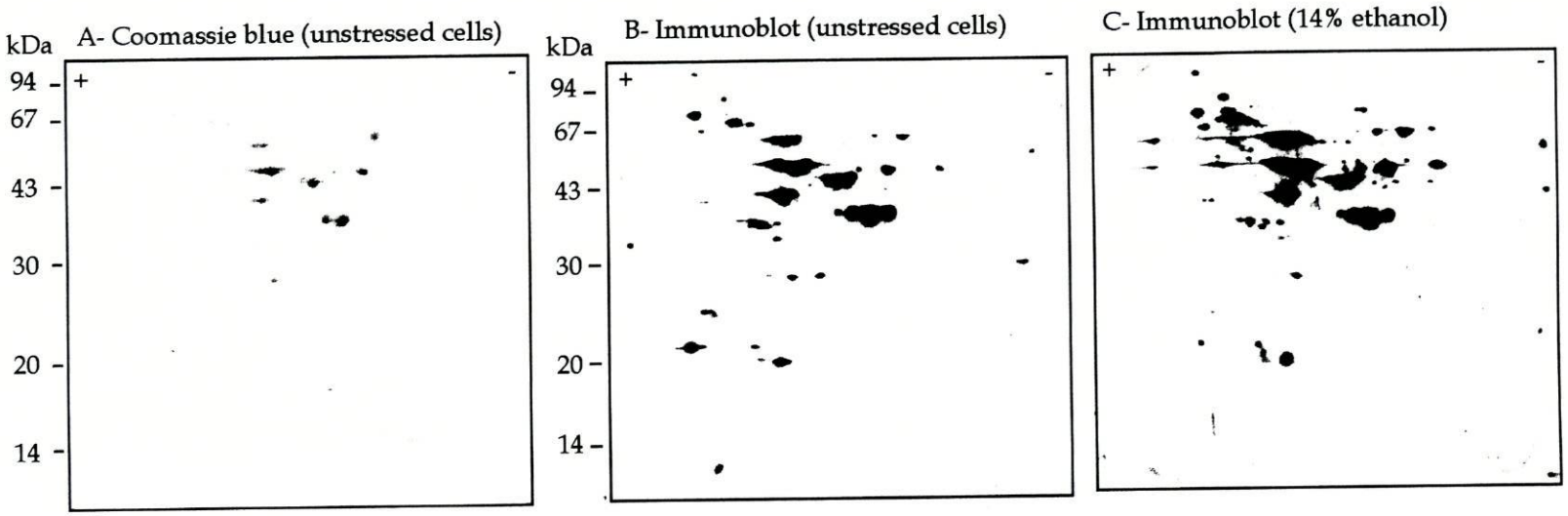

Fig. 8. Two-dimensional analysis of proteins from exponential phase *S. cerevisiae* DL1 cells growing in YPD. A- Coomassie blue staining of proteins from unstressed cells. A similar pattern was obtained for proteins from cells exposed to 14% ethanol. B, C- Immunodetection of carbonyls in proteins from unstressed cells (B) or from cells exposed to 14% ethanol for 60 min (C). Samples were processed as described in Fig. 4. The experiment was reproduced three times, using independent samples. A representative gel/blot is shown.

In this study, we analysed pro-oxidant effects of H_2O_2 and ethanol in *S. cerevisiae*, namely MDA concentration and protein carbonyls, as an index of lipid peroxidation and protein oxidation, respectively. It was previously shown that H_2O_2 increases MDA production (Steels *et al.*, 1994). Aiming to investigate the importance of lipid peroxidation on H_2O_2 toxicity, we determined MDA levels in cells hypersensitive to H_2O_2, namely *yap1Δ* and *yap1Δyap2Δ* mutants (Schnell *et al.*, 1992; chapter 2). Our results indicate that there is no correlation between H_2O_2 sensitivity (higher) and MDA production (similar) in *yap1Δ vs* wild type cells, and suggest that MDA is not a major factor in H_2O_2-induced cells death. Supporting this hypothesis is the observation that MDA pre-treatment does not induce any adaptative response to a subsequent H_2O_2 treatment and *vice versa* (Turton *et al.*, 1997). Nevertheless, we cannot rule out the hypothesis that H_2O_2 toxicity is correlated with lipid peroxidation, as MDA is one of many products generated during this highly complex, autocatalytic process (Halliwell and Guteridge, 1989). It was interesting to note a correlation between MDA production and H_2O_2 sensitivity in *yap1Δyap2Δ* double mutants: MDA levels were significantly higher than those observed in *yap1Δ* mutants, and H_2O_2 resistance of *yap1Δyap2Δ* double mutants is lower, compared to that of *yap1Δ* cells (chapter 2). These results suggest that Yap1p and Yap2p are both required for some protection from H_2O_2-induced MDA formation or for MDA detoxification. The higher production of MDA in *yap1Δyap2Δ* double mutants can be due to a deficient capacity to intercept ROS or to repair oxidised molecules. The role of Yap1p as a key mediator of oxidative stress tolerance is well established, since Yap1p directly regulates the transcription of at least five genes involved in antioxidant protection: *GSH1, GLR1, YCF1, TRX2,* and *TRR1,* encoding glutathione synthetase, glutathione reductase, a glutathione S-conjugate transporter, thioredoxin, and thioredoxin reductase, respectively (Wu and Moye-Rowley, 1994; Grant *et al.*, 1996b; Wemmie *et al.*, 1994a; Kuge and Jones, 1994; Morgan *et al.*, 1997). In addition, *YAP1* overexpression increases mRNA levels for nine additional genes that belong to the functional group of

oxidoreductases and might have an important protective role during oxidative stress (DeRisi *et al.*, 1997). The identification of Yap2p-dependent genes encoding antioxidant defences will help understand the role of Yap2p in oxidative stress resistance. It was recently shown that glutathione is essential for MDA resistance (Turton *et al.*, 1997); however, Yap2p does not regulate the transcription of *GSH1* gene, which encodes the rate limiting enzyme in glutathione biosynthesis (Stephen and Jamieson, 1997), indicating that glutathione deficiency cannot account for the increased H_2O_2 sensitivity of *yap1Δyap2Δ* mutants, compared to that of *yap1Δ* cells. The only genes so far identified as being regulated by both Yap1p and Yap2p are *PDR5* and *SNQ2*, encoding multidrug resistance ABC transporters, which are activated in response to heat shock; however, it is unlikely that they play an important role in MDA detoxification, as disruption of *PDR5* or *SNQ2* does not increase oxidative stress sensitivity (Miyahara *et al.*, 1996).

It was previously shown that H_2O_2 oxidises methionine residues in proteins (Moskovitz *et al.*, 1997); however, the identification of the proteins which are oxidised during H_2O_2 exposure has not been reported. Our results show that H_2O_2 induces a severe, non specific protein oxidation. Exposure of proteins from control cells to H_2O_2 *in vitro* did not induce protein oxidation (data not shown), indicating that carbonyl derivatives are not due to H_2O_2 *per se*. Hydroxyl radicals, produced by the metal-dependent (Fe^{2+} or Cu^{+}) decomposition of hydrogen peroxide, via the Fenton reaction, are probably responsible for the oxidation. A sublethal H_2O_2 pretreatment, which increases cellular resistance to a subsequent lethal stress (Collinson and Dawes, 1992), decreased but did not prevent carbonyl formation, suggesting a partial correlation between protein oxidation and H_2O_2-induced cell death. The accumulation of oxidised proteins does not seems to be, however, a major factor contributing to H_2O_2 toxicity. Indeed, *yap1Δyap2Δ* double mutants did not accumulate higher levels of carbonyls than wild type cells, when exposed to H_2O_2, in spite of their higher sensitivity to H_2O_2. These results rule out the

involvement of Yap1p and Yap2p in the transcriptional activation of genes encoding antioxidant defences that function in the repair of oxidised proteins.

The non-specific protein oxidation induced by H_2O_2 indicates that almost all metabolic functions are affected; however, some proteins seem to be more extensively modified, and, therefore, their cellular functions, and the metabolic pathways in which they are involved, are severely reduced. We have identified three of these proteins as being cyclophilin (Cph1p), triosephosphate isomerase (Tpi1p), and phosphoglycerate mutase (Pmg1p). Cyclophilins accelerate the folding of proteins, by catalysing cis-trans isomerisation of proline imidic peptide bonds (Craig *et al.*, 1993). Cph1p is not essential for cellular viability (Dolinski *et al.*, 1997); however, it has an important function during heat shock, as *CPH1* expression is induced by heat shock, and *CPH1* disruption decreases survival of the cells at high temperatures (Sykes *et al.*, 1993). Whether Cph1p is important for oxidative stress resistance remains to be clarified. Regarding Tpi1p and Pmg1p, both are glycolytic enzymes and, as expected, the growth of *tpi1*Δ and *pmg1*Δ mutants is severely inhibited by glucose (Krieger and Ernst, 1994; Gamo *et al.*, 1994). The oxidative modifications of Tpi1p and Pmg1p in cells growing on glucose may, therefore, have similar consequences, being a major factor contributing to H_2O_2-induced cell death.

Ethanol, unlike H_2O_2, is not an oxidant *per se*; however, a number of results suggest that ethanol increase ROS production: yeast cells enhance the activity of antioxidant defences, such as MnSOD and catalase T, in response to ethanol, and respiratory deficiency suppresses ethanol sensitivity of *sod2*Δ cells (Wieser *et al.*, 1991; Costa *et al.*, 1993, 1997). The induction of oxidative damages by ethanol is, therefore, likely to occur, and may contribute to protein denaturation and membrane leakage associated with ethanol toxicity (Casey and Ingledew, 1986; Piper, 1995). Our data show that ethanol, in contrast to H_2O_2, does not increase MDA production, even in *sod2*Δ cells, which are hypersensitive to ethanol. Although these results cannot rule out the hypothesis that ethanol causes lipid peroxidation, it does not seems to be a major factor in ethanol toxicity. Protein oxidation may, however, contribute to ethanol-induced

cell death. Indeed, we could detect a mild oxidation of a nine proteins. An increase of carbonyls levels has also been described in circulating proteins of alcoholics (Grattagliano *et al.*, 1996) and in the liver, testis and gastrointestinal mucosa of rats (Rouach *et al.*, 1997; Grattagliano *et al.*, 1997; Bagchi *et al.*, 1998), and results from the ethanol-induced production of superoxide and hydroxyl radicals (Bagchi *et al.*, 1998). The identification of the proteins oxidised during exposure to ethanol, and the analysis of their role in ethanol resistance, will contribute to our understanding of the molecular mechanisms underlying ethanol toxicity.

Conclusions and perspectives

It is well established that ethanol, one of the major end products of sugar fermentation, is toxic to yeast cells (van Uden, 1984). Cellular membranes seem to be preferential targets of ethanol, which interposes in lipids, decreasing the strength of lipophilic interactions between fatty acids and, therefore, membrane integrity (Prasad, 1985; Prasad and Rose, 1986). As a consequence, ethanol affects the activity of membrane proteins, inhibiting the transport of amino acids and sugars, and decreasing growth and fermentations rates (Thomas and Rose, 1979; Leão and van Uden, 1982, 1984; Carlson *et al.*, 1991).

The identification of factors able to reduce cellular damages induced by ethanol is of critical importance in biotechnological applications, as it will allow manipulation of yeasts in order to increase their industrial performance. A number of stress proteins are induced during the adaptive response to ethanol, including Hsp26 (Piper *et al.*, 1994), Hsp30 (Panaretou and Piper, 1992), Hsp70 (Piper *et al.*, 1994), Hsp104 (Sanchez *et al.*, 1992), and catalase T (Belazzi *et al.*, 1991; Wieser *et al.*, 1991); however, only Hsp104 is essential for the acquisition of ethanol tolerance. Moreover, the plasma membrane ATPase activity (Panaretou and Piper, 1990; Rosa and Sá-Correia, 1996), the accumulation of trehalose (Odumeru *et al.*, 1993), and changes in lipid composition of membranes, such as the increase of fatty acyl chain length, and of the concentration of ergosterol and mono-unsaturated fatty acids (Lees *et al.*, 1980; Beaven *et al.*, 1982; Del Castillo Agudo, 1992; Arneborg *et al.*, 1995; Kajiwara *et al.*, 1996), are also important for ethanol tolerance (Piper, 1995). Hsp104 probably promotes the resolubilisation and reactivation of proteins that have unfolded and aggregated during exposure to ethanol, as shown for heat shock (Parsell *et al.*, 1994). Trehalose is a disacharide that stabilises proteins and prevents their inactivation (Colaço *et al.*, 1992; Hottiger *et al.*, 1994). Hsp104 and trehalose functions seem, therefore, to complement each other, and it was recently shown that they have a synergistic protective role in thermotolerance (Elliot *et al.*, 1996).

This work aimed to contribute to our understanding of the molecular mechanisms underlying the acquisition of ethanol tolerance in *Saccharomyces*

cerevisiae. We have investigated the role of antioxidant defences, namely glutathione and superoxide dismutases, in resistance to ethanol, and searched for evidences that oxidative stress mediates ethanol toxicity in *Saccharomyces cerevisiae* cells.

Glutathione, in its reduced form, can function as a primary antioxidant defence, by scavenging reactive oxygen species, and as a secondary defence, by reducing disulphide bonds in oxidised proteins, or alkyl hydroperoxides produced during lipid peroxidation (reaction catalysed by glutathione peroxidase). Oxidised glutathione generated in these reactions is reduced by glutathione reductase. It has been shown that glutathione and glutathione reductase are induced by superoxide radicals or hydrogen peroxide, and both play an important role in the acquisition of oxidative stress resistance (Stephen *et al.*, 1995; Izawa *et al.*, 1995; Stephen and Jamieson, 1996, 1997; Grant *et al.*, 1996b; Izawa *et al.*, 1998). In contrast, our results clearly show that glutathione has no major role in the protection of yeast cells from ethanol toxicity. Glutathione levels remain constant in exponential phase cells exposed to a sublethal heat shock or ethanol stress, or when yeast cells grow to the post-diauxic phase, conditions that significantly increase ethanol resistance. Furthermore, mutants deficient in glutathione (*gsh1*Δ) are able to increase ethanol tolerance to levels similar to those of wild type cells.

Superoxide dismutases are a family of metalloproteins, which catalyse the dismutation of superoxide radicals into molecular oxygen and hydrogen peroxide. Yeast cells contain a cytosolic superoxide dismutase (CuZnSOD), encoded by *SOD1* gene, and a mitochondrial superoxide dismutase (MnSOD), encoded by *SOD2* gene. The expression of *SOD1* and *SOD2* genes is induced by oxidative stress conditions, such as hyperoxia, superoxide radicals, and hydrogen peroxide (Gregory *et al.*, 1974; Lee and Hassan, 1985; Westerbeek-Marres *et al.*, 1988; Schnell *et al.*, 1992; Galiazzo and Labbe-Bois, 1993; Jamieson *et al.*, 1994; chapter 2), and superoxide dismutases are essential for resistance to these oxidants (Bilinski *et al.*, 1985; van Loon *et al.*, 1986; Zhu and Scandalios, 1992). Our results show that ethanol resistance is specifically dependent on the

mitochondrial superoxide dismutase, either at the exponential or at the post-diauxic phase. As superoxide dismutases reduce superoxide radicals, which are mainly produced as by-products of mitochondrial respiration, it was suggested that ethanol enhances mitochondrial production of superoxide radicals. Consistent with this model, we have shown that respiratory deficiency significantly increases ethanol resistance of *sod2*Δ mutants. The molecular mechanism underlying the increased production of superoxide radicals in the mitochondria is not known. It may be due to uncoupling of the respiratory chain, caused by disorganisation of mitochondrial membranes induced by ethanol, or to an increase in $NADH/NAD^+$ ratio, which enhances electron flow in the respiratory chain, favouring the generation of superoxide radicals, as shown for mammalian cells (Nordmann *et al.*, 1992). In *S. cerevisiae* cells, *ADH2* gene, encoding alcohol dehydrogenase 2 that converts ethanol to acetaldehyde, is under glucose repression, being derepressed at the diauxic shift phase (Boy-Marcotte *et al.*, 1998). Ethanol may, therefore, increase $NADH/NAD^+$ ratio at the post-diauxic phase; however, that hypothesis can only be considered at the exponential phase if ethanol derepresses *ADH2* gene expression, which remains to be demonstrated. In that case, it would be important identify the factor(s) involved in the transcriptional activation of *ADH2* gene, and evaluate ethanol resistance in cells deficient in that factor or in Adh2p.

It was suggested that the compartmentalisation of MnSOD in mitochondria, where most of the superoxide radicals are produced, increases the efficiency of superoxide trapping, preventing its flux to the cytosol and, therefore, decreasing cellular damages. This hypothesis is supported by two observations: a) MnSOD activity increases 4 fold in exponential phase *sod1*Δ cells, resulting in a higher ethanol resistance; and b) despite the higher CuZnSOD activity of *sod2*Δ cells, these cells are hypersensitive to ethanol.

We have previously shown that the induction of MnSOD activity by heat shock is due to the synthesis *de novo* of the protein, whereas the induction by 8% ethanol involves post-transcriptional regulatory mechanisms and/or post-translational activation of the protein (Costa *et al.*, 1997). Similar mechanisms

are involved in the regulation of *SOD* gene expression during growth from the exponential to the post-diauxic phase. The activity of both superoxide dismutases increases during growth; however, the induction of CuZnSOD and MnSOD activity during growth from the exponential to the diauxic-shift phase is due to the synthesis *de novo* of the proteins, whereas their induction during growth from the diauxic-shift to the post-diauxic phase involves post-transcriptional regulatory mechanisms or post-translational activation of the proteins. Post-transcriptional regulatory mechanisms have been shown to occur in yeast cells. An increased translatability of mRNA-*RAS2*, mRNA-*ENO1*, mRNA-*RPB4* and mRNA-*BCY1*, and post-translational modifications of Bcy1p was observed as cells grow to post-diauxic phase (Brevario *et al.*, 1988; Jigami *et al.*, 1986; Werner-Washburne *et al.*, 1991). Furthermore, the CuZnSOD apoprotein is post-translationaly activated by copper during aeration of hypoxic cells (Galiazzo *et al.*, 1991), probably mediated by the copper chaperone Lys7p (Culotta *et al.*, 1997; Gamonet and Lauquin, 1998). As hypoxic cells accumulate higher levels of copper from the medium than aerobic cells (Strain and Cullota, 1996), Lys7p might be the limiting factor under hypoxic conditions that prevents the activation of CuZnSOD. Several hypothesis can be suggested as putative mechanisms involved in the induction of MnSOD activity by ethanol (in the exponential phase), and in the increase of CuZnSOD and MnSOD activity, during growth from the diauxic shift to the post-diauxic phase: a) post-translational activation of the apoproteins, mediated by metal chaperones (Lys7p for copper; unidentified protein(s) for manganese); b) post-translational modification of the proteins; and c) increased translation of mRNA-*SOD1* and mRNA-*SOD2*.

Despite being hypersensitive to ethanol, exponential phase *sod2*Δ mutants are able to slightly increase ethanol resistance when pretreated with a mild stress, or when they grow to the post-diauxic phase, indicating that other factors are involved in the acquisition of ethanol tolerance. One of these factors might be CuZnSOD, as *sod2*Δ cells, in contrast with wild type cells, adapt to ethanol by increasing CuZnSOD activity, either at the exponential or at the

post-diauxic phase. It is conceivable that CuZnSOD partially complements the deficiency of MnSOD activity, by scavenging, in the cytosol, superoxide radicals that escape from the mitochondria. It would be interesting to investigate if *SOD1* overexpression enhances the capacity of *sod2Δ* mutants to resist ethanol, and if *SOD1* disruption has the opposite effect. Catalase T, or other antioxidant defences able to decompose the H_2O_2 produced by dismutation of superoxide radicals catalysed by MnSOD or CuZnSOD, may also have an important role in ethanol resistance. In fact, the highest ethanol tolerance is achieved by the coordinated action of MnSOD and catalase T. The induction of *CTT1* gene expression and MnSOD activity is higher when exponential phase cells are exposed to a sublethal ethanol than in those exposed to a heat shock, and ethanol pretreatment confers higher ethanol tolerance than heat shock (Wieser *et al.*, 1991; Costa *et al.*, 1993; Schuller *et al.*, 1994). Moreover, MnSOD activity is higher at the post-diauxic phase than at the diauxic shift phase, and *CTT1* gene expression is only derepressed at the post-diauxic phase (Belazzi *et al.*, 1991), these cells being more tolerant to ethanol than diauxic shift cells. Plasma membrane H^+-ATPase, encoded by *PMA1* gene, and Hsp104 may also contribute to the acquisition of ethanol tolerance in *sod2Δ* cells. The H^+-ATPase pumps protons from the cytosol to the extracellular space, playing a critical role in the control of intracellular pH (Serrano, 1993). The induction of H^+-ATPase activity counteracts the intracellular acidification caused by ethanol, decreasing its inhibitory effects (Rosa and Sá-Correia, 1996). Hsp104 synthesis is also induced by ethanol, as well as during growth to the post-diauxic phase, being required for ethanol tolerance (Sanchez *et al.*, 1992; Werner-Washburne *et al.*, 1996). Hsp104 probably reactivates proteins unfolded by ethanol *per se*. Protein unfolding may also be due to oxidative modifications caused by reactive oxygen species generated during exposure to ethanol. In fact, we have shown that ethanol induces protein oxidation. It is, however, unlikely that Hsp104 promotes the reactivation of oxidised proteins, as they are irreversible modified (only methionine sulfoxide and disulphide bonds can be reduced to methionine and cysteine, respectively), being most probably

degraded by the ubiquitin/proteasome system. Consistent with this hypothesis, *UBI4* gene expression is induced by ethanol (Piper *et al.*, 1994), and mutants deficient in ubiquitin-conjugating enzymes have a higher sensitivity to ethanol (V. Costa and P. Moradas-Ferreira, unpublished).

It has been suggested that the increase of MnSOD activity may be directly or indirectly regulated by mitochondrial superoxide radicals flux *per se* (Hassan and Fridovich, 1977; Freeman and Crapo, 1981; Moody and Hassan, 1984; Westerbeek-Marres *et al.*, 1988). Our results support this hypothesis, as the increase of mRNA-*SOD2* levels, during heat shock, and the induction of MnSOD activity, by heat shock or 8% ethanol, are lower or no longer occur in respiration deficient cells. We also raised the hypothesis that H_2O_2, generated during dismutation of superoxide radicals, might mediate the induction of MnSOD activity by ethanol. This is not the case, as the increase of MnSOD activity in exponential phase cells exposed to H_2O_2 is due to an increased expression of *SOD2* gene. *SOD1* expression is also enhanced by H_2O_2. Notably, we have shown that H_2O_2-mediated transcriptional activation of *SOD1* is mediated by Yap1p, a bZIP transcription factor that plays a critical role in the protection against oxidative stress conditions, including H_2O_2, by directly regulating the expression of genes encoding antioxidant defences, such as *TRX2* (thioredoxin 2), *TRR1* (thioredoxin reductase), *GSH1* (γ-glutamylcysteine synthetase) and *GLR1* (glutathione reductase) (Kuge and Jones, 1994; Wu and Moye-Rowley, 1994; Grant *et al.*, 1996b; Morgan *et al.*, 1997). The regulation of *SOD1* expression by expression by Yap1p is consistent with the Yap1p-binding site present in *SOD1* gene promoter. Our results also suggest that Yap1p may be indirectly involved in post-translational activation of CuZnSOD, under physiological conditions. Whether this effect is mediated by the copper chaperone Lys7p has yet to be established; however, this is a strong possibility, since *LYS7* promoter contains a Yap1p-binding site (Horecka *et al.*, 1995).

Strikingly, the transcriptional activation of *SOD2* gene induced by H_2O_2 is partially dependent on Yap1p, in spite of the lack of a Yap1p-binding site in the promoter region. Moreover, Yap2p, a transcription factor of the Yap family

that shows strong homology to Yap1p (Bossier *et al.*, 1993), seems to have an important role by sustaining the induction of *SOD2* gene expression. These results suggest that Yap1p has an important role in early responses, while Yap2p is involved in late responses. Whether Yap1p and Yap2p dependent regulation of *SOD2* gene expression is mediated by the STRE present in its promoter, as suggested by Gounalaki and Thireos (1994) for *TPS2* gene, is unknown. A significant increase of mRNA-*SOD2* levels is still observed in *yap1Δ* and *yap1Δyap2Δ* cells exposed to H_2O_2, and might be mediated by Msn2p, Msn4p, or other unidentified transcription factor(s) that bind the STRE (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996). Our results also indicate an important, overlapping role of Yap1p and Yap2p in the post-translational activation of MnSOD during exposure to H_2O_2. The increase of MnSOD activity during H_2O_2 treatment is similar in wild type and *yap1Δ* or *yap2Δ* single mutants; however, it is abolished in *yap1Δyap2Δ* cells, despite the transient induction of mRNA-*SOD2*. The identification of manganese chaperones, or other factors mediating activation of MnSOD, will be an important challenge for a future work.

The impaired induction of MnSOD activity in *yap1Δyap2Δ* cells is correlated with a higher sensitivity of these mutants to 1.5mM H_2O_2. In fact, the low H_2O_2 tolerance of *yap1Δ* cells is further decreased by *YAP2* disruption. Moreover, despite the acquisition of H_2O_2 resistance by 0.1mM H_2O_2 pretreatment, the viability of *yap1Δyap2Δ* cells is severely reduced. These results indicate that Yap2p contributes to H_2O_2 resistance of *yap1Δ* cells. In agreement with these data, we raised the hypothesis that the higher sensitivity of *yap1Δ* and *yap1Δyap2Δ* mutants could result from the accumulation of oxidative damages. Both lipid peroxidation and protein oxidation were evaluated, by analysis of malondialdehyde concentration and carbonyl derivatives, respectively. Our results show no correlation between lipid peroxidation, when assessed by analysis of malondialdehyde concentration, and the higher sensitivity of *yap1Δ* cells to H_2O_2, suggesting that lipid

peroxidation is not a major factor contributing to the increased cell death. Supporting this hypothesis is the observation that malondialdehyde pretreatment does not induce any adaptative response to a subsequent H_2O_2 treatment, and *vice versa* (Turton *et al.*, 1997). We could find, however, a correlation between malondialdehyde production and the higher sensitivity of *yap1Δyap2Δ* mutants. These results suggest that Yap1p and Yap2p are both required for some protection from H_2O_2-induced malondialdehyde formation or for malondialdehyde detoxification. The role of Yap1p in the regulation of genes encoding antioxidant defences that intercept reactive oxygen species has been characterised in recent years; however, the role of Yap2p in oxidative stress response remains unknown. The identification of Yap1p- and Yap2p-dependent genes that encode defences involved in the repair of oxidised lipids, or in the detoxification of lipid peroxidation products, will be a major contribution to the understanding of the molecular mechanisms underlying oxidative stress protection.

The analysis of protein oxidation shows that H_2O_2 induces a severe, non specific protein oxidation. Oxidative modification of the proteins is probably due to hydroxyl radicals, produced by the metal-dependent (Fe^{2+} or Cu^{+}) decomposition of H_2O_2, via the Fenton reaction, since H_2O_2 *per se* does not induce carbonyls formation. Some of the proteins more extensively modified were identified as being cyclophilin (Cph1p), triosephosphate isomerase (Tpi1p), and phosphoglycerate mutase (Pmg1p). The function of these proteins, and the metabolic pathways in which they are involved, is severely reduced. Cph1p is a not essential chaperone (Dolinski *et al.*, 1997), which accelerates the folding of proteins, by catalysing cis-trans isomerisation of proline imidic peptide bonds (Craig *et al.*, 1993). Whether Cph1p is important for oxidative stress resistance, as it was shown for thermotolerance (Sykes *et al.*, 1993), remains to be clarified. Tpi1p and Pmg1p are glycolytic enzymes, being essential for growth on glucose (Krieger and Ernst, 1994; Gamo *et al.*, 1994). The oxidative modifications of Tpi1p and Pmg1p in cells growing on glucose may impair ATP production, decreasing the capacity of yeast cells to adapt and

recover from stress. The inhibition of glycolysis is likely to be a major factor contributing to H_2O_2-induced cell death. The H_2O_2-induced protein oxidation is attenuated but not protected by a sublethal H_2O_2 pretreatment, which increases cellular resistance to a subsequent lethal stress (Collinson and Dawes, 1992). Although these results indicate a partial correlation between protein oxidation and H_2O_2-induced cell death, the accumulation of oxidised proteins does not seems to be a major determinant of H_2O_2 toxicity, as carbonyls formation is not enhanced in *yap1*Δ*yap2*Δ mutants, despite their high sensitivity to H_2O_2. The degradation of oxidised proteins may, however, be essential for recovery from stress, since the accumulation of abnormal proteins may be toxic to the cells. The polyubiquitin/proteasome system may have an important function in the turnover of oxidised proteins (Cheng *et al.*, 1994; Watt and Piper, 1997), and the characterisation of its role in the oxidative stress protection is required.

One of the aims of this work was to identify oxidative modifications induced by ethanol. Ethanol, unlike H_2O_2, is not an oxidant *per se*; however, our results suggest that ethanol increases the production of reactive oxygen species, and, therefore, could induce oxidative damages. Our data show that ethanol does not increase MDA production, even in *sod2*Δ cells, which are hypersensitive to ethanol, suggesting that lipid peroxidation is not involved in ethanol toxicity. An hypothesis that should be explored is that lipid peroxidation may be restricted to mitochondria, which has been suggested as a target for ethanol damage (Aguilera and Benitez, 1985; Sá-Correia and van Uden, 1986). In contrast, we found that ethanol induces a mild protein oxidation. Preliminary results suggest that exposure to 8% ethanol prevents protein oxidation during a subsequent exposure to lethal ethanol concentrations, indicating a correlation between ethanol toxicity and the accumulation of oxidised proteins. An increase of carbonyls levels has also been described in circulating proteins of alcoholics (Grattagliano *et al.*, 1996) and in the liver, testis and gastrointestinal mucosa of rats (Rouach *et al.*, 1997; Grattagliano *et al.*, 1997; Bagchi *et al.*, 1998), and results from the ethanol-induced production of superoxide and hydroxyl radicals (Bagchi *et al.*, 1998).

The identification of the proteins oxidised during exposure to ethanol, and the analysis of their role in ethanol resistance, will contribute to our understanding of the molecular mechanisms underlying ethanol toxicity.

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