Protein S-thiolation targets glycolysis and protein synthesis in response to oxidative stress in the yeast *Saccharomyces cerevisiae*

Daniel SHENTON and Chris M. GRANT[1]

Department of Biomolecular Sciences, University of Manchester Institute of Science and Technology (UMIST), P.O. Box 88, Manchester M60 1QD, U.K.

The irreversible oxidation of cysteine residues can be prevented by protein S-thiolation, a process by which protein SH groups form mixed disulphides with low-molecular-mass thiols such as glutathione. We report here the target proteins which are modified in yeast cells in response to H_2O_2. In particular, a range of glycolytic and related enzymes (Tdh3, Eno2, Adh1, Tpi1, Ald6 and Fba1), as well as translation factors (Tef2, Tef5, Nip1 and Rps5) are identified. The oxidative stress conditions used to induce S-thiolation are shown to inhibit GAPDH (glyceraldehyde-3-phosphate dehydrogenase), enolase and alcohol dehydrogenase activities, whereas they have no effect on aldolase, triose phosphate isomerase or aldehyde dehydrogenase activities. The inhibition of GAPDH, enolase and alcohol dehydrogenase is readily reversible once the oxidant is removed. In addition, we show that peroxide stress has little or no effect on glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase, the enzymes that catalyse NADPH production via the pentose phosphate pathway. Thus the inhibition of glycolytic flux is proposed to result in glucose equivalents entering the pentose phosphate pathway for the generation of NADPH. Radiolabelling is used to confirm that peroxide stress results in a rapid and reversible inhibition of protein synthesis. Furthermore, we show that glycolytic enzyme activities and protein synthesis are irreversibly inhibited in a mutant that lacks glutathione, and hence cannot modify proteins by S-thiolation. In summary, protein S-thiolation appears to serve an adaptive function during exposure to an oxidative stress by reprogramming metabolism and protecting protein synthesis against irreversible oxidation.

Key words: glutathione, oxidative stress, protein S-thiolation, regulation of metabolism, yeast.

INTRODUCTION

Thiol (SH) groups play a remarkably broad range of roles in the cell, with their redox state affecting the activity and structure of many enzymes, receptors and transcription factors. Not surprisingly therefore, all organisms contain complex regulatory machinery to maintain the redox states of SH groups in both proteins and low-molecular-mass thiols (reviewed in [1–3]. Cysteine SH groups are among the most easily oxidized residues in proteins, and oxidation can cause intermolecular protein cross-linking and enzyme inactivation, eventually leading to cell death [4]. Such irreversible oxidation events can be prevented by protein S-thiolation, in which protein SH groups form mixed disulphides with low-molecular-mass thiols such as GSH [5,6]. This post-translational modification may serve an antioxidant role in the protection of protein SH groups against irreversible oxidation, or alternatively may serve a regulatory role analogous to other post-translational modifications such as protein phosphorylation.

We have shown previously that the basal levels of S-thiolation in yeast are maintained at low levels, but are increased following treatment with oxidants [7]. In addition, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) has been identified as the major target of S-thiolation. This protein modification was found to be highly regulated, since, despite a high degree of sequence homology (98 %), the Tdh3 but not the Tdh2 GAPDH isoenzyme was found to undergo S-thiolation. A model was suggested where both enzymes are required for survival during conditions of oxidative stress, playing complementary roles depending on their ability to undergo S-thiolation [8]. Further evidence that protein S-thiolation is tightly regulated in response to oxidative stress conditions was provided by the finding that both GAPDH isoenzymes are S-thiolated when H_2O_2 is added to cell-free extracts [9]. This indicates that cellular factors are likely to be responsible for the difference in GAPDH S-thiolation observed *in vivo* rather than any intrinsic structural differences between the GAPDH isoenzymes. Furthermore, this protein modification is not a simple response to the cellular redox state, since different oxidants were found to lead to different patterns of protein S-thiolation. SDS/PAGE revealed that whereas GAPDH is the major target for modification following treatment with hydroperoxides, GAPDH is unaffected following oxidative stress induced by exposure to the thiol oxidant diamide [9].

Several studies have suggested that protein modification by S-thiolation may be involved in the regulation of protein function and activity, including the HIV-1 protease [10], ubiquitin-conjugating enzymes in bovine retina cells [11] and DNA binding by the transcription factor c-Jun [12]. Protein S-thiolation must therefore be reversible, and *in vitro* studies have shown dethiolation can occur via direct reduction by GSH, as well as enzymically via both glutaredoxins and thioredoxins [5,13]. Glutaredoxin appears to be the most active dethiolase *in vitro* and, in addition, a recent study has shown a correlation between protein–gluthione mixed disulphide reduction and glutaredoxin activity in mammalian cells [14]. However, the basal levels of protein S-thiolation are unaffected in yeast mutants lacking the classical glutaredoxins, encoded by *GRX1* and *GRX2* [15], but are elevated in mutants lacking the thioredoxins, encoded by *TRX1* and *TRX2* [16]. Yeast, like all eukaryotes, contains a family of

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6PDH, glucose-6-phosphate dehydrogenase; 6PGDH, 6-phosphogluconate dehydrogenase; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; MALDI-TOF MS, matrix-assisted laser-desorption ionization–time-of-flight MS.

[1] To whom correspondence should be addressed (e-mail chris.grant@umist.ac.uk).

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glutaredoxin-related proteins (GRX3–5) which contain a single cysteine residue at their active sites [17]. Thus they would be unable to substitute for glutaredoxins as disulphide reductases, which requires a dithiol mechanism, but they could reduce protein-mixed disulphides in a monothiol mechanism [17,18]. We have found that dethiolation of GAPDH is slower in a strain deleted for *GRX5* compared with the wild-type strain [9] and a *grx5* mutant accumulates mixed disulphides under non-stressed conditions (D. Shenton and C. M. Grant, unpublished work). It is unclear at present whether Trx1, Trx2 or Grx5 play a direct or indirect role in protein S-thiolation.

A growing list of proteins that become S-thiolated in response to cellular stress have been identified in mammalian cells. These include metabolic enzymes, as well as structural and transport proteins (reviewed in [6]). More recently, proteomic approaches have allowed the global identification of a range of target proteins [19–21]. There does not appear to be any one unifying feature of these proteins, and the fact that not all SH-containing proteins are thiolated in response to an oxidative stress confirms that this protein modification must be tightly regulated. In this present study, we have identified the yeast proteins which are S-thiolated in response to H_2O_2 stress. Most strikingly, a range of glycolytic and related enzymes, as well as a range of translation factors, are identified, and we show that oxidative stress reversibly inhibits both glycolysis and protein synthesis.

EXPERIMENTAL

Yeast strains and growth conditions

The *Saccharomyces cerevisiae* strains used in this study were the wild-type CY4 (*MATa ura3-52 leu2-3 leu2-112 trp1-1 ade2-1 his3-11 can1-100*) and an isogenic strain deleted for *GSH1* (*gsh1*::*LEU2*) [22]. Strains were grown in rich YEPD medium [2 % (w/v) glucose, 2 % (w/v) bactopeptone and 1 % (w/v) yeast extract] or minimal SD medium [0.17 % (w/v) yeast nitrogen base without amino acids, 5 % (w/v) $(NH_4)_2SO_4$ and 2 % (w/v) glucose] supplemented with appropriate amino acids and bases: 2 mM leucine, 0.3 mM histidine, 0.4 mM tryptophan, 0.15 mM adenine and 0.2 mM uracil. Media were solidified by the addition of 2 % (w/v) agar. Oxidant sensitivity was determined by growing cells to exponential phase in SD medium at 30 °C, and treating with H_2O_2 for 30 min. Aliquots of cells were diluted into fresh YEPD medium and plated in triplicate on YEPD plates to obtain viable counts after 3 days of growth.

Analysis of protein S-thiolation

The analysis of protein S-thiolation in yeast has been described previously [8]. Briefly, yeast cells were grown to exponential phase in minimal SD medium and treated with 50 μg/ml cycloheximide for 15 min to inhibit cytoplasmic protein synthesis. The intracellular pool of low-molecular-mass thiols was radiolabelled by incubating cells with approx. 2.5 nM L-[^{35}S]cysteine/methionine for 1 h. Cells were then washed and resuspended in fresh SD media with 2 mM H_2O_2 to induce S-thiolation. Cell-free extracts were prepared in 50 mM Tris buffer, pH 7.4, containing 1 × Complete™ Mini protease inhibitor cocktail (Roche), by breaking cells with glass beads using a Minibead Beater for 30 s at 4 °C. Parallel extracts were prepared in buffer containing either 50 mM NEM (*N*-ethylmaleimide) to prevent thiolation during the sample preparation, or 25 mM DTT (dithiothreitol) to reduce any S-thiolated proteins. Proteins were separated by SDS/PAGE and visualized using a Typhoon 8600 Imaging system (Amersham Biosciences).

Identification of S-thiolated proteins

Protein extracts, prepared as described above, were precipitated in three or four vol. of cold (− 20 °C) acetone and left on ice for 1 h. Proteins were pelleted in a microcentrifuge for 15 min before being resuspended in 8 M urea, 4 % CHAPS, 1 % dithioerythritol, 2.5 mM EDTA and 2.5 mM EGTA. Samples treated with NEM were resuspended in the above buffer minus dithioerythritol. Proteins were separated in the first dimension by isoelectric focusing using pH 3–10 non-linear Immobiline DryStrip gels run over approx. 26 h to 90 000 V · h at 20 °C on an IPGphor unit (Amersham Biosciences). The Immobiline DryStrip gels were then transferred to 10 % polyacrylamide gels and proteins separated using a Bio-Rad Protean II system for 20 h at 45 V/gel. Gels were stained using colloidal Coomassie Blue (Sigma) and dried using a Bio-Rad Gelair drier. Proteins of interest were excised from gels and peptide mass fingerprints were generated using MALDI-TOF MS (matrix-assisted laser-desorption ionization–time-of-flight MS). Proteins were identified using the Mascot mass fingerprinting programme (http://www.matrixscience.com) to search the NCBInr and Swissprot databases. Modification of cysteine residues by S-glutathiolation was searched for as a variable modification in the Mascot peptide fingerprint search. The GSH modification option was obtained from the UNIMOD protein modifications for MS database (http://www.unimod.org).

Enzyme assays

Cells were disrupted in 20 mM phosphate buffer, pH 7.4, containing 0.5 mM PMSF by breaking cells with glass beads using a Minibead Beater for 30 s at 4 °C. GAPDH assays were performed according to the method of McAlister and Holland [23]. Aldolase, triose phosphate isomerase, alcohol dehydrogenase and aldehyde dehydrogenase assays were performed as described in [24]. Enolase activity was measured as in [25]. G6PDH (glucose-6-phosphate dehydrogenase) activity was measured as described by [26] and 6PGDH (6-phosphogluconate dehydrogenase) as described by [27]. Activity is expressed as μmol of NADH or NADPH formed or oxidized/min per μg of protein.

Measurement of protein synthesis rate

To measure the rate of protein synthesis, exponential-phase cells were pulse-labelled for 5 min with 85 μM L-[^{35}S]cysteine/methionine. Aliquots of cells were lysed by boiling in 20 % (w/v) trichloroacetic acid for 10 min and then placing on ice for 10 min. Proteins were precipitated on to Whatman GF/C glass microfibre filters and washed using cold 20 % (w/v) trichloroacetic acid. Radioactive incorporation was measured by scintillation counting radioactivity retained on the filters and expressed as c.p.m./min per A_{600} unit.

RESULTS

Identification of targets of protein S-thiolation in yeast

The basal levels of protein S-thiolation in exponential-phase yeast cells were very low, but increased in response to H_2O_2 at concentrations between 0.5 and 4 mM [8]. The observed pattern of S-thiolation is similar throughout this concentration range, but is maximal following a treatment with 2 mM H_2O_2. Thiolation results in protein S-thiolation of GAPDH as well as several unknown proteins. In order to identify these unknown proteins, the intracellular pool of low-molecular-mass thiols was radiolabelled as described in the Experimental section. Cells were then

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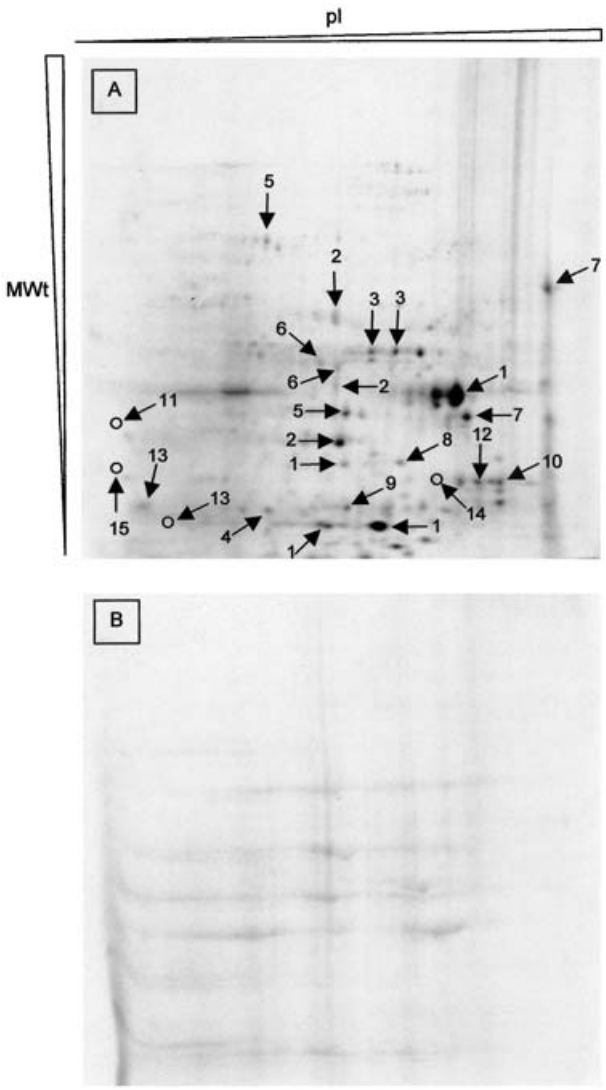

Figure 1 Two-dimensional gel analysis of S-thiolated proteins

Following ^{35}S radiolabelling of the intracellular pool of low-molecular-mass thiols, cells were treated with 2 mM H_2O_2 to induce S-thiolation. Cell extracts were prepared in the presence of NEM to prevent S-thiolation during the extraction procedure (**A**), or DTT to reduce any S-thiolated proteins (**B**). Proteins were separated based on pI (pH 3–10 isoelectric focusing) and molecular mass (MWt; SDS/10 %-PAGE) as indicated. Proteins were detected by PhosphorImager analysis and identified via MALDI-TOF MS. The numbers correspond to the identities listed in Table 1, and circles indicate proteins resolved on other gels.

challenged with 2 mM H_2O_2 for 30 min to induce S-thiolation. Cell extracts were prepared in the presence of NEM to prevent S-thiolation during the extraction procedure, or DTT to reduce any S-thiolated proteins, and separated by two-dimensional gel electrophoresis. Labelled proteins were visualized using a PhosphorImager and Figure 1 shows a representative experiment. Several S-thiolated proteins were detected following H_2O_2 treatment (Figure 1A), and radioactivity incorporation was confirmed to occur as a result of S-thiolation since it was reversed by treatment with DTT (Figure 1B). Spots were excised from the Coomassie-stained gel and analysed by MALDI-TOF MS. Table 1 lists the identity of 15 proteins which could be identified in one or more of three independent experiments. Two of these proteins (Tdh3 and Eno2) were identified as containing an S-glutathiolated peptide confirming that the protein modification is due to S-thiolation. The identified proteins fall into several different functional classes, but most strikingly, a range of glycolytic and related enzymes (Tdh3, Eno2, Adh1, Tpi1, Ald6 and Fba1), as well as translation factors (Tef2, Tef5, Nip1 and Rps5) were identified. We therefore investigated the cellular consequences of oxidative stress on glycolysis and protein synthesis.

Effect of oxidative stress on glycolytic enzymes

We have previously shown that S-thiolation of Tdh3 inhibits cellular GAPDH enzyme activity [8]. Similarly, other enzymic targets of S-thiolation may be inhibited by the formation of mixed disulphides with cysteine residues located at or near their active sites. We therefore examined enzyme activities following treatment of yeast cells with 2 mM H_2O_2 for 30 min (Figure 2A). As found previously, GAPDH activity was inhibited by approx. 90 % following treatment with H_2O_2. Similarly, enolase and alcohol dehydrogenase activities were inhibited by approx. 70 % following the H_2O_2 treatment. In contrast, aldolase, triose phosphate isomerase and aldehyde dehydrogenase were unaffected by the oxidant treatment.

We next measured enzyme activities in a strain deleted for *GSH1*, encoding the first step in GSH biosynthesis (Figure 2B). This strain is sensitive to oxidative stress due to the lack of GSH [22,28,29] and is unable to modify proteins by S-thiolation in response to oxidants [8]. The basal levels of enolase, alcohol dehydrogenase, aldolase and aldehyde dehydrogenase in the *gsh1* mutant were similar to those in the wild-type strain. In contrast, GAPDH activity was reduced by 2-fold and triose phosphate isomerase activity was increased by 2-fold in the *gsh1* mutant. However, the activity of all the glycolytic enzymes identified as targets of S-thiolation was inhibited in the *gsh1* mutant following exposure to H_2O_2, indicating that these enzymes are sensitive to oxidative stress conditions.

Enzyme inhibition of GAPDH, enolase and alcohol dehydrogenase is reversible

We have previously shown that protein S-thiolation induced in response to H_2O_2 reversibly inhibits the activity of GAPDH. GSH forms a mixed disulphide with the active-site cysteine, thus protecting the enzyme from inactivation caused by the oxidant. This inhibition is partially reversible when the oxidant is removed and GAPDH becomes dethiolated [8]. The inhibition of enolase and alcohol dehydrogenase activities may arise due to S-thiolation or irreversible oxidation of the enzyme active sites, both of which would inhibit enzyme activity. To distinguish between these two possibilities, cells were transferred into fresh media to follow the recovery of enzyme activity once the H_2O_2 stress was relieved. GAPDH activity was restored to approx. 50 % of the starting activity following a 2 h growth period (Figure 3A). Similarly, inhibition of enolase and alcohol dehydrogenase activities was reversible and activity was restored to approx. 60 % of the pre-stress activity levels. In contrast, little or no restoration of enzyme activity was seen in the *gsh1* mutant during the recovery period (Figure 3B).

Oxidative stress does not inhibit the entry of glucose equivalents into the pentose phosphate pathway

Given that the oxidative stress conditions used to induce S-thiolation result in an inhibition of key glycolytic enzymes, we examined whether these conditions also inhibited the entry of glucose equivalents into the pentose phosphate pathway. We therefore

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Table 1 Identities of S-thiolated proteins

Spot numbers of proteins correspond to those shown in Figure 1.

Spot no.	Standard name	Systematic name	Molecular function	Molecular mass (kDa)	pI	Sequence coverage (%)
1	Tdh3	YGR192c	GAPDH	35.7	6.96	13–46
2	Eno2	YHR174w	Phosphopyruvate hydratase	46.9	5.88	19–52
3	Adh1	YOL086c	Alcohol dehydrogenase	36.9	6.66	29–36
4	Tpi1	YDR050c	Triose phosphate isomerase	26.8	5.86	32–38
5	Ald6	YPL061w	Aldehyde dehydrogenase	54.4	5.18	21–24
6	Fba1	YKL060c	Fructose-bisphosphate aldolase	39.6	5.65	25
7	Tef2	YBR118w	Translation elongation factor 1α	50.0	9.72	20–41
8	Nip1	YMR309c	Translation initiation factor	93.2	4.69	22
9	Dst1	YGL043w	Positive transcription elongation factor	34.8	9.38	17
10	Mec1	YBR136w	Inositol/phosphatidylinositol kinase	273.3	8.37	2
11	Tef5	YAL003w	Translation elongation factor 1β	22.6	4.13	37
12	Pre6	YOL038w	Proteasome endopeptidase	28.4	6.92	20
13	Ssa2	YLL024c	Chaperone/heat-shock protein	69.5	4.77	13–16
14	Rps5	YJR123w	Small ribosomal subunit	25.0	9.11	30
15	–	YKL056cp	Unknown	18.7	4.41	44

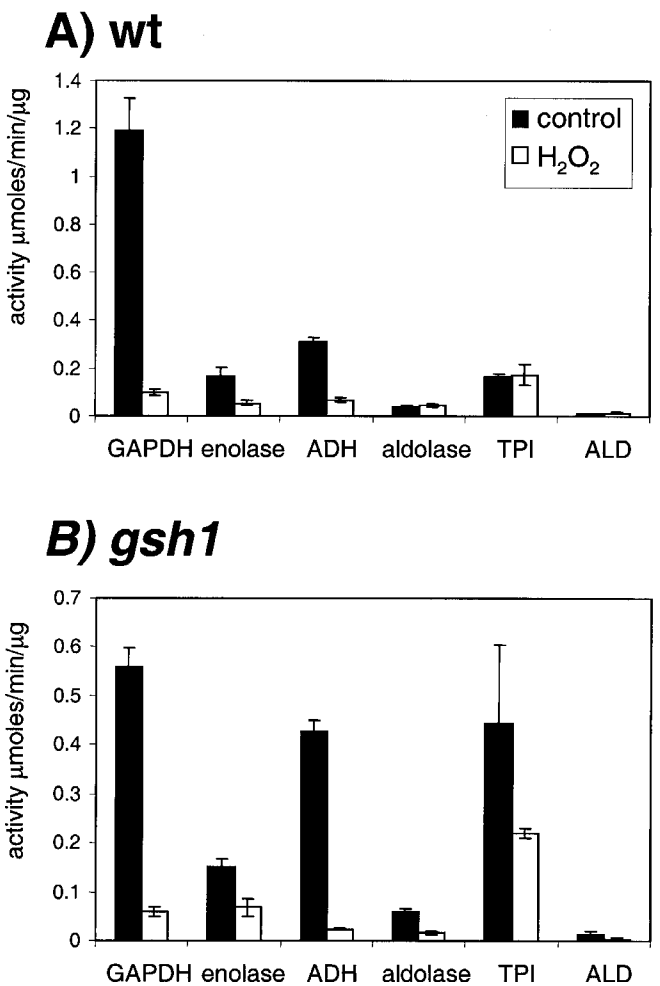

Figure 2 Effect of oxidative stress on glycolytic targets of S-thiolation

Cells were grown to exponential phase and treated with 2 mM H_2O_2 for 30 min. Cultures of the wild-type (**A**) and *gsh1* mutant (**B**) were assayed for GAPDH, enolase, alcohol dehydrogenase (ADH), aldolase, triose phosphate isomerase (TPI) and aldehyde dehydrogenase (ALD) activities. Values shown are the means ± S.E.M. from triplicate experiments.

investigated whether the concentrations of H_2O_2 used to induce S-thiolation of glycolytic enzymes would inhibit the activities of G6PDH and 6PGDH. Treatment of yeast cells with up to 2 mM H_2O_2 for 30 min was found to have no inhibitory effect on G6PDH or 6PGDH activities (Figure 4). We next examined whether these enzymes were affected by the loss of *GSH1*. Interestingly, G6PDH and 6PGDH levels were elevated by 5- and 2-fold respectively in the *gsh1* mutant during normal aerobic growth conditions. This may indicate that, in the absence of GSH, cells compensate by up-regulating the production of NADPH via the pentose phosphate pathway. G6PDH activity in the *gsh1* mutant was unaffected by any concentration of H_2O_2, indicating that this enzyme is particularly resistant to oxidative stress. In contrast, 6PGDH was inhibited 4-fold in the *gsh1* mutant in response to 2 mM H_2O_2, but was unaffected by lower levels of oxidant. Thus although protein S-thiolation induced by H_2O_2 would inhibit glycolysis, glucose equivalents could still be used to generate NADPH via the pentose phosphate pathway.

Effect of oxidative stress on protein synthesis

Given that we identified various translation factors as targets of protein S-thiolation, we investigated whether oxidative stress induced by H_2O_2 reversibly inhibited protein synthesis. The rate of protein synthesis was measured as the incorporation of [^{35}S]cysteine/methionine into proteins during a 5 min labelling period. Treatment with 2 mM H_2O_2 for 30 min reduced the rate of protein synthesis by approx. 95% in the wild-type strain (Figure 5A). Once the oxidant had been removed the rate of protein synthesis was almost fully restored within 3 h. Analysis of cell viability revealed that the oxidant treatment killed 55% of cells (Figure 5B). Surprisingly, viability was not restored during a 5 h recovery period and actually fell to 28% survival. These data indicate that protein synthesis is reversibly inhibited by oxidative stress and can be restored to normal levels once the oxidant is removed, despite continued cell death. Viability was, however, restored to 100% following a 24 h recovery period (results not shown). The oxidant treatment resulted in 99% loss of viability in the *gsh1* mutant, and protein synthesis was inhibited to a similar level as in the wild-type strain (Figure 5). However, there was no recovery of viability or restoration of protein synthesis during the 5 h recovery growth period.

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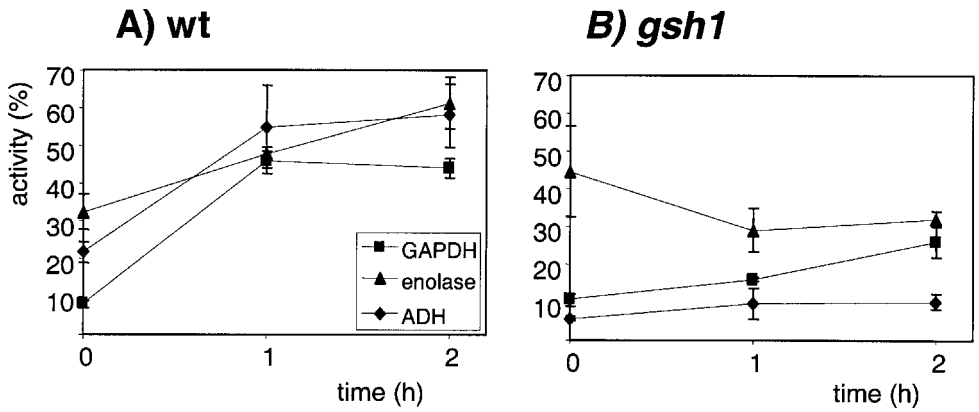

Figure 3 Recovery of GAPDH, enolase and alcohol dehydrogenase (ADH) activities following H_2O_2 stress

Cells were grown to exponential phase and treated with 2 mM H_2O_2 for 30 min to induce S-thiolation. Cells were then washed to remove the oxidant and resuspended in fresh SD medium to follow the recovery of enzyme activities after 1 and 2 h. Cultures of the wild-type (**A**) and *gsh1* mutant (**B**) were assayed for GAPDH, enolase and ADH activities. Means ± S.E.M. as shown.

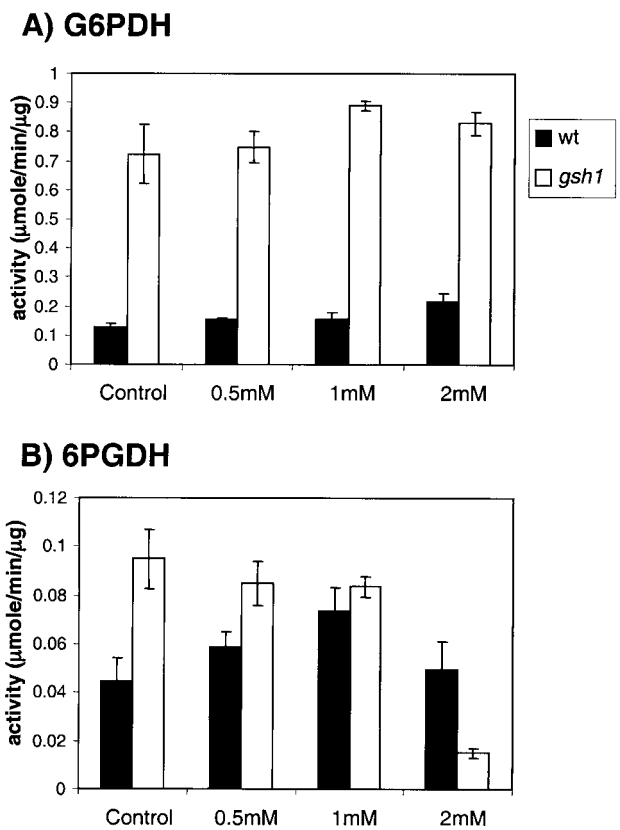

Figure 4 Activity of enzymes of the pentose phosphate pathway following oxidative stress

Cultures of the wild-type and *gsh1* mutant strain were grown to exponential phase and treated with 0.5, 1 or 2 mM H_2O_2 for 30 min. G6PDH (**A**) and 6PGDH (**B**) activities were assayed and values shown are the means ± S.E.M. from triplicate experiments.

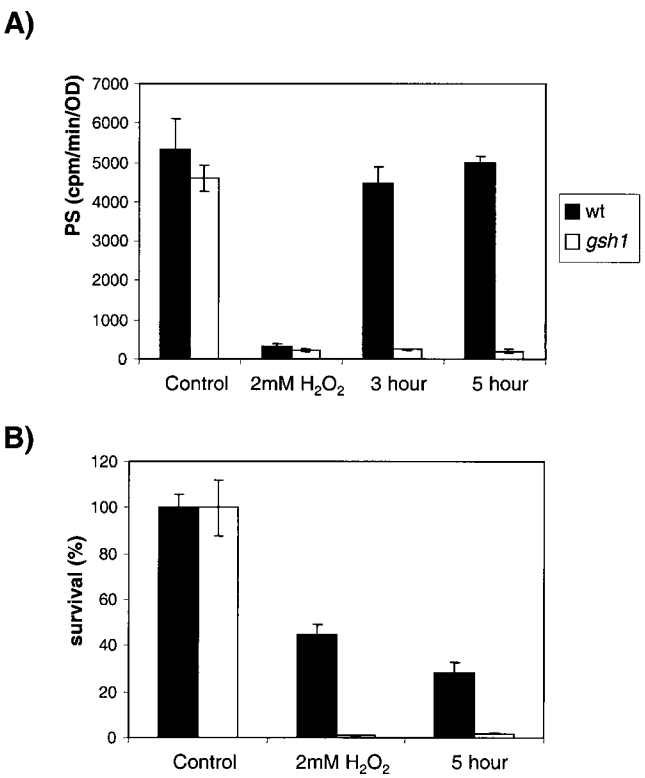

Figure 5 Oxidative stress reversibly inhibits protein synthesis

Cultures of the wild-type and *gsh1* mutant strain were grown to exponential phase in minimal SD media. (**A**) Aliquots of cell culture were used to measure the rate of protein synthesis (PS) by pulse labelling with [^{35}S]cysteine/methionine for 5 min. Results are shown for untreated cultures (control), following treatment with 2 mM H_2O_2 for 30 min, and following 3 and 5 h recovery in fresh SD medium in the absence of oxidant. (**B**) Cells were diluted and plated in triplicate on to YEPD medium to monitor cell viability. Percentage survival is expressed relative to the untreated control cultures (100 %); means ± S.E.M. as shown.

DISCUSSION

In this study, we have shown that protein S-thiolation, induced by exposure to 2 mM H_2O_2 for 30 min, modifies a range of proteins involved in various processes including glycolysis, translation, transcription, cell-cycle control, protein degradation and the heat-shock response. Further work will be required to determine whether protein S-thiolation plays any role in regulating these various processes. In addition to Tdh3, several glycolytic enzymes were identified as targets of S-thiolation, including Eno2, Adh1, Tpi1, Ald6 and Fba1. The concentration of H_2O_2 used in these experiments was found to inhibit the activities of GAPDH, enolase and alcohol dehydrogenase by at least 70 %. The inhibition of these enzymes would therefore effectively block the conversion

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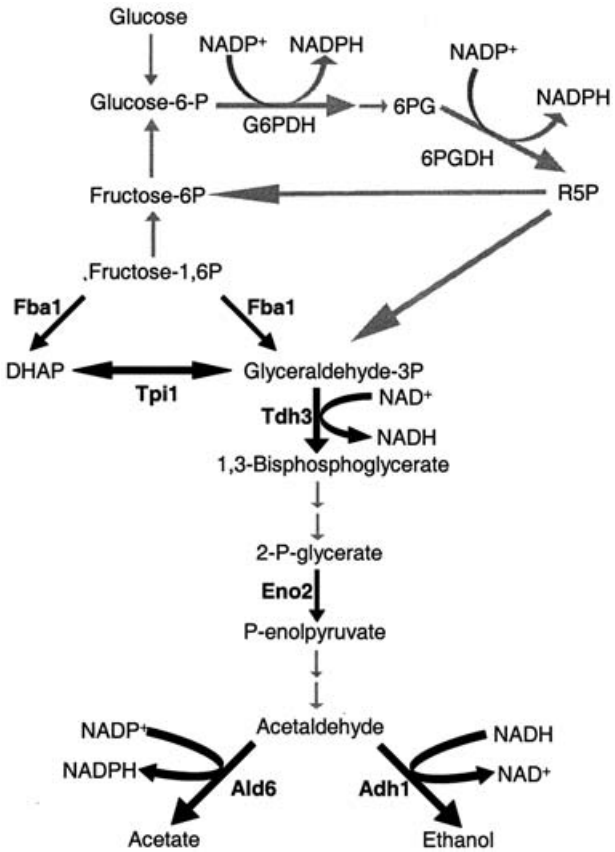

Scheme 1 Simplified version of glycolysis showing the NADPH-producing steps of the pentose phosphate pathway

Steps which are affected by S-thiolation are shown with black arrows. Inhibiting glycolytic flux during an oxidative stress would promote entry of glucose equivalents into the pentose phosphate pathway for NADPH production. 6GP, 6-phosphogluconate; R5P, ribulose 5-phosphate; DHAP, dihydroxyacetone phosphate.

of glucose into ethanol via glycolysis (Scheme 1). A previous study used metabolic labelling and two-dimensional gel electrophoresis to examine the changes in gene expression that occur in response to low adaptive concentrations of H_2O_2 [30]. Interestingly, Tdh3, Eno2, Adh1 and Ald6 were all identified as proteins whose synthesis is repressed in response to H_2O_2 exposure. Thus yeast cells respond to H_2O_2 by inhibiting glycolytic enzymes via S-thiolation, and switch off gene expression to prevent synthesis of any new enzymes. Combining regulation at the levels of protein modification and gene expression would provide a rapid means of reversibly inhibiting the flux through glycolysis.

The finding that protein S-thiolation targets particular glycolytic enzymes indicates that this protein modification may serve a regulatory role, rather than a simple function in protection of protein SH groups against irreversible oxidation. In addition, the observation that the Tdh3, and not the Tdh2, GAPDH isoenzyme is modified, despite their high degree of sequence homology, confirms that this protein modification is tightly regulated [9]. Yeast cells grown on glucose-based media preferentially grow by fermentation until the glucose is exhausted (reviewed in [31,32]). Thus regulating glycolysis in response to an oxidative stress would provide a powerful means of controlling cellular metabolism. It has been proposed previously that blocking glycolysis could be beneficial during conditions of oxidative stress since it would result in an increased flux of glucose equivalents through the pentose phosphate pathway leading to the generation of NADPH [33,34]. NADPH is important during exposure to oxidants since it provides the reducing power for antioxidant enzymes, including the GSH/glutaredoxin and thioredoxin systems [35]. G6PDH and 6PGDH catalyse the first two steps of the pentose phosphate pathway, which is the major source of cellular reducing power in the form of NADPH. G6PDH catalyses the key NADPH-production step and is known to play a role in protection against oxidative stress [36]. In addition, G6PDH and 6PGDH enzyme activities are induced in yeast cells in response to low adaptive concentrations (0.2 mM) of H_2O_2 [37]. We therefore examined whether the activity of G6PDH, along with 6PGDH, which catalyses the second NADPH generation step of the pathway, is affected by the peroxide treatment used in this study. G6PDH contains a single Cys residue and 6PGDH contains seven Cys residues, but both enzymes were unaffected by the H_2O_2 treatments that inhibited glycolytic enzymes. These data indicate that glucose equivalents could flow into the pentose phosphate pathway under these conditions, and generate NADPH (Scheme 1).

S-Thiolation of Fba1, Tpi1 and Ald6 did not affect cellular aldolase, triose phosphate isomerase or aldehyde dehydrogenase activities. This may indicate that the Cys targets of thiolation do not affect the active sites of these enzymes. Yeast contains a single aldolase and triose phosphate isomerase, whereas there are several aldehyde dehydrogenase isoenzymes. This means that we may not have been able to detect any inhibition of Ald6 in an enzyme assay using a crude cell extract. Inhibition of aldolase and triose phosphate isomerase would prevent ribose 5-phosphate generated from the oxidative phase of the pentose phosphate pathway from being recycled back to glucose 6-phosphate (Scheme 1). Modification of these enzymes may therefore play a regulatory role, and future experiments will be required to address this issue. An alternative branch point from glycolysis is the generation of glycerol from dihydroxyacetone phosphate. Interestingly, the synthesis of various glycerol cycle enzymes have been shown to increase in response to an adaptive dose of H_2O_2 [30]. However, we found no effect of the 2 mM H_2O_2 conditions used in our experiments on cellular glycerol levels, indicating that increased glycerol production does arise as a consequence of inhibiting glycolytic enzymes (results not shown).

Protein S-thiolation was found to target translation factors including elongation factors EF-1α (Tef2) and EF-1β (Tef5), initiation factor Nip1 (a subunit of initiation factor eIF3) and a small ribosomal subunit protein (Rps5). Furthermore, peroxide stress resulted in a rapid and reversible inhibition of protein synthesis, as measured by radiolabelling. It is unclear at present whether protein S-thiolation of any of the translation factors listed above accounts for this inhibition of protein synthesis. The H_2O_2 treatment used in these studies (2 mM, 30 min) caused a 55 % loss of viability in the wild-type strain. In addition, viability continued to decrease even after the oxidant was removed, presumably as a result of the continued generation of intracellular reactive oxygen species. However, the rate of protein synthesis was restored to the pre-treatment levels within a 3-h recovery period. These data indicate that the protein-synthetic machinery is protected against oxidative damage, despite the continued accumulation of cell damage resulting in a loss of viability. Yeast, like all eukaryotic cells, have a range of defence systems for dealing with oxidative stress. Inhibiting protein synthesis during exposure to an oxidant may provide a means of preventing continued gene expression under potentially error-prone conditions. It is known that exposure to oxidants can cause an arrest in the cell cycle [38], which, combined with the block in protein synthesis, might allow time for the cell to detoxify reactive oxygen species and their breakdown products.

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Once the reactive oxygen species have been removed or detoxified cells would then be able to resume cell division and protein synthesis.

The tripeptide GSH is an important molecule in the protection of yeast cells against damage induced by oxidative stress. Yeast mutants lacking *GSH1*, encoding the first step in GSH biosynthesis, are unable to grow in the absence of GSH and are sensitive to oxidative stress caused by H_2O_2 and various alkyl hydroperoxides [22,28,29]. We have shown previously that the *gsh1* mutant is defective in S-thiolation and is unable to modify Tdh3 by S-thiolation [8]. All of the glycolytic enzymes that were identified as targets of S-thiolation were oxidant-sensitive in the *gsh1* mutant. In addition, the *gsh1* mutant was unable to protect GAPDH, enolase and alcohol dehydrogenase against irreversible oxidation. Similarly, the H_2O_2 treatment used in these studies inhibited protein synthesis irreversibly. Thus the inability to protect glycolytic enzymes and translation factors against oxidation may partly explain the hypersensitivity of the *gsh1* mutant to oxidative stress.

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