The Saccharomyces cerevisiae Proteome of Oxidized Protein Thiols

CONTRASTED FUNCTIONS FOR THE THIOREDOXIN AND GLUTATHIONE PATHWAYS*

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Protein thiol oxidation subserves important biological functions and constitutes a sequel of reactive oxygen species toxicity. We developed two distinct thiol-labeling approaches to identify oxidized cytoplasmic protein thiols in Saccharomyces cerevisiae. In one approach, we used N-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide to purify oxidized protein thiols, and in the other, we used N-[14C]ethylmaleimide to quantify this oxidation. Both approaches showed a large number of the same proteins with oxidized thiols (∼200), 64 of which were identified by mass spectrometry. We show that, irrespective of its mechanism, protein thiol oxidation is dependent upon molecular O2. We also show that H2O2 does not cause de novo protein thiol oxidation, but rather increases the oxidation state of a select group of proteins. Furthermore, our study reveals contrasted differences in the oxidized proteome of cells upon inactivation of the thioredoxin or GSH pathway suggestive of very distinct thiol redox control functions, assigning an exclusive role for thioredoxin in H2O2 metabolism and the presumed thiol redox buffer function for GSH. Taken together, these results suggest the high selectivity of cytoplasmic protein thiol oxidation.

The amino acid cysteine subserves important biological functions due to the unique redox properties of the sulfur atom of its thiol side chain (1). By engaging in a wide variety of redox reactions and coordinating metals, cysteine serves as a key residue in enzyme catalysis, protein oxidative folding (2–4) and trafficking (5), and redox signaling and regulation (6–8). However, these unique redox properties also make the cysteine residue vulnerable to reaction with a wide spectrum of non-physiological electrophiles, especially the reactive oxygen and nitrogen species, potentially leading to unwanted redox modifications and protein loss of function (9).

The cysteine residue exists in vivo in the fully reduced free thiol form (–SH or –S−) and in different oxidation forms: the thyl radical (–S•); the disulfide bond (Cys–S–S–Cys); the sulfenic (–SOH), sulfonic (SO2H), and sulfonic (–SO3H) acid forms; and the S-nitrosylated form (–S–NO) (1, 7, 10). The cysteine thyl radical and cysteine sulfenic acid are very unstable because of their highly reactive nature and thus cannot be easily identified biochemically. In contrast, the cysteine sulfenic and sulfonic acids are irreversible forms of protein oxidation, although the cysteine sulfenic acid that forms in the peroxide-reducing enzyme peroxiredoxin is enzymatically retroreduced by sulfiredoxin (11, 12). Disulfide bonds are relatively stable, reversing to the reduced state by thiol-disulfide exchange with kinetics depending on the protein context (10). Their occurrence is thought to be restricted to specific subcellular compartments. In the endoplasmic reticulum, disulfide bond formation drives the correct folding of secreted proteins and is catalyzed by a FAD-containing sulfhydryl oxidase (Ero1) and protein-disulfide isomerase (Pdi1) (2–4). Protein import and folding in the mitochondrial intermembrane space are driven by disulfide bond formation by another recently identified redox relay involving the FAD-containing Ero1 oxidase and Mia40 (5, 13). In contrast, the cytoplasm is not thought to contain many protein–disulfide bonds due to its highly reducing nature caused by two potent NADPH-dependent thiol-reducing systems, the thioredoxin and GSH pathways (14, 15). Still, disulfide bonds are formed in this compartment at least in a few enzymes with a thiol oxidation step in their catalytic cycle such as ribonucleotide reductase and in redox-regulated proteins such as the Saccharomyces cerevisiae Yap1 and Escherichia coli OxyR H2O2 sensors (8). Cytoplasmic disulfide bonds are also found in the form of mixed disulfides between GSH and protein thiols, a modification referred to as S-thiolation (16–18). Protein S-thiolation protects oxidized protein thiols, whether in the thyl or sulfenic acid form, from further irreversible oxidation and has been proposed to regulate protein function.

The in vivo redox state of cysteine residues must reflect both their diverse redox functions and their potential modifications by oxidative and nitrosative stress. We sought to evaluate the scope of cytoplasmic protein thiol oxidation in S. cerevisiae with the aim of questioning the dogma of its reducing nature and of identifying new thiol redox-mediated physiological processes. We also evaluated the consequences of thiol redox pathway inactivation on protein thiol oxidation and questioned to what extent cysteine residues represent targets of oxidative stress caused by H2O2.

Because of their intrinsic reactivity, cysteine residues can undergo redox modification upon cell lysis, making it difficult to evaluate their true in vivo redox state. This problem can be circumvented by quenching the reactivity of thiol groups by acid cell lysis (19, 20), followed by differential labeling of reduced versus oxidized thiols with thiol-specific reagents. We used this general thiol-trapping technique together with two different thiol-specific reagents. The use of N-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide (biotin-HPDP)2 and N-(6-(biotinamido)hexyl)-3′-(2′-pyridyldithio)propionamide (biotin-HPDP)2

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allowed the purification of oxidized proteins and their identification by mass spectrometry (MS), whereas the use of N-[^14]Cethymaleimide (NEM) permitted a semiquantitative analysis of the extent of protein oxidation at the proteome level. We present our findings obtained from the combined use of these two redox proteomic approaches and discuss the possible cellular mechanisms underlying cytoplasmic disulfide bond formation.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—Experiments were performed with the *S. cerevisiae* wild-type strain Y252 (MATa ura3-52 lys2-801 amber ade-2-101 ochre trpl-1 ΔI leu2-Δ1), a derivative of YPH98 (21), and isogenic derivatives. The Δgsh1PRO2-ΔGsr1 (yeast SOGΔgsr1) strain has been described (22). Chromosomal gene inactivation was performed by oligonucleotide-mediated PCR replacement of entire coding regions and confirmed by PCR. Δtrrr1Δtrxx2 and Δtdtd3 were obtained by replacement with *KANMX4*, and Δtrxx1 with *URA3*. The triple mutant Δtrrr1Δtrxx2/Δtrxx1 was obtained by a cross between Δtrrr1 and Δtrxx1/Δtrxx2 and sporulation of the triple heterozygous diploid. Cells were grown at 30°C in 0.67% yeast nitrogen base, 0.1% casamino acids, and Tris-Cl (pH 8.8), three times with wash buffer A (8 M urea, 4% CHAPS, 25 mM Tris-Cl (pH 8.8)), three times with wash buffer B (8 M urea, 4% CHAPS, and 25 mM Tris-Cl (pH 8.8)), and once with elution buffer (8 M urea, 4% CHAPS, and 7 mM Tris). Biotinylated proteins were eluted with elution buffer (400 μl) containing DTT (20 mM). Eluted proteins were subjected to two-dimensional gel electrophoresis.

[^14]C/NEM Protein Labeling—[^14]C/NEM thiol labeling was carried out as described above for biotin-HPDP labeling, except that biotin-HPDP was replaced with 2 mM[^14]C/NEM (37.5 mCi/mmol; PerkinElmer Life Sciences). Extracts were trichloroacetic acid-precipitated to remove excess[^14]C/NEM, solubilized in elution buffer, and subjected to two- or one-dimensional gel electrophoresis. Where indicated, the denaturing buffer used was 100 mM Tris, 10 mM EDTA, and 1% SDS.

Two-dimensional Gel Electrophoresis—Extracts were adjusted to a 400-μl volume rehydration solution (8 M urea, 4% CHAPS, 20 mM DTT, 1% immobilized pH gradient buffer, and bromphenol blue) and loaded onto strip holders (Amersham Biosciences). Proteins were separated in the first dimension by isoelectric focusing using pH 3–10 nonlinear Immobiline DryStrip gels (18 cm) and run at 34 kV-h (50 μA/strip) for 20 h at 20°C on an IPGphor unit (Amersham Biosciences). In the second dimension, focused immobilized pH gradient strips were equilibrated for 15 min in equilibration buffer (6 M urea, 2% SDS, 50 mM Tris-Cl (pH 6.8), 20% glycerol, and bromphenol blue) containing DTT (50 mM) and for 15 min in the same buffer containing iodoacetamide (100 mM). Immobiline DryStrip gels were washed with electrophoresis buffer (Tris/glycine/SDS), transferred onto 13% polyacrylamide gels, and embedded in 0.5% (w/v) agarose containing Tris/glycine/SDS. SDS-PAGE migration was performed using an Etan DALTSix system for 18 h (1.5 watts/gel) at 30°C. For[^14]C/NEM-labeled extracts, gels were fixed, dried, exposed to sensitive screens, and analyzed using a Phosphorimager (Amersham Biosciences). For biotin-HPDP-purified proteins, gels were stained with colloidal Coomassie Blue (Sigma).

Western Blot Analyses—Trichloroacetic acid-precipitated extracts were solubilized in the presence of 50 mM NEM (Sigma). Protein extracts (20 μg) were dissolved in Laemmli buffer without β-mercaptoethanol, boiled, and loaded on SDS-polyacrylamide gel (15%). Separated proteins were blotted onto a nitrocellulose membrane, probed with either anti-Myc monoclonal antibody 9E10 or horseradish peroxidase-conjugated streptavidin, and revealed using an enhanced chemiluminescence Western blotting reagent kit (Amersham Biosciences).

Protein Identification by MALDI-TOF/MS—Protein spots of interest were excised from stained gels, cut into ~1-mm³ pieces, washed with water, dehydrated in 100% acetoni-trile, dried in a SpeedVac vacuum evaporator, and washed with ammonium bicarbonate (0.1 M) for 30 min at 56°C to remove the dye. Gels pieces were rehydrated for 45 min on ice in a solution containing 25 mM NH₄HCO₃, 5 mM CaCl₂, and 12.5 ng/μl trypsin. In-gel digestion was performed in the trypsin-free solution for 2 h at 37°C. The digest solution (0.5–1 μl) was applied to a sample plate, and an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid in 100% acetonitrile and 0.1% trifluoroacetic acid (1:1, v/v) was added. Air-dried droplets were desalted twice with 0.1% trifluoroacetic acid. Spectra were acquired using a Voyager-DE STR Biospectrometry workstation (Applied Biosystems) in positive ion reflector mode with an accelerating voltage of 20 kV. Masses were calibrated with external standards (Sequazyme peptide mass standard kit, Applied Biosystems) and with internal autolysis tryptic peptides. Proteins were identified by searching the Swiss Protein Data Base (available at prowl.rockefeller.edu/profound_bin/WebProFound.exe). Among proposed peptide search options, we included the two following modifications:
oxidation of methionines (peptide mass $\pm 16$ Da) and carbamidomethylation of cysteines (peptide mass $\pm 57$ Da). Calibrated monoisotopic peptide masses were searched against the Swiss Protein Non-redundant Protein Database using the Prowl program with a mass tolerance between 20 and 50 ppm. Identification was established by searches in all taxonomic categories with a Z-score $\geq 1.5$, except for Prx1, Hsp60, Ado1, Guk1, Ubc1, Cpr1, and Tma19, which had lower Z-scores.

RESULTS

Purification of Proteins with in Vivo Oxidized Thiols—We sought to identify yeast proteins bearing one or more oxidized cysteine residues (in the sulfenic acid or disulfide-bonded form) by adapting the biotin switch method aimed at $S$-nitrosylated proteins (24, 25). In brief, oxidized cysteine residues of crude yeast extracts were specifically labeled with biotin-HPDP and purified by streptavidin affinity chromatography (Fig. 1). HPDP is an absolute thiol-specific reagent that covalently and reversibly attaches to free thiol groups through a disulfide bridge. Two-dimensional Coomassie Blue-stained gels showed that numerous proteins were purified from exponentially growing cells by streptavidin affinity chromatography (Fig. 2A). We performed several controls for establishing the method specificity. (i) When biotin-HPDP was substituted with iodoacetamide, no proteins were recovered, indicating the absolute specificity of the purification for biotinylated proteins (Fig. 2B). (ii) When the reduction of disulfide bonds by DTT prior to the biotin-HPDP step was omitted in the purification scheme, only traces of proteins were eluted (Fig. 2C), indicating the HPDP labeling specificity for oxidized cysteine residues. The protein traces recovered may indicate unprompted disulfide bond reduction during the in vitro procedure and/or a low level of nonspecific HPDP protein labeling. We thus verified by Western blotting the extent of protein biotinylation of the same control extracts, i.e. iodoacetamide-blocked but not DTT-reduced, which showed the presence of minor trace signals, with the exception of a discrete 40-kDa band (Fig. 2F, lane 2) corresponding to Tdh3 (see below). Contrasting with the latter control is the heavy labeling of
iodoacetamide-blocked extracts that had been reacted with biotin-HPDP after DTT reduction (Fig. 2F, lane 1). We also directly assayed cysteine residue selectivity by testing whether the abundant enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH; Tdh3) could still be purified if it lacked its two cysteine residues. Cysteine-less Tdh3 was not purified using this experimental procedure, attesting the high selectivity of the method for cysteine residues (Fig. 2E). (iii) Finally, when DTT was omitted in the elution step, no or only barely detectable protein traces were recovered (Fig. 2D), indicating that only proteins bound to beads by a DTT-sensitive biotin attachment could be eluted, and this represents another demonstration of the cysteine selectivity of the purification scheme.

The purification method described here thus appears to be highly specific for proteins with oxidized cysteine residues. Furthermore, its protein yield is adapted to easy MS protein identification.

Identification of the Oxidized Proteome of Unstressed Cells—Coomassie Blue-stained two-dimensional gels of proteins purified from exponentially growing cells contained ~200 spots (Fig. 3A). We carried out an exhaustive MALDI-TOF-MS analysis of these proteins, thereby identifying about one-third of them (Fig. 3A and Table 1). The majority of the proteins identified were cytoplasmic (57/64), with very few resident proteins that were absent (data not shown). The proteins identified were sorted into functional classes that comprised reactive thioredoxin pathway (see below).

Several of these proteins have been iteratively identified in other in vivo protein thiol oxidation studies (see Table 1 for references). The peroxiredoxins (Tsa1 and Ahp1) are antioxidants that use a catalytic cysteine for peroxide reduction (26). Their oxidation in unstressed cells presumably reflects permanent enzyme cycling caused by reduction of endogenous peroxides. The presence of oxidized forms of thioredoxin reductase (Trr1) and protein-disulfide isomerase (Pdi1) is consistent with their thiol reductase functions, also indicating permanent enzyme cycling. Although copper/zinc-superoxide dismutase (Sod1)-mediated dismutation of $O_2^-$ does not involve a catalytic thiol, the mature enzyme carries a disulfide bond essential for activity (27). Thiol oxidation of the flavohemoglobin Yhb1, supposed to carry heme-dependent NO dioxygenase and reductase activities (28), is surprising in contrast and may indicate either involvement of a thiol in these activities or a new cysteine-based enzyme function. Carbohydrate metabolism and amino acid biosynthetic enzymes represent the largest oxidized protein group, among which is the key glycolytic enzyme GAPDH (Tdh3) (see “Discussion”). Oxidation of these enzymes probably relates, for many of them, to the presence of redox-reactive catalytic or metal-coordinating cysteine residues (Table 1).

Quantification of the Oxidized Proteome by $^{14}$C]NEM Labeling—The biotin-HPDP-based purification method did not yield information on the extent of protein oxidation. We thus set up a semiquantitative approach in which we used $^{14}$C]NEM as a labeling reagent and established a protein oxidation index ($I_{ox}$) by calculating the $^{14}$C signal value ratio of oxidized ($S_{ox}$) versus total ($S_{tot}$) protein thiols (Fig. 4A). Oxidized protein thiols were labeled by reacting $^{14}$C]NEM with extracts that had been first alkylated with unlabeled iodoacetamide and reduced with DTT (Fig. 4B). Total protein thiols were labeled by directly reacting $^{14}$C]NEM with DTT-reduced extracts (Fig. 4C). As expected, the two-dimensional gel autoradiogram of labeled total protein thiols carried much heavier signals (Fig. 4, compare B and C). The two-dimensional gels of $^{14}$C]NEM-labeled oxidized protein thiols were rigorously compared with the Coomassie Blue-stained two-dimensional gels of biotin-HPDP-purified proteins (Fig. 3, A and B), revealing very similar spot distributions and providing reciprocal proof of the specificity of the two methods. In particular, all oxidized proteins identified by MS were also visualized as $^{14}$C-labeled substrates. For calculating $I_{ox}$, we considered...
### TABLE 1
Oxidized protein identification

ROS, reactive oxygen species.

| Protein | Z-score | Location* | Cys | $I_{ox}$ (WT) | $I_{ox}$ ratio$^b$ | Previous identification$^c$ | Reactive thios | Ref. |
|---------|---------|-----------|-----|-------------|-----------------|---------------------------|----------------|-----|
| **Redox enzymes** | | | | | | | | |
| **ROS scavengers** | | | | | | | | |
| Sod1 (copper/zinc-superoxide dismutase) | 2.43 | C/M | 2 | 1.22 | 1.16 | 1.32 | + | Cys^{57}\text{–Cys}^{146} disulfide | 47 |
| Tsa1 (thiol-specific antioxidant) | 2.39 | C | 2 | 0.44 | 1.8 | 1.36 | + | Cys^{47}\text{–Cys}^{130} disulfide | 44, 45, 47–50 |
| Ahp1 (aldehyde hydroperoxide reductase) | 2.22 | C | 3 | 0.22 | 2.37 | 1.27 | + | Cys^{4}\text{–Cys}^{120} disulfide | 44 |
| **Sulfur oxidoreductases** | | | | | | | | |
| Trr1 (thioredoxin reductase) | 2.31 | C/N | 4 | 2.98 | 1.46 | 1.68 | + | Catalytic disulfide | 48 |
| Pdi1 (protein-disulfide isomerase) | 1.67 | C/N | 5 | | | | | |
| **Stress chaperones, protein folding** | | | | | | | | |
| Ssa2 (ATPase of the HSP70 family, vacuolar import) | 2.43 | C/V | 3 | 0.24 | 1.04 | 2.0 | + | | 18 |
| Sse1 (ATPase of the HSP70 family, component of the HSP90 complex) | 2.43 | C | 5 | 1.36 | | | + | | 48, 49 |
| Sbd1 (ATPase of the HSP70 family, protein folding) | 2.43 | C | 3 | 0.98 | | | + | | 48, 49 |
| Sti1 (HSP90 co-chaperone) | 2.39 | C | 3 | | | | | |
| Hsp60 (mitochondrial chaperone, mitochondrial import) | 0.59 | M | 5 | | | | + | | 49, 50 |
| **Carbohydrate and energy metabolism** | | | | | | | | |
| Hxk2 (hexokinase isoenzyme-2) | 2.43 | C/N | 4 | | | | | |
| Tpi1 (triose-phosphate isomerase) | 2.43 | C | 2 | 0.34 | 1.38 | 2.64 | + | Cys^{26}, folding and stability | 18, 47, 49 |
| **Pentose phosphate shunt** | | | | | | | | |
| **Glycerol/mannose metabolism** | | | | | | | | |
| Gpp1 (DL-glycerol-3-phosphatase, glycerol biosynthesis) | 2.43 | C/N | 3 | | 0.24 | | + | Cys^{35}, folding and stability | 18, 24, 44, 45, 47, 48 |
| Pmi40 (mannose-6-phosphate isomerase, protein mannosylation) | 2.29 | C/N | 5 | | | | | |
| Sec53 (phosphomannomutase, protein glycosylation/folding) | 2.36 | C | 3 | 0.47 | | | | |
| Psa1 (GDP-mannose pyrophosphorylase, cell wall biosynthesis) | 2.43 | C | 3 | | | | | |
| **Amino acid metabolism** | | | | | | | | |
| Sam1 (S-adenosylmethionine synthetase-1) | 2.43 | C | 5 | | 1.86 | | + | Cys^{47}\text{–Cys}^{203} in Fe–S cluster | 44, 46, 47 |
| Sam2 (S-adenosylmethionine synthetase-2) | 2.43 | C | 5 | | 1.5 | | | |
| Shm2 (serine hydroxymethyltransferase) | 2.37 | C | 5 | 1.77 | | + | | 44 |
| Met6 (cobalamin-independent methionine synthase) | 2.43 | C | 3 | 1.1 | | + | | 44 |
| Met17 (O-acetylhomoserine-O-acetylserine sulfhydrolase) | 2.40 | C | 1 | 1.24 | | | | |
two additional parameters. Oxidized spot $^{14}$C signal values not only reflect the amount protein in the oxidized form, but also the number of oxidized thiols/protein, a parameter lacking for most proteins. Furthermore, this number is not necessarily equal to the total number of cysteines/protein, thus giving an estimate of the approximate nature of the oxidized proteome after O$_2$ deprivation. Cells were grown to the exponential phase in the presence of 100% N$_2$ for 8 h before they were processed for $^{14}$C labeling (Fig. 5A). Two-dimensional gel spot intensities were dramatically decreased compared with those under the control aerobic conditions (Fig. 5A), and many of them almost disappeared. This visual impression was confirmed by the combined $I_{ox}$ spot values that decreased by a factor of 10 in two independent experiments. The $I_{ox}$ provides a relative protein oxidation scale within a two-dimensional gel.

**Oxygen Dependence of Protein Thiol Oxidation**—Molecular oxygen is expected to provide the main source of oxidizing equivalents for cellular thiol oxidation, either through reaction of thiols with O$_2$-derived H$_2$O$_2$ or through O$_2$-dependent catalysis. We thus evaluated the possible changes of the oxidized proteome after O$_2$ deprivation. Cells were taken into account in the oxidized proteome (Fig. 5B). Two-dimensional gel spot intensities were dramatically decreased compared with those under the control aerobic conditions (Fig. 5A), and many of them almost disappeared. This visual impression was confirmed by the combined $I_{ox}$ spot values that decreased by a factor of 10 in two independent experiments. The $I_{ox}$ provides a relative protein oxidation scale within a two-dimensional gel.

### TABLE 1—CONTINUED

| Protein    | Z-scores | Location | Cys | $I_{ox}$ (WT) | $I_{ox}$ ratio | $I_{ox}/$H$_2$O$_2$ | $I_{ox}/$H$_2$O$_2$ | Previous identification | Reactive thiols | Ref. |
|------------|----------|----------|-----|---------------|---------------|---------------------|---------------------|------------------------|------------------|------|
| Cys4       | 2.41     | C/M      | 1   | 2.05          | +             | Fe-S cluster        |                     |                        |                  | 44   |
| Leu1       | 2.43     | C        | 11  |               |               |                     |                     |                        |                  |      |
| Hom2       | 2.41     | C/N      | 9   |               |               |                     |                     |                        |                  |      |
| Gdi1       | 2.43     | C/N      | 6   | 1.9           |               |                     |                     |                        |                  |      |
| Lb52       | 2.41     | M        | 3   | 1.16          |               |                     |                     |                        |                  |      |
| Other metabolisms |        |          |     |               |               |                     |                     |                        |                  |      |
| Ado1       | 1.49     | C/N      | 3   | 1.9           |               |                     |                     |                        |                  |      |
| Ura1       | 2.41     | C        | 3   |               |               |                     |                     |                        |                  |      |
| Guk1       | 1.43     | C/N      | 1   |               |               |                     |                     |                        |                  |      |
| Ippl       | 2.43     | C        | 1   | 2.33          | +             |                     |                     |                        |                  | 50   |
| Protein translation |        |          |     |               |               |                     |                     |                        |                  |      |
| Ef1 (translation elongation factor-1$\beta$) | 2.40 | R | 1 | 0.26 | 0.6 | 0.61 | + | 18, 45, 49 |
| Tef1 (translation elongation factor-1$\alpha$) | 2.41 | R | 7 | 0.98 | 1.7 | 0.73 | + | 18, 45, 49 |
| Rpl12A (protein of the large (60 S) ribosomal subunit) | 2.38 | R | 1 | 0.5 | 1.3 | 0.17 | + | 18, 45, 49 |
| Tef51A (translation initiation factor-5A) | 1.85 | C/M/R | 2 | 0.06 | 0.95 | + | 18, 45, 49 |
| Rp30a (protein of the small (40 S) ribosomal subunit) | 2.43 | R | 1 | 0.5 | 1.3 | 0.17 | + | 18, 45, 49 |
| Rp55 (protein of the small (40 S) ribosomal subunit) | 2.35 | R | 1 | 0.33 | 1.02 | + | 18, 45, 49 |
| Asc1 (WD repeat-containing protein, translation regulation) | 2.38 | C | 2 | 0.08 | 1.55 | + | 18, 45, 49 |
| Proteases and proteasomes |        |          |     |               |               |                     |                     |                        |                  |      |
| Pep4 (vaccinal aspartyl protease, proteasine A) | 2.39 | V | 4 | 1.56 | 1.63 | 2.56 | + | 18, 45, 49 |
| Cdc48 (ATPase) | 2.08 | C/ER/N | 4 | 0.09 | 0.92 | + | 18, 45, 49 |
| Ubc1 (ubiquitin-conjugating enzyme-1) | 1.33 | P | 1 | 0.52 | 1.02 | + | 18, 45, 49 |
| Ubc4 (ubiquitin-conjugating enzyme-4) | 1.99 | P | 3 | 0.16 | 1.02 | + | 18, 45, 49 |
| Miscellaneous |        |          |     |               |               |                     |                     |                        |                  |      |
| Cpr1 (peptidylprolyl cis,trans-isomerase-1, cyclophilin) | 1.33 | C | 2 | 0.81 | + | 18, 45, 49 |
| Cpr6 (peptidylprolyl cis,trans-isomerase-6, cyclophilin) | 2.38 | C | 7 | 1.26 | + | 18, 45, 49 |
| Por1 (mitochondrial porin) | 2.43 | M | 2 | 1.02 | + | 18, 45, 49 |
| Gsp1 (GTP-binding protein) | 2.30 | C/N | 3 | 0.25 | 0.45 | 0.26 | + | 18, 45, 49 |
| Tma19 (hypothetical protein, unknown function) | 1.06 | C | 1 | 0.16 | 1.02 | + | 18, 45, 49 |

$^{a}$ C, cytoplasm; M, mitochondria; N, nucleus; R, ribosome; ER, endoplasmic reticulum; V, vacuole; P, proteasome.

$^{b}$ $I_{ox}$ ratio = $I_{ox}/I_{ox}/$H$_2$O$_2$, or $I_{ox}/I_{ox}/$H$_2$O$_2$ (values in boldface represent ratios $>$2).

$^{c}$ Previous identifications whether oxidized or S-thiolated (with the corresponding references).

$^{d}$ Proteins identified in the $\Delta$trr1/Δtrr1/Δtrr2 mutant.
sulfhydryl oxidase Ero1, which catalyzes Pdi1 oxidation (3). Sod1 was the only protein that did not have decreased oxidation. Sod1 disulfide bond formation is supposedly catalyzed by Erv1 (13) via Ccs1 (27, 30, 31), and Erv1 receives its oxidizing equivalents from cytochrome c rather than directly from molecular O2. Enough oxidized cytochrome c may remain in the mitochondria of anaerobically growing cells to maintain the essential function of Erv1.

**Effect of H2O2 on Protein Thiol Oxidation**—Although O2-dependent, it is not clear whether oxidation proceeds through reaction of thiols with O2-derived H2O2 or through O2-dependent catalysis. We thus sought to identify protein directly reacting with H2O2, which should carry solvent-exposed cysteines in the thiolate form (–S\(_{\text{H2O2}}\)), i.e. with a \(pK_a\) less than the intracellular pH (7). Exponentially growing wild-type cells were exposed to a mildly toxic concentration of H2O2 (1 mM) for 1 min, a time point not allowing protein expression changes to occur (33), and oxidized protein thiols were labeled with \([14C]\)NEM. Two-dimensional gel spot distribution was not different compared with untreated cells (Fig. 5, compare A and C). We thus quantified the H2O2-induced protein oxidation changes by calculating the matched spot \(I_{\text{ox}}\) ratio of H2O2-treated versus untreated samples \((I_{\text{ox(int)}/I_{\text{ox(WT)}}})\) (Table 1). We found no important differences for the majority of proteins (0.8 < \(I_{\text{ox}}\) ratio < 2), except for Ahp1 (2.37), Tdh3 (3.05), Ald6 (2.10), Cdc19 (2.12), Pdc1 (3.14), Cys4 (2.05), and Lpp1 (2.33), which had oxidation increases of 2-fold or more. In contrast, Ubc1 (0.52), Ubc4 (0.16), Ebf1 (0.6), Sec53 (0.47), and a protein of unknown function, Tma9 (0.45) had clearly decreased \(I_{\text{ox}}\) values, which could reflect irreversible thiol oxidation. Tsa1, which carries an H\(_2\)O\(_2\)-reactive thiolate, did not show such an increase (1.8), but an additional more acidic spot appeared (Fig. 5D, arrow). This new spot identified as Tsa1 by MS likely corresponds to its acidic cysteine sulfinate form occurring at this H\(_2\)O\(_2\) concentration (11). The persisting \(14C\) labeling of the Tsa1 acidic form indicates that the other of its two cysteines is in the disulfide-bonded form, probably with itself. One-dimensional SDS-PAGE separation of \([14C]\)NEM-labeled extracts of cells treated with H\(_2\)O\(_2\) at increasing concentrations (0.5–2 mM) visually confirmed the prominence of the Ahp1 and Tdh3 oxidation responses and, conversely, the paucity of the overall proteome response (Fig. 5E). This experiment also showed the decrease in Tsa1 dimer at elevated H\(_2\)O\(_2\) concentrations.

In conclusion and unexpectedly, H\(_2\)O\(_2\) does not apparently cause oxidation of novel proteins, but rather increases the oxidation state of a small number of select proteins that presumably carry H\(_2\)O\(_2\)-reactive thiolates. These results therefore suggest that cysteine oxidation is not a major mechanism of H\(_2\)O\(_2\) toxicity.

**The Oxidized Proteome of Cells with an Altered Thioredoxin Pathway**—Cytoplasmic protein thiols are maintained in a reduced state by the thioredoxin and GSH pathways. We analyzed the consequence of genetic ablation of either of these pathways on protein thiol oxidation. The cytoplasmic thioredoxin pathway comprises two thioredoxins (Trx1 and Trx2) and a thioredoxin reductase (TrxR1). We used a strain that lacks this entire pathway (Δtrxr1 Δtrx1 Δtrx2) and that combines...
phenotypes of both thioredoxin and thioredoxin reductase mutants. The oxidized proteome was analyzed by both 14C labeling and biotin-HPDP-based purification methods (Figs. 6 and 7). [14C]NEM-labeled two-dimensional gels showed differences compared with wild-type cells, with higher spot intensities and the appearance of several new spots (compare Figs. 6 (A and B) and 7 (A and B)). Furthermore, a few proteins appeared as doublet or triplet spots (Tsa1, Tsa2, Ahp1, Sod1, and Ccs1) that could correspond to undefined redox conformers or other covalent post-translational modifications. Two-dimensional gels of biotin-HPDP-purified proteins (Fig. 7, C and D) confirmed the new spots and enabled us to identify some of them (Table 1). These are the thiol-based peroxidases Tsa2 and Prx1 and the peroxiredoxin-like Gpx2, Mxr1, and Ccs1. Mxr1 is a methionine-sulfoxide reductase, reducing methionine sulfoxide by a thiol-based mechanism (34). Ccs1 is the copper chaperone for Sod1, also catalyzing Sod1 disulfide bond formation, and has been identified in a disulfide-linked form with Sod1 in vitro (27). $I_{ox}$ values were increased compared with the wild-type strain $I_{ox}$ values (Table 1), but only moderately with regard to the especially high intensities of some spots (Figs. 6B and 7B). Spot intensity changes and the appearance of new spots were contributed not only by increased protein oxidation, but also by increased protein levels, explaining the moderate $I_{ox}$ values changes. Increased steady-state pro-

3 M. B. Toledano, unpublished data.
tein levels were seen in Coomassie Blue-stained two-dimensional gels of total extracts (Fig. 7, E and F) and are likely the consequence of the constitutive Yap1 activation characterizing this strain (20, 35, 36). Indeed, except for Mxr1 and Ccs1, all proteins with increased levels (Fig. 7, B, D, and F, arrows) are Yap1-dependent (37).

Thus, if one considers only \(^{14}\text{C}\)NEM-labeled protein spot intensities, the proteins highly oxidized in thioredoxin-deficient cells are Sod1, Ccs1, Mxr1, Pep4, Pdi1, Ts1a, Ts2a, Ahp1, Prx1, and Gpx2. Note that by including five thiol peroxidases and Mxr1, these proteins make up most of the yeast \(\text{H}_2\text{O}_2\)-metabolizing enzymes. Here, the increased oxidation of these enzymes is consistent with their use of the thioredoxin pathway as the electron donor system for \(\text{H}_2\text{O}_2\) reduction (8, 26, 34, 38). Note also that \(\text{H}_2\text{O}_2\) itself does not cause such a high increase in the oxidation state of \(\text{H}_2\text{O}_2\)-metabolizing enzymes, pointing to the high efficiency of the thioredoxin pathway in \(\text{H}_2\text{O}_2\) reduction. Finally, note that accumulation of disulfide bonds appears to be selective rather than random in thioredoxin-deficient cells.

The \textit{The Oxidized Proteome of Cells with an Altered Glutathione Pathway}—The GSH pathway was tested with the \(\Delta\text{gsh1PRO2}\)-\(\Delta\text{glr1}\) strain (22), which carries deletions of both \(\text{GLR1}\) (encoding glutathione reductase) and \(\text{GSH1}\) (encoding \(\gamma\)-glutamylcysteine synthase, the rate-limiting enzyme in GSH biosynthesis) and which harbors a third mutation in the proline biosynthetic enzyme gene \(\text{PRO2}\), including five thiol peroxidases and Mxr1, these proteins make up most of the yeast \(\text{H}_2\text{O}_2\)-metabolizing enzymes. Here, the increased oxidation of these enzymes is consistent with their use of the thioredoxin pathway as the electron donor system for \(\text{H}_2\text{O}_2\) reduction (8, 26, 34, 38). Note also that \(\text{H}_2\text{O}_2\) itself does not cause such a high increase in the oxidation state of \(\text{H}_2\text{O}_2\)-metabolizing enzymes, pointing to the high efficiency of the thioredoxin pathway in \(\text{H}_2\text{O}_2\) reduction. Finally, note that accumulation of disulfide bonds appears to be selective rather than random in thioredoxin-deficient cells.

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\textbf{DISCUSSION}

Proteomic approaches aimed at identifying proteins with oxidized thiols are important as a means of discovering new redox-regulated processes and dissecting their interplay with the reduced cytoplasm. We have developed two such approaches in \textit{S. cerevisiae} that take advantage of the technique of differential thiol labeling, both targeting proteins with cysteine residues engaged in a disulfide bond, whether intra- or intermolecular, or with GSH. In one, we used biotin-HPDP (25) to purify oxidized protein thiols. In the other, we used \(^{14}\text{C}\)NEM as a thiol-labeling reagent to quantify the extent of protein thiol oxidation. Both approaches showed a large number of the same proteins (~200) with oxidized thiols. We identified 64 of these proteins, several of which had been previously identified in other redox proteomic studies (Table 1). Our data establish that, irrespective of its mechanism, protein thiol oxidation is dependent upon molecular \(\text{O}_2\). We have also shown that \(\text{H}_2\text{O}_2\) does not lead to \textit{de novo} protein thiol oxidation, but rather increases the oxidation state of a select group of oxidized proteins, indicating both high selectivity and a limited contribution of thiol oxidation to the toxicity of this oxidant. Our study also reveals contrasting differences in the oxidized proteome of cells upon inactivation of the thioredoxin or GSH pathway, with specific increases and general decreases in protein oxidation, respectively (see Fig. 6). These differences are suggestive of very different thiol redox control functions for these pathways, assigning an exclusive role for the thioredoxin pathway in \(\text{H}_2\text{O}_2\) metabolism and the presumed thiol redox buffer function for GSH (18, 41). Furthermore, none of these mutant strains revealed the presence of random disulfide bond accumulation, a condition that was expected to occur upon thiol redox control inactivation and referred to as disulfide stress (42).
formation (data not shown). Nonspecific background signals were also present in two recent differential thiol labeling studies (43, 44). Quantification of the extent of protein oxidation is complex. Our method, based on $^{14}$C signal value ratios of oxidized ($S_{ox}$) versus total ($S_{tot}$) protein thiols, reliably establishes a relative protein oxidation scale within two-dimensional gels, but is approximate when comparing two-dimensional gel series because of intrinsic experimental variations. In their identification of the $E. coli$ oxidized protein thiold proteome by differential $[^{14}]$C iodoacetamide thiol labeling, Leichert and Jakob (44) more appropriately calculated protein-specific activities by establishing ratios of spot $^{14}$C signal values to protein quanta measured by Coomassie Blue staining. We failed in using this method because $^{14}$C signals dramatically decreased upon Coomassie Blue staining, hampering measures.

The recognized biological importance of protein thiol oxidation has recently spurred several redox proteomic studies. Differential cysteine-trapping techniques identified oxidized protein thiols in $E. coli$ (44) and mammals (45) and protein thiol sulfenates using arsenite as a specific sulfenic acid reductant (46). Diagonal electrophoreses identified disulfide-bonded proteins (47, 48). Other studies identified S-thiolated proteins in yeast (18) and mammals (49, 50). Emerging from our and these studies is the iterative identification of the same proteins or protein functions, especially enzymes involved in $H_2O_2$ and carbohydrate metabolism, translation factors, and stress chaperones (see Table 1). One of these, the key glycolytic enzyme GAPDH (Tdh3), is redox-sensitive because of the highly reactive nature of its catalytic cysteine. GAPDH has been identified in vivo in the S-thiolated, disulfide-linked, and S-nitrosylated forms. S-Thiolation of GAPDH, which inactivates enzyme activity, has been proposed to regulate glycolytic fluxes during oxidative stress (18). Recently, NO-induced S-nitrosylation of GAPDH has been shown to trigger its binding to the ubiquitin ligase Siah, followed by nuclear translocation and apoptosis (51). GAPDH disulfide bond formation also promotes nuclear accumulation, and its extent correlates with Alzheimer disease progression (52). Protein translation factors were identified as disulfide-bonded (48) or S-thiolated (18) proteins. S-Thiolation of translation factors prevents the inhibition of protein synthesis during oxidative stress (18). The heat stress chaperone HSP70 family has been shown to form intramolecular and mixed disulfides (48) and to undergo S-thiolation (18, 49), but how these redox modifications influence protein function is not yet known. The ubiquitin-conjugating enzyme family, two members of which were identified here (Ubc1 and Ubc4), has been shown to undergo S-thiolation (49) and could be redox-regulated (53).

Identifying the same oxidized proteins in studies crossing species gives reciprocal credence to their results and also more importantly indicates the remarkable specificity and conservation of the protein thiol oxidation phenomenon. Also emerging from these redox proteomic studies is the important overlap between protein thiol oxidation and S-thiolation. This overlap has been pointed out by Cumming et al. (48) and is suggested by the analysis of the strain with an inactive GSH pathway showing a dramatic decrease in oxidation of all oxidized proteins identified in wild-type cells. Redox proteomics is providing novel knowledge of the cellular thiol redox reaction, but still, all different approaches have an important limitation in the inability to identify weakly expressed proteins. For instance, we failed to identify the redox-regulated transcription factor Yap1, the redox transducer Orp1/Gpx3, or the sulfiredoxin Srx1, proteins that are known to carry oxidized thiols (8, 11), at least in response to $H_2O_2$. Future efforts should aim to increase the sensitivity of redox proteomics to low abundant proteins, which should include many of those involved in redox-regulated processes.
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