The 20 S proteasome core purified from Saccharomyces cerevisiae is inhibited by reduced glutathione (GSH), cysteine (Cys), or the GSH precursor γ-glutamylcysteine. Chymotrypsin-like activity was more affected by GSH than trypsin-like activity, whereas the peptidylglutamyl-hydrlyzolyzing activity (casepase-like) was not inhibited by GSH. Cys-sulfenic acid formation in the 20 S core was demonstrated by spectral characterization of the Cys-S(=O)-4-nitrobenzo-2-oxa-1,3-diazole adduct, indicating that 20 S proteasome Cys residues might react with reduced sulfhydrys (GSH, Cys, and γ-glutamylcysteine) through the oxidized Cys-sulfenic acid form. S-Glutathionylation of the 20 S core was demonstrated in vitro by GSH-biotin incorporation and by decreased alkylation with monobromobimane. Compounds such as N-ethylmaleimide (S-sulfhydryl H alkylating), dimedone (SOH sulfenic acid H reactant), or 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (either -SH or -SOH reactant) highly inhibited proteasomal chymotrypsin-like activity. In vivo experiments revealed that 20 S proteasome extracted from H2O2-treated cells showed decreased chymotrypsin-like activity accompanied by S-glutathionylation as demonstrated by GSH release from the 20 S core after reduction with NaBH4. Moreover, cells pretreated with H2O2 showed decreased reductive capacity assessed by determination of the GSH/oxidized glutathione ratio and increased protein carbonyl levels. The present results indicate that at the physiological level the yeast 20 S proteasome is regulated by its sulfhydryl content, thereby coupling intracellular redox signaling to proteasome-mediated proteolysis.

The proteasome is an essential proteolytic complex in eukaryotic cells where it is responsible for the degradation of many cellular proteins. It plays an important role in cell-cycle regulation, cell signaling, including apoptosis, and elimination of abnormal proteins generated by mutation (1, 2) and oxidative damage (3–5). In recent years, many publications have reported the reversible S-glutathionylation of a discrete number of proteins (6). Protein S-glutathionylation seems to play an essential role in redox regulation. This form of regulation has direct effects on both enzyme activity and the ability of transcription and replication factors to bind DNA targets. The mechanism of protein glutathionylation has evolved in recent years from the strict belief that this event would take place solely when intracellular GSSG3 levels increased upon oxidative stress, with the formation of mixed disulfides between protein Cys-SH residues and GSSG (7, 8), to the present and more complete understanding of protein sulfhydryl chemistry and evidence showing formation of protein-Cys-SOH derivatives (9) that are prone to S-glutathionylation by reduced GSH (10).

Either individual enzyme activity or global cellular responses can be rapidly controlled by the oxidation of protein-Cys-SH residues (reviewed in Ref. 6) generating Cys-SH, Cys-SO2H, and Cys-SO3H acid forms (11), where Cys-SOH is susceptible to S-thionylation and reversibly reduced to Cys-SH (11–14). Cys-SH formation and S-glutathionylation during enzyme catalysis and redox signaling are novel cofactors in the context of redox regulation (14). In a recent publication (15) it was demonstrated that the reduced and oxidized forms of GSH modulate the chymotrypsin-like activity of purified 20 S proteasome extracted from mammalian cells. In the present report we show that the activity of the 20 S proteasome purified from the yeast Saccharomyces cerevisiae is also sensitive to GSH, though in a different way from that observed in the mammalian proteasome. The 20 S proteasome extracted from yeast is inhibited by reduced GSH and S-glutathionylated in vitro, as well as in vivo, when cells are submitted to oxidative challenge. Considering that the proteasome plays important role in cell signaling regulation by hydrolysis of many proteins involved in cascade events of the cellular regulatory pathways, it is not surprising that its activity may be regulated by its Cys-SH residues redox status.

MATERIALS AND METHODS

Chemicals and Reagents—Diethylenetriaminepentaacetic acid (DTPA), dimedone (5,5-dimethyl-1,3-cyclo-hexanone), dinitrophenylhydrazine, dithionitrobenzoic acid (DTNB), N-ethylmaleimide (NEM), fluorogenic substrates succinyl-Leu-Leu-Val-Tyr-MCA (s-LLVY-MCA) and t-butoxycarbonyl-Gly-Lys-Arg-MCA, γ-glutamylcysteine (GC), and streptavidin immobilized on 4% beaded agarose were purchased from Sigma. The fluorogenic substrate carboxbenzoxyl-Leu-Leu-Glu-MCA, the protease inhibitors lactacystin and tri-leucine vinyl sulfone, and monobromobimane (mBrB) were purchased from Calbiochem. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD) was purchased from Aldrich. All other reagents used were of analytical grade, and the water was purified with the Milli-Q system.

Yeast Strains and Growth—S. cerevisiae BY4741 strain (MATa his3Δ1 leu2Δ30 met15Δ0 uro3Δ0) was obtained from Euroscarf, Frankfurt, Germany. 1

1 The abbreviations used are: GSSG, oxidized glutathione; Cys-SH, reduced cysteine; Cys-SOH, Cys-sulfenic acid; Cys-SO2H, Cys-sulfonic acid; Cys-SO3H, Cys-sulfonic acid; DTNB, dithionitrobenzoic acid; DTPA, diethylenetriaminepentaacetic acid; DTT, dithiothreitol; GC, γ-glutamylcysteine; GSH, reduced glutathione; mBrB, monobromobimane; MCA, 4-methylcoumarin-7-amide; NEM, N-ethylmaleimide; s-LLVY-MCA, succinyl-Leu-Leu-Val-Tyr-MCA; HPLC, high pressure liquid chromatography; ANOVA, analysis of variance.
obtained from strains containing FLAGHis6-tagged PRE1; however, we
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proteasome was eluted from the agarose beads, when specified, by
in vivo
protocol. The final preparations were analyzed by SDS-PAGE

20 S proteasome preparations were treated with H2O2. Proteasome
chromatography was performed using a HiTrap™ chelating HP column
was determined by the difference between total GSH and GSSG.
Intracellular reduced GSH was determined by the reduction of 20 S

Extract and Purification of the 20 S Proteasome—Yeast cells were
disrupted according to the protocol described in Ref. 16. The 20 S
proteasome from untreated strain was purified by sequential chromatography
(Phenyl Sepharose, Sephadryl S-400, MonoQ column, Amersham Biosciences), and a monoQ column in an HPLC system. The purification was
performed according to a method described previously (17), except that gel filtration was used instead of a sucrose gradient, and the monoQ column was used in the HPLC instead of the fast protein liquid chromatography system. The 20 S proteasome core from the RJD1144 strain with a FLAGHis6-tagged PRE1 subunit was purified by affinity chromatography or, when specified, by immunoprecipitation. Affinity chromatography was performed using a HiTrap™ chelating HP column (Amersham Biosciences) attached to a P1 peristaltic pump (Amersham Biosciences) according to the manufacturer’s protocol. The proteasome was eluted from the column with 300 mM imidazole. Active fractions from the final steps of all preparations were assayed for the fluorogenic peptide s-LLVY-MCA and confirmed by inhibition with tri-locine vinyl sulfone. Final enriched fractions were pooled, concentrated, and reloaded twice in a Centricon CM-30 apparatus (Amicon). Purification by immunoprecipitation was performed according to the protocol described in Ref. 16 with the anti-FLAG® M2 affinity gel freeze-dried antibody (Sigma) immobilized on agarose beads. The 20 S proteasome was eluted from the agarose beads, when specified, by incubation with the FLAG® peptide (Sigma) according to the manufacturer’s protocol. The final preparations were analyzed by SDS-PAGE and by electrophoresis on a 5% non-denaturing polyacrylamide gel according to the protocol described in Ref. 17. The three different 20 S proteasome preparations described here were used for the assays used for this assay. The results shown below (in vivo and in vitro) refer to the preparations obtained from strains containing FLAGHis6-tagged PRE1; however, we did not observe any difference compared with preparations from strains containing untagged PRE1 (data not shown).

Hydrolysis Assay of Fluorogenic Peptides—20 S proteasome preparations were incubated at 37 °C in 10 mM Tris/HCl buffer, pH 7.8, containing 20 mM KCl and 5 mM MgCl2, here referred to as standard buffer. Incubation was started by the addition of 25–50 μM of the peptide (other additions are specified in the legends to the figures). The reaction was stopped by adding 4 volumes of 0.1 M sodium borate, pH 9, containing 7.5% ethanol. Fluorescence emission at 480 nm (excitation at 385 nm). MCA from the substrates was calculated from a standard curve of free MCA.

Oxidation of 20 S Proteasome with H2O2—When specified, purified 20 S proteasome preparations were treated with H2O2. Proteasome concentration in these experiments was typically 50 μg or as otherwise specified, and the preparation was incubated for 30 min at room temperature in the presence of 5 mM H2O2, with or without 100 μM DTPA in standard buffer. After incubation, excess H2O2 was removed by three cycles of centrifugation and redilution with standard buffer through Microcon CM-10 filters (Amicon). Aliquots of the 20 S proteasome were taken for further incubation or for hydrolysis assay after determination of protein concentration.

Reduction of 20 S Proteasome with NaBH4—After isolation of the 20 S core with the anti-FLAG antibody, as described above, the agarose beads were washed three times according to the manufacturer’s protocol. NaBH4 reduction was performed with the 20 S proteasome bound to the anti-FLAG-agarose bead complex incubated in the presence of 20 mM NaBH4 buffered in 50 mM Tris/HCl, pH 8.6, for 40 min at 37 °C. After the suspension was centrifuged, the supernatant was utilized for GSH determination (described below), and the beads were washed twice with standard buffer. The proteolysis assay, when specified, was performed with the 20 S proteasome still bound to the beads. GSH Determination—Intracellular GSH was extracted by lysing the cell pellets in 1 volume of glass beads and 2 volumes of 3.5% sulfosalicylic acid. The suspension was vortexed for 20 min at 4 °C in a multi-fold vortex and centrifuged at maximum speed in a microcentrifuge. This procedure was repeated twice, and the supernatants were combined. Total GSH, as well as GSSG, was assayed according to a protocol described previously (15). The determination was performed by reaction with DTNB in the presence of glutathione reductase and NADPH. Samples for GSSG measurement were incubated previously for 1 h with NEM after adjusting the pH to 7. GSH released from 20 S proteasome was detected as follows: 20 S proteasome isolated by immunoprecipitation was reacted with NaBH4 as described above. The supernatant recovered was filtered through a Microcon CM-10 filter. The filtrate was acidified to remove remaining NaBH4 (reaction with NaBH4 was performed at pH 8.6) and the pH was adjusted to 7 prior to the addition of DTNB, glutathione reductase, and NADPH. GSH detection was in detection in the presence of glutathione reductase and NADPH comparing in detection in the presence of DTNB only. GSH concentration was calculated from standard curves of GSH. Intracellular reduced GSH was determined by the reduction of total GSH and GSSG.

Proteasome S-Modification by the GSH-Biotin Derivative—H2O2-, treated or untreated 20 S proteasome (450 μg) was incubated with 5 mM GSH-biotin for 20 min at room temperature. Excess GSH-biotin was removed by filtration through a Microcon CM-10 filter followed by redilution three times in standard buffer. After washing, protein was diluted in standard buffer, mixed with streptavidin-agarose beads (100 μl/20 μg protein), and incubated for 60 min at 4 °C. The beads were washed by four cycles of centrifugation/redilution in standard buffer containing 150 mM NaCl, and the remaining protein was eluted from the beads by incubation for 30 min in standard buffer containing 0.1% SDS and 10 mM DTT, followed by centrifugation at maximum speed for 15 min. The supernatants were submitted to 12.5% SDS-PAGE after determination of protein concentration.

Cell Viability—Aliquots of the cell suspension were diluted to an A600 of 0.2 immediately after the treatments, followed by a further 5,000× redilution. Aliquots of 100 μl of the final suspension were spread on YPD-agar plates (five per sample) and incubated at 30 °C for 48 h. After incubation, colonies were counted manually. Cell viability assays were repeated at least five times.

Protein Determination—Protein concentration was determined with Bradford® reagent (Bio-Rad).

RESULTS

Yeast 20 S Proteasomal Activity Is Inhibited by Sulphydral Compounds—Purified 20 S proteasome core isolated from S. cerevisiae was assayed for hydrolysis with the s-LLVY-MCA substrate in the presence of GSH, GSSG, and Cys (Fig. 1). GSSG had a slight effect on 20 S proteasomal activity at any concentration tested. However, GSH or Cys strongly inhibited 20 S proteasomal activity. Neither the GSH precursor GC also inhibited chymotrypsin-like activity in a pattern similar to that obtained by Cys treatment (results not shown). The trypsin-like activity determined by the hydrolysis of the fluorogenic substrate β-butyroylcarbonyl-Leu-Lys-Arg-MCA was 50% decreased in the presence of 10 mM GSH (results not shown). The trypsin-like specific activity (μmol MCA/min/mg) represented less than 5% of the chymotrypsin-like activity measured under the experimental conditions described here (the same protocols were used for both determinations). The peptidylglutamyl-hydrolyzing activity assayed by the hydrolysis of the fluorogenic peptide carbobenzoxy-Leu-Leu-Glu-MCA was not affected by any of the sulphydryl compounds tested (GSH, GC, or Cys) at any concentration or by GSSG (result not shown).

One question raised by these results was how the reduced form of GSH and other thiol tested (Cys and GC), but not GSSG, inhibited 20 S proteasomal activity. Our working hypothesis was that Cys-SH residues in the 20 S proteasome structure are probably oxidized to the Cys-SOH form, susceptible to S-glutathionylation by GSH, as described elsewhere (6, 20), but not by GSSG, according to the reaction,
To test this hypothesis, purified 20 S proteasome preparations were incubated in the presence of 5 mM H₂O₂ and 100 μM DTPA. The iron chelator DTPA was used to prevent the Fenton reaction and consequently the generation of the very reactive hydroxyl radical, which may produce nonspecific protein oxidation, in addition to iron-catalyzed thiol oxidation and generation of several protein-sulfur derivatives (21). Thus, in the absence of iron or another transition metal, sulphydryl oxidation to Cys-SOH may prevail, though further oxidation of Cys-SOH to Cys-SO₂H and Cys-SO₃H is expected (11). According to our results, 20 S proteasome treatment with H₂O₂ in the presence of DTPA decreased chymotrypsin-like activity to 80% of the original level (Fig. 2A), whereas when H₂O₂ treatment was performed in the absence of DTPA the activity was reduced to 60% (data not shown). When 20 S proteasome was pretreated with H₂O₂ plus DTPA the chymotrypsin-like activity was much more affected by GSH (Fig. 2A). In fact, after H₂O₂/DTPA pretreatment, inhibition by GSH occurred at concentrations as low as 0.01 mM (35% inhibition), and GSH at 1 mM promoted stronger inhibition (60%; see Fig. 2A) than that verified without pretreatment (Fig. 1). In contrast, GSSG did not affect 20 S proteasome activity (Fig. 2A). These results are in agreement with our hypothesis in the reaction shown above.

In contrast to H₂O₂, DTT treatment enhanced proteasomal activity by about 15–20% (Fig. 2B). Moreover, it sensitized the proteasome to GSH incorporation after H₂O₂ treatment. When 20 S proteasome samples were treated with 10 mM DTT, followed by incubation with H₂O₂/DTPA, the chymotrypsin-like activity was even more sensitive to GSH when compared with samples not preincubated with DTT (Fig. 2A). In this condition, proteasomal activity was decreased to 50 and 5% in the presence of 0.5 or 1 mM GSH, respectively (Fig. 2B), whereas when the same GSH concentrations were employed without DTT pretreatment, chymotrypsin-like activity was decreased only to 60 and 40%, respectively (Fig. 2A). Cys-SH already present in 20 S proteasome was probably reduced to Cys-SH by DTT, with the consequent prevention of hyperoxidation of Cys-SOH to Cys-SO₂H or Cys-SO₃H by H₂O₂ treatment.

As expected according to our hypothesis described in the reaction shown above, when the 20 S core was pretreated only with DTT, no alteration by GSH was observed in its activity (Fig. 2B). Also, proteasome reduced by DTT was not inhibited by GSSG (result not shown) as could be expected, because GSSG is able to react with sulfhydryl groups to form mixed disulfides (7, 8). Reduced Cys-20 S might be prevented from reacting with GSSG by structural constraints imposed by nearby groups.

**Proteasomal Activity Is Inhibited by Cys-SH and Cys-SOH Reactants**—To demonstrate that modification of Cys-20 S core residues is responsible for the inhibition of chymotrypsin-like activity, we next tested this activity in the presence of Cys-SH and Cys-SOH reactants, such as NBD, dimedone, and NEM (Table I). We observed that chymotrypsin-like activity was inhibited 40 and 30% by 50 μM NBD and 1 mM NEM, respectively. NEM alkylates Cys-SH whereas NBD is incorporated into both Cys-SH and Cys-SOH. This might be, at least in part, the reason why NBD is more potent than NEM in terms of chymotrypsin-like activity inhibition. After inhibition by NBD, 20 S proteasomal activity was recovered by incubation with 5 mM DTT (result not shown). Because DTT treatment leads to NBD release (9), this result indicates that inhibition was promoted by Cys conjugation.

The specific Cys-SOH reagent dimedone (9) produced low inhibition when compared with the former reagents. Dimedone
promoted 23% inhibition only at 10 mM concentration. On the other hand, when purified 20 S proteasome preparations were pretreated with H2O2/DTPA prior to incubation with dimedone, we observed increased proteolytic inhibition compared with the inhibition observed in the absence of H2O2/DTPA pretreatment followed by dimedone incubation (results shown in Table I). Upon H2O2 pretreatment, 10 mM dimedone decreased chymotrypsin-like activity to 50% of that observed in H2O2 control samples.

Taken together, the results reported thus far indicate that any group located in Cys residues of the 20 S core decreases its hydrolytic activity, at least the chymotrypsin-like activity, which is considered the strongest of its activities (1). It seems that Cys residues in the 20 S proteasome must be reduced as much as possible to allow maximum activity. Nevertheless, these residues appear to oxidize easily to Cys-SOH.

**Cys-SOH Formation and S-Glutathionylation**—To demonstrate that Cys residues in the 20 S proteasome structure are oxidized to Cys-SOH, 20 S proteasome preparations were incubated with NBD. This compound reacts with Cys-SH, as well as with Cys-SOH. NBD adducts of Cys-SOH and Cys-SH can be distinguished by their spectra (9). The proteasome-Cys-S(O)-NBD adduct was generated by 20 S proteasome pretreatment with H2O2/DTPA followed by NBD incubation (Fig. 3, dashed line spectrum). This adduct showed maximum absorbance at 345 nm whereas the purified 20 S core not oxidized by H2O2 yielded the NBD adduct with a maximum absorbance at 420 nm (Fig. 3, solid line spectrum).

We also reacted denatured 20 S proteasome preparations with the −SH reactant mBrB (22). Denatured 20 S proteasome samples were preincubated with DTT or GSH. After incubation, DTT and GSH were removed, and the samples were treated overnight with 150 μM mBrB. Proteasome-Cys-bimane conjugates were detected by fluorescence emission recorded after removal of mBrB. DTT-reduced samples showed fluorescence emission at least twice as high as control samples, whereas GSH-treated samples showed reduced fluorescence, probably because 20 S proteasome mixed disulfides could not conjugate with mBrB (Fig. 4). This result indicates that GSH does not play the role of a reducing compound as DTT, but probably GSH was incorporated into the protein structure by S-conjugation.

To demonstrate effectively that the effect of GSH on proteasomal activity is because of S-glutathionylation, 20 S proteasome preparations were incubated with the GSH-biotin derivative according to the protocol described in the literature (18). This method allows the direct determination of protein S-glutathionylation, because biotinylated GSH-proteasome complexes can be isolated by the streptavidin affinity procedure. 20 S proteasome pretreatment with H2O2 increased GSH incorporation (Fig. 5). The protein concentration determined after protein elution from the streptavidin-agarose beads was 4-fold higher in samples pretreated with H2O2. Protein recovered from control and H2O2-treated samples after elution from the streptavidin beads was 10 and 40.5 μg, respectively (the amount of protein reacted with GSH-biotin was the same in both samples, i.e. 300 μg). This result is direct proof that 20 S proteasome is susceptible to S-glutathionylation by means of GSH addition to Cys-SOH. Because a significant amount of S-glutathionylated 20 S proteasome was detected in the control sample, part of its Cys residues were probably already oxidized to Cys-SOH (Fig. 5). It should be emphasized that the reaction with GSH-biotin was performed under non-denaturing conditions. The preparation was brought to denaturing conditions only after incubation with and removal of GSH-biotin to displace the 20 S core from the streptavidin-agarose beads. Thus the results described in Fig. 5 do not allow us to predict how many or which subunits of the 20 S core were S-glutathiony-

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**Table I**

| Proteasome incubation                               | MCA release μmol × min⁻¹ × mg⁻¹ |
|-----------------------------------------------------|---------------------------------|
| Control (no addition)                               |                                 |
| H2O2/DTPA-treated samples                          |                                 |
| NBD                                                 | 25.4 ± 1.4                      |
| Dimedone                                            | 20.3 ± 1.5                      |
| 1 μM                                                | 15.7 ± 0.8                      |
| 5 μM                                                | 24.1 ± 1.1                      |
| 10 μM                                               | 22.6 ± 1.8                      |
| 50 μM                                               | 19.6 ± 1.8                      |
| 10 mM                                               | 11.6 ± 1.0                      |
| NEM                                                 | 17.6 ± 1.0                      |
| 1 μM                                                | 10.4 ± 0.8                      |
| 5 mM                                                | 7.6 ± 0.6                       |

* p ≤ 0.0001 (ANOVA).

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**Fig. 3.** NBD-modified 20 S proteasome UV-visible spectra. The Cys-S(O)-NBD conjugate (dashed line) was generated by incubating denatured 20 S proteasome (40 μg) in standard buffer with H2O2/DTPA, as described under "Materials and Methods," followed by incubation with 100 μM NBD for 30 min. All reagents were washed out by filtration through Microcon CM-10 filters prior to absorbance measurements. The Cys-S-NBD conjugate (solid line) was generated by incubating denatured 20 S proteasome (40 μg) in standard buffer with 100 μM NBD. Proteasome was denatured by preincubation in 5 M guanidine solution buffered in 50 mM Tris/HCl, pH 7.5. Spectra were recorded with a Hitachi spectrophotometer.
in 500 mM guanidine buffered in 0.1 M Tris/HCl, pH 7.5. Fluorescence was recorded at 476 nm (excitation at 400 nm). Results are expressed as means ± S.D. of three independent experiments. *, p < 0.000025; **, p at least 0.001 (ANOVA).
Cells were grown in YPD medium to \( A_{600} \) of 0.6–0.8 followed by a further 1-h incubation with \( H_2O_2 \) added to the medium at the final concentrations shown. After incubation, aliquots were taken for cell viability assay. The remaining cells were harvested by centrifugation and washed twice with water. Cell extract preparation, 20 S proteasome purification by immunoprecipitation, reduction with NaBH\(_4\), and measurements of proteolysis, total and oxidized glutathione, GSH released from 20 S proteasome, and carbonyl proteins are described under "Materials and Methods." ND, not determined.

### Table III

| Cell viability, no. of colonies | H\(_2\)O\(_2\) | | | |
|--------------------------------|--------------|--------------|--------------|--------------|
| Proteasomal activity and redox parameters upon H\(_2\)O\(_2\) cell treatment | No addition | 0.1 | 0.5 | 1 |
| 20 S proteasome fraction | | | | |
| Before NaBH\(_4\), reduction | 100 ± 6 | 102 ± 3 | 50 ± 3\(^e\) | 36 ± 2\(^e\) |
| After NaBH\(_4\) reduction | 110 ± 7 | ND | 70 ± 4\(^e\) | ND |
| Proteasome-free cell extract\(^b\) | 100 ± 4 | 128 ± 9\(^e\) | 160 ± 7\(^e\) | 165 ± 8\(^e\) |
| GSH, nmol/mg\(^c\) | Not detected | ND | 1.40 ± 0.05 | ND |
| GSH/GSSG | 54 ± 3 | 66 ± 2\(^e\) | 37 ± 9\(^e\) | 29 ± 1\(^e\) |
| Protein carbonyl formation, nmol/mg\(^f\) | 20 ± 2 | 24 ± 2 | 27 ± 1\(^e\) | ND |

\(^a\) \( p \leq 0.00027 \) (ANOVA).
\(^b\) Results are expressed as percentage of control samples (No addition), set as 100.
\(^c\) Proteolysis was determined in the cell extract, free of the 20 S proteasome particle, recovered after incubation two times with anti-FLAG antibody.
\(^d\) GSH was released from samples of 20 S proteasome after reduction with NaBH\(_4\), as described in footnote c.
\(^e\) The protein fraction from cell extracts was precipitated with 20% trichloroacetic acid. All results are means ± S.D. of four to five independent experiments.
\(^f\) \( p = 0.027 \) (ANOVA).

is an intermediate form involved in redox regulation and catalysis by protein sulfhydryl groups (reviewed in 6). Distinct fates for protein Cys-SOH have been considered (11, 14) whereby S-glutathionylation would be one of the mechanisms modulating protein activity. Protein S-glutathionylation is a reversible process, and there is considerable evidence that GSH release is controlled enzymatically, probably by glutaredoxin (29).

In this manuscript we describe the mechanism by which 20 S proteasome is glutathionylated in vitro. Taken together, those results indicate that during moderate oxidative stress Cys residues inside the 20 S proteasome core might be oxidized to Cys-SOH, reversibly protected by S-glutathionylation, and most likely deglutathionylated after cellular recovery from oxidative stress. After incubation 20 S proteasome was purified from the cell extract by immunoprecipitation and assayed for proteolysis before and after reduction with NaBH\(_4\). Table III shows that one to two molar GSH was released per mol of 20 S proteasome, consistent with the known stoichiometry of S-glutathionylation of 20 S proteasome in vivo (15). As shown in footnote c, the GSH/GSSG ratio upon H\(_2\)O\(_2\) cell treatment.

The reason for the partial recovery of proteasome activity after NaBH\(_4\) treatment (Table III) is probably the reduction of Cys-SOH or Cys-SSG to Cys-SH. However, other oxidative processes are responsive to reduction by NaBH\(_4\), e.g. formation of carbonyl and Schiff bases (19), but in this case the original amino acid structures are not regenerated as expected for Cys-SOH and Cys-SSG. GSH release from the 20 S core after reduction with NaBH\(_4\) (Table III) is strong evidence that proteasomal activity might be regulated by S-glutathionylation under intracellular oxidative conditions, and consequently decreased reductive capacity, here attested by a reduced GSH/GSSG ratio upon H\(_2\)O\(_2\) cell treatment.

Taken together, results obtained in vitro after cell treatment with H\(_2\)O\(_2\) indicate that loss of reductive cellular capacity, according to GSH/GSSG ratios and protein carbonyl levels, is associated with loss of 20 S proteasomal activity and with its S-glutathionylation. These data are an indication of redox modulation of 20 S proteasome activity.

### DISCUSSION

It is becoming increasingly apparent that many oxidant-sensitive proteins are S-glutathionylated in response to intracellular redox status (6, 18, 27) (reviewed in Ref. 28). Cys-SOH...
cytoplasm associated with the nuclear endoplasmic reticulum network (31).

It is interesting to point out that GSH distribution inside yeast cells is still not clear. Although it has been demonstrated already that GSH is distributed inside the mammalian nucleus at concentrations as high as in the cytoplasm (32), its presence inside the yeast nucleus is not clear, neither is the distribution of glutaredoxin isoforms inside yeast cells clear thus far. A mammalian nuclear isoform was described already (33, 34) whereas information on yeast nuclear isoforms is still lacking. Information on yeast nuclear isoforms is clear thus far. A yeast nuclear isoform was described already (33, 34)

Another example of redox regulation and proteasome distribution and its role in the yeast cell, as discussed above, elucidation of GSH distribution inside yeast cells is an important matter to corroborate the findings discussed here.

Another important difference suggested in the literature (30, 31) is that yeast proteasome is always capped by the 19 S regulatory unit in contrast to the finding that its counterpart, the mammalian 20 S proteasome, is found in 3-4 fold excess over the 19 S regulatory unit. In our opinion, even if it is true that the yeast proteasome is always capped with the 19 S regulator, this does not rule out the possibility that the catalytic unit represented by 20 S proteasome is regulated independently of either protein ubiquitinylation or substrate recognition by the 26 S proteasome. Our results indicated that redox regulation by glutathionylation is important in vitro (Table III). Other examples of redox regulation and S-glutathionylation have been described for other metabolic processes (27, 35, 36). Our results lead us to speculate that 20 S proteasomal activity can be modulated by S-glutathionylation through the increased presence of oxidants, which would be used in signaling processes. Besides, transient 20 S proteasome inhibition would decrease the hydrolysis of proteins responsible for redox signaling, e.g. AP-1-like factors. AP-1-like proteins are sensors of the redox state of the cell (37) and, as already demonstrated in mammalian cells, are degraded by the proteasome (38).

The metabolic advantage of protein S-glutathionylation in redox signaling is the prevention of irreversible oxidation of the Cys thiol group to Cys-SO2H or Cys-SO3H, permitting protein reactivation by reduction. Such mechanism might work in parallel to the main mechanism controlling proteasome-mediated proteolysis, i.e. ubiquitinylation. The relationship between proteasome glutathionylation and ubiquitinylation of substrates remains to be established.

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*J. Biol. Chem.* 2003, 278:679-685.
doi: 10.1074/jbc.M209282200 originally published online October 29, 2002

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