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20S proteasome activity is modified via S-glutathionylation based on intracellular redox status of the yeast *Saccharomyces cerevisiae*: Implications for the degradation of oxidized proteins

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ABSTRACT

Protein S-glutathionylation is a post-translational modification that controls many cellular pathways. Recently, we demonstrated that the α5-subunit of the 20S proteasome is S-glutathionylated in yeast cells grown to the stationary phase in rich medium containing glucose, stimulating 20S core gate opening and increasing the degradation of oxidized proteins. In the present study, we evaluated the correlation between proteasomal S-glutathionylation and the intracellular redox status. The redox status was controlled by growing yeast cells in distinct carbon sources which induced respiratory (glycerol/ethanol) or fermentative (glucose) metabolism. Cells grown under glycerol/ethanol displayed higher reductive power when compared to cells grown under glucose. When purified from cells grown in glucose, 20S proteasome α5-subunit exhibited an intense anti-glutathione labeling. A higher frequency of the open catalytic chamber gate was observed in the S-glutathionylated preparations as demonstrated by transmission electron microscopy. Therefore, cells that had been grown in glucose displayed an increased ability to degrade oxidized proteins. The results of the present study suggest that 20S proteasomal S-glutathionylation is a relevant adaptive response to oxidative stress that is capable to sense the intracellular redox environment, leading to the removal of oxidized proteins via a process that is not dependent upon ubiquitylation and ATP consumption.

Introduction

The proteasome is the main nuclear and cytosolic proteolytic system that degrades intracellular proteins, and it exists in distinct forms. The 26S proteasome degrades proteins that have been modified by a poly-ubiquitin chain and that are involved in major cellular functions related to signaling, cell cycle regulation and homeostasis. The 26S proteasome is composed of a catalytic unit, termed the 20S proteasome (20S),[1] and the 19S regulatory unit, which is coupled to one or both sides of the 20S. The proteasome also exists in a form that is devoid of regulatory subunits, which is termed the free 20S. In mammalian and yeast cells, the 20S accounts for one-third of the total proteasome [1–3]. The degradation of oxidatively modified proteins is considered to be one of the major roles of the 20S [4–6], which is of importance because few repair systems for oxidatively modified amino acid residues exist. The relevance of 20S-mediated degradation of oxidized proteins has been demonstrated in various cellular models to be a ubiquitin- and ATP-independent process [7–10].

The 20S is cylindrical in shape and is composed of four heptameric rings that are ordered as follows: $\alpha_7\beta_7\beta_7\alpha_7$. The outer α rings are responsible for proteasome gating, and the two β rings contain three catalytic sites disposed in the β1, 2 and 5 subunits, summarizing six active sites in both β-heptamers. The opening of the 20S gate is favored by coupling of the 19S unit, or alternatively, by other activators, such as the 11S and PA200 [11]. The binding of poly-ubiquitylated substrates to the 19S regulatory unit promotes

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[1] *Abbreviations used:* BSA, bovine serum albumin; 2-DE PAGE, two-dimensional poly-acrylamide gel electrophoresis; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; Glu, yeast cells grown in glucose-synthetic medium; Gly, yeast cells grown in glycerol/ethanol-synthetic medium; GR, glutathione reductase; Grx2, recombinant glutaredoxin 2; GSH, glutathione; GSSG, oxidized glutathione; HRP, horse radish peroxidase; OD, optical density; 20S, 20S proteasome; 26S, 26S proteasome; TEM, transmission electron microscopy; Trr, thioredoxin reductase; Trx, thioredoxin.

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gate opening, as previously demonstrated by the increased peptidolytic activity of the 20S [12]. In addition, ATP binding to the ATPase hexameric ring, located in the base of the 19S unit, independent of ATP hydrolysis, is important for gate opening [13].

All of the free 20S core particles within the cells were previously assumed to be in the closed gating conformation, although evidence has indicated that oxidized and disordered proteins can be degraded by the 20S proteasome *in vitro* and *in vivo* [7,14]. We recently demonstrated that S-glutathionylation of the 20S proteasomal α-subunits promotes gate opening in a 19S-independent manner, increasing the degradation of oxidized proteins [15].

Cysteinyl-redox modification is a widespread enzymatic and regulatory mechanism that is coupled to intracellular redox signaling [16,17]. Protein S-glutathionylation is among these modifications and is related to many regulatory pathways, ranging from gene expression to the modification of transcription factors to enzymatic modulation [18–20].

Our present hypothesis is that the post-translational modification of the 20S via S-glutathionylation may play an antioxidant role, controlling oxidized protein levels inside the cells. Accordingly, we observed that the regulation of the 20S by S-glutathionylation is correlated with the intracellular redox state. Therefore, in the present study, we evaluated the correlation between the intracellular redox status, proteasomal S-glutathionylation and the removal of oxidized proteins.

Materials and methods

Materials

Reagents were obtained from the following sources: anti-DNP antibody, azo-modified Bovine Serum Albumin (azo-BSA), dithiothreitol (DTT), H_2O_2, reduced glutathione (GSH) and yeast glutathione reductase from Sigma; anti-20S (subunits β1 and β7), anti-ubiquitin and anti-19S (Rpt4 subunit) antibodies from Merck; the molecular weight markers for SDS–PAGE from GE Healthcare; anti-actin and anti-ubiquitin antibodies from Santa Cruz Biotechnology, Inc.; anti-glutathione antibody from Arbor Assay; anti-actin antibody from ICM; the Bradford protein assay reagent from Bio-Rad; Amplex Red from Molecular Probes; and horse-radish peroxidase (HRP) from Pierce. Anti-Grx2, anti-Trr1 and anti-Trx3 antibodies were generated in the Instituto Butantan via rabbit immunization with yeast recombinant proteins, as previously described [21].

Yeast strain and growth

The *Saccharomyces cerevisiae* RJD1144 strain (MATa his3Δ200 leu2-3,112, lys2-801 trp1Δ63 ura3-52 $PRE1^{FH}$::Ylplac211 URA3), which was derived from JD47-13C, was kindly donated by Dr. Raymond Deshaies, Division of Biology, Caltech, Pasadena, CA. This strain contains a 20S Pre1 subunit that is tagged with a flag peptide sequence and a polyhistidine tail [22]. The cells were diluted to an OD_{600} of 0.1, cultured in synthetic medium containing either 2% glucose (Glu) or 2% glycerol plus 2% ethanol (Gly) at 30 °C with reciprocal shaking and harvested after a 48 h incubation. Growth to the stationary phase was monitored using a growth curve (Suppl. Data, Fig. S1).

Extraction and purification of the 20S proteasome

The 20S was purified using either affinity chromatography through a continuous gradient of imidazole in an HPLC system (Äkta Purifier GE Healthcare) or immunoprecipitation with the ANTI-FLAG® M2 Affinity Gel Freezer-safe (Sigma), as previously described [23].

Intracellular measurements of total and oxidized glutathione, oxygen consumption, and H_2O_2 removal

Intracellular GSH and GSSG extraction and determination were performed as previously described [23]. Oxygen consumption was measured using a Clark-type electrode in a glass cuvette that was equipped with magnetic stirring, as described by Monteiro et al. [24]. To evaluate the total H_2O_2 removal activity, 5–1000 μg of the protein extracts of freshly lysed yeast cells were incubated with 0.1 M phosphate buffer at pH 7.0 for 3 min at 37 °C in the presence of 2 mM H_2O_2. A 2.5 μl aliquot was collected and diluted into 200 μl of buffer containing 50 μM Amplex Red and 1.0 U/ml of horseradish peroxidase (HRP). Fluorescence emission was recorded at 587 nm (excitation at 563 nm).

Two-dimensional gel electrophoresis (2-D PAGE)

Purified 20S preparations (50 μg) were subjected to 2-D PAGE to separate the 20S subunits. The first dimension was carried out on 7 cm IPG strips on a 4–7 pH gradient, as specified in the legends of the figures. After strip rehydration for 16 h at room temperature, IEF was conducted in gradient mode on an IPGphor 3 IEF system (GE Healthcare) according to the manufacturer's recommendations. The second dimension was performed on a 12.5% SDS–PAGE, and the protein spots were visualized using Coomassie Brilliant Blue R-250 staining. The proteasomal subunits were identified using Maldi-TOF-MS fingerprinting, as described by Silva et al. [15]. When specified, the subunits that had been separated using 2-D PAGE electrophoresis were transferred to nitrocellulose membranes for anti-GSH labeling.

Immunoblotting

Immunoblotting was performed as outlined in the protocol that was enclosed in the ECL™ Western Blotting System packaging (*GE* Healthcare). After being labeled with the primary antibody, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and the protein signals were detected using Enhanced Chemiluminescence Western Blotting Detection Reagents (*GE* Healthcare). The band intensities were quantified using the Histogram tool of the AdobePhotoshop software version 6.0. The dilutions of the primary antibodies were as follows: Anti-GSH, 1:500; anti-Actin, 1:750; anti-DNP, 1:5000; anti-20S, 1:1000; anti-19S, 1:1000; anti-Ub, 1:200; Anti-Trr1, 1:2000; anti-Trx3, 1:2000; and anti-Grx2, 1:1500.

Proteolysis determination

Total extracts of cells that had been grown in glucose and glycerol/ethanol-containing media were utilized to evaluate the degradation of the azo-BSA_{ox} derivative, as follows: Total extract (200 μg of total protein) was incubated for 2 h at 37 °C, followed by protein precipitation with 20% TCA and centrifugation. The supernatants were collected for absorbance measurements at 412 nm. When specified, 1 mg of the total cellular extracts was utilized to immunoprecipitate the 20S using anti-FLAG Sepharose resin [23]. After a 2-h incubation, the resin was washed 3 times with standard buffer containing 150 mM NaCl. After washing, the samples were resuspended in standard buffer and incubated in the presence of azo-BSA_{ox} for 2 h at 37 °C followed by centrifugation, and the supernatants were collected for absorbance determinations. The cell extracts were also assayed after immune-depletion of the 20S and are representative of the cellular hydrolytic ability in the

absence of the 20S. Azo-BSA was oxidized by incubation of the azo-modified BSA in the presence of 5 mM H_2O_2 for 30 min at room temperature and the remaining H_2O_2 was removed by cycles of filtration and redilution through YM-10 micro-filters (Millipore).

Negative staining of the 20S particles using TEM

TEM quantitative analyses of the purified 20S preparations were manually performed by counting the frequencies of the open or closed structures from proteasomal populations that were obtained from two independent preparations of both growth conditions in glucose- and glycerol/ethanol-containing media. The counting was performed on 100 panels (50–60 core particles in each panel) from each preparation. The possibility of saturated images was excluded because the microscope was operated under similar light conditions and many of the images that were obtained contained both the closed and open conformations.

Results

20S proteasome S-glutathionylation is determined by yeast cell growth conditions

In the present work, *S. cerevisiae* cells were grown to the stationary phase in the presence of two different carbon sources, glucose (Glu) and glycerol/ethanol (Gly), to obtain distinct intracellular redox conditions. In the presence of glucose (Glu), fermentative conditions were achieved, with the repression of the expression of antioxidant defense-related genes via catabolite repression [25–27]. In contrast, cells grown in the presence of glycerol/ethanol (Gly) consumed higher levels of O_2 via the mitochondrial respiratory chain, which was accompanied by the increased production of reactive oxygen species, but also with increased antioxidant defense activity. Therefore, the conditions of growth in the presence of glucose or glycerol/ethanol adopted in the present work do not represent same steps of stationary phase (Suppl. Data Fig. S1) but they are representative of diverse intracellular redox conditions, as shown below.

The 20S was isolated from these cells and subjected to 2-D PAGE followed by an immunoblot analysis with anti-GSH antibody. Fig. 1A and B represent the 2-DE pattern of the 20S stained by Coomassie Blue, whose subunits were previously identified using Maldi-TOF analysis [15, Suppl. Data Fig.S2]. The anti-GSH immunoblotting revealed intense labeling of the α5 subunit of the 20S that was purified from cells grown in glucose-containing medium (Fig. 1C). Remarkably, distinct labeling was detected in 20S preparations from cells grown in glycerol/ethanol (Fig. 1D). Later preparations displayed discrete labeling probably of α1 or of β7 subunits, which were not detected in the 20S that had been extracted from cells grown either in synthetic medium containing glucose (Fig. 1C) or YPD [15]. Both preparations were incubated with glutaredoxin 2 (Grx2). As previously described [21], this oxidoreductase provoked deglutathionylation of the 20S, as revealed using 2-D PAGE followed by anti-GSH immunolabeling (Fig. 1E and F). As expected, α5 labeling after Grx2 treatment was significantly decreased (Fig. 1E) when compared to that shown in Fig. 1C, whereas S-glutathionylation seen in Fig. 1E, completely disappeared (Fig. 1F). The α5-subunit S-glutathionylation of proteasome purified from cells grown in synthetic medium containing glucose was also confirmed with an anti-S-glutathionylated α5-C76 antibody (Suppl. Data Fig. S3).

S-glutathionylation of the α5 subunit of the 20S is related to gate opening [15]. Therefore, gate status in 20S preparations was analyzed using TEM. In these experiments, we observed that 70 ± 2% of the 20S that was purified from cells that had been grown

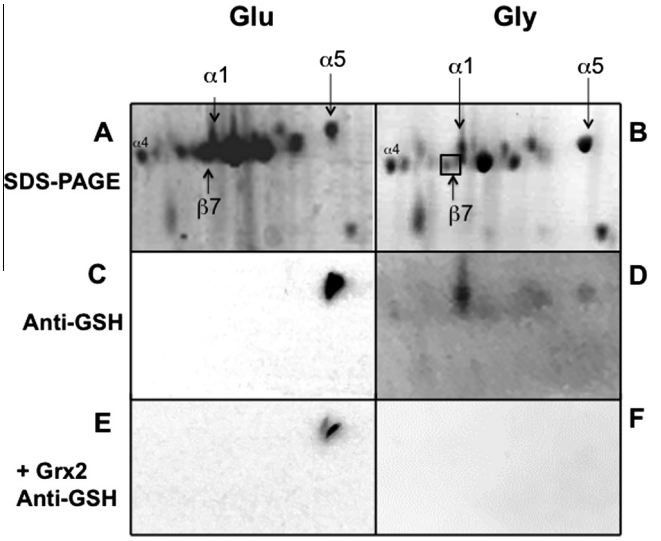

Fig. 1. (A) 20S proteasomal subunits that were separated by 2-D gel electrophoresis from yeast cells that were grown to the stationary phase using glucose and glycerol/ethanol as carbon sources. (A and B) show the representative 2-D PAGEs of the 20S (30 μg) that was purified from yeast cells that had been grown in glucose (*Glu*)- and glycerol/ethanol (*Gly*)-containing media and stained with Coomassie blue. The pH range was 4–7 from left to right. The subunits, as indicated, were identified using Maldi-Tof analyses (Suppl. Data Fig. S2). (C and D) represent the results of the western blot analyses of the same 20S preparations using the anti-GSH antibody. (E and F) display these same preparations after incubation for 30 min with Grx2 (60 μg), as described in the "Experimental procedures" section, prior to anti-GSH labeling. The α5-subunit, as indicated (C and E), was deduced by superposing membranes and 2D-gels.

in glucose was in the open gate conformation (Fig. 2A) in contrast to 15 ± 1% found in cells grown in glycerol/ethanol (Fig. 2B). To demonstrate that the open proteasomal conformation is related to S-glutathionylation-mediated α5 subunit modification, the 20S samples obtained from cells grown in glucose were treated with 10 mM DTT, a sulfhydryl-reductant, followed by TEM analyses. The frequency of the open conformation after DTT treatment decreased to 30%, whereas prior to treatment, it had represented 70% of the total structures examined.

Whether α1- or β7-subunit is S-glutathionylated in 20S purified from cells grown into glycerol-containing medium is under investigation in our lab. In any case, these posttranslational modifications are most likely not related to gating regulation, as their redox modification were not observed in any preparation of the 20S obtained from cells that had been grown in glucose. In these cells, S-glutathionylation of the α5-subunit was clearly related to the increased open gate conformation [15 and present results].

Intracellular reductive ability and oxidative protein damage are dependent upon yeast growth conditions

To correlate the intracellular reductive capability with proteasomal S-glutathionylation and proteasomal gate opening, we examined a series of parameters related to mitochondrial and redox metabolism. Although the total glutathione levels were not found to significantly differ between the two growth conditions, the GSH/GSSG ratio in the cells grown in Gly-containing medium was at least 2-fold the ratio found in cells grown in Glu-containing medium (Table 1), indicating that in the absence of catabolic repression, yeast cells displayed more reductive conditions.

As expected for respiratory conditions, the cells that had been grown in the presence of glycerol/ethanol consumed approximately 4-fold more oxygen than the cells that had been grown in the presence of glucose (Table 1). Moreover, the cellular ability

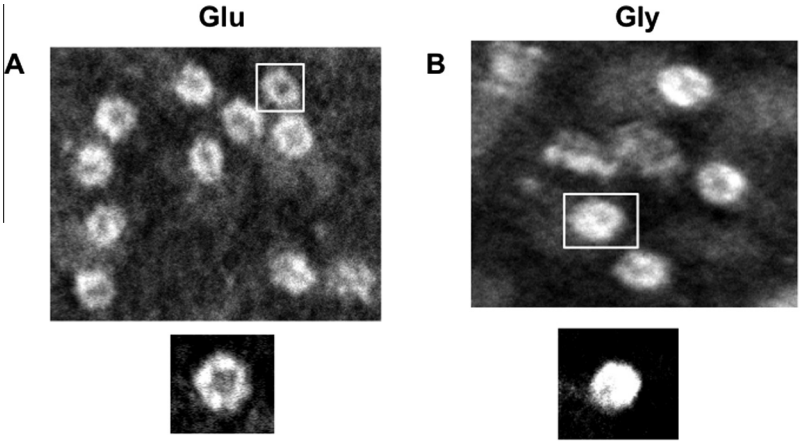

Fig. 2. Transmission electron microscopy (TEM) of 20S preparations that were purified from yeast cells grown to the stationary phase using glucose and glycerol/ethanol as carbon sources. The images shown are representative of the top views of the 20S purified from yeast cells that had been grown in (A) glucose- and (B) glycerol/ethanol-containing media. The structures marked by squares are highlighted below each panel.

to remove H_2O_2 was found to be approximately 12-fold higher under these conditions (Table 1), in accordance with the higher levels of antioxidant enzymes that were anticipated in the absence of repression by glucose [16].

In addition, we determined the protein levels of distinct enzymatic antioxidant pathways. Cytoplasmic Trr1, which maintains Trxs in the reduced form, and Grx2 concentrations were evaluated (Fig. 3). Based on our previous data, cytosolic Trxs and Grx2 were hypothesized to play important roles in keeping the 20S deglutathionylated [21]. Trx3 was also evaluated because it is representative of mitochondrial metabolism. The increased pool of Trx3, in combination with increased oxygen consumption, could also be considered to be a parameter that is associated with the expected increase in mitochondrial biogenesis and oxidative phosphorylation activity in cells grown in medium containing glycerol/ethanol (Fig. 3 and Table 1). The expression of Grx2 was also measured using RT-PCR and was found to be twofold higher in Gly cells as compared to Glu (Suppl. Data Fig. S4), corroborating the elevated levels of Grx2 that were observed using western blotting (Fig. 3).

Next, the levels of oxidative protein damage were evaluated by measuring the carbonyl protein levels [28] because carbonyl proteins have been described to be substrates for the 20S [5]. A comparatively increased pool of carbonylated proteins was detected in cells grown in Gly-containing medium (Fig. 4A, *Anti-DNP*), which represented an apparent contrast, given the fact that those cells displayed higher reductive properties (Table 1) and increased levels of antioxidant defense enzymes (Fig. 3). Therefore, we hypothesized that the proteasomal degradation of oxidized proteins would be higher in cells grown in Glu, according to the gate conformation status.

Table 1
Redox parameters in yeast cells grown using glucose and glycerol/ethanol as carbon sources during the stationary phase. The cells were grown to the stationary phase in synthetic medium containing glucose or glycerol/ethanol, as described in the "Materials and methods" section.

	Glucose	Glycerol
GSH/GSSG	40 ± 7	91.5 ± 15*
Total GSH (μmoles/g of wet pellet)	7 ± 2	9 ± 1.5
H_2O_2 removal activity (nmoles/μg of cellular protein)	1.5 ± 0.05	20 ± 0.5*
Oxygen consumption (μmoles/min)	2.5 ± 0.1	9.5 ± 0.15**

All results are displayed as the means ± SD of 3–4 independent experiments.
* $p < 0.00027$.
** $p < 0.025$.

To establish a correlation with proteasomal S-glutathionylation, the total pool of S-glutathionylated proteins was also evaluated. As depicted in Fig. 4B (*Anti-GSH*), higher levels of S-glutathionylated proteins were found in the cells grown in Glu, which was in agreement with the 20S S-glutathionylation that was observed under the same growth conditions (Fig. 1), as well as with a lower reductive capability (Table 1). Notably, the levels of protein carbonylation (Fig. 4A) were not found to be positively correlated with S-glutathionylation. This observation is aligned with our hypothesis that protein S-glutathionylation is associated with the mild loss of intracellular reductive ability, and likely indicates that this process plays a regulatory and protective role, which may mediate the degradation of oxidized proteins by the S-glutathionylated 20S. As demonstrated above (Fig. 3), relevant deglutathionylases were also present in higher amounts in cells grown in Gly, corroborating the decreased levels of S-glutathiolated proteins in such condition (Fig. 4B).

Taken together, these data indicated that intracellular reductive parameters (GSH/GSSG ratio, GRX2 expression, Grx2, Trx3 and Trr1 levels, and the ability to remove H_2O_2) were found to be inversely correlated with proteasome S-glutathionylation, indicating that when cells are grown in Gly-containing medium, the ability of the 20S to degrade oxidized proteins is inhibited (the gate is closed), leading to the accumulation of carbonylated proteins.

Basal conditions for the degradation of poly-ubiquitinylated proteins

The overall levels of ubiquitinylated proteins, and the concentrations of the 20S and 19S proteasomal units, were also evaluated under both growth conditions. While 20S concentrations were not significantly altered under either condition (Fig. 5, *Anti-20S* and Suppl. Data Fig. S4), the concentrations of the 19S unit were 35% higher in the cells that had been grown in the presence of Gly (Fig. 5, *Anti-19S*). The lower levels of poly-ubiquitinylated proteins (Fig. 5, *Anti-ubiquitin*) in the cells that had been grown in the presence of Gly were consistent with the higher levels of the 26S, which is capable of degrading such modified polypeptides. However, we cannot rule out the possibility of decreased activity of proteins responsible for the poly-ubiquitinylation process (E1–E3) that would imply on reduced levels of poly-ubiquitinylated proteins. The mRNA levels corresponding to a subunit of the 20S (PRE2, the gene encoding the β5 catalytic subunit) were also evaluated using RT-PCR. According to these results, PRE2 expression did not significantly vary under either condition (Suppl. Data Fig. S4), though a discrete increase in its expression was observed

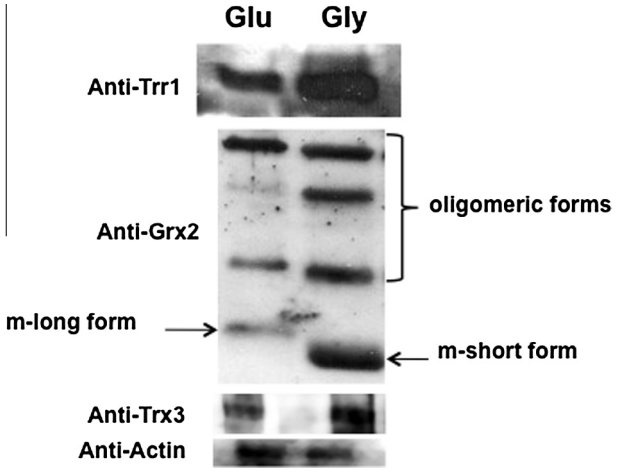

Fig. 3. The oxidoreductase systems of yeast cells grown to the stationary phase using glucose and glycerol/ethanol as carbon sources. Total cellular extracts (20–50 μg protein) from cells that had been grown to the stationary phase in glucose- (*Glu*) or glycerol/ethanol-containing media (*Gly*) were analyzed using immunoblotting. All the results shown were obtained from samples of the same cellular extract and are representative of three independent experiments. *Trr1*, cytosolic thioredoxin reductase; *Trx3*, mitochondrial thioredoxin and *Grx2*, mitochondrial long- and short-forms of glutaredoxin 2 (*m-long form* and *m-short form*, respectively; [21]). Sample of the anti-actin immunoblotting, utilized as a loading control for the preparations, was taken from the same cellular extract.

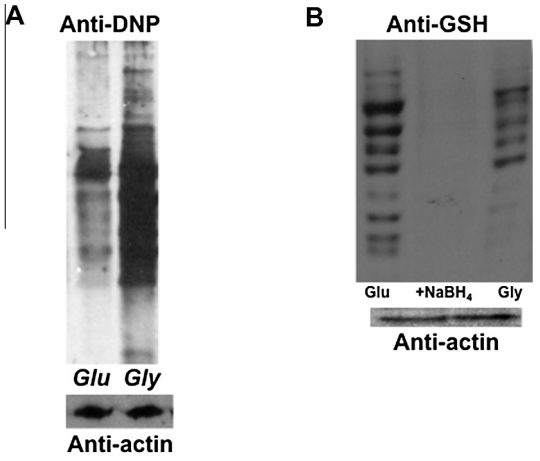

Fig. 4. Immunoblotting analyses of the carbonylated and S-glutathiolated protein pool of yeast cells grown to the stationary phase using glucose and glycerol/ethanol as carbon sources. (A) Yeast extracts that were obtained from cells that had been grown to the stationary phase in glucose- (*Glu*) or glycerol/ethanol-containing media (*Gly*) were reacted with 5 mM DNPH. After pH neutralization, the samples (20 μg) were subjected to 10% SDS–PAGE and transferred to a nitrocellulose membrane, followed by blotting against anti-DNP. (B) Extracts (50 μg) that were obtained from the same cellular growth conditions that were described above from independent experiments were subjected to 10% SDS–PAGE and transferred to a nitrocellulose membrane, followed by blotting against anti-GSH.+*NaBH*$_4$ refers to the samples that were obtained from cells that had been grown in glucose (*Glu*) and were treated with 1 mM $NaBH_4$. These samples served as a control for anti-GSH specificity. Samples of the anti-actin immunoblottings shown were taken from the same cellular extracts utilized for the anti-DNP and anti-GSH analyses.

in cells grown in the presence of glucose (not statistically significant). The importance of the inter-conversion between proteasomal conformations is still a poorly studied process. Recently, uncoupling between the 20S core particle and the 19S regulatory unit was described [10,29], and the augmented expression of the 20S unit [30] was observed to be a cellular adaptation to oxidative stress. Our results indicated that the free 20S is prevalent in cells grown in Glu-containing medium, whereas the 26S is more abundant in cells grown in Gly-containing medium though these conditions are not related to an acute production of oxidative imbalance. Instead, the conditions assayed in the present work are probably an adaptive response to oxidative stress.

Degradation of oxidized BSA by the 20S redox forms

As previously mentioned, the 20S is the primary proteolytic system responsible for the degradation of oxidized proteins [31]. Therefore, we investigated the proteolytic ability of the 20S, which was purified from cells that were grown under both growth conditions, to degrade oxidized proteins. As expected based on our previous results, the proteolysis rates of oxidized albumin (BSA_{ox}) were 55% higher in the Glu-derived extracts than in the Gly-derived extracts (Table 2, first column). Proteolysis rates were evaluated by incubating the samples with azoBSA_{ox}. To unequivocally attribute these activities to proteasomal particles, the 20S

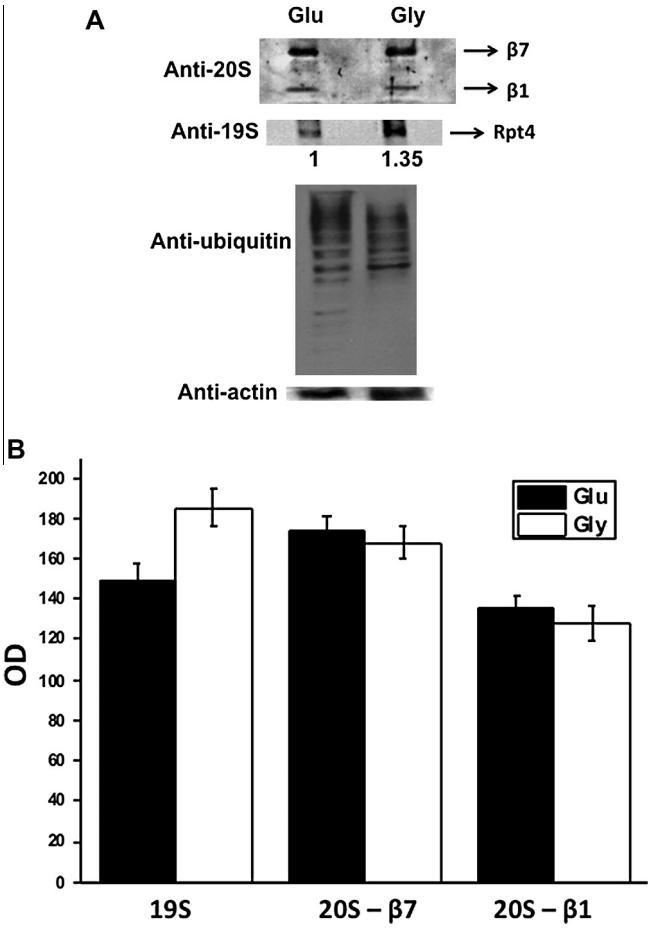

Fig. 5. The levels of the proteasomal subunits (19S and 20S) and the pool of ubiquitylated proteins in yeast cells grown to the stationary phase using glucose and glycerol/ethanol as carbon sources. (A) Yeast extracts (50 μg total protein) were obtained from cells that were grown to the stationary phase in glucose- (Glu) or glycerol/ethanol-containing media (Gly) and were immunoblotted, as shown. The proteasomal concentration was determined by immunoblotting against the β7- and β1-20S subunits of the catalytic core, and the Rpt4 subunit of the19S regulatory particle. The total levels of ubiquitylated proteins were assessed using immunoblotting with anti-Ub. Anti-actin immunoblotting was performed as a loading control and is displayed here. All samples for the immunoblottings shown were taken from the same cellular extracts (B) The values that are shown in the plot are reported as the relative optic density (*OD*) of the blottings shown in (A) and normalized by the anti-actin immunoblotting. The results are expressed as the means ± SD of 3 independent experiments.

Table 2
Proteolytic abilities of yeast cells. Total extracts of cells that had been grown in glucose (*Glu*)- and glycerol/ethanol (*Gly*)-containing media were utilized to evaluate the degradation of the azo-BSA_{ox} derivative, as described in "Experimental procedures". Proteolytic activity was determined in the *Total extract*, with the 20S proteasome captured onto anti-FLAG resin (*20S*) and in the total protein extract of the immune-depleted 20S (third column). Therefore, these extracts are representative of the cellular hydrolytic ability in the absence of the 20S. The 20S proteasome was recovered from the anti-FLAG Sepharose resin and was subjected to SDS–PAGE, where it served as a control for the 20S concentration in the hydrolytic assay (Suppl. Data, Fig. 3S).

	Total extract (A_{412}/mg of total protein)	20S[1] (A_{412})	Total extract after removal of the 20S (A_{412}/mg of total protein)
Gly	0.125 ± 0.010	1.0 ± 0.1	0.063 ± 0.005
Glu	0.195 ± 0.015*	3.50 ± 0.25**	0.065 ± 0.010

The results are expressed as the means ± SD of three independent experiments.
[1] The results were calculated by setting the determined minor absorbance (*Gly* samples) to 1 after considering the total 20S levels in both samples to be identical, as shown in Suppl. Data (Fig. 3S).
* $p < 0.003$ (ANOVA).
** $p < 0.000043$ (ANOVA).

was immobilized on anti-FLAG Sepharose resin. Again, the proteolysis rates were increased in anti-FLAG 20S-isolated preparations (3.5×) from cells that had been grown in the presence of Glu in comparison with similar preparations that were grown in the presence of Gly (Table 2, second column). Finally, the proteolytic activities in the proteasome immune-depleted extracts were determined to test their ability to degrade oxidized BSA_{ox} (Table 2, third column). As predicted, the proteolysis rates did not differ between the samples. These results unequivocally indicated that higher rates of protein degradation by the 20S core particle occurred when the cells were grown in Glu-containing medium, which was likely the result of α5-subunit S-glutathionylation and gate opening.

Discussion

The yeast cell is an interesting model for performing redox metabolism studies because its intracellular redox state can be manipulated according to the growth phase and the carbon source. Glucose is thought to repress the expression of many genes related to mitochondrial biogenesis and antioxidant defenses [32]. Thus, the yeast cell represents an important physiological model for the investigation of signaling and regulation by redox mechanisms.

Small number of Cys residues of the 20S is redox modulated inside cells [15]. Indeed, S-glutathionylation of two Cys residues on the α5 subunit correlates with the opening of the catalytic chamber, thereby facilitating protein entrance, as evidenced by the increased degradation of oxidized proteins. The results of the present study indicated that the increased breakdown of oxidized proteins in less reductive environments is consistent with the idea of an adaptive response to oxidative stress.

Interestingly, peptidase activities of the 20S are decreased when the 20S is S-glutathionylated (21, 23, 33). Accordingly, in the present work we found that the 20S chymotrypsin-like activity was 5-fold increased in 20S samples purified from cells grown in glycerol/ethanol medium when compared to samples grown in glucose (Table 2S). This apparently is in contradiction with the fact that S-glutathionylated 20S is more active in degrading oxidizing proteins. Same samples when incubated in the presence of Grx2 showed a significant increase (90%) in the site-specific hydrolytic activity of the 20S extracted from cells grown in glucose whereas same procedure rendered less than 10% of increased activity of the 20S extracted from cells grown in glycerol/ethanol. This was expected since the content of S-glutathionylated 20S was higher in Glu than in Gly condition. We had observed before [15,21] that S-glutathiolation promotes an allosteric effect to the proteasomal catalytic sites that inhibited the peptidase activities, as assessed by fluorogenic peptides. We hypothesize that the site-specific activity modification observed would determine the generation of diverse peptide fragments of same protein depending on proteasomal redox state. The nature of this allosteric effect remains to be identified but it is clearly distinct to the gate conformational effect.

S-glutathionylation is an important post-translational modification that is involved in redox cellular processes. The identification of proteasome S-glutathionylation in other organisms, such as plants, rats and humans [33–35], has provided important evidence of the ubiquitous relevance of this post-translational modification of the 20S. The increased degradation of oxidized proteins that is induced by 20S S-glutathionylation may be important in all eukaryotic cells and may play a key role in antioxidant defense. 20S S-glutathionylation may also prevent the formation of insoluble protein aggregates, which is a cytotoxic hallmark of oxidative stress-dependent processes, such as aging and neurodegenerative diseases.

Oxidized proteins have been observed to activate the 20S particle *in vitro* [30]. Indeed, convincing reports in the literature have demonstrated that the proteasomal degradation of oxidized proteins in mammalian cells is not dependent upon ubiquitin conjugation [7–10,29]. Wang and co-workers [10] demonstrated that the decoupling of the 20S and its 19S regulatory particle occurs in oxidative intracellular environments, favoring the degradation of oxidized proteins that are not poly-ubiquitylated. In agreement with this idea, a decreased ratio of the 19S and 20S particles were observed (Fig. 5) under glucose-containing medium during which oxidized azoBSA degradation was intensified (Table 2). The mechanism by which oxidized proteins are recognized and interact with the 20S particle for degradation is still under investigation. The loss

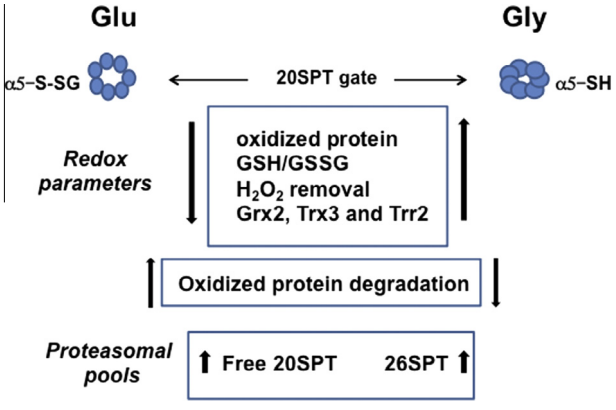

Fig. 6. The intracellular redox state and protein metabolism based on yeast growth conditions. The α5-subunit of the 20S from cells that had been grown in the presence of glucose (Glu) was S-glutathionylated and the open conformation was the most prevalent conformation, while the closed form of the 20S from cells grown in the presence of glycerol/ethanol (Gly) was the most prevalent conformation. The growth conditions determine the intracellular redox state of the 20S, which in turn modifies the rate of degradation of oxidized and poly-ubiquitinylated proteins. In addition, the proteasomal pools (the free 20S and the 26S) are dependent upon the cellular redox state.

of secondary structure, and thus increased surface hydrophobicity, is assumed to be the starting-point for proteasomal interaction [14,36,37]. We hypothesize that increased protein interaction with the 20S proteasome via oxidation, together with the opening of the catalytic chamber by proteasomal S-glutathionylation and the disassembly of the 26S proteasome, are concerted processes that allow cells to cope with the increased levels of oxidized proteins during the loss of reductive capability. In any case, it is possible that the free 20S and the 26S interact in a collaborative manner to resolve distinct stressful conditions. The discovery of thioredoxin-like proteins in the 19S regulatory unit suggests that 26S may also be subject to redox regulation by a still unknown mechanism [38–40]. Our proposal is that 20S and 26S would degrade proteins in distinct redox environments such as the scheme described in Fig. 6.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.abb.2014.05.002.

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