Role of glutaredoxin 2 and cytosolic thioredoxins in cysteinyl-based redox modification of the 20S proteasome

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The yeast 20S proteasome is subject to sulfhydryl redox alterations, such as the oxidation of cysteine residues (Cys-SH) into cysteine sulfenic acid (Cys-SOH), followed by S-glutathionylation (Cys-S-SG). Proteasome S-glutathionylation promotes partial loss of chymotrypsin-like activity and post-acidic cleavage without alteration of the trypsin-like proteasomal activity. Here we show that the 20S proteasome purified from stationary-phase cells was natively S-glutathionylated. Moreover, recombinant glutaredoxin 2 removes glutathione from natively or in vitro S-glutathionylated 20S proteasomes, allowing the recovery of chymotrypsin-like activity and post-acidic cleavage. Glutaredoxin 2 deglutathionylase activity was dependent on its entry into the core particle, as demonstrated by stimulating S-glutathionylated proteasome opening. Under these conditions, deglutathionylation of the 20S proteasome and glutaredoxin 2 degradation were increased when compared to non-stimulated samples. Glutaredoxin 2 fragmentation by the 20S proteasome was evaluated by SDS–PAGE and mass spectrometry, and S-glutathionylation was evaluated by either western blot analyses with anti-glutathione IgG or by spectrophotometry with the thiol reactant 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. It was also observed in vivo that glutaredoxin 2 was ubiquitinated in cellular extracts of yeast cells grown in glucose-containing medium. Other cytoplasmic oxidoreductases, namely thioredoxins 1 and 2, were also active in 20S proteasome deglutathionylation by a similar mechanism. These results indicate for the first time that 20S proteasome cysteinyl redox modification is a regulated mechanism coupled to enzymatic deglutathionylase activity.

Oxidation of protein cysteine residues into sulfenic acid (Cys-SOH) and the subsequent S-glutathionylation of these residues during enzyme catalysis and redox signaling have been increasingly accepted as commonly occurring events in redox regulation [1–9]. This reversible mechanism is believed to play a regulatory role in enzyme catalysis and binding of transcription factors to DNA targets, among other processes. The first step in protein-Cys-SH oxidation generates Cys-SOH, which is prone to S-glutathionylation by

Abbreviations
20S PT, 20S proteasome core; AMC, 7-amido-4-methylcoumarin; CDL, cardiolipin; Cys-SOH, cysteine sulfenic acid; GR, glutathione reductase; Grx2, recombinant glutaredoxin 2; Grx2C30S, mutant glutaredoxin 2; GSH, glutathione; HED, hydroxyethylidisulfide; NBD, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; n-PT, natively S-glutathionylated 20S proteasome; PT-SG, in vitro S-glutathionylated 20S proteasome; PT-SH, dithiotreitol-treated 20S proteasome; RS, reductive system for Grx2 containing 2 mM NADPH, 0.3 U mL–1 GR and 0.5 mM GSH; s-LLVY-AMC, succinyl-Leu-Leu-Val-Tyr-AMC; Trr1, recombinant thioredoxin reductase 1; z-ARR-AMC, carbobenzoxy-Ala-Arg-Arg-AMC; z-LLE-AMC, carbobenzoxy-Leu-Leu-Glu-AMC.
sulfhydryls, e.g. glutathione (GSH); otherwise, the oxidation continues to further generate the cysteine sulfonic (Cys-SO₂H) and cysteine sulfonic (Cys-SO₃) acid forms [5,10]. Glutaredoxins [9,11,12], as well as thioredoxins [13], are postulated to be directly responsible for deglutathionylation in yeast cells. The first function assigned to glutaredoxins was the reduction of intramolecular disulfide bonds in the ribonucleotide reductase of thioredoxin-deleted Escherichia coli strains [14]. Since then, biochemical and genetic approaches have provided evidence for a protective role of glutaredoxins under oxidative conditions and during redox signaling, e.g. GSH-dependent reduction of protein-mixed disulfides by means of its so-called deglutathionylase activity in various eukaryotic cells [9,11,12,15–17].

Yeast possesses two dithiolic (Grx1 and Grx2) and five monothiolic glutaredoxins. These isoforms differ in their location and response to oxidative stress, among other factors [9,11,18–22]. Evidence indicates that Grx2 is the main glutathione-dependent oxidoreductase in yeast, whereas Grx1 and Grx5 may be required during certain stress conditions or after the formation of particular mixed disulfide substrates [11,12].

We have shown previously that yeast Cys-20S proteasomal residues are S-glutathionylated in vitro by reduced glutathione if previously oxidized to Cys-SOH [8]. Moreover, this mechanism was shown to be responsible for a decrease in proteasomal chymotrypsin-like activity. Here, we show that the 20S proteasome core purified from stationary-phase cells is also S-glutathionylated under basal conditions, and that Grx2 was able to dethiolate the 20S core. Another interesting finding is that the resulting deglutathionylation process restores proteasomal chymotrypsin-like activity and post-acidic cleavage concomitant with Grx2 degradation by the 20S particle. We also show that cytoplasmic thioredoxins 1 and 2 play similar roles. Both isoforms were able to deglutathionylate the 20S core, allowing rescue of proteasomal activities.

**Results**

**20S proteasome is natively S-glutathionylated**

We demonstrated previously that the 20S proteasome core (PT) is S-glutathionylated when cells are challenged with H₂O₂ [8]. We began the present investigation by verifying whether the 20S PT is also natively S-glutathionylated. Remarkably, the 20S core purified from cells grown to stationary phase in glucose-enriched medium was natively S-glutathionylated, as assessed by western blotting using anti-GSH (Fig. 1A, n-PT). By comparing the in vitro proteasome S-glutathionylation (PT-SG) to that observed in preparations obtained from cells grown to stationary phase (n-PT), we observed that the 20S particle was not fully S-glutathionylated in vivo when compared to the in vitro process (Fig. 1A). The in vitro assay results indicated that the potential for S-glutathionylation of 20S proteasome subunits is much higher than that observed inside cells (Fig. 1A). Moreover, the 20S core purified from cells grown to stationary phase in glucose-containing medium was more greatly S-glutathionylated when compared to preparations obtained...
from cells grown in glycerol/ethanol-containing medium (Fig. 1B, lanes Glu and Gly, respectively). As a control, samples purified from cells grown in glucose were treated with 10 mM dithiothreitol before loading onto the gel utilized for the immuno-blot assay (Fig. 1B, lane dithiothreitol). After dithiothreitol treatment, 20S proteasome S-glutathionylated bands were completely absent. The purified 20S PT SDS/PAGE profile is shown in supplementary Fig. S1 (lane 2).

As shown previously [23] and confirmed in our laboratory, intracellular reductive ability is higher when yeast cells are grown in glycerol/ethanol-enriched medium (data not shown). Glucose is known to repress expression of genes related to antioxidant defenses and mitochondrial biogenesis [24,25], but glycerol/ethanol growth conditions only support respiratory growth and maintain antioxidant defenses at increased levels [23]. Together with increased antioxidant parameters, we found that the chymotrypsin-like activity of purified 20S proteasome obtained from cells grown in glycerol/ethanol was five times that of preparations obtained from cells grown in glucose-containing medium, with no alteration of 20S proteasome levels (data not shown). These results suggest that proteasomal activity might be modulated according to intracellular redox modifications.

20S proteasome deglutathionylation by Grx2

The observation that the 20S core purified from stationary-phase cells was already S-glutathionylated, together with our data showing that S-glutathionylation of the 20S core particle varies according to the metabolic conditions of yeast cells (Fig. 2 and Demasi M & Silva GM unpublished results), provide strong evidences that this redox alteration plays an important physiological role. Our next goal was to identify an enzymatic mechanism that is able to modulate the proteasomal activity by redox modifications, e.g. deglutathionylation. Based on reports in the literature, Grx2 is one of the enzymes responsible for GSH-dependent deglutathionylase activity in yeast cells [11], and, in addition, Grx2 co-localizes with the proteasome in the cytosol. Thus, recombinant Grx2 was evaluated for its ability to deglutathionylate PT-SG obtained through a multi-step procedure as described in Experimental procedures. Preparations from each step (oxidized, in vitro S-glutathionylated and Grx2-treated samples) were reacted with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD). The Cys-S(O)–NBD conjugate (solid line) or NBD-reacted S-glutathionylated 20S core (dotted line) were generated by reaction of 100 μM NBD with H2O2 or GSH-treated proteasome preparations (described in Experimental procedures) denatured using 5 M guanidine. The Cys-S–NBD conjugate (dashed line) was generated by incubation of S-glutathionylated 20S PT with Grx2 in the presence of the RS (2 mM NADPH, 0.3 U·mL⁻¹ GR and 0.5 mM GSH), followed by reaction with NBD. Excess NBD was removed by filtration as described previously [8]. Spectra were recorded as indicated. (B) Anti-GSH blotting. The in vitro S-glutathionylated 20S PT was prepared as described in Experimental procedures. Samples (20 μg PT-SG) were incubated for 30 min at 37 °C under the indicated conditions in a final volume of 40 μL and applied to 12.5% SDS–PAGE for immuno-blot analysis. RS, sample incubated in the presence of 0.5 mM GSH, 2 mM NADPH and 0.3 U·mL⁻¹ GR without Grx2; PT-SG, sample incubated without the RS or Grx2; Grx2-incubated, samples incubated in the presence of the RS plus Grx2 at the indicated concentrations. Anti-FLAG, loading control performed as described in Experimental procedures on the same membranes utilized for anti-GSH blotting.

Fig. 2. Recombinant Grx2 deglutathionylase activity on S-glutathionylated 20S PT. (A) Assay with the sulfhydryl and sulfenic acid reactant 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD). The Cys-S(O)–NBD conjugate (solid line) or NBD-reacted S-glutathionylated 20S core (dotted line) were generated by reaction of 100 μM NBD with H2O2 or GSH-treated proteasome preparations (described in Experimental procedures) denatured using 5 M guanidine. The Cys-S–NBD conjugate (dashed line) was generated by incubation of S-glutathionylated 20S PT with Grx2 in the presence of the RS (2 mM NADPH, 0.3 U·mL⁻¹ GR and 0.5 mM GSH), followed by reaction with NBD. Excess NBD was removed by filtration as described previously [8]. Spectra were recorded as indicated. (B) Anti-GSH blotting. The in vitro S-glutathionylated 20S PT was prepared as described in Experimental procedures. Samples (20 μg PT-SG) were incubated for 30 min at 37 °C under the indicated conditions in a final volume of 40 μL and applied to 12.5% SDS–PAGE for immuno-blot analysis. RS, sample incubated in the presence of 0.5 mM GSH, 2 mM NADPH and 0.3 U·mL⁻¹ GR without Grx2; PT-SG, sample incubated without the RS or Grx2; Grx2-incubated, samples incubated in the presence of the RS plus Grx2 at the indicated concentrations. Anti-FLAG, loading control performed as described in Experimental procedures on the same membranes utilized for anti-GSH blotting.

measurement. When the 20S core was oxidized with H2O2, sulfenic acid was formed (Fig. 2A, solid line). However, the sulfenic form of the 20S core cysteine residues completely disappeared when H2O2-oxidized 20S preparations were treated with GSH (Fig. 2A,
S-glutathionylated proteasome by Grx2 would increase chymotrypsin-like activity and post-acidic cleavage. Next, our goal was to verify whether reduction of S-glutathionylation when compared to the incubation with 5 µg Grx2. The molar ratios of PT : Grx2 were 1 : 10 and 1 : 20, respectively. To evaluate the effect of the GSH-dependent reductive system on deglutathionylation, proteasomal preparations were incubated in standard buffer containing the reductive system but not Grx2 (Fig. 2B, RS). The reductive system had no effect on 20S PT deglutathionylation.

Taken together, the results shown in Fig. 2 provide direct evidence that Grx2 is capable of partly deglutathionylating the 20S proteasome.

**Grx2 increases chymotrypsin-like activity and post-acidic cleavage of the S-glutathionylated 20S proteasome**

To demonstrate to what extent S-glutathionylation interferes with proteasomal activity, site-specific activities were determined using n-PT and in vitro S-glutathionylated PT-SG and PT-SH preparations (Fig. 3). Chymotrypsin-like proteasomal activities from n-PT and PT-SG preparations were 62% and 45% of that observed in the PT-SH preparation, respectively, whereas the post-acidic cleavage in the n-PT and PT-SG preparations was 50% and 35%, respectively, of that in PT-SH preparations (Fig. 3; samples indicated by –). As observed previously [8], the trypsin-like activity was not modified by any redox modification of the core. The results shown in Fig. 3 (samples indicated by –) demonstrate that proteasomal activities are inversely correlated to the extent of S-glutathionylation.

As discussed above, chymotrypsin-like activity and post-acidic cleavage were decreased by S-glutathionylation. Next, our goal was to verify whether reduction of S-glutathionylated proteasome by Grx2 would increase modified proteasomal activities to the levels of the PT-SH preparation. As expected, Grx2 pre-incubation with S-glutathionylated forms of the 20S proteasome (n-PT and PT-SG) resulted in increased chymotrypsin-like activity and post-acidic cleavage (Fig. 3; samples indicated by +). The activities in the PT-SH preparation did not change after incubation with Grx2. If the dithiothreitol-reduced proteasomal activity (PT-SH) is taken as the maximum attainable (100%), chymotrypsin-like activity for n-PT was 63% recovered after incubation with Grx2, whereas the recovery was 48% for PT-SG. Post-acidic cleavage for the PT-SG and n-PT preparations was totally recovered after incubation with Grx2. Again, trypsin-like proteasomal activity was not modified by any of the treatments performed here. Taken together, the results presented so far indicate that S-glutathionylation and Grx2 modulate post-acidic cleavage and chymotrypsin-like activity by modifying the redox state of proteasomal cysteine residues.

Similar experiments to those described above were performed using cytosolic thioredoxins, and they also

![Fig. 3. Effect of Grx2 on proteasomal hydrolytic activities. To test for the recovery of proteasomal chymotrypsin-like activity and post-acidic cleavage after pre-incubation with Grx2, the indicated proteasomal preparations (50 µg/200 µL) were immobilized on anti-GSH IgG (Fig. 2B). PT-SG was incubated with two concentrations of recombinant Grx2 in the presence of the GSH-dependent reductive system, as described in Experimental procedures. As seen in Fig. 2B, S-glutathionylated bands of the 20S core (PT-SG) significantly decreased after incubation in the presence of Grx2 (Grx2-incubated), and incubation with 10 µg Grx2 increased proteasome deglutathionylation when compared to the incubation with 5 µg Grx2. The molar ratios of PT : Grx2 were 1 : 10 and 1 : 20, respectively. To evaluate the effect of the GSH-dependent reductive system on deglutathionylation, proteasomal preparations were incubated in standard buffer containing the reductive system but not Grx2 (Fig. 2B, RS). The reductive system had no effect on 20S PT deglutathionylation.

Taken together, the results shown in Fig. 2 provide direct evidence that Grx2 is capable of partly deglutathionylating the 20S proteasome.](image-url)
exhibited deglutathionylase activity towards 20S PT as evaluated by both anti-GSH probing and NBD assay of similar proteasome preparations (Fig. 4A,B, respectively). An immunoblot analysis performed after incubation of n-PT preparations with Trx1 revealed that the time course of proteasomal deglutathionylation was as short as 15 min, and 30 min incubation did not change the extension of deglutathionylation when these blots (Fig. 4A, 15 and 30) were compared to the control sample of n-PT (Fig. 4A, St). Anti-20S PT

![Image](https://example.com/image)

**Fig. 4.** Deglutathionylation of 20S proteasome preparations by recombinant Trx1 and Trx2. (A) n-PT preparations (20 μg) were mixed with Trx1 (3 μg) plus 2 mM NADPH and 0.5 μg Trr1 and incubated at 37 °C for 15 or 30 min (lanes indicated by 15 and 30, respectively) or kept on ice (lane indicated by 0). Samples were analyzed by western blotting with anti-GSH as described in Fig. 1. St, control n-PT preparation incubated for 30 min at 37 °C in the absence of Trx1. Anti-20SPT, loading control performed with the same membranes utilized for anti-GSH blotting. (B) PT-SH, PT-SOH (PT-SH after treatment with hydrogen peroxide) and PT-SG preparations were generated as described in Experimental procedures. The Cys–S–NBD (solid line), Cys–S(OH)–NBD (dashed line) conjugates and the NBD-reacted S-glutathionylated 20S core (dashed/dotted line) were generated from 100 μg PT-SOH or PT-SG preparations. The Cys–S–NBD conjugate (dotted line) was obtained after incubation of PT-SG (100 μg) with Trx1 or Trx2 (1 μg) in the presence of 2 mM NADPH and 0.5 μg Trr1 per 100 μL (final concentration), followed by dilution in 5 M guanidine and reaction with NBD. Results shown are representative of three independent experiments. (C) Effect of Trx1 and Trx2 on the recovery of chymotrypsin-like proteasomal activity. One microgram of PT-SH, PT-SOH or PT-SG, as indicated, was assayed for hydrolysis of the fluorogenic peptide s-LLVY-AMC (10 μM), as described in Experimental procedures. PT-SG samples (50 μg) were incubated for 30 min in the presence of Trx1 (1 μg) or Trx2 (1 μg) plus 2 mM NADPH and 0.5 μg Trr1 per 100 μL. Aliquots (1 μg) of Trx1- and Trx2-treated PT-SG were removed for the hydrolytic assay. The results shown are means ± SD and represent six independent experiments. Asterisks indicate P values of < 0.000012 (ANOVA) compared to PT-SG samples.
The proteasome concentration in the assays was five times the concentration utilized in the experiments shown in Fig. 4B. Thus, we concluded from this set of experiments that formation of the Cys–NBD adduct after incubation of PT-SG preparations with thioredoxins (as shown in Fig. 4B) most likely occurred through deglutathionylation.

Next we performed assays to test whether thioredoxins could recover the hydrolytic activity of S-glutathionylated proteasome preparations. Recovery of the chymotrypsin-like activity of in vitro S-glutathionylated core (PT-SG) by Trx1 and Trx2 was very similar (Fig. 4C). The chymotrypsin-like activity of PT-SG preparations compared to that obtained from dithiothreitol-reduced preparations (PT-SH) was 71% and 77% after incubation with Trx1 and Trx2, respectively. These results were very close to those obtained with Grx2 (63%), as described above.

**Mechanism of deglutathionylation**

One question raised during the experiments described above was whether the oxido-reductases exerted their effects by reducing only mixed disulfides located on the surface of the 20S core particle, or whether they were also able to enter the latent 20S PT to reduce cysteine residues inside the catalytic chamber. By analyzing structural features of yeast 20S PT from the Protein Data Bank (PDB identification 1RYP), we determined that only a few cysteine residues among the total of 72 are exposed to the environment: 10 solvent-accessible cysteines were determined to be present on the surface, with some of them being totally exposed and others slightly buried but still solvent-accessible. All of the other cysteine residues are either buried in the skeletal structure or exposed to the internal catalytic chamber environment. Therefore, we investigated whether Grx2 enters the core particle. Assuming that Grx2 must be at least partially degraded to reach inside the proteasome, we first evaluated Grx2 degradation using SDS–PAGE (Fig. 5A). Degradation of Grx2 was achieved by incubating n-PT with Grx2 in standard buffer for 2 h (Fig. 5A, lane 2) or by proteasomal stimulation with 0.0125% SDS (Fig. 5A, lane 4). As a control, proteasomal preparations were heated to 100 °C (Fig. 5A, lane 3) prior to incubation with Grx2 and compared to standard Grx2 incubated in standard buffer lacking proteasome (Fig. 5A, lane 1); no proteolysis was seen. Degradation by the proteasome was determined by the decreased intensity of Grx2 bands as evaluated by measurement of optical density. When incubated in standard buffer, n-PT was able to degrade about 70% of Grx2 (Fig. 5B). It is well established that 20S PT is activated by SDS at low concentrations [26]. When 0.0125% SDS was added to the buffer (Fig. 5A, lane 4), Grx2...
degradation was increased to 98% when compared to the standard band for Grx2. The same results were obtained with the other de glutathionylases assayed, Trx1 and Trx2. As shown in Fig. 6C, both Trx1 and Trx2 were degraded by the proteasome (molar ratios for n-PT : Trx1 and n-PT : Trx2 were 1 : 10 and 1 : 20, respectively).

To evaluate whether Grx2 degradation was a non-specific process, Grx2, commercially available cytochrome c, recombinant peroxidase Ohr (organic hydroperoxide resistance protein), ovalbumin and bovine casein at similar concentrations were incubated with n-PT (supplementary Fig. S2). We selected cytochrome c because of its well-known resistance to degradation by the latent form of the 20S particle [27,28], and because its molecular mass (12 kDa) is close to that of recombinant Grx2 (14.1 kDa), eliminating the possibility of size- or protein diameter-specific degradation. The organic hydroperoxide resistance protein Ohr (17 kDa) was tested because of its cysteinyl-based active site [29,30]. Ovalbumin is a larger protein (44 kDa) that is known to be degraded in vitro by 20S PT only when denatured [31,32]. Moreover, we compared the degradation of all proteins with that of casein, which has a low secondary structure content and is easily hydrolyzed by the 20S core. After incubation and prior to application to SDS–PAGE, n-PT was removed by filtration. The only two proteins degraded by 20S PT were Grx2 and casein (supplementary Fig. S2), indicating a specific proteolytic process, probably correlated to the structural characteristics of Grx2 and its interaction with 20S PT. All of the other proteins tested here were resistant to degradation, in agreement with the view that the latent form of the 20S PT recognizes specific features in target proteins. These results gave further support to the notion that Grx2 deglutathionylase activity plays a regulatory role in 20S PT activities.

We next analyzed Grx2 fragmentation using mass spectrometry, by incubating Grx2 in standard buffer for 30 min or 2 h in the presence of n-PT. After incubation, standard Grx2 and fragments recovered by filtering the incubation mixture through 100 kDa cut-off micro filters were processed for MS analysis, as described in Experimental procedures. Grx2 degradation by the core, as shown by SDS–PAGE (Fig. 5A), was confirmed by the MS analysis (Table 1 and supplementary Fig. S3). We selected cytochrome c because of its well-known resistance to degradation by the latent form of the 20S particle [27,28], and because its molecular mass (12 kDa) is close to that of recombinant Grx2 (14.1 kDa), eliminating the possibility of size- or protein diameter-specific degradation. The organic hydroperoxide resistance protein Ohr (17 kDa) was tested because of its cysteinyl-based active site [29,30]. Ovalbumin is a larger protein (44 kDa) that is known to be degraded in vitro by 20S PT only when denatured [31,32]. Moreover, we compared the degradation of all proteins with that of casein, which has a low secondary structure content and is easily hydrolyzed by the 20S core. After incubation and prior to application to SDS–PAGE, n-PT was removed by filtration. The only two proteins degraded by 20S PT were Grx2 and casein (supplementary Fig. S2), indicating a specific proteolytic process, probably correlated to the structural characteristics of Grx2 and its interaction with 20S PT. All of the other proteins tested here were resistant to degradation, in agreement with the view that the latent form of the 20S PT recognizes specific features in target proteins. These results gave further support to the notion that Grx2 deglutathionylase activity plays a regulatory role in 20S PT activities.
any fragmentation after 2 h incubation in standard buffer at 37 °C (supplementary Fig. S3A). As shown in supplementary Fig. S3B, after 30 min incubation with the proteasome, a 4898 kDa Grx2 fragment was generated (Table 1). Although Grx2 fragmentation was greatly increased after the 2 h incubation when compared to the 30 min incubation (supplementary Fig. S3C and Table 1), the 4898 kDa peptide remained intact. It is noteworthy that almost all the fragments detected after the 2 h incubation, possess the active site (47CPYC51; Table 1). Most probably, these N-terminal fragments are correctly structured and retain oxidoreductase activity as the CPYC domain appears in the inner core of most of them.

To corroborate the results shown above, we tested whether deglutathionylation by Grx2 is increased when its entry into the catalytic chamber is stimulated. Cardiolipin is a well-established proteasome activator that is capable of stimulating 20S core particle entry [33]. Our hypothesis was that cardiolipin would have a synergistic effect on Grx2-dependent deglutathionylation by increasing Grx2 core entry. Therefore, after incubation of 20S PT with cardiolipin and Grx2, samples were analyzed by SDS–PAGE (Fig. 6A,B) and western blot using antibody against GSH (Fig. 6C), in parallel with proteasomal activity measurement in order to confirm catalytic recovery (Table 2).

It was found that activation of the 20S core by cardiolipin increased Grx2 degradation by 30% according to optical density measurements when compared to its degradation by the 20S PT but not stimulated by cardiolipin (Fig. 6A, lanes 3 and 2, respectively, and Fig. 6B). In parallel, deglutathionylation by Grx2 (evaluated by anti-GSH blotting analysis) in the presence of cardiolipin was greatly enhanced (Fig. 6C). It is noteworthy that, with increasing incubation time, the effect of cardiolipin was much more pronounced when compared to proteasome samples solely incubated with Grx2 for the same duration of incubation (Fig. 6C). These results strongly suggest that proteasome deglutathionylation is dependent on Grx2 entry into the catalytic chamber. The results shown in Table 2 confirm the cardiolipin stimulatory effect on 20S PT deglutathionylation, showing increased chymotryptsin-like activity and post-acidic proteasomal cleavage after simultaneous incubation of proteasome preparations with cardiolipin and Grx2. The results obtained showed 25% and 65% increased chymotryptsin-like activity and 61% and 100% increased post-acidic cleavage of n-PT and PT-SG preparations, respectively, when compared to samples incubated solely in the presence of Grx2. In all of the experiments described, after a 30 min pre-incubation with 20S core particle, Grx2 and cardiolipin were removed.

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Table 2. Effect of Grx2 on chymotrypsin-like activity and post-acidic cleavage of the natively and in vitro S-glutathionylated 20S PT pre-incubated with cardiolipin. Natively (n-PT) and in vitro (PT-SG) S-glutathionylated proteasome preparations in 20 mM Tris/HCl, pH 7.5 (20 μg 100 μL⁻¹) were pre-incubated for 5 min with cardiolipin (1.75 μg 1 μL⁻¹ proteasome) followed by addition of Grx2 plus the RS. After 30 min at 37 °C, samples were filtered through YM-100 microfilters and washed three times with standard buffer. Proteasome recovered on the microfilter membrane was incubated (1 μg 100 μL⁻¹) with the indicated substrates (each at 50 μM). Fluorescence emission (440 nm; excitation 365 nm) was determined after 45 min incubation at 37 °C. All results are means ± SD and are expressed as nmol AMC released per μg proteasome per min. As controls, n-PT preparations were incubated in standard buffer in the absence of Grx2 or pre-treatment with cardiolipin (CDL), or pre-incubated with CDL in the absence of Grx2. Asterisks indicate a P value < 0.00034 compared to same proteasomal samples incubated in the presence of Grx2 without CDL (ANOVA).

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<td>n-PT</td>
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<td>14 ± 1</td>
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<tr>
<td>PT-SG/Grx2</td>
<td>37 ± 2.5</td>
<td>18 ± 1.0</td>
</tr>
<tr>
<td>+ CDL</td>
<td>61 ± 4.5*</td>
<td>36 ± 3.5*</td>
</tr>
</tbody>
</table>

Table 1. Peptides derived from in vitro degradation of Grx2 by the 20S proteasome and identified by mass spectrometry. Samples were prepared as described in Experimental procedures. Results shown were obtained as described for supplementary Fig. S3.
by cycles of filtration and re-dilution, as described in the legend to Table 2, immediately prior to hydrolytic activity measurement. This procedure ensured that the increased post-acidic cleavage and chymotrypsin-like activity observed after 20S PT incubation with Grx2 in the presence of cardiolipin were due to increased deglutathionylation rather than cardiolipin-dependent proteasomal-stimulated activity, as previously reported when 20S PT activity was determined during incubation with cardiolipin [33]. To control the cardiolipin washing procedure, proteasomal catalytic activity was determined with samples not incubated with Grx2. Under these conditions, proteasomal activity was not increased after washing cardiolipin from the reaction mixture when compared to proteasomal activity determined in samples of untreated 20S PT (Table 2). Our conclusion from this set of experiments was that cardiolipin-stimulated Grx2 entry into the core increased 20S PT deglutathionylation. These results suggest that cysteine residues located inside the core are critical for redox regulation through S-glutathionylation.

Glutaredoxins with two cysteines in the active site possess two activities: mono- and dithiolic [9]. Therefore, we performed experiments with the Grx2C30S mutant, which lacks the C-terminal cysteine residue and retains only monothiolic activity. Grx2C30S activity determined using hydroxyethylthiolsulfide (HED) as a substrate, as described in the Experimental procedures, was 70% of that with the wild-type protein (data not shown). Monothiolic Grx2C30S was also able to deglutathionylate n-PT, although to a lesser extent than wild-type Grx2 (supplementary Fig. S4, C30S and WT, respectively). The active C30S mutant was also degraded by the 20S PT (data not shown). Therefore, monothiolic glutaredoxins should be considered as potential proteasomal deglutathionylases.

Grx2 is ubiquitinated in vivo

To determine whether Grx2 ubiquitination takes place at the physiological level, we next analyzed the presence of Grx2–ubiquitin complexes in crude cellular extract from yeast grown to stationary phase in glucose-enriched medium. During ubiquitination, up to six molecules of ubiquitin (8.5 kDa) can be added to form a polyubiquitin chain. We performed the experiments by immunoprecipitating Grx2 from the crude cellular extracts, followed by anti-ubiquitin and anti-Grx2 western blotting analyses (Fig. 7). Blotting with anti-Grx2 serum under reducing conditions showed the short (11.9 kDa) and long (15.9 kDa) forms of Grx2 (Fig 7, anti-Grx2). The band at 20 kDa is compatible with the size of mono-ubiquitinated short Grx2 isoforms (cytolic and mitochondrial matrix) [34], as the same band was seen in the anti-ubiquitin blot (Fig. 7, anti-Ub). Blotting of the same samples with anti-ubiquitin revealed the presence of higher molecular mass complexes (above 50 kDa), compatible with poly-ubiquitinated Grx2 isoforms (Fig. 7, anti-Ub). These bands were not visualized in the anti-Grx2 blotting, most probably because they represent poly-ubiquitinated isoforms with a low concentration of Grx2. These results are the first demonstration that Grx2 is ubiquitinated in vivo.

Discussion

Sulphydryl groups play a critical role in the function of many proteins, including enzymes, transcription factors and membrane proteins [35]. In a previous report, we concluded that oxidative stress induced proteasome glutathionylation and loss of chymotrypsin-like activity [8]. Now, we show that the S-glutathionylation and deglutathionylation processes represent biological redox regulation of 20S PT under basal conditions. We also showed the existence of regulatory mechanisms (best characterized in the case of Grx2) that are able to deglutathionylate the core particle, leading to
concomitant recovery of proteolytic activities. Our data show that two cytosolic thioredoxins also have the same effects on the 20S particle (Fig. 4). Furthermore, in principle, monothiolic glutaredoxins might also dethiolate the core, based on the ability of mutant Grx2C30S to perform this activity (supplementary Fig. S4). The existence of multiple pathways to dethiolate 20S PT may represent a highly tuned process to regulate this protease complex.

The data present in Figs 5 and 6 indicate that either Grx2, Trx1 and Trx2 must enter the latent 20S core to deglutathionylate proteosomal cysteine residues and recover proteasomal activities (Figs 3 and 4C). Moreover, as Grx2 entry into the 20S core particle increased, deglutathionylation and recovery of proteasomal activities were significantly improved (Fig. 6C and Table 2). Therefore, a question to be raised is whether these oxido-reductases undergo catalytic cycles during proteasomal deglutathionylation since they are degraded by the core. We do not have a definitive answer so far. Based on the results obtained by mass spectrometry analysis, a considerable proportion of Grx2 was not cleaved even after 2 h incubation (supplementary Fig. S3C). Furthermore, as noted above, it is possible that the 4898 kDa peptide detected after 30 min incubation that contains the conserved CXXC motif retains dethiolase activity. Nevertheless, the central point addressed here is that Grx2 is involved in redox regulation of the proteasome, either by an enzymatic or chemical reaction. The details of this process will be further investigated.

As already demonstrated in mammals, some proteins are able to enter the 20S core particle, whereas, for others, only partial structural loss or the existence of poorly structured domains allow free entry [36,37]. Crystallographic modeling shows that the molecular architecture of Grx2 consists of a four-stranded, mixed β-sheet and five α-helices. The β-sheet forms the central core of the protein, with helices 1 and 3 located on one side of the sheet and helices 2, 4 and 5 located on the other side [38] (Discola KF & Netto LES, unpublished results). Most probably, a specific interaction of particular domains of these oxido-reductases stimulates 20S PT opening to allow their entry. Additionally, glutaredoxins and thioredoxins share a common fold, the so-called thioredoxin fold [39], and isoforms of both oxido-reductase families (Grx2, Trx1 and Trx2) are able to deglutathionylate the 20S PT. The recognition of structural features in Grx2, Trx1 and Trx2 by 20S PT indicates that the deglutathionylase activity reported here represents a relevant signaling event. We are presently investigating whether that common feature is related to their easy entry into the latent 20S particle.

According to our data, Grx2 is ubiquitinated inside cells (Fig. 7). Although Grx2 degraded by the 20S PT in vitro, the present findings show that degradation of Grx2 might be controlled by ubiquitination at the physiological level. Reports in the literature raise the possibility that proteins that can freely enter the 20S PT can be degraded by both ubiquitin-dependent and -independent processes [37].

**Experimental procedures**

**Materials**

Anti-FLAG IgG, cardiolipin (CDL), dithionitrobenzoic acid, diethylenetriaminepentaaetic acid, dithiothreitol, N-ethylmaleimide, GSH, glutathione reductase (GR), NaBH4 and Tris(2-carboxy-ethyl) phosphine hydrochloride were purchased from Sigma (St Louis, MO, USA). Anti-20S PT serum, cytochrome c from equine heart and the fluorogenic substrates carbobenzoxy-Leu-Leu-Glu-AMC (z-LLE-AMC), carbobenzoxy-Ala-Arg-Arg-AMC (z-ARR-AMC) and succinyl-Leu-Leu-Val-Tyr-AMC (s-LLVY-AMC) were obtained from Calbiochem (Darmstadt, Germany). Molecular mass markers for SDS-PAGE and Protein A-Sepharose 4B Fast Flow were obtained from Amersham Biosciences (Piscataway, NJ, USA). NBD and HED were purchased from Aldrich (St. Louis, MO, USA). AMC (7-amido-4-methylcoumarin) was purchased from Fluka (Buchs Switzerland).

Anti-GSH serum was obtained from Invitrogen (Carlsbad, CA, USA). Anti-ubiquitin monoclonal serum was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Bradford protein assay reagent was purchased from Bio-Rad (Hercules, CA, USA). Sinapinic acid (matrix) and myoglobin (MS standard) were part of the ProteoMass kit (Sigma).

**Yeast strain and growth**

*Saccharomyces cerevisiae* JD1144 (MATa his3A200 leu2-3,112 lys2-801 trp1Δ63 ura3-52 PRE1ΔH::Ylplac211 URA3) derived from strain JD47-13C was kindly donated by R. Deshaies (Division of Biology, Caltech, Pasadena, CA, USA). In this strain, the 20S proteasome Pre1 subunit is tagged with the FLAG peptide sequence and a polyhistidine tail, which allows single-step purification [40]. Cells were cultured in glucose-enriched YPD medium (4% glucose, 1% yeast extract and 2% peptone) at 30 °C with reciprocal shaking, and harvested after 60 h incubation.

**Extraction and purification of the 20S proteasome**

The 20S PT was purified by nickel-affinity chromatography or by immunoprecipitation with anti-FLAG® M2 affinity gel freezer-safe (Sigma) as described previously [8].

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G. M. Silva et al.
Non-tagged 20S PT was purified by conventional multi-step chromatography as described previously [8]. 20S proteasome preparations obtained by affinity chromatography were utilized in all experiments. Preparations obtained by conventional chromatography were utilized as controls for the tagged 20S particle. The purity of 20S PT preparations was confirmed by SDS-PAGE and non-denaturing PAGE as described previously [8].

Proteasome activity determination by hydrolysis of fluorogenic peptides

Fluorogenic peptides (AMC, 7-amido-4-methylcoumarin as the fluorescent probe) were utilized for determination of proteasomal activity, as described elsewhere [41]. s-LLVY-AMC was utilized as a standard peptide to assess the chymotrypsin-like activity of the core, z-LLE-AMC for the post-acidic cleavage and z-ARR-AMC for the trypsin-like activity [41]. 20S PT (0.5–3 μg) was incubated at 37 °C in 20 mM Tris/HCl buffer, pH 7.5, herein referred to as standard buffer. Incubation was started by the addition of 10–50 μM of peptide. Fluorescence emission was recorded at 440 nm (excitation at 365 nm). The amount of AMC released from the substrates was calculated using a standard curve of free AMC.

Reduction, oxidation and S-glutathionylation of the 20S proteasome

Preparations of purified 20S PT (500–1000 μg) extracted from cells grown in glucose-containing medium were incubated overnight at 4 °C in 20 mM Tris buffer, pH 7.5, containing 300 mM dithiothreitol. Then, proteasome preparations were passed through a HiTrap desalting column to remove dithiothreitol, according to the manufacturer’s protocol (Amersham Biosciences). Eluted protein fractions were tested for the presence of dithiothreitol by reaction with dithionitrobenzoic acid. Enriched protein fractions, identified by reactivity to Bradford reagent and for which dithiothreitol reactivity was decreased, were selected for further use. Combined fractions were filtered and concentrated through Microcon YM-100 filters (Millipore, Billerica, MA, USA). These preparations are referred to here as dithiothreitol-reduced 20S proteasome (PT-SH). Aliquots of these preparations were oxidized by incubation in standard buffer in the presence of 5 mM H₂O₂ and 100 μM diethylene-triaminepentaacetic acid for 30 min at room temperature. After incubation, excess H₂O₂ was removed by two cycles of centrifugation at 8000 g for 15 mins at room temperature, and re-dilution through Microcon YM-100 filters. S-glutathionylated 20S proteasome (PT-SG) was obtained by incubation of oxidized 20S proteasome (PT-SOH) aliquots (100 μg) at room temperature for 20 min in the presence of 5–10 mM GSH. Afterwards, GSH was removed by four cycles of centrifugation at 8000 g for 15 mins at room temperature, and re-dilution through Microcon filters. The S-glutathionylated core used for the assays described here was either the natively S-glutathionylated proteasome(n-PT) purified from cells grown to stationary phase in glucose-enriched medium or the in vitro S-glutathionylated core (PT-SG), as described above. After determination of protein concentration, aliquots of PT-SH, n-PT or PT-SG preparations were used for further incubation, immunoblot analyses, SDS-PAGE and hydrolytic assays.

Cloning and expression of yeast GRX2

Cloning of the yeast GRX2, its expression in E. coli, and Grx2 purification have been described previously [20]. The recombinant protein is tagged with an N-terminal polyhistidine sequence. Purified Grx2 was analyzed by SDS-PAGE. Grx2 activity was determined spectrophotometrically by measuring the reduction of 0.5 mM HED in the presence of 0.5 mM GSH, 0.1 mM NADPH and 0.3 U·mL⁻¹ GR at 37 °C, and following the disappearance of NADPH at 340 nm. All of the experiments with Grx2 were controlled by assaying non-tagged protein (thrombin-treated Grx2). No difference between tagged and non-tagged Grx2 was observed.

Cloning, expression and purification of yeast thioredoxin reductase 1 (Trt1)

Cloning of the yeast TRR1, its expression in E. coli, and Trt1 purification have been described previously [42]. The recombinant protein was tagged with an N-terminal polyhistidine sequence.

Cloning, expression and purification of yeast Txr1 and Txr2

The trxl and trc2 genes were amplified by PCR from yeast genomic DNA (Research Genetics, Invitrogen), as described previously [43]. PCR products were cloned into the NdeI and SpeI restriction sites of pET17b expression vector (Novagen, Darmstadt, Germany). E. coli BL21 (DE3) cells were transformed with pET17b/trx1 or pET17b/trx2 vectors. Protein purification was performed as described previously [43].

Incubation of S-glutathionylated 20S proteasome with Grx2 and thioredoxins

S-glutathionylated 20S PT (20–50 μg), obtained either by growing cells to stationary phase in 4% glucose (n-PT) or by in vitro S-glutathionylation (PT-SG), was incubated at 37 °C for 15–120 min in the presence of Grx2 (5–15 μg) in...
0.1 mL standard buffer containing 0.5 mM GSH, 2 mM NADPH and 0.3 U mL\(^{-1}\) GR, herein referred to as the reductive system (RS). Incubation with thioredoxins was performed under the same conditions with standard buffer containing 2 mM NADPH and 0.5 μg Trx1 per 100 μL (final concentration). When specified, 1.75 μg CDL per μg 20S PT was added to the mixture. After incubation, samples were filtered by four cycles of centrifugation at 8000 g for 15 mins at room temperature, and re-dilution through YM-100 Microcon filters. Control samples were incubated in the presence of all reagents except Grx2, Trx1, Trx2 or CDL.

**SDS–PAGE analysis of proteins**

SDS–PAGE was performed as described previously [44]. Protein preparations, after incubation under the indicated conditions (described in figure legends), were mixed with gel loading buffer (60 mM Tris/HC1, pH 6.8, containing 25% glycerol, 2% SDS and 0.1% bromophenol blue) and frozen until applied to the gel. Gels were stained either by Coomassie brilliant blue or by the silver staining method, as described previously [44].

**Immunoprecipitation with anti-Grx2 serum**

Grx2 immunoprecipitation from yeast cell lysates was performed as follows: pellets (150–200 mg) of yeast cells were disrupted by vortexing cells mixed with 1 volume of glass beads and 2 volumes of standard buffer, containing 500 mM NaCl plus 1 μL protease inhibitor cocktail (Calbiochem) per 20 mg cellular pellet. After 10 cycles of 2 min vortexing and 1 min resting on ice, the cell lysate was centrifuged at 15 000 g for 40 min. The supernatant was used for immunoprecipitation. After pre-clearing the cell lysate (1 mg protein) in 200 μL of pre-treated Protein A–Sepharose, the supernatant obtained after centrifugation was used for incubation with anti-Grx2 serum (1 : 50 dilution). Incubation was performed for 1 h in a cold room at 8°C on a rotary shaker and transferred to 200 μL Protein A–Sepharose for a further 15 h incubation. Samples were then centrifuged at 10 000 g, and the Protein A–Sepharose beads were washed five times with 500 μL Tris/NaCl buffer. After removing the final supernatant, 100 μL SDS–PAGE sample buffer was added to the immunoprecipitate, and samples were heated for 10 min at 100 °C. After centrifugation, at 15 000 g for 10 mins at room temperature samples were applied to 12.5% SDS–PAGE. Grx2 immunoprecipitates were immunooblotted with anti-Grx2 and anti-ubiquitin serum.

**Immunoblotting**

Immunoblotting was performed using the ECL™ western blotting system (Amersham Biosciences) according to the manufacturer’s protocol. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and protein signals were detected using enhanced chemiluminescence western blotting detection reagents (Amersham Biosciences). Proteasome samples analyzed using anti-GSH serum were mixed with gel loading buffer containing 10 mM N-ethylmaleimide. As a control, samples of PT-SH were run in parallel to all experiments shown and no background signal was observed. The loading control was evaluated by anti-FLAG blotting, as follows: the same membranes utilized for anti-GSH blotting were incubated overnight with 10 mM Tris(2-carboxy-ethyl) phosphine hydrochloride in NaCl/Pi (100 mM Tris, pH 7.5 containing 200 mM NaCl). Next, membranes were washed five times with NaCl/Pi followed by the anti-FLAG blotting. Dilutions of antibodies were as follows: 1 : 200 (anti-ubiquitin), 1 : 1000 (anti-GSH and anti-20S PT) and 1 : 2000 (anti-Grx2 and anti-FLAG).

**Optical density measurements**

When specified, optical density measurements were performed using IMAGEQUANT software from Molecular Dynamics (Sunnyvale, CA, USA).

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**References**


### Supplementary material

The following supplementary material is available online:

**Fig. S1.** SDS/PAGE profile of 20S PT preparations. 12.5% SDS–PAGE of 20S PT stained with Coomassie brilliant blue (lane 2). Lane 1 contains molecular mass markers.

**Fig. S2.** *In vitro* degradation of standard proteins by the 20S PT. Five micrograms of each protein, except casein (10 µg), were incubated for 2 h at 37 °C in standard buffer with 2.5 µg n-PT.

**Fig. S3.** MALDI-TOF analyses of Grx2 after incubation with natively S-glutathionylated 20S PT.

**Fig. S4.** Comparative 20S PT deglutathionylation by Grx2C30S and wild-type Grx2.

**Doc. S1.** MALDI-TOF analyses of Grx2.

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