Comparative resistance of the 20S and 26S proteasome to oxidative stress

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Oxidatively modified ferritin is selectively recognized and degraded by the 20S proteasome. Concentrations of hydrogen peroxide (H_2O_2) higher than 10 μmol/mg of protein are able to prevent proteolytic degradation. Exposure of the protease to high amounts of oxidants (H_2O_2, peroxynitrite and hypochlorite) inhibits the enzymic activity of the 20S proteasome towards the fluorogenic peptide succinyl-leucine-leucine-valine-tyrosine-methylcoumarylamide (Suc-LLVY-MCA), as well as the proteolytic degradation of normal and oxidant-treated ferritin. Fifty per cent inhibition of the degradation of the protein substrates was achieved using 40 μmol of H_2O_2/mg of proteasome. No change in the composition of the enzyme was revealed by electrophoretic analysis up to concentrations of 120 μmol of H_2O_2/mg of proteasome. In further experiments, it was found that the 26S proteasome, the ATP- and ubiquitin-dependent form of the proteasomal system, is much more susceptible to oxidative stress. Whereas degradation of the fluorogenic peptide, Suc-LLVY-MCA, by the 20S proteasome was inhibited by 50% with 12 μmol of H_2O_2/mg, 3 μmol of H_2O_2/mg was enough to inhibit ATP-stimulated degradation by the 26S proteasome by 50%. This loss in activity could be followed by the loss of band intensity in the non-denaturing gel. Therefore we concluded that the 20S proteasome was more resistant to oxidative stress than the ATP- and ubiquitin-dependent 26S proteasome. Furthermore, we investigated the activity of both proteases in K562 cells after H_2O_2 treatment. Lysates from K562 cells are able to degrade oxidized ferritin at a higher rate than non-oxidized ferritin, in an ATP-independent manner. This effect could be followed even after treatment of the cells with H_2O_2 up to a concentration of 2 mM. The lactacystin-sensitive ATP-stimulated degradation of the fluorogenic peptide Suc-LLVY-MCA declined, after treatment of the cells with 1 mM H_2O_2, to the same level as that obtained without ATP stimulation. Therefore, we conclude that the regulation of the 20S proteasome by various regulators takes place during oxidative stress. This provides further evidence for the role of the 20S proteasome in the secondary antioxidative defences of mammalian cells.

INTRODUCTION

Over the past few years a large number of publications [1–12] have reported on the relationship between protein oxidation and proteolysis. These studies were conducted using various cell types including erythrocytes, reticulocytes, and haemopoietic precursor cells [1–4]; *Escherichia coli* [1,2]; rat muscles *in vitro* [1,4]; hepatocytes [8–10]; and purified proteins and proteases *in vitro* [1–9]. These studies have concluded that proteins are inherently susceptible to oxidative damage, and that oxidative damage alters proteolytic susceptibility. Furthermore, these studies have consistently demonstrated that relatively mild oxidative damage increases proteolytic susceptibility (and degradation) whereas extensive oxidative damage causes decreased proteolysis, due to cross-linking, aggregation, and decreased solubility [1–5]. The multicatalytic proteinase, proteasome, appears to be the major proteolytic enzyme involved in the removal of oxidized proteins, although Matthews et al. [13] have questioned the role of proteasome in degrading oxidatively modified proteins. Two recent studies from our group, involving proteasome depletion using antisense techniques, revealed the role of proteasome in the degradation of oxidized proteins in Clone 9 liver cells [10] and K562 haemopoietic cells [11]. These studies found no change in the capacity of these cells to degrade foreign proteins or fluorogenic peptide substrates after treatment with oxidants, suggesting that the existing cellular proteasome content was sufficient to cope with new oxidant-generated protein substrates [10–12].

There is little knowledge about the effect of oxidants on the activity of the proteasome itself, except for a report by Strack et al. [14] which reported changes in the peptidase and proteinase activity after hydrogen peroxide (H_2O_2) and $FeSO_4$-EDTA-ascorbate treatment. Possible dissociation/reassociation with the PA28 activator was suggested [14], however, no information on the susceptibility of the 26S form of the multicatalytic proteinase towards oxidants is available. Both the 20S 'core' proteasome and the ATP-stimulated ubiquitin-dependent 26S proteasome appear to be responsible for the degradation of various abnormal cellular proteins. While involvement of the 20S proteasome in the degradation of oxidant treated proteins has been suggested by several authors [1–12], the ATP-stimulated ubiquitin-dependent 26S proteasome complex may play a larger role in the degradation of other abnormally folded proteins [15,16].

We undertook the present investigation with two major goals: first, to test the inhibitory effect of various oxidants on the activity of the 20S proteasome, and second, to test whether the 20S or the 26S proteasome is more susceptible to inactivation by oxidants.

MATERIALS AND METHODS

Isolation of the multicatalytic proteinases

The 20S and 26S multicatalytic proteinases were isolated from erythrocytes of outdated human blood conserves as described by Hough et al. [17]. Erythrocytes were lysed in Hepes buffer (10 mM, pH 7.0) supplemented with 1 mM dithiothreitol, 1 mM $MgCl_2$ and 1 mM ATP (all final concentrations). After the

Abbreviations used: Suc-LLVY-MCA, succinyl-leucine-leucine-valine-tyrosine-methylcoumarylamide; TCA, trichloroacetic acid; I_{50}, concentration giving 50% inhibition.

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removal of membranes and non-lysed cells by centrifugation, 20% (v/v) glycerol was added to the supernatant. Both proteinases were isolated by DEAE-cellulose chromatography, glycerol-density gradient centrifugation and separation on a Mono Q column using an FPLC system [17]. In the case of the 20S proteasome separation, ATP, $MgCl_2$ and glycerol were omitted in order to achieve a higher yield.

Treatment of proteins with oxidants

Ferritin (Sigma, Deisenhofen, Germany) was used as a model proteolytic substrate. To increase its proteolytic susceptibility by oxidative modification, ferritin was treated with various concentrations of H_2O_2 in 20 mM phosphate buffer, pH 7.4, for 2 h at room temperature. The protein was then dialysed for 16 h at 4 °C against 2 litres of 5 mM phosphate buffer, pH 7.4, containing 10 mM KCl, with one exchange of the dialysis fluid after 3 h. Only dialysed protein (either oxidized or control) was used for proteolysis measurements. The oxidant resistance of the 20S and 26S proteasome complexes was tested with H_2O_2, peroxynitrite ($ONOO^-$) and an equimolar solution of hypochlorite/hypochlorous acid (OCl^-/HOCl). Exposure of the proteasome complexes to H_2O_2 was carried out in the presence of 20 mM phosphate buffer, pH 7.4. Exposure to OCl^-/HOCl and $ONOO^-$ was conducted in 20 mM Hepes, pH 7.4. All oxidant exposures were carried out for 30 min at room temperature; OCl^-/HOCl and $ONOO^-$ were not detectable after this incubation. The remaining H_2O_2 was removed by the addition of 0.5 μg of catalase (Sigma, Deisenhofen, Germany).

Proteolysis measurements

The degradation of ferritin was measured by incubating 200 μg of the substrate protein with 7 μg of proteasome in a proteolysis buffer containing 50 mM Hepes, pH 7.8, 20 mM KCl, 5 mM MgOAc and 1 mM dithiothreitol. The degradation assay was performed for 2 h at 37 °C. The reaction was stopped by the addition of an equal volume of ice-cold 20% (w/v) trichloroacetic acid (TCA). After centrifugation (15 min, 14000 ***g***), the supernatants containing primary amines were neutralized using 1 M Hepes, pH 7.8. Fluorescamine (0.3 mg/ml in acetone) was added to the neutralized supernatants mixed thoroughly by vortex. The fluorescence was quantified at 390 nm excitation and 470 nm emission, using leucine as a standard. Proteolysis was calculated by subtraction of the blank values (substrate without proteasome and proteasome without substrate) from the release of free primary amines measured.

^{3}H-labelled ferritin was used as the substrate for the assessment of ferritin degradation by K562 cell lysates. The protein was radiolabelled by reduced methylation with [^{3}H]formaldehyde and sodium cyanoborohydride, as described by Jentoft and Dearborn [18], and then extensively dialysed. The [^{3}H]ferritin was either undamaged or oxidatively modified as described above. For proteolysis measurements, [^{3}H]ferritin was added to centrifuged cell lysates and proteolysis buffer as described previously [4]. The percentage degradation was calculated after TCA-precipitation, using 3% (w/v) bovine serum albumin as a carrier, as: (acid-soluble counts − background counts)/(total counts − background counts) × 100.

The peptidase activity of the proteasome preparations was measured by mixing proteasome with 30 μl of a 2 mM stock solution (in DMSO) of the fluorogenic peptide succinyl-leucine-leucine-valine-tyrosine-methylcoumarylamide (Suc-LLVY-MCA) in a final volume of 300 μl. The mixture was incubated at 37 °C for 1 h and then the reaction was stopped by the addition of an equal volume of ice-cold ethanol, followed by 10 volumes of 125 mM borate buffer, pH 9.0. Peptidase activity was monitored by the release of the fluorescent MCA moiety, measured at 380 nm excitation and 440 nm emission. For all measurements of ATP-stimulated proteolysis, 5 mM $MgCl_2$ and 5 mM ATP were added to reaction mixtures containing ATP-depleted cell lysates. Lactacystin was used at a final concentration of 5 μM.

Gel electrophoresis

One-dimensional SDS/PAGE was performed by the method of Schaegger and von Jagow [19] using a 12.5% separating gel containing 8 M urea. Electrophoresis was standardized using prestained low-molecular-mass standards (Bio-Rad, Munich, Germany).

Electrophoresis under non-denaturing conditions was performed as described by Hough et al. [17]. Briefly, the relevant proteinase was diluted with 100 mM Tris/HCl, pH 6.8 and 20% (v/v) glycerol. Proteins solutions (3 μg) were loaded into each lane. A 3% stacking gel and 4.5% separating gel were used and the separation was carried out overnight at 600 Vh and 4 °C. After electrophoresis, the gel was incubated in 50 mM Tris, pH 7.8, containing 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA and 10% (v/v) glycerol, for 15 min at 37 °C. Afterwards, the gel was mounted on a light box (emitting light: 366 nm) and the gel was overlaid with a 200 μM Suc-LLVY-MCA solution containing 5 mM ATP. The fluorescence was photographed between 10 min and 1 h after exposure to the fluorogenic peptide.

Cell culture

K562 cells (human chronic myelogenous leukaemia) were obtained from American Tissue and Cell Culture (A.T.C.C., CCL 243). The cells were cultured in 90% RPMI 1640 medium, supplemented with 10% (v/v) fetal bovine serum. Cells were initially seeded at a density of 0.4×10^6 cells/ml. Some cells were exposed to H_2O_2 for 30 min at 37 °C in PBS, pH 7.4, on the third day of growth. After exposure to oxidative stress these cells were washed twice and then lysed by repeated cycles of freezing and thawing, in a solution consisting of 0.25 M sucrose, 25 mM Hepes, 10 mM $MgCl_2$, 1 mM EDTA and 1 mM dithiothreitol.

RESULTS

Degradation of oxidized ferritin by the 20S proteasome

After exposure of ferritin to H_2O_2 a 7-fold increase in degradation by the 20S proteasome was evident (Figure 1). Whereas mild oxidative stresses (up to 10 μmol of H_2O_2/mg of ferritin) consistently increased proteolytic susceptibility, higher concentrations (20 μmol of H_2O_2/mg of ferritin and above) significantly decreased the degradation of ferritin by the 20S proteasome. These results are in close agreement with the conclusions reached previously by our group [1–5,10–12] and others [6–9] with different protein substrates. For further investigation of the influence of various oxidants on the proteolytic activity towards the proteasome we used either undamaged ferritin or ferritin modified by exposure to 10 μmol of H_2O_2/mg of ferritin, the substrate with the highest proteolytic susceptibility.

Inhibition of the 20S proteasome activity by H_2O_2

To study the influence of H_2O_2 on the activity of the multicatalytic 20S proteasome, the enzyme was incubated for 30 min with H_2O_2. The activity of the enzyme was tested using the fluorogenic peptidase substrate, Suc-LLVY-MCA. In addition, we measured the degradation of untreated ferritin and oxidized ferritin. The

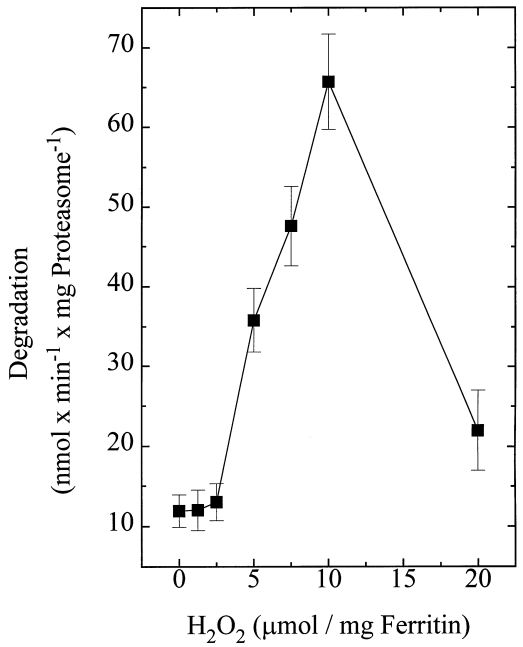

Figure 1 Degradation of H_2O_2-modified ferritin by the isolated 20S proteasome complex

Ferritin was either untreated or exposed to various concentrations of H_2O_2 as described previously [3,11]. Ferritin (1 mg/ml) was oxidized during a 2 h incubation in 20 mM phosphate buffer, pH 7.2. After oxidative modification the protein was extensively dialysed against 5 mM phosphate buffer, pH 7.2 containing 10 mM KCl. To measure proteolytic degradation the dialysed ferritin was incubated for 2 h with the 20S proteasome complex at 37 °C. The proteolysis buffer, reaction conditions, and the detection of free amino groups were as described in the Materials and methods section. Quantification of TCA-soluble amino groups generated by proteolysis was performed by reaction with fluorescamine, using leucine as a standard. The values are means ± S.E.M. for six independent experiments.

Table 1 Inhibition of the isolated 20S and 26S proteasome complexes by oxidants

The 20S and 26S proteasome complexes were isolated as described in the Material and methods section. In the case of the 26S proteasome, only preparations exhibiting an ATP stimulation of more than 2.5-fold were used. The 20S proteasome preparations were not contaminated by regularity complexes, such as PA28 or PA700 (ATP-stimulated regulator). Ferritin was either untreated (non-oxidized) or was treated with 10 μmol of H_2O_2/mg and both forms of the protein were then extensively dialysed. Oxidant (H_2O_2, OCl^-/HOCl, or $ONOO^-$) treatment of proteasome was performed as described in the Materials and methods, section. Values reported below represent the oxidant concentration required to achieve 50% inhibition (I_{50}) of the hydrolysing activity against either untreated ferritin or oxidized ferritin, or against the fluorogenic peptide Suc-LLVY-MCA. The I_{50} values are means ± S.E.M. calculated from five independent experiments. In the case of the 26S proteasome only the inhibition of the ATP-stimulated portion of the total proteolytic activity was taken into account.

	Oxidant concentration giving 50% inhibition (μmol/mg of proteasome)			
	20S Proteasome			26S Proteasome
	Non-oxidized ferritin	Oxidized ferritin	Suc-LLVY-MCA	Suc-LLVY-MCA
H_2O_2	40.1 ± 1.7	41.8 ± 5.9	12.6 ± 2.1	3.10 ± 0.68
Peroxynitrite	0.75 ± 0.18	0.78 ± 0.03	0.87 ± 0.08	0.052 ± 0.003
Hypochlorite	0.34 ± 0.06	0.41 ± 0.02	0.39 ± 0.03	0.10 ± 0.01

peptidase activity of the 20S proteasome declined after exposure to H_2O_2, with 50% inhibition (I_{50}) occurring at 12.6 μmol of H_2O_2/mg of proteasome (Table 1). The degradation of both untreated ferritin and oxidized ferritin also declined with increasing exposure of the 20S proteasome to H_2O_2. This decrease in proteinase activity (degradation of ferritin or oxidized ferritin) followed sigmoidal kinetics, with the I_{50} occurring at about 40 μmol of H_2O_2/mg of proteasome (Table 1). The inhibition curves were comparable for both untreated and oxidized ferritin.

The concentration of H_2O_2 required for 50% inhibition of the proteasome was about 4-fold higher than that required to produce a maximal increase in the proteolytic susceptibility of the ferritin substrate (Table 1). Therefore, the multicatalytic 20S proteasome seems to be able to catalyse the removal of oxidized proteins under conditions *in vivo*, as reported by our group earlier [10,11].

Changes in proteasome structure during oxidant exposure

To test whether inhibition of the 20S proteasome by H_2O_2 was due to the modification of amino acids or to decomposition of the quaternary structure of the multimeric enzyme complex, a series of non-denaturing PAGE, SDS/PAGE and activity gel studies were performed. Figure 2(A) shows the activity of the 20S proteasome at various H_2O_2 concentrations. We were able to detect Suc-LLVY-MCA peptidase activity at 40 μmol of H_2O_2/mg of protein, a concentration 3.2-fold higher than the I_{50} reported in Table 1 for this fluorogenic peptide. At 120 μmol of H_2O_2/mg of protein (10-fold more than the I_{50}) activity in the overlay gels could no longer be detected. Coomassie Blue staining revealed that from 0 to 40 μmol of H_2O_2/mg of protein, there were no changes in staining intensity, under either non-denaturing or denaturing electrophoretic conditions (Figures 2B and 2C). We concluded that the individual proteasome subunits were undamaged at H_2O_2 concentrations below 40 μmol/mg of protein, but that significant amino acid oxidative modifications occurred at higher concentrations. The diminished Coomassie Blue staining may be the result of damage to tryptophan and tyrosine residues.

Inhibition of the 20S proteasome activity by OCl^- and $ONOO^-$

Apart from H_2O_2, other oxidants appear to play major roles in biological systems. We therefore tested the effects of sodium hypochlorite (at physiological pH this is a mixture of hypochlorite and hypochlorous acid) and $ONOO^-$ on the activity of the 20S proteasome. Both oxidants inhibited the activity of the 20S proteasome in a concentration-dependent manner (results not shown). In both cases the inhibition of peptidase activity and proteinase activity was comparable. The I_{50} for inactivation of proteasome activity by $ONOO^-$ was approximately 0.8 μmol of $ONOO^-$/mg of proteasome, and for OCl^-/HOCl the I_{50} was 0.4 μmol of (OCl^-/HOCl)/mg of proteasome (Table 1). Therefore, on a molar basis, OCl^-/HOCl was the most potent oxidant-inhibitor of the 20S proteasome, being more than 100-fold more effective than H_2O_2.

Inhibition of the 26S proteasome and ATP-stimulated proteolysis by oxidants

To test the resistance of the 26S proteasome to oxidants, we exposed the isolated complex to H_2O_2, $ONOO^-$ and OCl^-/HOCl (Table 1). To determine the oxidant-sensitivity of the ATP-stimulated 26S proteasome, we always used proteasome preparations with at least a 2.5-fold ATP-stimulating effect on the degradation of the fluorogenic peptide Suc-LLVY-MCA. It was reported earlier [1–5,10] that the degradation of oxidized proteins *in vitro* was not stimulated by ATP (in fact ATP was mildly

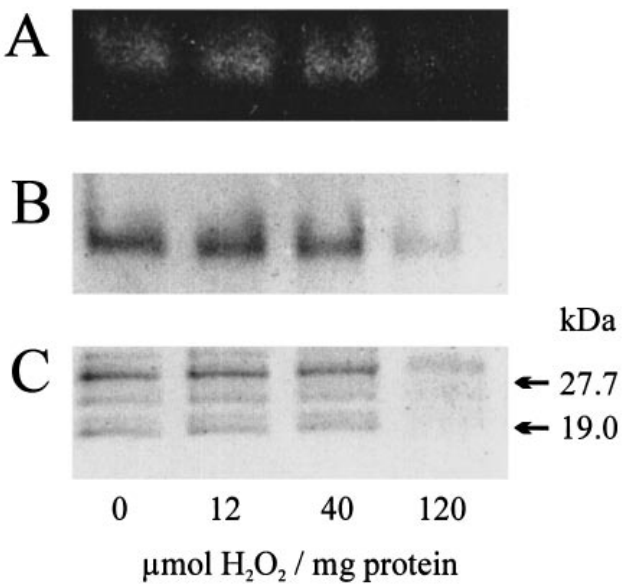

Figure 2 Non-denaturing PAGE and SDS/PAGE of the 20S proteasome complex after treatment with H_2O_2

The experimental conditions for H_2O_2 treatment and electrophoresis are described in the Materials and methods section. Panels (**A**) and (**B**) represent analysis of the isolated 20S proteasome by non-denaturing PAGE, whereas panel (**C**) shows the 20S proteasome after denaturing electrophoresis. Panel (**A**) demonstrates the fluorescence of the proteasome band after overlaying the gels with 200 μM of the peptide substrate Suc-LLVY-MCA, dissolved in 50 mM Tris (pH 7.8), 25 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol and 0.1 mM EDTA. Panel (**B**) shows the Coomassie Blue stained bands of the proteasome after treatment with various concentrations of H_2O_2. Panel (**C**) presents the Coomassie Blue stained bands of the same samples shown in panels (**A**) and (**B**). The molecular masses of known standards are indicated on the right. The electrophoresis data shown are representative of three independent experiments.

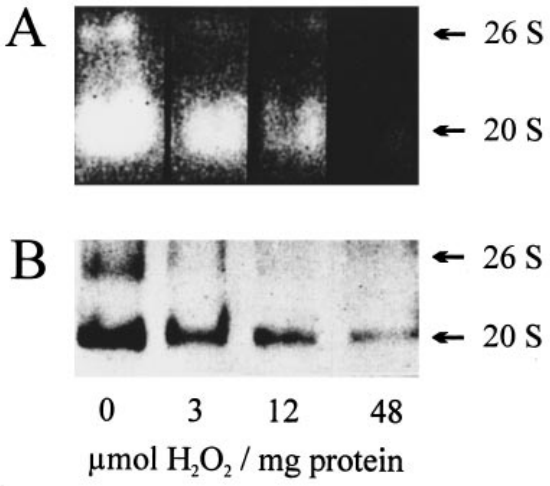

Figure 3 Non-denaturing PAGE of the isolated 20S and 26S proteasome complexes after treatment with hydrogen peroxide

The experimental conditions for H_2O_2 treatment and PAGE are described in the Materials and methods section and in the legend to Figure 2. Panel (**A**) is an activity gel showing the proteolytic activity of each proteasome complex after non-denaturing PAGE. The Suc-LLVY-MCA substrate overlay was performed as described in the legend to Figure 2, with the exception that 5 mM ATP and 5 mM $MgCl_2$ were added to the buffer. Panel (**B**) shows the Coomassie Blue stained proteasome bands after non-denaturing PAGE of the H_2O_2-treated enzyme complex. The migration positions of isolated 20S and 26S proteasome preparations (without incubation times) are indicated. The gels shown are representative of several experiments.

Table 2 Effects of exposure to H_2O_2 on the subsequent degradation of proteins in lysates of K562 cells

K562 cells were exposed to H_2O_2 in PBS for 30 min, harvested immediately, and cell lysates prepared as described in the Materials and methods section. [^{3}H]Ferritin was either untreated (non-oxidized) or treated with 10 μmol of H_2O_2/mg of protein and both forms were then extensively dialysed. For proteolysis measurements oxidized or untreated [^{3}H]ferritin were added to the centrifuged cell lysates. After incubation at 37 °C for 2 h the percentage degradation of [^{3}H]ferritin was determined as described in the Materials and methods section. The results are means ± S.D. of three independent experiments.

	Non-oxidized ferritin		Oxidized ferritin	
Exposure of K562 cells	−ATP	+ATP	−ATP	+ATP
No exposure	0.95 ± 0.13	1.19 ± 0.22	2.45 ± 0.22	2.13 ± 0.24
30 min, PBS	1.11 ± 0.11	1.17 ± 0.15	2.21 ± 0.20	2.20 ± 0.18
30 min, 1 mM H_2O_2	1.10 ± 0.15	1.22 ± 0.15	2.55 ± 0.19	2.36 ± 0.26
30 min, 2 mM H_2O_2	1.18 ± 0.13	1.12 ± 0.12	1.95 ± 0.35	1.90 ± 0.22

inhibitory) and that oxidized proteins were poor substrates for the ATP-stimulated 26 S proteasome *in vitro*. Therefore, in our present studies of 26 S proteasome inactivation by oxidants, we measured only the degradation of the fluorogenic peptide Suc-LLVY-MCA. The I_{50} values for H_2O_2, OCl^-/HOCl and $ONOO^-$ were 3.1, 0.1 and 0.052 μmol/mg of proteasome, respectively (Table 1). Therefore, the ATP-stimulated peptidase activity of the 26S proteasome was about 4-fold more susceptible to H_2O_2 and OCl^-/HOCl, and more than 16-fold more susceptible to $ONOO^-$, than was the (ATP-independent) peptidase activity of the 20S proteasome. The inactivation of ATP-stimulated 26S proteasomal proteolysis by H_2O_2 (an I_{50} of 3.1 μmol/mg of proteasome, Table 1) occurred at a 3-fold lower H_2O_2 concentration than that required to produce a maximal increase in the susceptibility of ferritin to degradation by the 20S proteasome (10 μmol/mg protein, see Figure 1).

Figure 3 shows the influence of H_2O_2 on the 26S proteasome. Figure 3(A) shows an activity gel for degradation of the fluorogenic peptide Suc-LLVY-MCA, in which two distinct bands, representing the 20S proteasome and the 26S proteasome can be seen. The ATP-stimulated 26S proteasome is always contaminated by the 20S proteasome. This is probably the result of dissociation of the 26S proteasome to yield the 20S proteasome and the ATP-dependent activator [17] during the 2 h incubation at 37 °C, and the overnight run of the electrophoresis itself. This small contamination did not present great difficulties, however, even at concentrations of H_2O_2 as low as 3.0 μmol/mg of protein one can see a clear decline in the activity in the band of the 26S proteasome in non-denaturing gel electrophoresis (Figure 3A). This decline was concentration-dependent and at 48 μmol/mg of protein no activity of the 26S proteasome remained. This decline in proteolytic activity was accompanied by a loss of the 26S proteasome Coomassie-Blue-stainable band as shown in Figure 3(B). These results indicate that the 26S proteasome complex is inactivated at relatively low H_2O_2 concentrations.

20S and 26S proteasome activities in K562 cells after H_2O_2 treatment

K562 cells increase protein turnover after oxidant exposure and selectively degrade oxidized proteins [10–12]. The proteolytic capacity of lysates from these cells for oxidized proteins seems to be unaffected by up to 1 mM H_2O_2 [11]. The results reported in Table 2 show that, although there is a significantly higher

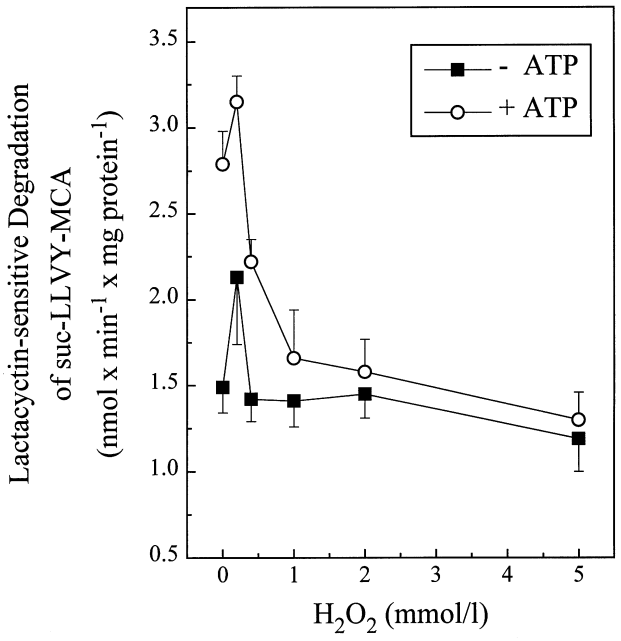

Figure 4 ATP-stimulated and ATP-independent lactacystin-sensitive proteolytic activities in K562 cell lysates after treatment of intact cells with H_2O_2

Cells were cultured, harvested and treated for 30 min with H_2O_2 as described in the Materials and methods section. Cell lysates were prepared by repeated cycles of freezing and thawing over a 1 h period. ATP levels in the cell lysates were measured to be less than 1% of initial values (insufficient to support ATP-stimulated protein degradation). The peptidase activity was determined in the presence or absence of ATP as described in Materials and methods section. The fluorescence of free MCA was measured at 380 nm and 440 nm, and was quantified using an MCA standard. The values are means ± S.E.M. for three independent experiments; S.E.M.s were always less than 10%.

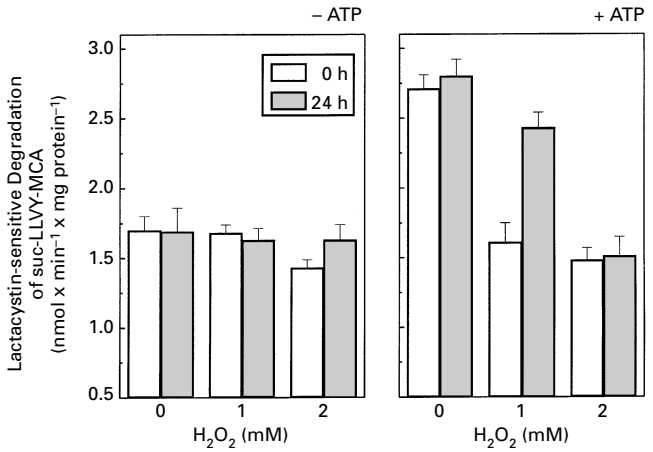

Figure 5 Recovery of 26S proteasome activity in K562 cell lysates after treatment of the cells with H_2O_2

The experimental conditions for H_2O_2 treatment and electrophoresis are described in the Materials and methods section and in the legend to Figure 4. The lactacystin-sensitive proteolytic degradation of Suc-LLVY-MCA in the absence and presence of ATP is presented. The data are means ± S.E.M. of six independent experiments.

degradation rate for oxidized compared with untreated ferritin, even exposure of K562 cells to 2 mM H_2O_2 did not result in diminished degradation of the radiolabelled protein substrates. In order to measure the proteasomal activity in the cell lysate we used the proteasome-specific inhibitor lactacystin. The data presented in Figures 4 and 5 represent the lactacystin-sensitive contribution to Suc-LLVY-MCA degradation. The ATP-independent degradation of Suc-LLVY-MCA at a concentration of 0.2 mM H_2O_2 showed moderate stimulation (Figure 4). We next tested the activity of the ATP-stimulated portion of the total proteolytic activity in K562 cell lysates after exposure of the cells to various concentrations of H_2O_2. A drastic decline in ATP-stimulated proteolytic activity against the fluoropeptide Suc-LLVY-MCA occurred after treatment of the cells with H_2O_2 (Figure 4). After exposure of the cells to 1 mM H_2O_2 it was no longer possible to detect any stimulation of proteolytic activity by ATP. As described in previous studies [11], K562 cells are able to preferentially degrade oxidized proteins at these concentrations of H_2O_2.

Long-term recovery of 26S proteasome activity in K562 cells after H_2O_2 treatment

To test whether the ATP-stimulated proteolytic activity of K562 cells was irreversibly damaged by H_2O_2 treatment, we incubated K562 cells for an additional 24 h after oxidative stress. The results presented in Figure 5 reveal almost complete restoration of 26S proteasome activity 24 h after treatment with 1 mM H_2O_2 and at least partial restoration after treatment with 2 mM H_2O_2. We found that 80% of the ATP-stimulated activity of K562 cell lysates towards the fluoropeptide was restored 24 h after treatment of the cells with 1 mM H_2O_2. The activity of the 20S proteasome did not show any significant change after treatment with 1 or 2 mM H_2O_2.

DISCUSSION

The proteasome complex exists in both an ATP-independent 20S (670–700 kDa) form and an ATP-stimulated 26S (2000 kDa) form in mammalian cells [20,21]. Our previous work [1–5,10–12], the work of Rivett [8,9] with primary hepatocytes and the work of Stadtman's group [7,22,23], provided experimental evidence that the ATP-independent 20S (670–700 kDa) 'core' proteasome complex is the form of the enzyme complex that recognizes and selectively degrades oxidatively damaged protein substrates. The ATP-independent degradation of oxidized proteins was also demonstrated by Waxman's group [14,24]. What happens to the 20S and the 26S proteasome during oxidative stress, and which of the two complexes is more resistant to oxidative damage, has not been well studied. Strack et al. [14] recently reported activation of the 20S proteasome by H_2O_2 and postulated the involvement of thiol oxidation, as well as dissociation and reassociation of the proteasome, with the PA28 regulator complex.

We felt it was important to test whether the 20S or 26S proteasome is affected by oxidation. We report that the 20S 'core' proteasome is quite resistant to H_2O_2 exposure, although the complex can be inhibited by quite low concentrations of $ONOO^-$, and even lower concentrations of OCl^-/HOCl. OCl^-/HOCl is able to fragment polypeptide backbones [25] and fragmentation may also have occurred in the case of the proteasome subunits. With H_2O_2 exposure we observed no fragmentation of polypeptides, using up to 40 μmol of H_2O_2/mg of protein, so it can be assumed that the inhibition of proteolytic activity caused by H_2O_2 was due to amino acid side-chain oxidation. Since we did not see significant loss of protein in the

20S proteasome-band (non-denaturing PAGE) at concentrations of H_2O_2 below 40 μmol/mg of protein, disintegration of the multimeric complex can also be excluded. At higher H_2O_2 concentrations, however, a loss of band staining was found in both non-denaturing and SDS/PAGE, suggesting either fragmentation of the polypeptides or a loss of Coomassie Blue staining due to modification of the amino acids, or both.

At none of the H_2O_2 concentrations studied could we find evidence for the activation of the proteolytic activity, using the isolated 20S proteasome, as reported by Strack et al. [14]. The effect of H_2O_2 on the 20S proteasome not associated with the PA28 activator is, therefore, either negligible or directed towards an inhibition of the enzyme at higher concentrations of H_2O_2. In contrast to the experiments performed by us, Strack et al. [14] used a 20S proteasome–PA28 regulator complex. Using lysates of K562 cells we did find moderate activation (Figure 4) of the ATP-independent proteolysis of Suc-LLVY-MCA, which appears to confirm the results of Strack et al. [14]. It seems that the activation by H_2O_2 found by Strack et al. is not a result of the direct action of H_2O_2 on the 20S proteasome, but rather of an effect on the PA28 activator or the activator–proteasome interaction.

In general the 26S proteasome was several times more sensitive than the 20S proteasome to the oxidants employed in this study. An especially drastic inhibition was found using $ONOO^-$, possibly suggesting effects of $ONOO^-$ on the ATP-stimulated regulator, as earlier described for other ATPase complexes. Strong inhibition of the 26S proteasome, even by low concentrations of oxidants, demonstrated the lower resistance of the 26S complex to oxidation, in comparison with the 20S proteasome which appears to be responsible for the degradation of oxidized proteins [2–4,6–12,26]. Using K562 cells exposed to oxidative stress, we observed an inhibition of the 26S proteasome at low oxidant concentrations that had no inhibitory effect on the 20S proteasome (in fact such low concentrations were slightly stimulatory). The 20S proteasome activity remained unchanged after H_2O_2 exposure of up to 2 mM. These results are in agreement with our previous data on the degradation of metabolically radiolabelled oxidized proteins in K562 cells [11]. The total proteolytic activity against oxidized proteins in K562 cell lysates remained constant up to 6 h after treatment of the cells with 1 mM H_2O_2 [11]. In the experiments presented here we found no drastic changes in the degradation of the fluorogenic peptide, Suc-LLVY-MCA, by the 20S proteasome after treatment of the K562 cells with up to 2 mM H_2O_2, although the 26S proteasome was already almost completely inhibited by this concentration of H_2O_2, as seen by the loss of the ATP-stimulated share of proteolytic activity in the cell lysates.

These data support our contention that ATP-independent proteolysis, catalysed by the 20S proteasome, is normally responsible for the degradation of oxidized proteins *in vivo*, and that the ATP-stimulated 26S proteasome does not significantly contribute to the hydrolysis of oxidized proteins during intracellular oxidative stress. Additionally, in experiments *in vitro* the inactivation of the 26S proteasome occurred at H_2O_2 concentrations significantly below those which maximally increased the proteolytic susceptibility of protein substrates. Therefore, one can conclude that, both *in vitro* and *in vivo*, the 20S proteasome is relatively resistant towards oxidants, whereas the 26S proteasome is easily inhibited by oxidation.

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