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

Thomas REINHECKEL*†, Nicole SITTE*, Oliver ULLRICH*, Ulrike KUCKELKORN†, Kelvin J. A. DAVIES|| and Tilman GRUNE*†

*Clinics of Physical Medicine and Rehabilitation, Medical Faculty (Charité), Humboldt University Berlin, Schumannstr. 20/21, D-10099, Berlin, Germany, †Institute of Biochemistry, Medical Faculty (Charité), Humboldt University Berlin, D-10098 Berlin, Germany, ||Department of Experimental Surgery, Medical Faculty, University of Magdeburg, D-39120 Magdeburg, Germany, and ||Ethel Percy Andrus Gerontology Center, The University of Southern California, Los Angeles, CA 90089-0191, U.S.A.

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 proteasome 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

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.
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, MgCl₂ 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 H₂O₂ 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 H₂O₂, peroxynitrite (ONOO⁻) and an equimolar solution of hypochlorite/hypochlorous acid (OCl⁻/HOCl). Exposure of the proteasome complexes to H₂O₂ 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 H₂O₂ 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 quantitated 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.

³H-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 [³H]formaldehyde and sodium cyanoborohydride, as described by Jentoft and Dearborn [18], and then extensively dialysed. The [³H]ferritin was either undamaged or oxidatively modified as described above. For proteolysis measurements, [³H]ferritin was added to centrifuged cell lysates and proteolysis buffer as described previously [4]. The percentage degradation was calculated after TCA-precipitation, using 5 % (w/v) bovine serum albumin as a carrier, as: (acid-soluble counts – background counts)/(total counts – background counts) × 100.

Peptide activity of the proteasome preparations was measured by mixing proteasome with 30 μl of a 2 M 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 MgCl₂ 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 precasted 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 incubat in 50 mM Tris, pH 7.8, containing 25 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 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 × 10⁶ cells/ml. Some cells were exposed to H₂O₂ 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 MgCl₂, 1 mM EDTA and 1 mM dithiothreitol.

RESULTS

Degradation of oxidized ferritin by the 20S proteasome

After exposure of ferritin to H₂O₂ a 7-fold increase in degradation by the 20S proteasome was evident (Figure 1). Whereas mild oxidative stresses (up to 10 μmol of H₂O₂/mg of ferritin) consistently increased proteolytic susceptibility, higher concentrations (20 μmol of H₂O₂/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 H₂O₂/mg of ferritin, the substrate with the highest proteolytic susceptibility.

Inhibition of the 20S proteasome activity by H₂O₂

To study the influence of H₂O₂ on the activity of the multicatalytic 20S proteasome, the enzyme was incubated for 30 min with H₂O₂. 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
peptidase activity of the 20S proteasome declined after exposure to H$_2$O$_2$, with 50% inhibition (I$_{50}$) occurring at 12.6 µmol of H$_2$O$_2$/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$_2$O$_2$. This decrease in proteasome activity (degradation of ferritin or oxidized ferritin) followed sigmoidal kinetics, with the I$_{50}$ occurring at about 40 µmol of H$_2$O$_2$/mg of proteasome (Table 1). The inhibition curves were comparable for both untreated and oxidized ferritin.

The concentration of H$_2$O$_2$ 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 vitro, 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$_2$O$_2$ 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$_2$O$_2$ concentrations. We were able to detect Suc-LLVY-MCA peptidase activity at 40 µmol of H$_2$O$_2$/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$_2$O$_2$/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$_2$O$_2$/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$_2$O$_2$ 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.

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

<table>
<thead>
<tr>
<th>Oxidant concentration giving 50% inhibition (µmol/mg of the proteasome)</th>
<th>20S Proteasome</th>
<th>26S Proteasome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-oxidized ferritin</td>
<td>Oxidized ferritin</td>
<td>Suc-LLVY-MCA</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>40.1±1.7</td>
<td>41.8±5.9</td>
</tr>
<tr>
<td>Peroxynitrite</td>
<td>0.75±0.18</td>
<td>0.78±0.03</td>
</tr>
<tr>
<td>Hypochlorite</td>
<td>0.34±0.06</td>
<td>0.41±0.02</td>
</tr>
</tbody>
</table>

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

Apart from H$_2$O$_2$, 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 proteasome 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$^-$/HOCI the I$_{50}$ was 0.4 µmol of (OCl$^-$/HOCI)/mg of proteasome (Table 1). Therefore, on a molar basis, OCl$^-$/HOCI was the most potent oxidant-inhibitor of the 20S proteasome, being more than 100-fold more effective than H$_2$O$_2$.

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$_2$O$_2$, ONOO$^-$ and OCl$^-$/HOCI (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
Table 2  Effects of exposure to H₂O₂ on the subsequent degradation of proteins in lysates of K562 cells

<table>
<thead>
<tr>
<th>Exposure of K562 cells</th>
<th>Non-oxidized ferritin</th>
<th>Oxidized ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td>No exposure</td>
<td>− ATP</td>
<td>+ ATP</td>
</tr>
<tr>
<td>30 min, PBS</td>
<td>0.95 ± 0.13</td>
<td>1.19 ± 0.22</td>
</tr>
<tr>
<td>30 min, 1 mM H₂O₂</td>
<td>1.10 ± 0.15</td>
<td>1.22 ± 0.15</td>
</tr>
<tr>
<td>30 min, 2 mM H₂O₂</td>
<td>1.18 ± 0.13</td>
<td>1.12 ± 0.12</td>
</tr>
</tbody>
</table>

K562 cells were exposed to H₂O₂ in PBS for 30 min, harvested immediately, and cell lysates prepared as described in the Materials and methods section. [³H]Ferritin was either untreated (non-oxidized) or treated with 10 μmol of H₂O₂/mg of protein and both forms were then extensively dialysed. For proteolysis measurements oxidized or untreated [³H]ferritin were added to the centrifuged cell lysates. After incubation at 37 °C for 2 h the percentage degradation of [³H]ferritin was determined as described in the Materials and methods section.

The results are means ± S.D. of three independent experiments.

20S and 26S proteasome activities in K562 cells after H₂O₂ 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 H₂O₂ [11]. The results reported in Table 2 show that, although there is a significantly higher 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 I₅₀ values for H₂O₂, OCl⁻/HOCI 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 26 S proteasome was about 4-fold more susceptible to H₂O₂, OCl⁻ and HOCI, and more than 16-fold more susceptible to ONOO⁻ than was the (ATP-independent) peptidase activity of the 20 S proteasome. The inactivation of ATP-stimulated 26 S proteasomal peptidolysis by H₂O₂ (an I₅₀ of 3.1 μmol/mg of proteasome, Table 1) occurred at a 3-fold lower H₂O₂ concentration than that required to produce a maximal increase in the susceptibility of ferritin to degradation by the 20 S proteasome (10 μmol/mg protein, see Figure 1).

Figure 3 shows the influence of H₂O₂ on the 26 S proteasome. Figure 3(A) shows an activity gel for degradation of the fluorogenic peptide Suc-LLVY-MCA, in which two distinct bands, representing the 20 S proteasome and the 26 S proteasome can be seen. The ATP-stimulated 26 S proteasome is always contaminated by the 20 S proteasome. This is probably the result of dissociation of the 26 S proteasome to yield the 20 S 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 H₂O₂ as low as 3.0 μmol/mg of protein one can see a clear decline in the activity in the band of the 26 S proteasome in non-denaturing gel electrophoresis (Figure 3A). This decline was concentration-dependent and at 48 μmol/mg of protein no activity of the 26 S proteasome remained. This decline in proteolytic activity was accompanied by a loss of the 26 S proteasome Coomassie-Blue-stainable band as shown in Figure 3(B). These results indicate that the 26 S proteasome complex is inactivated at relatively low H₂O₂ concentrations.

The results presented in Figure 2 demonstrate the fluorescence of the proteasome band after treatment with various concentrations of H₂O₂. Panel (A) shows the Coomassie Blue stained bands of the proteasome after non-denaturing electrophoresis. Panel (B) shows the 20 S proteasome after treatment with 1 mM EDTA. 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 several experiments.

The experimental conditions for H₂O₂ 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 MgCl₂ were added to the buffer. Panel (B) shows the Coomassie Blue stained proteasome bands after non-denaturing PAGE of the H₂O₂-treated enzyme complexes. The migration positions of isolated 20 S and 26 S proteasome preparations (without incubation times) are indicated. The gels shown are representative of several experiments.

Figure 2  Non-denaturing PAGE and SDS/PAGE of the 20S proteasome complex after treatment with H₂O₂

The experimental conditions for H₂O₂ treatment and electrophoresis are described in the Materials and methods section. Panels (A) and (B) represent analysis of the isolated 20 S proteasome by non-denaturing PAGE, whereas panel (C) shows the 20 S 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 MgCl₂, 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 H₂O₂. 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.
degradation rate for oxidized compared with untreated ferritin, even exposure of K562 cells to 2 mM H₂O₂ 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 H₂O₂ 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 H₂O₂. A drastic decline in ATP-stimulated proteolytic activity against the fluoropeptide Suc-LLVY-MCA occurred after treatment of the cells with H₂O₂ (Figure 4). After exposure of the cells to 1 mM H₂O₂, 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 H₂O₂.

Long-term recovery of 26S proteasome activity in K562 cells after H₂O₂ treatment

To test whether the ATP-stimulated proteolytic activity of K562 cells was irreversibly damaged by H₂O₂ 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 H₂O₂ and at least partial restoration after treatment with 2 mM H₂O₂. 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 H₂O₂. The activity of the 20S proteasome did not show any significant change after treatment with 1 or 2 mM H₂O₂.

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 H₂O₂ 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 H₂O₂ 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 H₂O₂ exposure we observed no fragmentation of polypeptides, using up to 40 μmol of H₂O₂/mg of protein, so it can be assumed that the inhibition of proteolytic activity caused by H₂O₂ 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$_2$O$_2$ below 40 $\mu$mol/mg of protein, disintegration of the multimeric complex can also be excluded. At higher H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$. 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 remains constant up to 6 h after treatment of the cells with H$_2$O$_2$. The total proteolytic activity against oxidized proteins in K562 cell lysates [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$_2$O$_2$, although the 26S proteasome was already almost completely inhibited by this concentration of H$_2$O$_2$, 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 vitro, 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$_2$O$_2$ 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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