Protection from oxidative inactivation of the 20 S proteasome by heat-shock protein 90

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Heat-shock protein 90 (Hsp 90) has been implicated in both protection against oxidative inactivation and inhibition of the multicatalytic proteinase (MCP, also known as 20 S proteasome). We report here that the protective and inhibitory effects of Hsp 90 depend on the activation state of the proteasome. Hsp 90 (and also α-crystallin) inhibits the *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity (Cbz = benzyloxycarbonyl; MCA = 7-amido-4-methylcoumarin) when the rat liver MCP is in its latent form, but no inhibitory effects are observed when the MCP is in its active form. Metal-catalysed oxidation of the active MCP inactivates the Ala-Ala-Phe-MCA-hydrolysing (chymotrypsin-like), *N*-Boc-Leu-Ser-Thr-Arg-MCA-hydrolysing (trypsin-like; Boc = t-butyloxycarbonyl), *N*-Cbz-Leu-Leu-Glu-β-naphthylamine-hydrolysing (peptidylglutamyl-peptide hydrolase) and *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activities, whereas these activities are actually increased when the MCP is in its latent form. Hsp 90 protects against oxidative inactivation of the trypsin-like and *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activities of the MCP active form, and α-crystallin protects the trypsin-like activity. The specificity of the Hsp 90-mediated protection was assessed by a quantitative analysis of the two-dimensional electrophoretic pattern of MCP subunits before and after oxidation of the MCP, in the presence or absence of Hsp 90. Treatment of the FAO hepatoma cell line with iron and ascorbate was found to inactivate the MCP. Hsp 90 overexpression obtained by challenging the cells with iron was associated with a decreased susceptibility to oxidative inactivation of the MCP trypsin-like activity. Depletion of Hsp 90 by using antisense oligonucleotides resulted in an increased susceptibility to oxidative inactivation of the MCP trypsin-like activity, providing evidence for the physiological relevance of Hsp 90-mediated protection of the MCP.

INTRODUCTION

Oxidative damage to cellular components, including protein, has been implicated in age- and disease-related impairment of cellular functions. In certain tissues the level of oxidized protein increases with age and/or in response to oxidative stress [1–4]. The multicatalytic proteinase (MCP) or 20 S proteasome is believed to have a key role in the degradation of damaged proteins and consequently to be important in the regulation of the steady-state level of altered proteins in the cell [5,6]. Under oxidative stress, an increase in intracellular proteolysis of oxidized protein, attributed to the MCP, is observed (reviewed in [7]). However, no significant increase in the level of proteasome subunits has been found [8,9]. This might indicate that an induced increase in proteolysis on oxidative stress is mainly the result of an increased proteolytic susceptibility of the oxidized protein. In addition, an activation of the MCP remains a possibility. Because MCP is abundant and is important in ubiquitin-dependent protein degradation [10–12], antigen presentation [13,14] and cell cycle regulation [15,16], its hydrolytic properties should be regulated. Different effectors of the proteasome have been described, e.g. proteasome activators PA 28 and PA 700 and proteasome inhibitors PI 200 and PI 240 [17–22]. As Tsubuki et al. [23] first demonstrated, heat-shock protein 90 (Hsp 90) is an inhibitor of the *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity (Cbz = benzyloxycarbonyl; MCA = 7-amido-4-methylcoumarin) of bovine brain MCP. Wagner and Margolis [24,25] have also observed that α-crystallin and Hsp 90 are co-purified with the MCP from bovine lens; they have also shown that α-crystallin inhibits the *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity and provides limited protection of MCP activity from heat inactivation. MCP, isolated in a latent form, can be activated by various treatments, such as incubation with polylysine or fatty acids, binding to PA 28 activator, heating, freezing, storage in the absence of glycerol, addition of low concentrations of SDS or dialysis against water [17,26–29]. The manner by which the different treatments activate the MCP is still unclear but suggests that the enzyme might also be subject to regulation *in vivo*. We previously reported that Hsp 90 can protect the *N*-Boc-Leu-Ser-Thr-Arg-MCA-hydrolysing (also referred to as trypsin-like; Boc = t-butyloxycarbonyl) activity of the MCP from oxidative inactivation [30]. To determine whether the activation state of the MCP is affecting the way in which Hsp 90 and α-crystallin inhibit the MCP and protect it from oxidant-induced inactivations, we have analysed the effects of these proteins on four peptidase activities of the latent and active forms of purified rat liver MCP before and after metal-catalysed oxidation. To probe the specificity of the interaction of Hsp 90 with the MCP, quantitative analysis of the two-dimensional electrophoretic pattern of MCP subunits, before and after oxidative treatment,

Abbreviations used: Bis-ANSA, bis-8-anilino-1-naphthalenesulphonic acid; Boc, t-butyloxycarbonyl; Cbz, benzyloxycarbonyl; GS, glutamine synthetase; Hsp 90, heat-shock protein 90; MCA, 7-amido-4-methylcoumarin; MCP, multicatalytic proteinase; PA, proteasome activator.

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was performed in the presence and in the absence of Hsp 90. Finally, to address the physiological relevance of the Hsp 90-mediated protection of the proteasome, the peptidase activities of the MCP were measured in FAO cells subjected to oxidative stress before and after induction or depletion of Hsp 90 in the cells.

EXPERIMENTAL

Reagents and chemicals

Ala-Ala-Phe-MCA, *N*-Boc-Leu-Ser-Thr-Arg-MCA, *N*-Cbz-Leu-Leu-Glu-β-naphthylamine, MCA, β-naphthylamine and α-crystallin were purchased from Sigma Chemical Co. Bis-8-anilino-1-naphthalenesulphonic acid (Bis-ANSA) was obtained from Molecular Probes. *N*-Cbz-Leu-Leu-Leu-MCA was synthesized as described [31,32].

Purification of the MCP and Hsp 90

Male Fischer 344 rats (8 months old) were obtained from Iffa Credo (L'Arbresle, France). The rats were killed, their abdominal cavities were opened and their livers were excised and frozen immediately in liquid nitrogen and stored at -70 °C. The livers were thawed at 4 °C and homogenized in 20 mM Hepes/0.1 mM EDTA/1 mM 2-mercaptoethanol (pH 7.8) (4 ml/g of liver) with a Potter–Elvehjem glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 25000 ***g*** for 2 h at 4 °C. The MCP was purified as described previously [33]. The Hsp 90 was purified by the same procedure. In brief, the liver homogenate supernatant was subjected to an $(NH_4)_2SO_4$ cut (between 35% and 60% saturation). The first ion-exchange chromatography was performed on a TosoHaas DEAE-5PW column, with a Beckman Gold liquid chromatograph. Purification was achieved in two additional chromatographic steps: ion-exchange chromatography on a Mono-Q HR 5/5 column (Pharmacia) and gel-filtration chromatography on a Superose 6 column (Pharmacia). Purified MCP was dialysed against 0.1 M Hepes, pH 7.8, to obtain the latent form of the enzyme, and against water to obtain the active form.

Protein and peptidase assays

Protein concentrations were determined by the bicinchoninic acid assay (Pierce). Assays of the chymotrypsin-like, trypsin-like, peptidylglutamyl-peptide hydrolase and *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activities were performed with fluorogenic peptides Ala-Ala-Phe-MCA, *N*-Boc-Leu-Ser-Thr-Arg-MCA, *N*-Cbz-Leu-Leu-Glu-β-naphthylamine and *N*-Cbz-Leu-Leu-Leu-MCA respectively. The incubation mixture contained 5 μg of MCP in 0.1 M Hepes, pH 7.8, with the appropriate peptide substrate concentration in a final volume of 200 μl. Incubation was at 37 °C for 20 min, at which point the reaction was stopped with 300 μl of acid or ethanol. After the addition of 2 ml of distilled water, the fluorescence was monitored on a Perkin–Elmer LS-5B spectrofluorimeter. The excitation and emission wavelengths were 350/440 nm and 333/410 nm for amino-methylcoumarin and β-naphthylamine products respectively.

Escherichia coli glutamine synthetase (GS) (a gift from Dr. R. L. Levine, NHLBI, NIH, Bethesda, MD, U.S.A.) was prepared and oxidized as described [34]. The number of amino groups on short peptides generated by proteolysis of oxidized GS by the MCP was monitored with the fluorescamine assay as described previously [33]; a calibration curve was performed with glycine as standard amino acid carrying one amino group.

Thermal inactivation

Before thermal inactivation, the MCP was diluted in 0.1 M Hepes, pH 7.8, giving a final concentration of 0.1 mg/ml and incubated at 55 °C. At the indicated times, 50 μl aliquots were added to 75 μl of 0.1 M Hepes, pH 7.8, on ice to terminate the thermal inactivation. Residual peptidase activities were determined at 37 °C after addition of the substrates as described above.

Bis-ANSA binding

Latent or active MCP (12 μg) in 0.1 M Hepes, pH 7.8, was added to 1 ml of 10 μM Bis-ANSA. The fluorescence emission spectra between 400 and 600 nm (excitation wavelength 370 nm) of the mixtures were monitored with a Perkin–Elmer LS-5B spectrofluorimeter.

Metal-catalysed oxidation of the MCP

Before metal-catalysed oxidation, the MCP was dialysed against 0.1 M Hepes, pH 7.8, or water. To initiate the oxidation reaction, the MCP was diluted to 0.1 mg/ml in 0.1 M Hepes, pH 7.8, in the presence of 0.1 mM $FeCl_3$ and 25 mM ascorbate at 37 °C. At indicated times, 50 μl aliquots were diluted in 200 μl of 0.1 M Hepes, pH 7.8, containing the fluorogenic peptide substrate; residual peptidase activities were determined at 37 °C.

Gel electrophoresis and Western blots

SDS/PAGE was performed by the Laemmli method [35] on a 12% (w/v) separating gel followed by Coomassie staining or Western blotting. The molecular mass standards used were Rainbow Markers (14.3–200 kDa) from Amersham. Western blot experiments were performed with 12 μg of total cellular proteins and performed as described previously [30]. For two-dimensional electrophoresis, the first dimension was done with Immobilines Drystrips (pH gradient 4–7; length 18 cm) with the Multiphor II system (Pharmacia) as follows: 24 μg aliquots of purified proteasome were diluted 1:5 in sample buffer [9 M urea/4% (v/v) Nonidet P40/2% pharmalytes (pH 3–10)/65 mM dithiothreitol/Bromophenol Blue (0.02%, w/v)] loaded on the Drystrip rehydrated in rehydration buffer [8 M urea/2% (v/v) Nonidet P40/0.5% pharmalytes (pH 3–10)/65 mM dithiothreitol/Bromophenol Blue (0.02%, w/v)] and then focused for 50000 V·h. After focusing, the Immobilines Drystrips were equilibrated for 10 min in equilibration buffer [50 mM Tris/HCl (pH 6.8)/6 M urea/30% (v/v) glycerol/2% (w/v) SDS] supplemented with 1% (w/v) dithiothreitol, and for 10 min in equilibration buffer containing 2.5% iodoacetamide. The second dimension was performed with SDS/PAGE [11.5% (w/v) gel] with the Protean II two-dimensional multi-cell system (Bio-Rad). Proteins were stained with silver nitrate [36] and the gel was digitized with a JX-330 scanner (Sharp). Computerized two-dimensional analysis was performed on a SPARC station 5 microcomputer (Sun Microsystems). After spot detection and quantification, two-dimensional gel patterns were edited and matched with the PDQUEST software package (PDI, Huntington Station, NY, U.S.A.).

Cell culture, exposure to metal-catalysed oxidation and treatment with antisense oligonucleotides

The FAO rat hepatoma cell line was provided by Dr. C. Deschatrette (Institut Pasteur, Paris, France). The cells were

Table 1 Proteolytic activities of the latent and active forms of MCP

Proteolytic activities of purified latent and active forms of MCP were assayed as described in the Experimental section. Specific activities (nmol/min per mg) were determined with 20 μM Ala-Ala-Phe-MCA (AAF-MCA), 20 μM *N*-Boc-Leu-Ser-Thr-Arg-MCA (LSTR-MCA), 100 μM *N*-Cbz-Leu-Leu-Glu-β-naphthylamine (LLE-NA), 20 μM *N*-Cbz-Leu-Leu-Leu-MCA (LLL-MCA) or 16 μM oxidized GS. The values reported are averages of those obtained in three separate experiments and the results were reproducible within $\pm$20%.

	Specific activity (nmol/min per mg)	
Proteolytic activities and substrates	Latent form	Active form
Chymotrypsin-like (AAF-MCA)	3.9	4.3
Trypsin-like (LSTR-MCA)	1.0	2.6
Peptidylglutamyl-peptide hydrolase (LLE-NA)	12.6	31.8
Leu-Leu-Leu-MCA-hydrolysing (LLL-MCA)	0.6	1.7
Oxidized protein degradation (oxidized GS)	3.0	21.7

plated at a density of 5×10^4 cells/cm² and grown for 2 days at 37 °C in COON's modified medium (Polylabo) supplemented with 5% (v/v) fetal calf serum, 1% (w/v) glutamine and 1% (w/v) antibiotics (penicillin/streptomycin mix). Subconfluent cells were then exposed to metal-catalysed oxidation by the addition of 25 mM ascorbate and 0.1 mM $FeCl_3$ in 0.5 mM ADP to the medium. After various durations of treatment, cells were harvested and disrupted by five 5 s sonications in 0.25 M Tris/HCl, pH 7.8. After centrifugation at 20000 ***g*** for 20 min, the supernatant containing total soluble cellular proteins was collected. Peptidase activities were measured with 50 μg of total proteins in 0.1 M Hepes, pH 7.8, and the appropriate peptide substrate concentration in a final volume of 200 μl. MCP proteolytic activity was determined as the difference between total activity and the remaining activity of the crude extract in the presence of the proteasome inhibitor MG132 (*N*-Cbz-Leu-Leu-leucinal).

To induce Hsp 90 synthesis before exposure to metal-catalysed oxidation, cells were first subjected to pretreatment overnight with 0.1 mM $FeCl_3$. Depletion of Hsp 90 in the cells was achieved by antisense experiments. Cells were plated at a density of 5×10^4 cells/cm² and treated daily for 5 days with 10 μM Hsp 90 sense (5′-TCAAGATGCCTGAGGAAGTG-3′) or antisense (5′-CACTTCCTCAGGCATCTTGA-3′) 5′-aminodeoxynucleotides and 10 μg/ml of Lipofectine® (Gibco BRL) in serum-free medium. Every day, 5 h after treatment, serum was added to a final concentration of 5%. Next, the cells were either exposed to metal-catalysed oxidation or used as a control; 5 h after exposure the cells were collected and the peptidase activities were measured.

RESULTS

Peptidase activities and structural properties of the latent and active forms of MCP

The latent form of MCP was purified from the livers of 8-month-old Fisher 344 rats. The active form of MCP was prepared by dialysis against water [29]. Because the rates for peptide hydrolysis were different in the latent and active forms of MCP, four peptidase activities (chymotrypsin-like, trypsin-like, peptidylglutamyl-peptide hydrolase and *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing) were assayed for each form with fluorogenic peptides. The active form showed respectively 150%, 160% and 180% increases in specific activity over that of the latent form for the following three activities: peptidylglutamyl-peptide hydrolase, trypsin-like and *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity (Table 1). In addition, the active form of MCP showed a 7-fold increase in its ability to degrade oxidized GS compared with that of latent form MCP.

Activation of the MCP has been associated with structural modifications of some MCP subunits [29,37,38]. Therefore we compared the pattern of subunits for the two forms by analytical SDS/PAGE; no significant change was detected between the latent and active forms of MCP (results not shown). However, thermal treatment of the active form of MCP at 55 °C resulted in a biphasic inactivation process for the four peptidase activities, whereas for the latent form activities decreased in a monophasic manner. As an example, Figure 1(A) shows the thermal in-

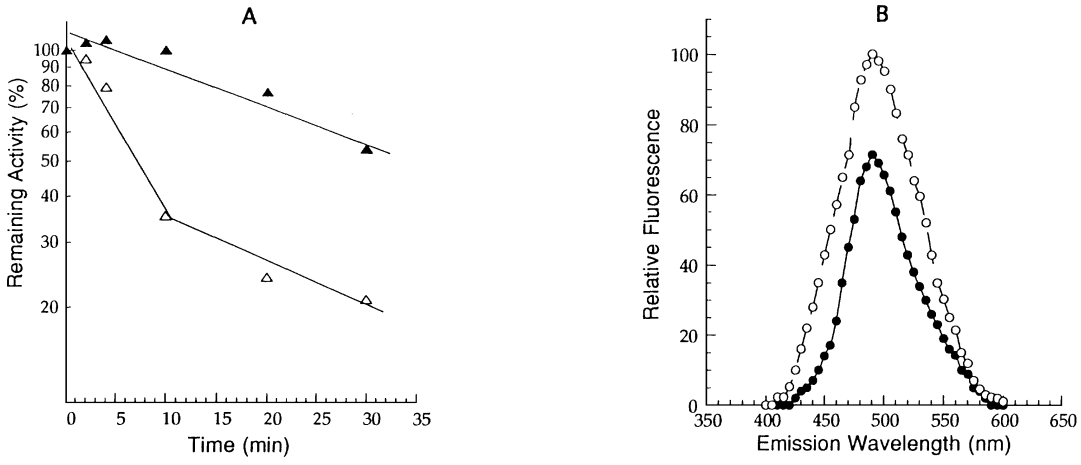

Figure 1 Thermal inactivation of the latent and active forms of MCP and binding to Bis-ANSA

(**A**) MCP at 0.1 mg/ml was incubated in 0.1 M Hepes, pH 7.8, at 55 °C. At indicated times the *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity was assayed as described in the Experimental section; 100% activity was defined as the peptidase activity at zero time of the thermal inactivation. Symbols: ▲, latent form of MCP; △, active form of MCP. (**B**) Fluorescence emission spectra from 400 to 600 nm (excitation wavelength 370 nm) of Bis-ANSA (10 μM) in the presence of the latent (●) and active (○) form of MCP (12 μg/ml). In each panel a typical experiment taken from three independent experiments is shown.

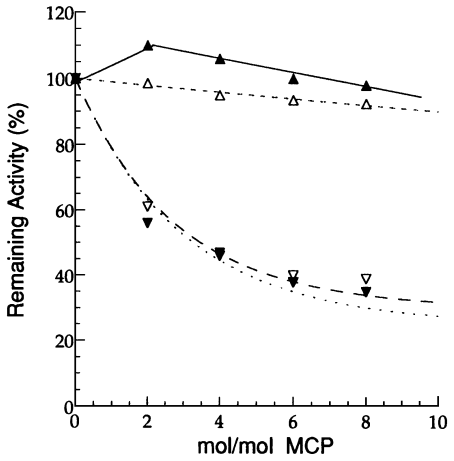

Figure 2 Effects of Hsp 90 and α-crystallin on hydrolysis of Cbz-Leu-Leu-Leu-MCA by MCP

Hydrolysis of Cbz-Leu-Leu-Leu-MCA by the latent and active forms of MCP (5 μg) was measured, as described in the Experimental section, in the presence of Hsp 90 and α-crystallin. The Figure shows the average of two separate experiments. Latent form MCP in the presence of Hsp 90 (▽) and α-crystallin (▼), active form MCP in the presence of Hsp 90 (△) and α-crystallin (▲).The activity of the MCP in the absence of the proteins was taken as 100%.

activation of the *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity for the latent and active forms of MCP. To detect whether any conformational change might be associated with the transition from the latent to the active form of MCP, we studied the hydrophobic regions accessible to solvents by monitoring the binding of the hydrophobic probe Bis-ANSA. When bound to protein, Bis-ANSA exhibits an increase in fluorescence [39,40]. Experiments with Bis-ANSA showed that the active form exhibited greater Bis-ANSA binding than the latent form (Figure 1B).

Effect of Hsp 90 and α-crystallin on the Leu-Leu-Leu-MCA-hydrolysing activity

The presence of Hsp 90 and α-crystallin on analytical SDS/PAGE gels of purified MCP has been noted in several laboratories. Furthermore, it has been shown that Hsp 90 and α-crystallin are specific endogenous inhibitors of the *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity of MCP [23,25]. These results suggest that Hsp 90 and α-crystallin are MCP regulators that can modulate its activity *in vivo*. We tested, *in vitro*, the ability of these two proteins to inhibit the *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity of latent and active MCP. As shown in Figure 2, only the latent form of MCP was inhibited by Hsp 90 and α-crystallin, but no inhibitory effect was observed for the active form.

Effect of Hsp 90 and α-crystallin on the latent and active forms of MCP during metal-catalysed oxidation

We have previously reported that Hsp 90 protects the MCP against inactivation by metal-catalysed oxidation [30]. To test whether this inactivation and protection depend on MCP conformation, the active and latent forms of MCP were incubated with an iron/ascorbate system, and various peptidase activities were determined as a function of incubation time (see the Experimental section). Metal-catalysed oxidation of the active form of MCP resulted in a decrease in the peptidase activities (Figure 3B). In contrast, an increase in these activities was observed for the latent form (Figure 3A). This increase, however, was not associated with an increase in the ability of the latent form to degrade oxidized GS (results not shown). Figures 4(A) and 4(B) show that Hsp 90 protected the trypsin-like and the *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activities of the active form of MCP against oxidative inactivation, whereas α-crystallin

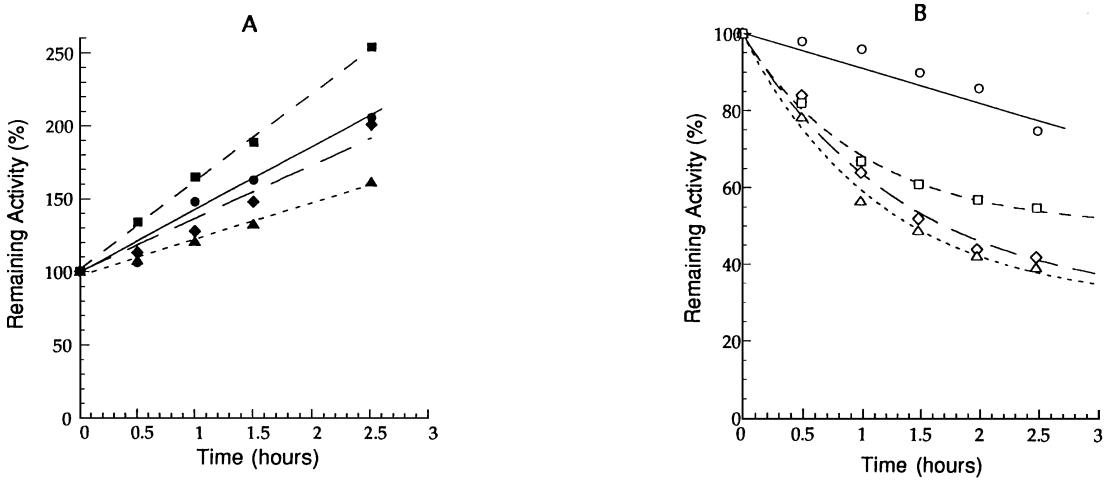

Figure 3 Peptidase activities after metal-catalysed oxidation of the latent and active forms of MCP

The latent and active forms of MCP at 0.1 mg/ml in 0.1 M Hepes, pH 7.8, were incubated with 0.1 mM $FeCl_3$/25 mM ascorbate at 37 °C, and at the indicated times the peptidase activities were assayed as described in the Experimental section; 100% activity was defined for each peptidase activity as the peptidase activity at zero time of oxidation. (**A**) Activities of the latent form of MCP: ●, chymotrypsin-like activity; ■, trypsin-like activity; ◆, peptidylglutamyl-peptide hydrolase activity; ▲, *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity. (**B**) Activities of the active form of MCP: ○, chymotrypsin-like activity; □, trypsin-like activity; ◇, peptidylglutamyl-peptide hydrolase activity; Δ, *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity. In each panel a typical experiment taken from three independent experiments is shown.

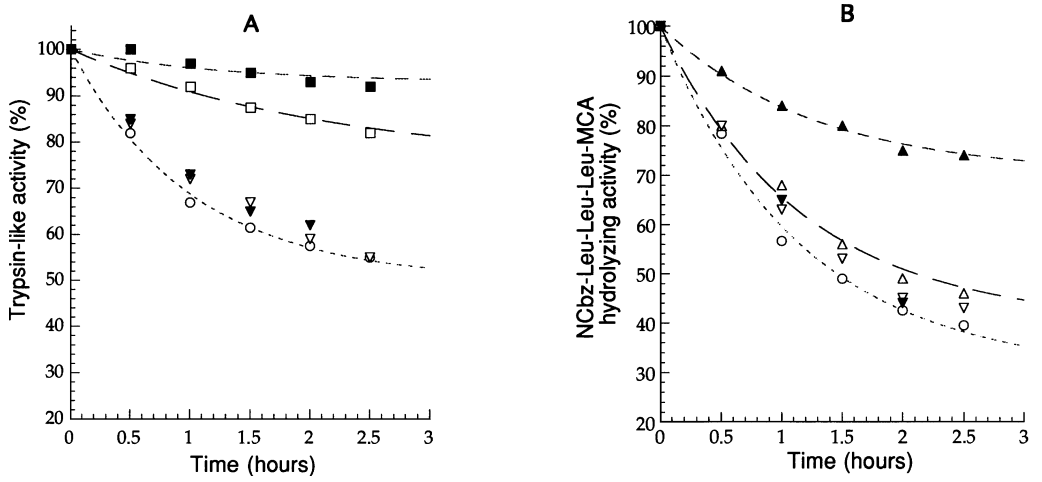

Figure 4 Peptidase activities after metal-catalysed oxidation of the active form of MCP in the absence and in the presence of Hsp 90 or α-crystallin

The active form of MCP at 0.1 mg/ml in 0.1 M Hepes, pH 7.8, was incubated with 0.1 mM $FeCl_3$/25 mM ascorbate at 37 °C in the absence and in the presence of a 4-fold molar excess of Hsp 90 (0.08 mg/ml) and α-crystallin (0.45 mg/ml). At the indicated times the trypsin-like and *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activities were assayed as described in the Experimental section; 100% activity was defined for each peptidase activity as the activity of the MCP at zero time of oxidation. (**A**) Trypsin-like activity of active MCP alone (○) and in the presence of Hsp 90 (■) or α-crystallin (□); (**B**) *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity of active MCP alone (○) and in the presence of Hsp 90 (▲) or α-crystallin (△). Thyroglobulin (▽) and glucose-6-phosphate dehydrogenase (▼) were used as control proteins. In each panel a typical experiment taken from three independent experiments is shown.

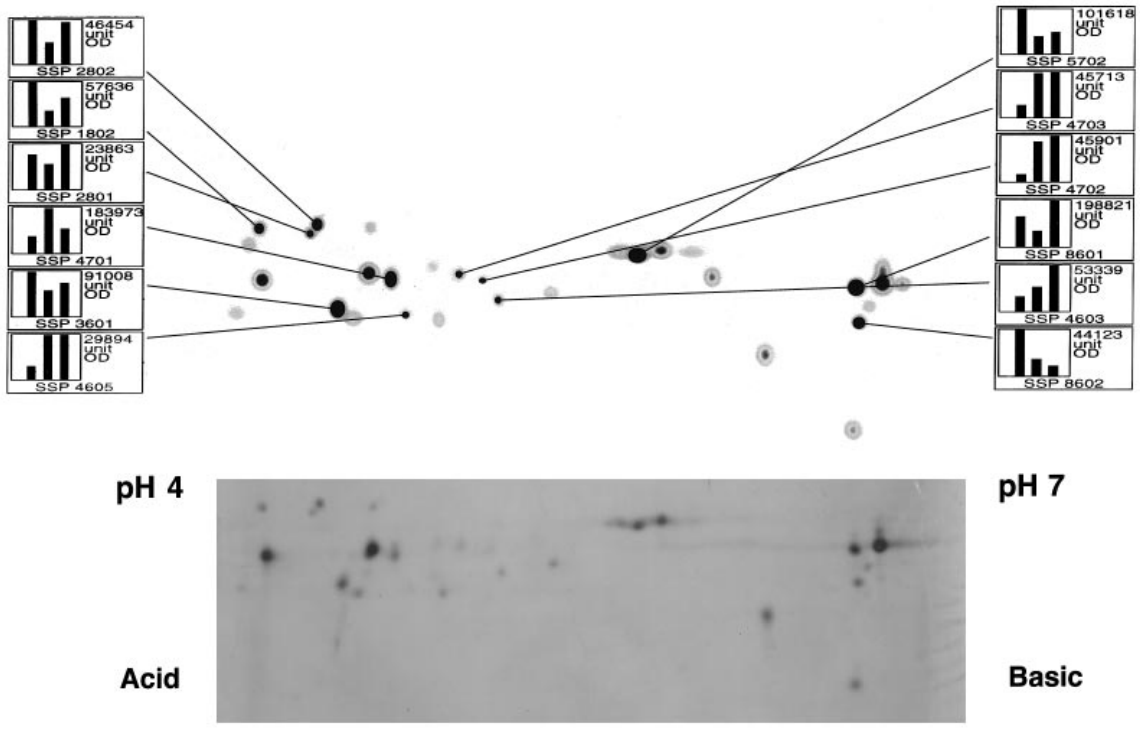

Figure 5 Two-dimensional gel electrophoresis pattern of proteasome subunits

Two different samples of the active form of MCP before and after treatment with iron and ascorbate, in the absence and in the presence of a 4-fold molar excess of Hsp 90, were subjected to two-dimensional electrophoresis and the gels were stained with silver and analysed as described in the Experimental section. The pattern of protein spots obtained after two-dimensional electrophoresis is shown on a typical picture of the stained gel (bottom panel) and its computerized counterpart (top panel). To select unambiguous modifications in the pattern of proteasome subunits, the level of significance was set as a 2-fold variation in the protein spot intensity. After metal-catalysed oxidation, a decrease in intensity of seven protein spots and an increase in intensity of five spots was found: see insets, bars at the left (untreated) and the middle (treated). Among these protein spots, five of them (nos. SSP 2802, SSP 1802, SSP 2801, SSP 4701 and SSP 8601) were no longer affected when iron and ascorbate treatment was performed in the presence of Hsp 90: see the corresponding insets, bars at the left (untreated), the middle (treated, minus Hsp 90) and the right (treated, plus Hsp 90).

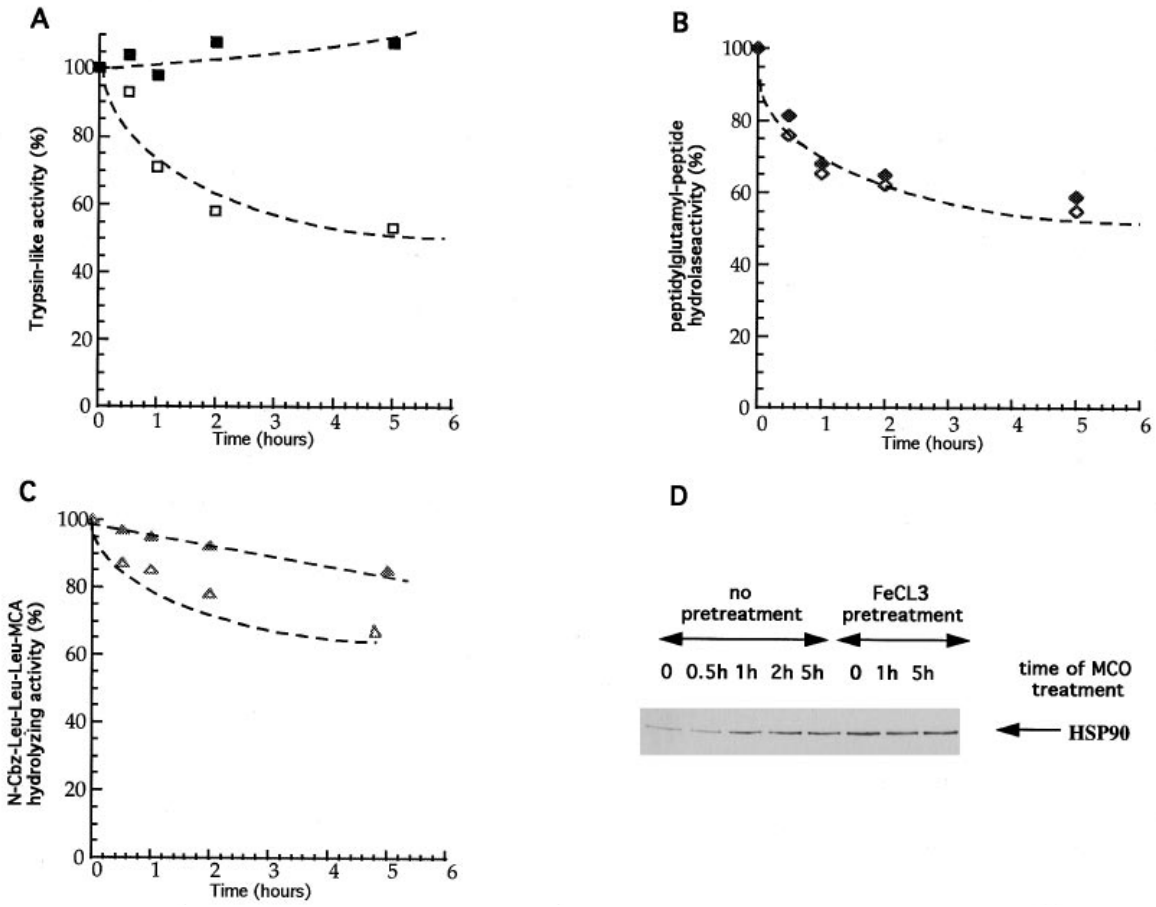

Figure 6 Peptidase activities of the MCP after metal-catalysed oxidation in FAO hepatoma cells pretreated or not by $FeCl_3$

FAO hepatoma cells were either pretreated or not pretreated with 0.1 mM $FeCl_3$ for 16 h and 0.1 mM $FeCl_3$/25 mM ascorbate was then added to the cells and, at the indicated times, crude homogenates were prepared and peptidase activities of the MCP were assayed as described in the Experimental section; 100% was defined for each peptidase activity as the value obtained at zero time of treatment. Results in (**A**–**C**) are averages of two separate experiments. (**A**) Trypsin-like activity; (**B**) peptidylglutamyl-peptide hydrolase activity; (**C**) *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity. Open symbols are used for naive cells and filled symbols for $FeCl_3$-preincubated cells. (**D**) Western blot revealed with anti-(Hsp 90) polyclonal antibody, showing the Hsp 90 content in homogenates after different durations of iron/ascorbate (MCO, metal-catalysed oxidation) treatment of naive cells (0, 0.5, 1, 2 and 5 h) and of cells that had previously been exposed to $FeCl_3$ (0, 1 and 5 h).

protected only the trypsin-like activity. No protection was observed in the controls with thyroglobulin (600 kDa) and glucose-6-phosphate dehydrogenase (110 kDa) (see Figure 4).

Structural changes of the MCP subunits on treatment with iron/ascorbate in the absence and in the presence of Hsp 90 analysed by two-dimensional electrophoresis

The ability of Hsp 90 to protect the active form of MCP from oxidative inactivation was monitored at a structural level by two-dimensional electrophoresis (see the Experimental section). To optimize the resolution and the analysis of the protein spots from the different preparations, the first dimension was performed on 18 cm Immobilines (pH 4–7) with the Multiphor II system. A 2-fold change in the intensity of a protein spot was interpreted as a significant modification in the pattern of MCP subunits. The active form of MCP was exposed to metal-catalysed oxidation with an iron/ascorbate system for 2 h, as described in the Experimental section, in the absence and in the presence of a 4-fold molar excess of Hsp 90. As shown in Figure 5, 26 spots corresponding to proteasome subunits were detected on two-dimensional gels with all MCP preparations. Treatment of MCP with iron and ascorbate resulted in a significant variation in intensity of 12 protein spots, reflecting the occurrence of oxidative modifications to MCP subunits. In the presence of Hsp 90, out of the 12 spots whose intensity varied on oxidation, 7 were affected in the same way, whereas the other 5 were no longer affected. These results show that the MCP subunits related to these five spots are protected by Hsp 90 from modification by metal-catalysed oxidation.

Peptidase activities of the MCP after metal-catalysed oxidation of FAO cells: effect of Hsp 90 induction in the cells

To investigate further the implication of Hsp 90 in the protection of the 20 S proteasome from oxidative inactivation *ex vivo*, the peptidase activities of the MCP taken from FAO rat hepatoma cells were monitored after various treatments of the cells. Cells were grown at 37 °C in serum-supplemented medium for 2 days and then were subjected to metal-catalysed oxidation by the addition of 0.1 mM $FeCl_3$ and 25 mM ascorbate to the medium. After various durations of treatment, cells were harvested, protein homogenates were prepared and three peptidase activities of the MCP were assayed. The results, shown in Figures 6(A–C),

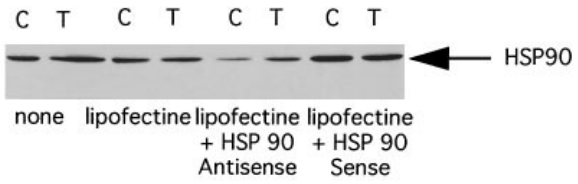

Figure 7 Hsp 90 content in cell homogenates monitored by Western blotting

Total proteins (12 μg) from cells treated (T) or not (C) with Lipofectine, Lipofectine plus sense oligonucleotide, or Lipofectine plus antisense oligonucleotide were subjected to SDS/PAGE, electrotransfer and Western blotting as described in the Experimental section. The Western blot was revealed with anti-(Hsp 90) polyclonal antibody and shows a decreased content of Hsp 90 in antisense-treated cells compared with the different control cells (sense-treated, Lipofectine-treated and untreated cells).

Table 2 Trypsin-like activity of the MCP after metal-catalysed oxidation in FAO hepatoma cells treated or not with Hsp 90 antisense oligonucleotide

Cells, subjected or not for 5 days to a daily addition of Lipofectine, Lipofectine plus sense oligonucleotide, or Lipofectine plus anti-sense oligonucleotide, were then exposed to metal-catalysed oxidation and the MCP trypsin-like activity was determined as indicated in the Experimental section; 100% activity was defined in each case as the MCP trypsin-like activity of cells not subjected to metal-catalysed oxidation. Values are means $\pm$ S.E.M. for three independent experiments.

Hsp 90 oligonucleotide	Trypsin-like activity in iron/ascorbate-treated cells (% of unoxidized controls)
None	86.6 $\pm$ 7.5
Lipofectine	88.5 $\pm$ 8.8
Lipofectine + Hsp 90 anti-sense	50.6 $\pm$ 7.2
Lipofectine + Hsp 90 sense	86.2 $\pm$ 8.5

indicate that all the activities tested were affected by metal-catalysed oxidation within 30 min. After 5 h of treatment, trypsin-like, peptidylglutamyl-peptide hydrolase and *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activities of MCP in the homogenates declined in each case by 60–50% compared with the control. This finding suggests that in FAO cells, MCP is present in an active form, owing to its sensitivity to oxidative inactivation, as opposed to the latent form. In addition, as observed for the purified active form of MCP, thermal inactivation of the MCP in cell homogenates was found to exhibit a biphasic profile (results not shown).

Because iron overload has been described to induce the synthesis of Hsp 90 in rat renal tubular cells [41], the FAO rat hepatoma cells were first challenged with 0.1 mM $FeCl_3$ to induce Hsp 90 overexpression, then treated with iron and ascorbate. The enhanced expression of Hsp 90 was monitored by Western blotting with anti-(Hsp 90) polyclonal antibody (Figure 6D). In these cells, both the trypsin-like and the *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activities were protected from oxidative inactivation compared with the control cells (Figures 6A and 6C). In contrast, the peptidylglutamyl-peptide hydrolase activity remained sensitive to oxidative inactivation in Hsp 90-over-expressing cells, as in the control cells (Figure 6B).

Effect of Hsp 90 depletion in FAO cells on the susceptibility of the MCP trypsin-like activity to oxidative inactivation

Decreased expression of Hsp 90 in cells was achieved by using antisense oligodeoxynucleotides directed against the initiation codon region of the protein. Cells were grown for 5 days and treated daily with Hsp 90 sense or antisense aminodeoxy-nucleotides and Lipofectine in serum-free medium for 5 h, after which serum was added to a final concentration of 5% (v/v). The Western blot analysis of the cell homogenates (Figure 7) shows a decrease in Hsp 90 content in antisense-treated cells. In all control cells (sense-treated, Lipofectine-treated or untreated), iron and ascorbate treatment had a slight inhibitory effect on MCP trypsin-like activity (Table 2). Probably because of different growth conditions, these cells were more resistant to oxidation than the cells grown for 2 days described above. In contrast, in Hsp 90-depleted cells, this activity was strongly inhibited (Table 2). The peptidylglutamyl-peptide hydrolase activity was also assayed but no increased susceptibility to oxidative inactivation was observed for Hsp 90-depleted cells (results not shown).

DISCUSSION

Proteins are inherently susceptible to oxidative damage, which can alter their biological function and susceptibility to proteolysis. Oxidative damage to protein is implicated in a number of physiological and pathological processes and is known to affect intracellular protein turnover [7,42]. For these reasons the study of the intracellular proteolytic systems subjected to oxidative processes is a significant issue. The MCP is the major system involved in the degradation of oxidized proteins [5–9]. We have previously shown that Hsp 90 protects MCP against inactivation by metal-catalysed oxidation [30], and both Hsp 90 and α-crystallin have been described as specific inhibitors of the the *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activity of MCP [23,25]. In the present study we have found that this activity is affected only when the MCP is in its latent form but that no inhibitory effect of either protein is observed when the MCP is in its active form. The results also show that after metal-catalysed oxidation the MCP is either activated or inactivated, depending on the form of the MCP. Hsp 90 and α-crystallin protect some peptidase activities (trypsin-like and *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing) of the active form of MCP against oxidative inactivation.

Activation of the latent form of MCP has been associated with different treatments that probably induce conformational changes and/or mimic MCP activators. This suggests that MCP activity could also be regulated *in vivo*. After dialysis against water, the MCP was converted into an active form, evidenced by an increase in its peptidase activities. As a result of its activation, the ability of the MCP to degrade oxidized protein was also enhanced. We have found that latent MCP can be distinguished from water-dialysed active MCP on the basis of its thermostability and ability to bind the hydrophobic fluorescent probe Bis-ANSA. Indeed, active MCP is less thermostable and binds more Bis-ANSA than latent MCP. In addition, latent MCP was completely protected by α-crystallin from thermal inactivation at 55 °C, whereas active MCP is only partly protected; Hsp 90 had no protective effect for either form (results not shown). These results favour the possibility that activation of the MCP involves a conformational change of its subunits. The change in the structure might imply an opening of the α-subunits ring that facilitates substrate entry or a modification of the β-subunits that enhance their proteolytic activity.

When the latent form of MCP is subjected to metal-catalysed oxidation, an increase in the peptidase activities is observed. This observation is supported by the recent finding of Strack et al.

[43], who have shown that $FeSO_4$/EDTA/ascorbate treatment activates peptidase and casein-hydrolysing activities in the MCP. We did not observe an increased degradation of oxidized GS. This result might be explained by differences in the protein substrate and/or oxidative treatments. When the active form of MCP is subjected to metal-catalysed oxidation, the peptidase activities decrease. In this case, Hsp 90 and α-crystallin act as protectors of two peptidase activities (trypsin-like or *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activities) against oxidative inactivation.

To assess the specificity of the protection of MCP by Hsp 90, the subunit patterns of MCP were analysed by two-dimensional electrophoresis after metal-catalysed oxidation of the active form of MCP in the absence and in the presence of Hsp 90. As shown in Figure 5, the intensities of 12 protein spots are significantly decreased or increased when MCP is subjected to metal-catalysed oxidation but only five of them are no longer modified when Hsp 90 has been incubated with MCP. This finding indicates that MCP subunits corresponding to these five protein spots are protected by Hsp 90 as a result of its direct binding or induced conformational changes. On the basis of previous assignments of the protein spots to MCP subunits [44,45] it is tempting to speculate on the specific subunits that might be protected by Hsp 90. Indeed, among the protein spots that are protected by Hsp 90, spot no. 8601 might be related to subunit C9. This α-type subunit is carrying a KEKE motif that has been suggested by Realini and co-workers [46,47] to promote the association between MCP subunits and specific protein ligands such as regulators and activators. Hsp 90 does exhibit a KEKE motif; α-crystallin, which is a multimer, also carries a short related sequence (REEK) located at the C-terminal end of its polypeptide chain. In the subunit arrangement in mammalian proteasome [48], subunit C9 interacts directly with subunits MB1 and Z, two β-type subunits that have been proposed to carry the trypsin-like activity of the MCP [49,50]. Therefore the protection of the trypsin-like activity of the MCP could be explained by the interaction of Hsp 90 with subunit C9, which would result in the protection of subunits Z and MB1 against oxidation.

The observed protection of several activities of the active form MCP by Hsp 90 or α-crystallin could be of physiological relevance if the MCP exists as an active form during oxidative stress. We have therefore monitored the MCP peptidase activities in FAO hepatoma cells after iron/ascorbate-induced oxidative stress, in three different physiological states: after the induction of Hsp 90 synthesis, after the depletion of Hsp 90, and in the control. After oxidative stress, all three MCP peptidase activities tested were inactivated, indicating that MCP exists in a form related to the active form rather than the latent form. Evidence for protection of the trypsin-like or *N*-Cbz-Leu-Leu-Leu-MCA-hydrolysing activities by Hsp 90 is supported by the results obtained when Hsp 90 content has been increased in $FeCl_3$-challenged cells. In such cells, these two activities become much more resistant to oxidative inactivation than in control cells. In fact, the trypsin-like activity is no longer inactivated. Additional support for the physiological relevance of the protective role of Hsp 90 on MCP trypsin-like inactivation by oxidants has been provided by the fate of this activity in Hsp 90-depleted cells. In this situation, the susceptibility of the MCP trypsin-like activity to oxidative inactivation is increased markedly compared with the control cells.

That Hsp 90 might have a crucial role in increasing resistance to injury caused by iron-overload-induced oxidative stress has been recently proposed by Fukuda et al. [41]. They observed an enhanced expression of Hsp 90 in rat renal tubular cells after an intraperitoneal injection of ferric nitrilotriacetate. The implication of α-crystallin in protection against oxidative stress has also been suggested by Mehlen et al. [51], who reported that the constitutive expression of heat-shock protein 27 or αB-crystallin confers resistance to oxidative-stress-induced cytotoxicity in murine L929 fibroblasts. Furthermore, according to Wagner and Margolis [25], there is an age-dependent association of bovine lens MCP with both Hsp 90 and α-crystallin, and an age-related decrease in some peptidase activities of the MCP including trypsin-like activity. Because oxidative damage to protein has been involved in age-related decline of enzyme activity, decreased association of Hsp 90 or α-crystallin with bovine lens MCP on aging might result in a decreased protection of MCP against oxidative inactivation and therefore explain, at least in part, the age-related decrease in MCP hydrolytic activities.

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