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High affinity Zn^{2+} inhibitory site(s) for the trypsin-like peptidase of the 20S proteasome

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ABSTRACT

The effect of Zn^{2+} on three major peptidase activities of the 20S proteasome purified from *Xenopus* oocytes was kinetically investigated. An extremely low concentration of Zn^{2+} (μM range) strongly inhibited the trypsin-like activity of the 20S proteasome which was fully recoverable by the addition of EDTA. The concentration of Zn^{2+} for half-maximum inhibition ($K_{0.5}$) was 0.60 μM which was at least 10 times lower than that of any other divalent cation tested and essentially the same as for proteasomes purified from various other organisms indicating that the inhibition is highly Zn^{2+}-specific, reversible, and common to the proteasome regardless of its source. Zn^{2+} at concentrations below 100 μM instantaneously activated the chymotrypsin-like and PGPH activities, and the Zn^{2+} concentration for half-maximum activation was found to be 42–48 μM. These activities were time-dependently inactivated by submillimolar concentrations of Zn^{2+}. The inactivation rates were dependent on the concentration of Zn^{2+} and reached a maximum of 1.60–2.40 min^{-1} for the three peptidase activities under the conditions used. The Zn^{2+} concentration for half-maximum inactivation was found to be 0.70–1.23 mM. This time-dependent inactivation was not reversed by the addition of EDTA or DTT and might not be accompanied by the dissociation of subunits of the 20S proteasome indicating that all activities are inactivated by an identical phenomenon. These results reveal the three types of effects of Zn^{2+} on the 20S proteasome.

The degradation of intracellular protein plays a major role in a variety of cellular processes [1–5]. The proteasome system has been demonstrated in several cellular functions, including selective protein degradation, differentiation, cell-cycle progression, protein quality control, apoptosis [6–8]. The proteasome is a multicatalytic proteinase complex comprising two units, the 20S proteasome and the 19S regulatory unit. The 20S proteasome degrades unfolded proteins in an ATP- and ubiquitin-independent manner [9] which has three well-characterized peptidase activities, trypsin-like, chymotrypsin-like, and postglutamyl peptide hydrolytic (PGPH)[1] activities, that are associated with three distinct subunits, β5, β2, and β1, respectively [10–12]. During aging, oxidatively damaged proteins appear to aggregate and accumulate to abnormally high levels in cells [13,14]. The oxidized proteins are mainly degraded by the 20S proteasome [15]. Therefore, the 20S proteasome has been described as a secondary antioxidative defense mechanism which degrades non-functional oxidized proteins [16].

Over the past few years, a large number of articles have demonstrated that alterations in cellular metal ion concentrations play a fundamental role in neurological diseases like Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, and Prion diseases [17–19]. In AD, Zn^{2+} content is abnormally high in various parts of the brain [20,21]. Postmortem analyses of brain samples from AD patients showed a decrease in the proteolytic activity of the 20S proteasome by approximately 50% [22,23]. Since Zn^{2+} is an essential micronutrient with multiple structural and regulatory cellular functions [24] and the inhibition of the proteasomes function by Zn^{2+} may result in alterations of the cell cycle or cellular homeostasis, we have focused our attention on the enzymatic function of the 20S proteasome.

Various metal ions affect the catalytic properties of the 20S proteasome or the mechanisms involved in the protein degradation by the proteasome system [25,26]. Zn^{2+} is of particular interest, because it seems to be a more generalized effector of enzyme function and has so far been the only divalent cation to display invariably an inhibitory effect on proteasome [27–29]. The detail of the Zn^{2+}-induced specific inhibition (or activation) of catalytic

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[1] *Abbreviations used:* Suc, succinyl; Boc, *t*-butyloxycarbonyl; *Z*, benzyloxycarbonyl; MCA, 4-methylcoumaryl-7-amide; AMC, 7-amino-4-methylcoumarine; PGPH, peptidyl glutamyl peptide hydrolase.

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activities of the 20S proteasome has not yet been characterized. Therefore, the kinetics was examined in this study to investigate the Zn^{2+}-induced inhibition (or activation) of the three peptidase activities of the 20S proteasome. The results obtained suggest the existence of high-affinity inhibitory binding site(s) of Zn^{2+} for the trypsin-like peptidase of the 20S proteasome.

Materials and methods

Chemicals

The synthetic fluorescent peptide substrates, Suc-LLVY-MCA, Boc-LRR-MCA, and *Z*-LLE-MCA, were purchased from Peptide Institute (Osaka, Japan). The $ZnCl_2$ was purchased from Wako Pure Chemicals Ltd (Osaka, Japan). EDTA and DTT were from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade.

Purification of the 20S proteasome

The 20S proteasome was purified from the cytosol of various organisms by successive chromatography on Superose-6, DE52-cellulose, phenyl-Sepharose CL-4B, and DE52-cellulose again. The cytosolic fraction (5 ml) was applied to a Superose-6 column (2.7 × 98 cm) in a 15 mM Tris–HCl, pH 7.5, 20% glycerol, and 10 mM 2-mercaptoethanol buffer (TGM buffer). The active fractions were pooled and applied to a DE52-cellulose column (1.2 × 10 cm) in TGM buffer containing 100 mM KCl. The column was washed with the same buffer and then eluted with a linear gradient of 100–300 mM KCl in TGM buffer. The active fractions were pooled and applied to a phenyl Sepharose CL-4B column (1.5 × 5 cm) in TGM buffer containing 1.25 M KCl. The absorbed materials were eluted with a linear gradient of 1.25 to 0.5 M KCl in TGM buffer. The active fractions were pooled, diluted to 100 mM KCl in TGM buffer, and applied to a second DE52-cellulose column (1.2 × 5 cm). After a wash with TGM buffer containing 100 mM KCl, the column was eluted with a linear gradient of 100–300 mM KCl in TGM buffer. The active fractions were pooled and concentrated through a small DE52-cellulose column in TGM buffer. To examine the purity and the subunit composition of the 20S proteasome, the concentrated sample was subjected to SDS–PAGE and non-denaturing-PAGE. This preparation gave at least nine typical 20S proteasome bands in SDS–PAGE and a single band in non-denaturing-PAGE. The sample was stored at −80 °C. No loss of activity was detected for at least 6 months.

Activity assays

The activity of peptidyl substrate hydrolysis was measured at 37 °C for 45 min in a total volume of 2 ml containing 50 mM Tris–HCl, pH 8.5, 1.43 pM 20S proteasome, 10 μM peptidyl substrates [Suc-LLVY-MCA for chymotrypsin-like activity (hereafter termed as LLVY-hydrolysis), *Z*-LLE-MCA for PGPH activity (LLE-hydrolysis) and Boc-LRR-MCA for trypsin-like activity (LRR-hydrolysis)], various concentrations of Zn^{2+} or EDTA, 1 mM 2-mercaptoethanol, and 1% DMSO. The reaction was started by adding peptidyl substrate and various concentrations of Zn^{2+} to the reaction mixture containing 20S proteasome. After incubation for various periods, the reactions were stopped by adding 200 μl of reaction buffer containing 10% SDS. Then, the fluorescence of the released AMC was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm by a fluorescence spectrophotometer (F-3000, Hitachi, Tokyo). The activity to hydrolyze the peptidyl substrate was calculated as nmol of substrate hydrolyzed per mg of protein (20S proteasome) per min.

Protein concentration

The protein concentration was measured by the method of Bradford with BSA as a standard and the purity was checked by non-denaturing-PAGE and SDS–PAGE [30].

Electrophoresis

The non-denaturing-PAGE was carried out as described by Laemmli [31] using a continuous 4% acrylamide gel without SDS in the reagents and running buffer.

Analysis of the Zn^{2+}-induced changes of peptidase activities of the 20S proteasome

The Zn^{2+} concentration-dependent changes of three kinds of peptidase activities of the 20S proteasome throughout the text has been analyzed by fitting the kinetic data to a theoretical curve. The following equation was used for the analysis of Zn^{2+}-induced inactivation, where V_{inact} is the rate of inactivation, K_m is the Michaelis constant, V_{max} is the maximum velocity, and $[Zn^{2+}]_0$ is the total concentration of zinc ion in the reaction solution.

$$V_{inact} = \frac{V_{max}}{1 + K_m/[Zn^{2+}]_0}$$

Graphs and fits of the kinetic data were obtained by nonlinear regression using the program Origin, version 7.5 (Japan).

Results

Zn^{2+} inhibits LRR-hydrolysis intensively and specifically

Although the inhibitory effect of Zn^{2+} on the catalytic activities of the 20S proteasome has been demonstrated in millimolar or submillimolar concentration range [32–34], the phenomenon involved is still unknown. We found a strong inhibition of Boc-LRR-MCA hydrolysis (LRR-hydrolysis) of the 20S proteasome by an extremely low (μM) concentration of Zn^{2+} (Fig. 1A). The inhibition was instantaneous with the addition of Zn^{2+} and dependent on its concentration. After the addition of Zn^{2+}, the activity for LRR-hydrolysis was stable during the reaction time measured. The Zn^{2+} concentration for half-maximum inhibition ($K_{0.5}$) of LRR-

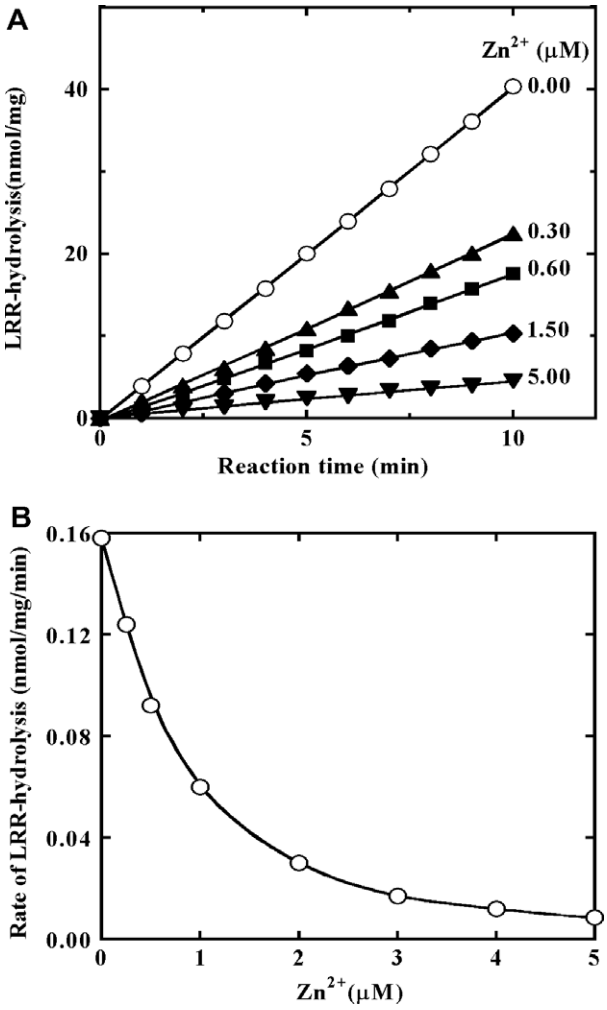

Fig. 1. Inhibition of LRR-hydrolysis by Zn^{2+}. Reactions were carried out at 37 °C for 45 min in a total volume of 2 ml containing 50 mM Tris–HCl, pH 8.5, 1.43 pM 20S proteasome, 1% DMSO, 10 μM Boc-LRR-MCA and various concentrations of Zn^{2+}(μM): 0 (○), 0.3 (▲), 0.6 (■), 1.5 (♦), and 5 (▼). Reactions were started by adding Boc-LRR-MCA and Zn^{2+} to the enzyme solution and stopped by adding 200 μl of reaction mixture containing 10% SDS at various reaction times. (A) The hydrolyzed Boc-LRR-MCA was measured as described in Materials and methods and plotted against reaction time. (B) Rates of LRR-hydrolysis at the addition of Zn^{2+} were determined from the results of (A) and plotted against the Zn^{2+} concentration. Essentially the same results were obtained for other independent experiments. The LRR-hydrolysis was completely inhibited by Zn^{2+} as shown in (B) and the Zn^{2+} concentration for half-maximum inhibition was determined as 0.60 μM.