Free Radical Biology and Medicine 67 (2014) 304–313

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contributions

Peptides that activate the 20S proteasome by gate opening increased oxidized protein removal and reduced protein aggregation

Francisco H. Dal Vechio [a], Fernanda Cerqueira [c], Ohara Augusto [c], Robson Lopes [b], Marilene Demasi [a,*]

[a] Laboratório de Bioquímica e Biofísica, Instituto Butantan, São Paulo, SP 05503-001, Brazil
[b] Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, São Paulo, SP, Brazil
[c] Laboratório Especial de Toxinologia Aplicada, Instituto Butantan, São Paulo, SP 05503-001, Brazil

ARTICLE INFO

Article history:
Received 14 August 2013
Received in revised form
1 November 2013
Accepted 18 November 2013
Available online 27 November 2013

Keywords:
Proteasome
Therapeutic peptides
Protein oxidation
Protein aggregation
ALS
Free radicals

ABSTRACT

The proteasome is a multicatalytic protease that is responsible for the degradation of the majority of intracellular proteins. Its role is correlated with several major regulatory pathways that are involved in cell cycle control, signaling, and antigen presentation, as well as in the removal of oxidatively damaged proteins. Although several proteasomal catalytic inhibitors have been described, very few activators have been reported to date. Some reports in the literature highlight the cellular protective effects of proteasome activation against oxidative stress and its effect on increased life span. In this work, we describe a peptide named proteasome-activating peptide 1 (PAP1), which increases the chymotrypsin-like proteasomal catalytic activity and, consequently, proteolytic rates both in vitro and in culture. PAP1 proteasomal activation is mediated by the opening of the proteasomal catalytic chamber. We also demonstrate that the observed proteasomal activation protected cells from oxidative stress; further, PAP1 prevented protein aggregation in a cellular model of amyotrophic lateral sclerosis. The role of 20SPT gate opening underlying protection against oxidative stress was also explored in yeast cells. The present data indicate the importance of proteasomal activators as potential drugs for the treatment of pathologies associated with the impaired removal of damaged proteins, which is observed in many neurodegenerative diseases.

The proteasome is the main machinery involved in the degradation of intracellular proteins. It is a multimeric and multicatalytic complex composed of a central cylinder, called the 20S proteasome (20SPT). The 20SPT is composed of four heptameric rings, named α and β. Two central β-rings carry the catalytic sites, which are located in three of the seven polypeptide chains, and two α-rings flank the central catalytic core [1]. The major regulatory particle, the 19S unit, is usually coupled to the 20S core on one or both sides. The 19S unit is responsible for the recognition of polyubiquitylated substrates as well as their deubiquitylation, unfolding, and translocation to the catalytic chamber. The complex that is formed by the coupling of the 20SPT to the 19S unit is called the 26S proteasome (26SPT). The 26SPT is involved in the degradation of proteins that participate in many essential cellular functions, such as cell cycle control, differentiation, signal transduction, antigen presentation, inflammatory response, and apoptosis. The protein ubiquitylation system, which involves complex enzymatic machinery together with the 26SPT, is essential for the maintenance of cellular homeostasis [2]. On the other hand, the catalytic activity of the 20SPT in cells in an oxidative environment or under many other stressful conditions is fundamental for the removal of damaged proteins [3]. It is well accepted, and further evidence is presented in this work, that the removal of damaged proteins, mainly oxidized proteins, is achieved through a process that is not dependent on polyubiquitylation [4,5]. It has been reported that 20–30% of the proteasomes in cells are free of regulatory particles [6,7] and are most likely engaged in the degradation of oxidized, unstructured, and misfolded proteins.

Proteasomal catalytic inhibitors were first described in the early 1990s [8]. Because proteasomal inhibition promotes apoptosis, this finding implicated the proteasome as an important cellular target for the development of new drugs [9]. However, proteasomal

Abbreviations:: ALS, amyotrophic lateral sclerosis; AMC, 7-amido-4-methylcoumarin; *z*-ARR-AMC, *z*-Ala-Arg-Arg-amido-4-methylcoumarin; ChT-L, chymotrypsin-like proteasomal activity; DMF, *N*,*N*-dimethyl formamide; DMSO, dimethyl sulfoxide; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; FALS, familial amyotrophic lateral sclerosis; FITC, fluorescein isothiocyanate; HOBt, hydroxybentriazole; MS, mass spectrometry; NMM, 4-methylmorpholine; PAP, proteasome-activating peptide; PI, propidium iodide; 20SPT, 20S proteasome; 26SPT, 26S proteasome; SOD, superoxide dismutase; *suc*-LLVY-AMC, *suc*-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; WT, wild type

* Corresponding author. Fax: +55 11 37267222x2018.
E-mail address: marimasi@butantan.gov.br (M. Demasi).

0891-5849/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.freeradbiomed.2013.11.017

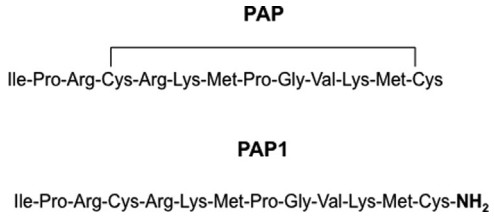

Fig. 1. The sequence of PAP and its open structure with the C-terminal modified by amidation (PAP1).

activators have been poorly described to date. The few studies reported in the literature demonstrate the benefits of proteasomal activation, notably its potential to increase cellular life span [10,11]. In those studies, intracellular proteasomal activation was achieved by gene manipulation [12,13] or by in vitro and cell treatment with peptides and organic compounds [14,15]. Oleuropein, which activates the proteasome in vitro, fails to activate it within cells, despite promoting the induction of proteasomal expression [16].

Here, we describe an activator of the proteasome (proteasome-activating peptide: PAP and PAP1, closed and open conformations, respectively; Fig. 1) that significantly increases the chymotrypsin-like (ChT-L) catalytic activity, and consequently proteolysis rates, of the proteasome, as determined in vitro. Moreover, fibroblasts incubated with PAP1 and challenged with H_2O_2 were protected from death, and PAP1 prevented protein aggregation in a cellular model of amyotrophic lateral sclerosis. In both cell models, we observed a decreased pool of oxidized proteins after oxidative challenge when cells were preincubated with PAP1. As shown here, PAP1 promotes the opening of the 20SPT chamber without any apparent effect on the intracellular pool of polyubiquitylated proteins. Thus, our data suggest that the major PAP1 cellular target is the free 20SPT pool. The role of 20SPT gate opening in the removal of damaged proteins was also explored in yeast cells.

Materials and methods

Reagents

The human and rabbit 20S proteasomes were purchased from Affinity BioReagents (Rockford, IL, USA). Anti-Cu,Zn superoxide dismutase 1 (SOD1; code 574597) antibody, dithiobenzoic acid (DTNB), *suc*-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (*suc*-LLVY-AMC), and *z*-Ala-Arg-Arg-amido-4-methylcoumarin (*z*-ARR-AMC) were purchased from Calbiochem (San Diego, CA, USA). Anti-actin (code SC-7210) and anti-ubiquitin (code SC-8017) antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-dinitrophenol (DNP) antibody (code D-9656), casein–fluorescein isothiocyanate (FITC), 2,4-dinitrophenylhydrazine (DNPH), DNase, and RNase were purchased from Sigma (St. Louis, MO, USA). *N,N*-dimethyl formamide (DMF), hydroxybentriazole (HOBt), 4-methylmorpholine (NMM), and *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium tetrafluoroborate (TBTU) were purchased from Merck (Darmstadt, Germany). Bradford reagent was obtained from Bio-Rad (Hercules, CA, USA). Cell culture media and complements were obtained from Invitrogen (Grand Island, NY, USA). Secondary antibodies were obtained from GE Healthcare (Buckinghamshire, UK). All other reagents were of analytical grade.

PAP synthesis

PAP and modified structures were either purchased from ANASPEC (Fremont, CA, USA) or synthesized in our lab as described by Atherton and Sheppard [17] in an automated bench-top simultaneous multiple solid-phase synthesizer (PSSM 8 system from Shimadzu Co., Kyoto, Japan) using the Fmoc procedure for solid-phase peptide synthesis. Briefly, the sequential coupling of protected amino acids was performed with HOBt, TBTU, and NMM on preloaded Fmoc-amino acid Wang resin (Merck). Fmoc cleavage was performed with 50% morpholine (v/v) in DMF. The resin-bound peptides were cleaved and deprotected with a TFA/thioanisole/EDTA/phenol/water (82.5/5/2.5/5v/10v) mixture at room temperature for 6 h. After filtration, TFA mixtures were concentrated by argon flow and precipitated with cold diethyl ether. The crude peptide was purified by reverse-phase chromatography (Shim-Pack Prep-ODS, Shimadzu) in semipreparative HPLC. The purity and identity of peptides were confirmed by liquid chromatography–mass spectrometry (MS) and analytical HPLC in two different solvent systems. The final products were lyophilized, stored at −20 °C, and dissolved in Milli-Q water just before use. To render the cyclic structure, the linear sequence was dissolved in 0.1 M NH_3HCO_3 as described by Yamashiro et al. [18]. The cyclization was monitored by HPLC and MS and reaction with the sulfhydryl reactant DTNB. The stability of the PAP1 open structure over time was monitored by MS and reaction with DTNB.

Yeast strain and growth

The *Saccharomyces cerevisiae* RJD1144/JD 122 (MATa his3Δ200 leu2-3,112, lys2-801 trp1Δ63 ura3-52 $PRE1^{FH}$::Ylplac211 URA3) strain, derived from the JD47-13C strain, was kindly donated by Dr. Raymond Deshaies (Division of Biology, Caltech, Pasadena, CA, USA). The RJD1144 strain contained the 20S proteasome PRE1 gene modified with the FLAG peptide and a polyhistidine tail sequence [19]. The cells were cultured in YPD medium containing 4% glucose (referred to as YPD) at 30 °C with reciprocal shaking and harvested after 60 h of incubation. The SUB556 strain, derived from SUB62, was kindly donated by Dr. Michael H. Glickman (Department of Biology, Technion–Israel Institute of Technology, Haifa, Israel). The 20SPT in the SUB556 strain contains N-terminal deletions in both the α3 and the α7 subunits [20].

Extraction and purification of the 20S proteasome

The 20SPT from the RJD1144 strain was purified by nickel-affinity chromatography with a continuous gradient of imidazole using HPLC (Akta Purifier; GE Healthcare Life Sciences). The final preparations were passed through a PD10-desalting column according to the manufacturer's protocol (GE Healthcare). Mammalian 20S proteasome preparations were obtained from horse erythrocytes by a three-step chromatographic procedure as described in Demasi et al. [21].

Proteasomal activity in vitro and in the cellular extracts

Proteasomal activity was measured using fluorogenic peptides (AMC as the fluorescent probe). *suc*-LLVY-AMC was used as a standard peptide to access the chymotrypsin-like activity of the core and *z*-ARR-AMC for the trypsin-like (T-L) activity. The 20S proteasome (0.5–3 μg 100 μl^{-1}) was incubated at 37 °C in 20 mM Tris–HCl buffer, pH 7.5, herein referred to as standard buffer. The incubation was initiated by the addition of 10–50 μM peptides. The fluorescence emission was recorded at 440 nm (with excitation at 365 nm). The in vitro assays consisted of a 15-min preincubation of purified proteasome preparations with PAP derivatives, followed by the hydrolytic assay described above. The proteasomal activity was determined in cell extracts by incubating 50-mg aliquots of cellular protein at 37 °C with the same substrates described above at 125 μM (final concentration), and the emission was recorded for 45 min. In parallel, similar samples were previously incubated (15 min) in the presence of