Volume 326, number 1,2,3, 215–218 FEBS 12690 July 1993

Thermoplasma acidophilum proteasomes degrade partially unfolded and ubiquitin-associated proteins

Thorsten Wenzel and Wolfgang Baumeister

Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, W-8033 Martinsried, Germany

Received 7 May 1993; revised version received 24 May 1993

It is shown that proteasomes from the archaebacterium *Thermoplasma acidophilum* selectively degrade substrate proteins partially unfolded by phenylhydrazine- or hydrogen peroxide-treatment. Surprisingly, the pre-incubation of the substrate proteins with ubiquitin is also sufficient to render them susceptible to proteolytic degradation by proteasomes. We propose that, upon spontaneously associating with the substrate protein, ubiquitin exerts a chaotropic effect on it; this may involve the exposure of hydrophobic segments of the polypeptide chain which are recognized by the binding sites of the proteasome.

Proteasome; Ubiquitin; *Thermoplasma acidophilum*; Proteolytic degradation; Oxidant-damaged proteins

1. INTRODUCTION

ATP-ubiquitin-dependent proteolysis plays a crucial role in the selective elimination of abnormal or of partially unfolded proteins and in the modulation of the levels of short-lived regulatory proteins (for recent reviews see [1–5]). Considerable progress has been made over the past few years in elucidating the enzymatic mechanisms of ubiquitin activation and ligation to substrate proteins (for a recent review see [6]). Although it is widely accepted now that the 26S proteasome is the key proteolytic system, much less is known about the structure of the conjugate degrading machinery, the mechanism by which it recognizes the conjugate, the precise proteolytic mechanism(s) and its regulatory elements. The 20S proteasome is regarded as the catalytic core of the 26S complex. It has been shown previously that the 20S proteasome degrades partially unfolded substrate proteins [7–10]. In this communication, we show that ubiquitin is capable of associating spontaneously with substrate proteins, such as hemoglobin, rendering them susceptible to proteolytic degradation. This suggests that ubiquitin has the capacity to induce unfolding, possibly by intercalation into the tertiary structure of the substrate proteins.

Correspondence address: W. Baumeister, Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, W-8033 Martinsried, Germany. Fax: (49) (89) 8578-2641.

Abbreviations: HPLC, high performance liquid chromatography; NMec, 4-methyl-7-coumarylamide; SDS, sodium dodecyl sulfate; Suc, succinyl; Ub, ubiquitin; Hb, hemoglobin.

2. MATERIALS AND METHODS

2.1. *Isolation, purification and activity assay of proteasomes*

Proteasomes from either *T. acidophilum* or recombinant proteasomes, expressed in *E. coli* [11], were isolated and purified as described previously [12]. Routinely, proteolytic activities of isolated proteasomes were measured by incubating 0.1 ml of the enzyme solution with 0.1 ml substrate solution at 37°C for 1 h. 20 μM Suc-Leu-Leu-Val-Tyr-NMec (Bachem Biochemica GmbH, Heidelberg), which was used as a test substrate, was dissolved in 20 mM Tris-HCl, 1 mM EDTA and 1 mM NaN_3, pH 7.5. The reaction was terminated with 1 ml of 100 mM monochloracetate and 100 mM sodium acetate, pH 4.3. Cleavage of the peptide substrate was monitored by measuring the release of 4-methyl-7-coumarylamide fluorometrically [13].

2.2. *Degradation of phenylhydrazine-treated hemoglobin*

For the degradation of human hemoglobin-A_0 (Sigma Chemie GmbH, Deisenhofen) by proteasomes, different amounts of proteasomes were incubated in a volume of 1 ml containing 50 mM potassium phosphate buffer (pH 7.5) and 1 mg of substrate protein pretreated with 1 mM phenylhydrazine for 1 h at 0°C. The reaction was terminated after a 2 h incubation period at 37°C by adding cold perchloric acid to a final concentration of 0.5 M. Following 30 min in an ice-bath, the insoluble material was removed by centrifugation. The supernatant was neutralized with 0.05 ml of 10 M KOH and insoluble perchlorate was removed [9]. The proteolytic cleavage of the substrate protein was analyzed by gel electrophoresis and HPLC (see below).

2.3. *Degradation of hydrogen peroxide-treated proteins*

Human hemoglobin-A_0 and α-lactalbumin from bovine milk (Sigma Chemie GmbH, Deisenhofen) were incubated at concentrations of 1 mg/ml in 1 ml containing 50 mM Tris-HCl (pH 7.5) and 30 mM hydrogen peroxide for 1 h at room temperature [8]. To stop damaging proteins by oxidation, solutions were dialyzed against 5 litres of 50 mM potassium phosphate at pH 7.5 for 24 h. Proteolytic cleavage of the substrate proteins was analyzed by gel electrophoresis and HPLC (see below).

2.4. *Degradation of proteins in the presence of ubiquitin*

Human hemoglobin-A_0 and α-lactalbumin from bovine milk were

incubated at concentrations of 1 mg/ml in 1 ml containing 50 mM Tris-HCl (pH 7.5) with different concentrations of ubiquitin from bovine red blood cells (Sigma Chemie GmbH, Deisenhofen), ranging from 1 to 10 mg. The proteolytic cleavage was initiated by the addition of 5 mg of proteasomes. All other experimental details are given in the figures and tables.

2.5. *Gel electrophoresis and HPLC*

Electrophoresis in 16% polyacrylamide tricine–sodium dodecyl sulphate-containing gels was performed according to the method of Schägger and von Jagow [14]. The gel was run at 40 mA for 3 h at room temperature. It was fixed with 12% trichloracetic acid and stained with 0.05% (w/v) Coomassie G-250 dissolved in 20 ml of ethanol and 80 ml of water, containing 10% ammonium sulphate and 2% phosphoric acid.

High performance liquid chromatography was performed on an HPLC System Gold from Beckman, containing a programmable solvent module 125 and a diode array detector module 168. For protein separation, a TSK G 2000 SW column (7.5 × 600 mm) from LKB was used. The elution from TSK G 2000 molecular sieve was performed with 0.1% trifluoroacetic acid in 30% (v/v) acetonitrile in water at a flow rate of 1 ml/min. The eluate was monitored by measuring the absorbance at 206 nm and 280 nm. Chromatography was carried out at 20°C.

3. RESULTS AND DISCUSSION

3.1. *Degradation of phenylhydrazine-treated hemoglobin-A_o*

It had been shown previously that exposure of erythrocytes to 1 mM phenylhydrazine greatly enhances the susceptibility of erythrocyte proteins to degradation by proteasomes [7,9,15]. While untreated native hemoglobin is not noticeably degraded by proteasomes, *Thermoplasma acidophilum* proteasomes as well as the recombinant proteasomes are capable of degrading phenylhydrazine-treated human hemoglobin-A_o to an extent that only a broad band in the low molecular weight range remains (Fig. 1). HPLC of the proteolytic breakdown products shows numerous peaks in the molecular weight range between 1 and 5 kDa (data not shown). A massive change in the secondary structure of hemoglobin, i.e. a loss of α-helical content is induced by phenylhydrazine treatment as indicated by CD-spectra (data not shown). It turned out impossible to subject the peptide fragments to automated Edman degradation; probably, phenylhydrazones are formed with the peptide backbone upon phenylhydrazine treatment.

3.2. *Degradation of hydrogen peroxide-treated proteins*

Cells are stressed by exposure to hydrogen peroxide. The proposition that oxygen radical-mediated oxidation of different amino acid residues of proteins is a marking step in protein turnover has been confirmed by several observations (for a recent review see [16]). Actually, many common proteases degrade oxidized proteins more rapidly than the unoxidized forms.

In order to examine the hypothesis that proteasomes contribute to the breakdown of oxidant-damaged proteins [10,17–19], we have chosen human hemoglobin-A_o and α-lactalbumin from bovine milk as substrate proteins. After incubating the substrate proteins with 30 mM hydrogen peroxide in 50 mM Tris-HCl at pH 7.8 for 1 h at room temperature, it was important to dialyse the solutions against a buffer for at least 24 h, as described in section 2, in order to prevent damage of proteasomes by hydroxyl radicals. After dialysis and incubation with proteasomes, proteolytic degradation of hemoglobin and α-lactalbumin have been measured. As shown in Fig. 2, hydrogen peroxide-treated hemoglobin was degraded by proteasomes over a time period of about 18 h. Higher molecular aggregates, which formed upon hydrogen peroxide-treatment, were degraded as well as the nonaggregated hemoglobin. A broad band of peptides centred around 1 kDa was detected by HPLC (Fig. 2). As with the phenylhydrazine treated samples, the activity of proteasomes from *Thermoplasma acidophilum* and of recombinant proteasomes was indistinguishable.

3.3. *Degradation of proteins in the presence of ubiquitin*

Proteolysis in the cytosol and in the nucleus requires exact signalling mechanisms and highly specific proteolytic systems recognizing only the proteins marked for degradation. This is provided by the ubiquitin-dependent proteolytic system [4,20–22]. We have recently shown that ubiquitin occurs in *Thermoplasma acidophi-*

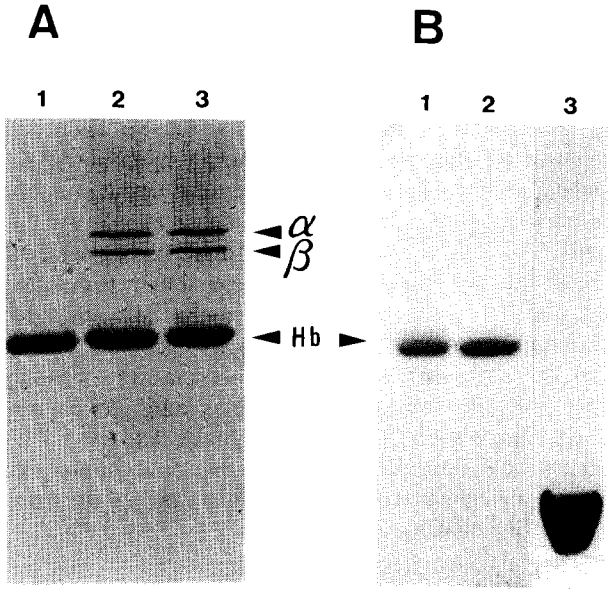

Fig. 1. Degradation of phenylhydrazine-treated hemoglobin-A_o by 20S proteasomes. Panel A shows in lane 1 untreated hemoglobin (5 μg) and in lanes 2 and 3 respectively hemoglobin-A_o (5 μg) exposed to proteasomes (1 μg) for 1 h and 24 h. Panel B shows the proteolytic cleavage of phenylhydrazine-treated hemoglobin: (lane 1) 5 μg untreated hemoglobin-A_o; (lane 2) phenylhydrazine-treated hemoglobin-A_o without exposure to proteasomes; (lane 3) 10 μg phenylhydrazine-treated hemoglobin-A_o incubated with 1 μg proteasomes for 2 h. The positions of the α- and β-subunits of *Thermoplasma acidophilum* proteasomes are indicated.

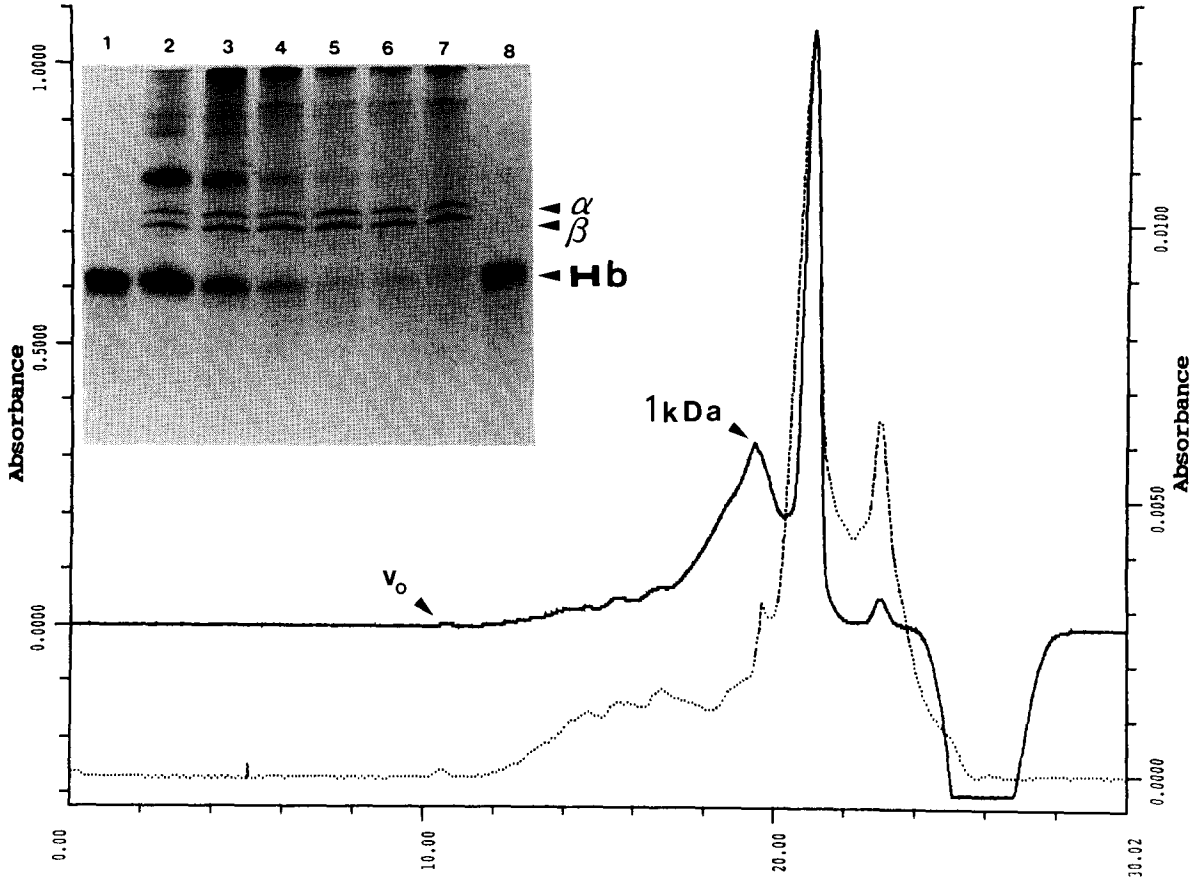

Fig. 2. Degradation of H_2O_2-treated hemoglobin by 20S proteasomes. Reaction mixtures contained in a volume of 1 ml: 1 mg hemoglobin-A_0, treated with 30 mM H_2O_2 for 1 h, 50 mM potassium phosphate (pH 7.5) and 5 μg of proteasomes. Following incubation at 60°C, the samples (50 ml) were subjected to electrophoresis on a 16% polyacrylamide-SDS gel. The time increments from lane 2 (0 h) to lane 7 (30 h) were 6 h. Lanes 1 and 8 are controls. The positions of the α- and β-subunits of the proteasome and the position of the hemoglobin are indicated. Degradative products could not be measured by gel electrophoresis. The 24 h-digest (50 μl) was analyzed by HPLC using a TSK G 2000 SW column (7.5 × 600 mm). The chromatogram shows a broad peak of cleavage products with a mass maximum at approximately 1000 Da (solid line absorbance at 206 nm, broken line absorbance at 280 nm).

lum, making it likely that ubiquitin-dependent proteolysis has an evolutionary antecedent in archaebacteria (Wolf et al., submitted). Whether or not an ubiquitin-conjugating machinery of a complexity similar to eukaryotic cells exists is as yet unknown.

We have exposed hemoglobin and α-lactalbumin, pre-incubated with ubiquitin from bovine red blood cells, to proteasomes. In the presence of ubiquitin both proteins were degraded. As shown in Fig. 3, hemoglobin becomes completely degraded over a period of 12 h. The rate of proteolytic cleavage depends on the amount of ubiquitin present. Ubiquitin itself is not degraded by proteasomes and no effect has been detected upon adding ATP or haemin, as observed in the case of 26S complex (data not shown). When using synthetic peptides, such as Suc-Leu-Leu-Val-Tyr-NMec (see section 2) as a substrate, the presence of ubiquitin had no effect on the activity of proteasomes, ruling out that ubiquitin exerts its activating effect by a direct interaction with the proteasome.

The hemoglobin- and α-lactalbumin-ubiquitin associates can be subjected to HPLC without dissociating them, indicating that ubiquitin spontaneously forms rather stable complexes with hemoglobin and α-lactalbumin. Upon complex formation, the substrate proteins become susceptible to proteolytic cleavage. The extent of proteolytic degradation is comparable to the degradation of the oxidant-damaged proteins suggesting that ubiquitin induces similar unfolding. The extent of proteolytic cleavage points to a broad specifity of the *Thermoplasma acidophilum* proteasome which is classified as chymotryptic [23]. The preponderance of cleavage products with molecular weights in the range of 1 kDa may be taken as an indication for the existence of a molecular ruler in the proteasome. One could envisage that simply the distance between the multiple active sites in the complex which has an $\alpha_{14}\beta_{14}$-stoichiometry [12] provides such a ruler. Supposing that the catalytic centers are located at the β-subunits, this distance will fall into the range between 2 nm and 4.2 nm, depending on

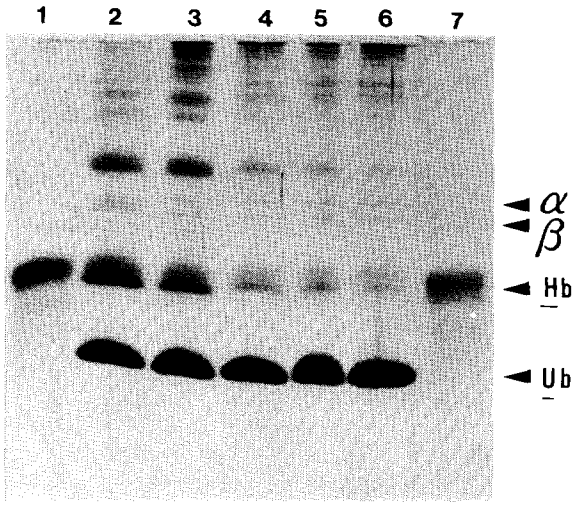

Fig. 3. Ubiquitin-dependent degradation of hemoglobin by 20S proteasomes. Reaction mixtures contained in a volume of 1 ml: 0.5 mg hemoglobin, 1 mg ubiquitin, 50 mM Tris-HCl (pH 7.5) and 5 μg proteasomes (α- and β-subunits are indicated). Following incubation at 60°C, the samples (50 μl) were subjected to electrophoresis; the time increments from lane 2 (0 h) to lane 6 (24 h) were 6 h. Lanes 1 and 7 are controls.

their exact location, i.e. on the inside of the barrel-shaped complex or more towards the periphery. This hypothesis is particularly attractive in the view of the putative role of proteasomes in antigen processing.

The proposition that ubiquitin acts as a 'chaotropic' polypeptide unfolding target molecules, offers a link between the degradation of oxidant-damaged and ubiquitin-conjugated substrate molecules. It suggests that unfolded segments of the polypeptide chain exposing hydrophobic residues act as 'recognition signals'. In this context, it is interesting to note that the molecular architecture of proteasomes and of some molecular chaperones is strikingly similar [24]; both must be capable of distinguishing unfolded forms of a protein from the correctly folded forms. One could imagine that proteasomes possess binding grooves or pockets mimicking the hydrophobic interior of a protein, similar to the molecular chaperone BiP (hsp 70), where the existence of such binding sites is inferred from affinity measurements with random sequence peptides [25].

Acknowledgements: The authors wish to thank Mr. P. Zwickl for help in preparing the recombinant proteasomes and Mr. J. Peters for his help with the HPLC.

REFERENCES

[1] Hershko, A. and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807.
[2] Goldberg, A.L. and Rock, K.L. (1992) Nature 357, 375–379.
[3] Hochstrasser, M. (1992) Curr. Opin. Cell Biol. 4, 1024–1031.
[4] Hilt, W. and Wolf, D.H. (1992) Mol. Microbiol. 6, 2437–2442.
[5] Rechsteiner, M., Hoffman, L. and Dubiel, W. (1993) J. Biol. Chem. 268, 6065–6068.
[6] Jentsch, S. (1992) Annu. Rev. Genet. 26, 179–207.
[7] Fagan, J.M., Waxman, L. and Goldberg, A.L. (1986) J. Biol. Chem. 261, 5705–5713.
[8] Pacifici, R.E., Salo, D.C. and Davies, K.J.A. (1989) J. Free Radicals Biol. Med. 7, 521–536.
[9] Sacchetta, P., Battista, P., Santarone, S. and Di Cola, D. (1990) Biochim. Biophys. Acta 1037, 337–343.
[10] Fagan, J.M. and Waxman, L. (1992) J. Biol. Chem. 267, 23015–23022.
[11] Zwickl, P., Lottspeich, F. and Baumeister, W. (1992) FEBS Lett. 312, 157–160.
[12] Pühler, G., Weinkauf, S., Bachmann, L., Müller, S., Engel, A., Hegerl, R. and Baumeister, W. (1992) EMBO J. 11, 1607–1616.
[13] Dahlmann, B., Kühn, L. and Reinauer, H. (1983) FEBS Lett. 160, 243–247.
[14] Schägger, H. and von Jagow, F. (1987) Anal. Biochem. 166, 368–379.
[15] Boches, F.S. and Goldberg, A.L. (1982) Science 215, 978–980.
[16] Stadtman, E.R. (1992) Science 257, 1220–1225.
[17] Kirschner, R.J. and Goldberg, A.L. (1982) Fed. Proc. 41, 865.
[18] Fagan, J.M., Waxman, L. and Goldberg, A.L. (1985) Proceedings of the 13th International Congress of Biochemistry, abstract MO278, p. 96, August 25–30, 1985, Elsevier, Amsterdam, The Netherlands.
[19] Davies, K.J.A. and Goldberg, A.L. (1987) J. Biol. Chem. 262, 8227–8234.
[20] Eytan, E., Ganoth, D., Armon, T. and Hershko, A. (1989) Proc. Natl. Acad. Sci. 86, 7751–7755.
[21] Seufert, W. and Jentsch, S. (1992) EMBO J. 11, 3077–3080.
[22] Hoffman, L., Pratt, G. and Rechsteiner, M. (1992) J. Biol. Chem. 267, 22362–22377.
[23] Dahlmann, B., Kuehn, L., Grziwa, A., Zwickl, P. and Baumeister, W. (1992) Eur. J. Biochem. 208, 789–797.
[24] Zwickl, P., Pfeifer, G., Lottspeich, F., Kopp, F., Dahlmann, B. and Baumeister, W. (1990) J. Struct. Biol. 103, 197–203.
[25] Flynn, G.C., Pohl, J., Flocco, M.T. and Rothman, J.E. (1991) Nature 353, 726–730.