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Involvement of proteasomes (multicatalytic proteinase) in ATP-dependent proteolysis in rat reticulocyte extracts

Keiji Tanaka and Akira Ichihara

Institute for Enzyme Research, University of Tokushima, Tokushima 770, Japan

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The role of proteasomes, particles with latent multicatalytic proteinase, in ATP-dependent proteolysis in rat reticulocyte extracts was examined. Removal of proteasomes from the extracts by immunoprecipitation caused almost complete inhibition of ATP-dependent degradation of [^{3}H]methylcasein, without affecting ATP-independent proteolysis. Peptide fragments of [^{3}H]casein, obtained by cyanogen bromide cleavage, were rapidly degraded in an ATP-independent fashion and this activity was not affected by removal of the proteasomes. These results suggest that proteasomes are involved in ATP-dependent proteolysis in the extracts and that they catalyze the initial cleavage of large proteins.

Multicatalytic proteinase; ATP; Proteolysis; Reticulocyte; Proteasome

1. INTRODUCTION

The degradation of intracellular proteins requires metabolic energy [1,2], and ATP has actually been shown to stimulate proteolysis in reticulocyte extracts [3]. It is now generally believed that ATP is required for two distinct steps of intracellular protein degradation: (i) covalent binding of ubiquitin to protein substrates, a step that renders the proteins susceptible to proteolytic attack [2]; and (ii) proteolysis per se catalyzed by ATP-dependent proteases [4–7]. ATP-stimulated proteases of high molecular mass have been partially purified from various mammalian sources [8–11], but studies of these enzymes have been hampered by the limited purity of the preparations. More recently, a multicatalytic protease of similarly large molecular size has been purified by us [12–16] and others [17–20] from a wide variety of eukaryotic organisms ranging from yeast to man. However, the protease activity of these large particles, termed 'proteasomes' [14,16], is not believed to be involved in the ATP-dependent proteolytic system, since it does not require ATP [12,18–20]. Nevertheless, the possibility that it is involved in ATP-dependent proteolysis cannot be excluded in view of the fact that some properties of the activity of proteasomes, such as its sensitivity to inhibitors, broad substrate specificity and weakly alkaline pH optimum, resemble those of the ATP-dependent proteolytic activity in reticulocyte extracts. Here, we report evidence that this multicatalytic protease actually takes part in ATP-dependent proteolysis in reticulocyte extracts.

Correspondence address: A. Ichihara, Institute for Enzyme Research, University of Tokushima, Tokushima 770, Japan

2. MATERIALS AND METHODS

Reticulocyte extracts were prepared as described [4] except that Wistar rats (weighing 200–250 g) were used as a source of reticulocytes. [^{3}H]Methylcasein was prepared by reductive methylation [21]. Proteolytic activity was assayed as in [4,12]. The assay mixture (final volume, 200 μl) consisted of 50 μl reticulocyte extract, 50 mM Tris-HCl (pH 7.8), 10 μg [^{3}H]methylcasein, 1 mM DTT and 5 mM $MgCl_2$, with or without 5 mM ATP. After incubation for 2 h at 37°C, the reaction was terminated by addition of 0.8 ml of 10% trichloroacetic acid and the radioactivity of the acid-soluble fragments liberated was determined. Antiserum against rat liver proteasomes was raised in a rabbit by immunization with purified proteasomes as described in [12] and the IgG fraction was prepared from the serum by protein A-Sepharose chromatography. For immunoprecipitation experiments, 0.3 ml reticulocyte extract or 50 μg purified proteasomes [12] was treated for 60 min at room temperature with preimmunized

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control IgG or anti-proteasome IgG at the indicated concentrations. The mixture was then incubated at 4°C for 100 min with *Staphylococcus aureus* cells in an amount corresponding to a 2-fold excess of the binding capacity of the added IgG. The precipitate formed was removed by centrifugation and the proteolytic activity of the supernatant fraction was measured.

3. RESULTS

Ouchterlony analysis has shown that anti-rat liver proteasome IgG can recognize the enzyme in various rat tissues [12] but is less reactive or unreactive with proteasomes from other species, such as yeast and humans [16]. As shown in fig.1, neither control IgG nor anti-proteasome IgG affected the protease activity of purified proteasomes, suggesting that the IgG did not compete with [^{3}H]casein as substrate and that the enzyme remained catalytically active upon formation of an enzyme-antibody complex. Removal of the immuno complex by immunoprecipitation with *S. aureus* cells, however, resulted in inhibition of the protease activity, the extent of inhibition depending on the amount of IgG added. About 200 μg anti-proteasome IgG was required to remove 50 μg of enzyme completely.

Proteolysis in rat reticulocyte extracts was stimulated 4–5-fold by addition of ATP, as reported for rabbit reticulocyte extracts [3–7]. ATP hydrolysis seemed to be essential for this effect, because β,γ-methylene-ATP or ATP added to the system in the absence of Mg^{2+} had no stimulatory effect (not shown). We next studied the effect of anti-proteasome IgG on proteolysis. Although the addition of the antibody had no effect on proteolysis, removal of the enzyme-antibody complex by immunoprecipitation with *S. aureus* cells resulted in almost complete loss of the ATP-dependent [^{3}H]casein-degrading activity, without affecting the ATP-independent proteolysis (table 1). Control IgG did not affect the proteolysis in either the presence or absence of ATP. As shown in fig.2, complete inhibition of the ATP-dependent activity was achieved by the addition of 200 μg IgG, an amount that can precipitate about

Table 1

Effects of ATP and immunoprecipitation of proteasomes on [^{3}H]casein degradation in reticulocyte extracts

Treatment	[^{3}H]Casein hydrolysis (% per h)		ATP-dependent activity (% per h)
	− ATP	+ ATP	
None	3.11	11.63	8.52
Control IgG	2.87	11.20	8.33
Anti-proteasome IgG	2.56	10.02	7.46
Control IgG + *S. aureus* cells	2.39	11.82	8.74
Anti-proteasome IgG + *S. aureus* cells	2.68	2.84	0.30

Treatment of reticulocyte extracts with antibody (200 μg IgG) and assay of proteolytic activity were carried out as described in section 2. Values are means for triplicate determinations

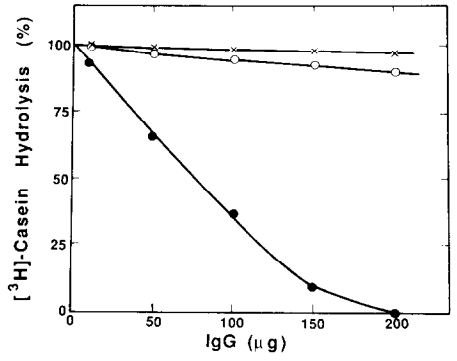

Fig.1. Immunoprecipitation of purified proteasomes with polyclonal antibody. The enzyme (50 μg) was treated with the indicated amounts of control IgG (×), anti-proteasome IgG (○) or the antibody plus *S. aureus* cells (●) as described in section 2. Values are expressed as percentages of the activity without IgG. Values are for protease activity with [^{3}H]casein as substrate, but similar results were obtained for the degradation of various fluorogenic oligopeptides.

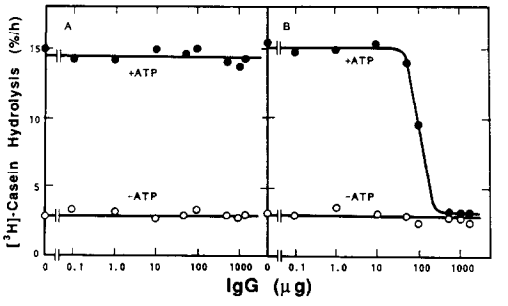

Fig.2. Dose-dependent effect of immunoprecipitation of proteasome on [^{3}H]casein degradation with or without ATP in reticulocyte extracts. (A) Control IgG, (B) anti-proteasome IgG.

Table 2

Effect of immunoprecipitation of proteasomes on degradation of cyanogen bromide fragments of [^{3}H]casein in reticulocyte extracts

Treatment	Unmodified casein (% per h)		Cyanogen bromide fragments (% per h)	
	− ATP	+ ATP	− ATP	+ ATP
Control IgG	1.73	8.89	6.48	9.87
Anti-proteasome IgG + *S. aureus* cells	1.68	1.84	7.04	10.68

[^{3}H]Casein (5×10^{10} dpm/mg) was treated with cyanogen bromide. Equivalent amounts of cyanogen bromide fragments (50000 dpm/μg) and unmodified casein were used for assays. Values are means for two independent determinations

50 μg purified proteasomes. From the proteasome content of rat reticulocytes, determined previously by quantitative enzyme immunoassay [12], we estimated that the extract used contained 50 μg proteasomes. These results suggested that proteasomes were responsible for ATP-dependent degradation of [^{3}H]casein.

As the rapid degradations of insulin and glucagon in the reticulocyte extracts were not enhanced by the addition of ATP (unpublished), it was of interest to examine the effect of ATP on the proteolysis of small peptides. As shown in table 2, the breakdown of peptide fragments, produced from [^{3}H]casein by cyanogen bromide treatment, was enhanced only slightly (about 50% stimulation) by ATP and this enhanced degradation was unaffected by immunoprecipitation of the proteasomes. SDS-polyacrylamide gel electrophoresis indicated that these peptide fragments used as substrates had molecular masses no greater than 16 kDa (not shown). These findings suggested that proteasomes catalyzed the initial cleavage of large polypeptide chains into smaller fragments in an ATP-dependent fashion, and that the smaller fragments were then degraded further by ATP-independent proteolytic activities.

4. DISCUSSION

Our finding that the specific removal of proteasomes from a reticulocyte extract by immunoprecipitation resulted in almost complete loss of activity for ATP-dependent proteolysis of [^{3}H]casein (table 1 and fig.2) indicates clearly that this multicatalytic protease is involved in the ATP-dependent process. McGuire et al. [11] recently reported similar results on ATP-dependent proteolysis in BHK21/13 cell extracts. An enigma is how proteasomes, which do not require ATP for catalytic activity, can catalyze an ATP-dependent reaction. A likely explanation is that proteasomes in cell extracts have ATP-dependent activity, but that this ATP dependency is lost during their purification. This possibility is supported by the fact that partially purified ATP-dependent proteases obtained from reticulocytes [9] and other mammalian cells [8,10,11] were large molecules with similar chromatographic behavior and pH optima to proteasomes, and like proteasomes were stabilized by glycerol. Further supporting evidence is the fact that proteasomes exist in cell extracts in a latent form and can be activated by such compounds as poly(L-lysine), *N*-ethylmaleimide, and SDS [12]. Probably, ATP can also reversibly activate the latent enzyme. Hough et al. [9] recently suggested that an ATP/ubiquitin-dependent protease in reticulocytes is a complex of a multicatalytic protease and a component(s) that confers ATP dependency on the protease. If this is so, then it is conceivable that purified proteasomes have lost this component(s) during their purification. At any rate, further studies are needed to obtain a conclusive picture of the mechanism by which proteasomes catalyze ATP-dependent proteolysis.

Some partially purified ATP-dependent proteases from mammalian sources have been shown to degrade ubiquitin-conjugated, but not unubiquitinated, proteins [9,10]. However, there is a report that in reticulocyte extracts even proteins with modified amino termini, which are thus unable to bind covalently to ubiquitin, are actively degraded in an ATP-dependent fashion [4]. Furthermore, an ATP-dependent protease from erythroleukemia cells has been shown to catalyze proteolysis without the aid of ubiquitin [8]. Therefore, these ATP-dependent proteases seem to be of two types, ubiquitin-dependent and -independent, in spite of their similarities described above. However, McGuire et al. [11] reported that macropain, a proteasome-like protease, is involved

in both ubiquitin-dependent and -independent proteolysis in BHK21/13 cell extracts. This finding indicates the need for studies on whether ubiquitin is necessary for proteasome-catalyzed breakdown of [^{3}H]casein in reticulocyte extracts.

As proteolysis of cyanogen bromide fragments of [^{3}H]casein in reticulocyte extracts was only slightly stimulated by ATP and unaffected by removal of proteasomes (table 2), the multicatalytic protease probably catalyzes only the initial cleavage of large polypeptide chains, and is not responsible for the breakdown of smaller peptides. In fact, the proteolytic products formed from large proteins by the action of proteasomes have been shown to have molecular masses no greater than 2–3 kDa [12]. The peptide fragments produced by the action of proteasomes appeared to be degraded further by the action of other proteases and peptidases, probably in an ATP-independent fashion. Thus, the nonlysosomal cytosolic route of protein breakdown seems to involve multiple enzymes that cause sequential cleavage of proteins to amino acids.

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