Branched-chain-amino-acid-preferring peptidase activity of the lobster multicatalytic proteinase (proteasome) and the degradation of myofibrillar proteins

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The multicatalytic proteinase (MCP or proteasome) is a large proteolytic complex that contains at least five catalytic components: the trypsin-like, chymotrypsin-like, peptidylglutamyl-peptide hydrolase (PGPH), branched-chain-amino-acid-preferring (BrAAP) and small-neutral-amino-acid-preferring activities. We have shown that brief heating of the lobster muscle proteasome activates a proteolytic activity that degrades casein and myofibrillar proteins and is distinct from the trypsin-like, chymotrypsin-like and PGPH components. Here we identify the BrAAP activity as a catalytic component involved in the initial degradation of myofibrillar proteins *in vitro*. This conclusion is based on the following. (1) The BrAAP component was activated by heat-treatment, whereas the other four peptidase activities were

not. (2) The BrAAP and proteolytic activities showed similar sensitivities to cations and protease inhibitors: both were inhibited by 3,4-dichloroisocoumarin, chymostatin, *N*-ethylmaleimide and Mg^{2+} , but were not affected by leupeptin, phenylmethanesulphonyl fluoride or Li^+ . (3) The BrAAP activity was inhibited most strongly by casein substrates and troponin; conversely, the troponin-degrading activity was inhibited by the BrAAP substrate. Another significant finding was that incubation of the heat-activated MCP in the presence of chymostatin resulted in the limited cleavage of troponin-T₂ (45 kDa) to two fragments of 41 and 42 kDa; this cleavage was completely suppressed by leupeptin. These results suggest that under certain conditions the trypsin-like component can cleave endogenous protein.

INTRODUCTION

The multicatalytic proteinase (MCP), or proteasome, is a large highly conserved multi-subunit complex that plays a central role in intracellular proteolysis of eukaryotic cells [for reviews, see Orlowski (1990), Goldberg and Rock (1992), Tanaka et al. (1992), Rechsteiner et al. (1993), and Rivett (1993)]. The complex has at least five activities, which cleave synthetic peptides on the carboxyl side of acidic, basic, hydrophobic, branched-chain and small neutral amino acid residues; these are the peptidylglutamyl-peptide hydrolase (PGPH), trypsin-like, chymotrypsin-like, branched-chain-amino-acid-preferring (BrAAP) and small-neutral-amino-acid-preferring (SNAAP) activities respectively (Orlowski et al., 1993; Cardozo et al., 1994). The proteasome is often isolated from tissues in a latent or basal state and can be activated by a variety of conditions and treatments. For example, low concentrations of SDS and fatty acids activate the PGPH and BrAAP activities (Wilk and Orlowski, 1983; Orlowski and Michaud, 1989; Arribas and Castano, 1990; McDermott et al., 1991; Orlowski et al., 1991, 1993; Pereira et al., 1992b; Yu et al., 1993), which is associated with conformational changes in the complex (Saitoh et al., 1989; Djaballah et al., 1993). Dialysis against water or Tris/HCl buffer lacking EDTA, sulphated glycolipids, polycations, *N*-acetylimidazole or Mg^{2+} also activate the complex (McGuire et al., 1989; Mellgren, 1990; Yu et al., 1991, 1993; Ohkubo et al., 1991; Pereira et al., 1992b). In addition, endogenous activators (Dubiel et al., 1992; Chu-Ping et al., 1992a, 1994; Yukawa et al., 1993) and inhibitors (Li et al., 1991; Chu-Ping et al., 1992b; Li and Etlinger, 1992; Driscoll et al., 1992) of the proteasome have been isolated. These data

suggest that the proteasome is under allosteric regulation and that conformational changes induced by these allosteric modulators alter the catalytic properties of the complex.

The lobster proteasome can assume three distinct catalytic states *in vitro*, which we have termed the basal, SDS-activated and heat-activated MCPs (Mykles and Haire, 1991). The basal MCP is the form purified directly from muscle; the SDS-activated MCP is the form in the presence of 0.03 % SDS; and the heat-activated MCP is the form when the basal MCP is heated at 60 °C for 1 min (Mykles, 1989b; Mykles and Haire, 1991; Clark et al., 1991). The activation with SDS is reversible; when the SDS is removed, the complex reverts to the basal form. In contrast, the activation by heat-treatment is irreversible. If, however, the heat-activated form is incubated briefly with SDS, it can be converted back to the basal form when SDS is removed. The three forms of the enzyme differ in enzymic properties. The basal MCP has relatively high trypsin-like activity and low chymotrypsin-like and PGPH activities (Mykles and Haire, 1991). SDS inhibits the trypsin-like and chymotrypsin-like activities and stimulates the PGPH activity. The heat-activated MCP shows enhanced activity against protein substrates, such as casein and myofibrillar proteins (Mykles, 1989a; Mykles and Haire, 1991). These results suggest that the proteasome plays a role in moulting-induced claw muscle atrophy, during which at least 40 % of the protein is degraded [see Mykles (1992) for review].

Given the importance of the proteasome in intracellular proteolysis, a major focus has been the identification of the catalytic components that cleave endogenous polypeptide substrates. Activities that carry out initial cleavages are especially important, since they may constitute the rate-limiting step in the

Abbreviations used: AP-N, aminopeptidase-N; β NA, β -naphthylamide; BrAAP, branched-chain-amino-acid preferring; DCI, 3,4-dichloroisocoumarin; FITC, fluorescein isothiocyanate; MCP, multicatalytic proteinase (EC 3.4.99.46); MHC, major histocompatibility complex; NEM, *N*-ethylmaleimide; pAB, *p*-aminobenzoate; PGPH, peptidylglutamyl-peptide hydrolase; PMSF, phenylmethanesulphonyl fluoride; pNA, *p*-nitroanilide; SNAAP, small-neutral-amino-acid-preferring; Tn, troponin; Z, benzyloxycarbonyl.

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complete hydrolysis of a protein to peptides and amino acids. Orłowski et al. (1993) have shown that the BrAAP activity is involved in the degradation of endogenous peptides, such as neurotensin and the oxidized B chain of insulin *in vitro*. Our own studies of the lobster proteasome show that the degradation of myofibrillar proteins by the heat-activated MCP is not consistent with the properties of the trypsin-like, chymotrypsin-like or PGPH components, suggesting that heat-treatment induces a fourth catalytic component that degrades proteins (Mykles and Haire, 1991). The present study characterizes the BrAAP and SNAAP activities of the three forms of the lobster proteasome. We used purified troponin as a model substrate, since it is degraded only by the heat-activated form (Mykles and Haire, 1991). The effects of cations, protease inhibitors and protein substrates are consistent with the BrAAP activity being the principal catalytic component involved in the initial hydrolysis of myofibrillar proteins *in vitro*.

EXPERIMENTAL

Materials

Lobsters (*Homarus americanus*) were purchased from local supermarkets. Z-GPALA-pAB, Z-GPALG-pAB and Z-GPAGG-pAB (Z, benzyloxycarbonyl; pAB, *p*-aminobenzoate; one-letter amino acid codes) were gifts from Dr. Marian Orłowski, Mount Sinai School of Medicine. Z-LLE- β NA (β NA, β -naphthylamide), Z-GGL-pNA (pNA, *p*-nitroanilide), α -casein, leupeptin, chymostatin, aminopeptidase N (AP-N), fluorescein isothiocyanate (FITC), 3,4-dichloroisocoumarin (DCI) and *N*-ethylmaleimide (NEM) were purchased from Sigma, Z-GGR- β NA was from Bachem, and phenylmethanesulphonyl fluoride (PMSF) from Boehringer Mannheim. All other reagents were of at least analytical grade and were purchased from Baxter Scientific Products, Sigma, Bio-Rad or United States Biochemical.

The proteasome was purified from lobster striated muscles as described previously (Mykles, 1989b) and stored on ice in Buffer A (20 mM Tris/acetate, pH 7.5, 20 mM KCl, 1 mM EDTA, 1 mM dithiothreitol). Troponin was purified from lobster deep abdominal muscle as described by Ojima and Nishita (1986) and stored either at -45°C in LiCl/glycerol buffer (0.2 M LiCl, 0.5 mM NaHCO_3 , 2.5 mM 2-mercaptoethanol and 50% glycerol) or on ice in LiCl buffer (0.4 M LiCl, 1 mM NaHCO_3 and 5 mM 2-mercaptoethanol). Protein was quantified by fluorescence emission spectroscopy (Avruch and Wallach, 1971).

M.s. analysis

The hydrolysis products of the BrAAP and SNAAP activities were analysed by m.s. Reaction mixtures (200 μl), containing 20 mM Hepes/NaOH (pH 8), 20 μg of heat-activated MCP and either 0.4 mM Z-GPALG-pAB or 0.4 mM Z-GPAGG-pAB, were incubated for 4 days at room temperature. Control reactions contained 20 mM Hepes/NaOH (pH 8) and substrate. Reactions were stopped with 50 μl of 30% (w/v) trichloroacetic acid, dried with a Speed-Vac, dissolved in 50% (v/v) acetonitrile, and analysed with a VG Platform single-quadrupole mass spectrometer equipped with an electrospray interface (Fisons Instruments).

Effects of protease inhibitors on the catalytic properties of lobster proteasome

One series of experiments quantified the effects of DCI on the peptidase and proteinase activities of the basal, SDS-activated and heat-activated MCPs. Reaction mixtures (200 μl) contained

20 mM Hepes/NaOH (pH 8), MCP (10 μg), and Z-GGR- β NA (0.2 mM) or Z-GGL-pNA (0.2 mM), or Z-LLE- β NA (0.2 mM), or Z-GPALA-pAB (0.2 mM) and AP-N (0.05 unit; 1.7 μg of protein); FITC-casein (44 μg ; 118 nmol of FITC/mg of casein); or [^{14}C]methylcasein (10000 c.p.m.; 7670 c.p.m./ μg of casein). The proteasome was heat-activated at 60°C for 1 min as described previously (Mykles, 1989b; Mykles and Haire, 1991). Reactions assaying the activities of the SDS-activated form contained 0.03% SDS. The proteasome was preincubated with DCI for 30 min at room temperature before the addition of substrate. Reactions were incubated for 1 h at room temperature; the generation of products was linear over this incubation interval. Control reactions contained substrate and AP-N (no MCP) or substrate alone. Free β NA was measured by fluorescence emission spectroscopy as described by Mykles and Haire (1991); free pNA and pAB were measured by the diazotization procedure as described by Wilk and Orłowski (1980); trichloroacetic acid-soluble ^{14}C -labelled products were measured as described by Mykles and Haire (1991).

A second series of experiments quantified the effects of leupeptin, chymostatin, PMSF, NEM, LiCl, and MgCl_2 on the trypsin-like, chymotrypsin-like, PGPH, BrAAP and [^{14}C]methylcaseinolytic activities of the complex. The assay procedure was the same as described above, except that the reaction mixtures contained 5 μg of MCP and were incubated for 2 h at room temperature. Reactions for the measurement of proteinase activity contained 20000 c.p.m. of [^{14}C]methylcasein. Basal MCP was used for the assays of trypsin-like and chymotrypsin-like activities; SDS-activated MCP (0.03% SDS included in the reaction mixture) was used for the assay of PGPH activity; and heat-activated MCP was used for the assays of BrAAP and [^{14}C]methylcaseinolytic activities. The MCP was preincubated for 30 min at room temperature with leupeptin, chymostatin, PMSF or NEM before the addition of substrate. LiCl and MgCl_2 were added to reaction mixtures immediately before the addition of substrate.

Competition experiments

One series of experiments quantified the effects of protein substrates on the trypsin-like, BrAAP and PGPH activities. Reaction mixtures (200 μl) contained 5 μg of MCP, 20 mM Hepes/NaOH (pH 8), 0.2 mM peptide substrate, and various amounts (0–50 μg) of α -casein, methylcasein [α -casein methylated as described by Mykles (1989a), except that unlabelled formaldehyde was used], FITC-casein or troponin. Heat-activated MCP and Z-GPALA-pAB were used for the assay of BrAAP activity, SDS-activated MCP (0.03% SDS) and Z-LLE- β NA for the assay of PGPH activity, and basal MCP and Z-GGR- β NA for the assay of trypsin-like activity. Since both the trypsin-like and PGPH activities were inhibited by LiCl (see Table 2), the concentration of LiCl in the reactions containing troponin was kept constant; the final concentrations of the LiCl/glycerol buffer components were 18 mM LiCl, 0.04 mM NaHCO_3 , 0.2 mM 2-mercaptoethanol and 4.6% glycerol. After incubation for 2 h at room temperature, pAB and β NA were quantified as described above. In a separate series of experiments in which free FITC was added to reaction mixtures, we showed that FITC did not interfere with the detection of free pAB and β NA (results not shown).

A second series of experiments quantified the effects of peptide substrates on the degradation of troponin by the heat-activated MCP. Hydrolysis of troponin was quantified by SDS/PAGE, since preliminary experiments showed that incubation of the heat-activated MCP with troponin did not produce tri-

chloroacetic acid-soluble products. Reaction mixtures (50 μ l) contained 5 μ g of heat-activated MCP, 20 mM Hepes/NaOH (pH 8) and 40 μ g of troponin, with or without 0.4 mM Z-GGR- β NA, Z-LLE- β NA or Z-GPALA-pAB; the final concentrations of the LiCl buffer were 50 mM LiCl, 0.1 mM NaHCO₃, and 0.6 mM 2-mercaptoethanol. As shown in Table 2, 50 mM LiCl did not inhibit the BrAAP or [¹⁴C]methylcaseinolytic activities of the heat-activated form. After incubation for 4 h at room temperature, the reactions were stopped by addition of 50 μ l of SDS sample buffer and heating at 90 °C for 3 min. Samples equivalent to 2.5 μ l of the original reaction were separated on discontinuous SDS/10%-polyacrylamide gels and stained with ammoniacal silver (Mykles, 1988).

Effects of inhibitors on troponin degradation

The reaction conditions and gel analysis were the same as described above, except that heat-activated MCP was preincubated for 30 min at room temperature with leupeptin (0.05 mM), chymostatin (0.05 mg/ml), PMSF (1 mM), DCI (0.05 mM) or NEM (0.5 mM) before the addition of substrate; SDS (0.03 %) or MgCl₂ (25 mM) was added immediately before the addition of substrate. Reactions (50 μ l) were incubated for 4 h at room temperature and stopped with 50 μ l of SDS sample buffer.

RESULTS

The basal form of the lobster proteasome hydrolysed both Z-GPALA-pAB and Z-GPAGG-pAB in the presence of AP-N, showing that the complex possesses BrAAP and SNAAP activities (Table 1). Appearance of free pAB was absolutely dependent on the presence of AP-N in the reaction, showing that the initial cleavage by the proteasome is at an internal site. M.s. analysis showed that cleavage occurred at the peptide bond on the carboxyl side of Leu in the BrAAP substrate and of the second Gly in the SNAAP substrate; the masses of the products were 491.3 and 435.0, which are consistent with the expected masses of Z-GPAL and Z-GPAG, respectively. The Pro at the P₃ position is required for the specific hydrolysis of these peptides by the BrAAP and SNAAP activities, respectively (Cardozo et al., 1994). The SNAAP activity was not characterized further, since it was not activated by SDS or heat-treatment (results not shown).

Since both SDS and DCI stimulate the BrAAP and proteolytic activities of the bovine and chicken proteasomes (Pereira et al., 1992a; Cardozo et al., 1992; Strack et al., 1992; Vinitsky et al.,

1992; Orłowski et al., 1993; Yu et al., 1993), we determined the effects of these compounds on the lobster proteasome (Table 1). Neither DCI nor SDS activated the BrAAP activity of the basal MCP. However, heat-treatment stimulated the BrAAP activity (Table 1), as well as the hydrolysis of [¹⁴C]methylcasein (Table 1) and FITC-casein (results not shown). SDS inhibited the trypsin-like and chymotrypsin-like activities but stimulated the PGPH activity, whereas DCI inhibited the chymotrypsin-like, BrAAP and PGPH activities (Table 1).

The above results suggested that the BrAAP component is involved with the hydrolysis of proteins by the heat-activated MCP. To examine this further, we carried out a series of competition experiments, in which the effects of protein substrates on the BrAAP, PGPH and trypsin-like activities were quantified. For all three substrates, the BrAAP activity was inhibited the most (Figure 1a), whereas the trypsin-like activity was inhibited the least (Figure 1c), by increasing amounts of protein; inhibition of the PGPH activity (Figure 1b) was intermediate between the inhibition of the BrAAP and trypsin-like activities. FITC-casein was more effective in suppressing the three peptidase activities than was casein or methylcasein. The inhibition by FITC-casein was not caused by the small amount of free FITC present in the preparation, since addition of free FITC to the reaction mixtures had no effect on the peptidase activities (results not shown).

A second series of competition experiments determined the effects of troponin, a myofibrillar protein that is degraded only by the heat-activated MCP (Mykles and Haire, 1991), on the trypsin-like, chymotrypsin-like, PGPH and BrAAP activities of the complex (Figure 2). The concentration of LiCl in the reaction mixtures was kept low (18 mM) to minimize inhibition of the PGPH activity (see below; LiCl was included in the buffer to prevent precipitation of the troponin). The BrAAP activity was more sensitive to troponin than were the trypsin-like and PGPH activities. In contrast, the chymotrypsin-like activity was not inhibited by troponin.

A third series of competition experiments determined the effects of peptide substrates on the hydrolysis of troponin. Tn-T₂ and Tn-I₁, -I₃ and -I₅ are the major isoforms of troponin-T and troponin-I, respectively, in lobster fast muscle (Mykles, 1985a,b). Tn-T₂ and Tn-I isoforms were hydrolysed in the control reaction containing MCP and troponin, resulting in a decrease in the relative band densities (Figure 3, lane b). We have shown that hydrolysis of these proteins produces four major fragments (12–16 kDa), which migrated at the dye front (Mykles and Haire, 1991). Z-LLE- β NA and Z-GPALA-pAB inhibited the hydrolysis of troponin (Figure 3, lanes d and e), whereas Z-GGR- β NA had no effect (Figure 3, lane c).

Table 1 Effect of DCI on the catalytic activities of the basal, heat-activated and SDS-activated forms of lobster proteasome

Activities were measured as described in the Experimental section in 20 mM Hepes/NaOH (pH 8). Z-GGR- β NA, Z-GGL-pNA, Z-LLE- β NA and Z-GPALA-pAB were used to measure the trypsin-like, chymotrypsin-like, PGPH and BrAAP activities, respectively. The MCP was preincubated for 30 min at room temperature with or without 50 μ M DCI before addition of substrate. Data are presented as means $\pm$ 1 S.E.M.; the S.E.M. is not given if less than 0.01. Units are μ mol of peptide hydrolysed/h per mg of MCP and μ g of [¹⁴C]methylcasein hydrolysed/h per mg of MCP for peptidase and proteinase activities, respectively. The SNAAP activity of the lobster proteasome was low (about 0.02 μ mol/h per mg of MCP) and was not affected by SDS or heat-treatment.

Substrate	Basal		Heat-activated		SDS-activated	
	– DCI	+ DCI	– DCI	+ DCI	– DCI	+ DCI
Z-GGR- β NA	1.57 $\pm$ 0.05	1.44 $\pm$ 0.02	1.30 $\pm$ 0.08	1.40 $\pm$ 0.05	0.04	0.03
Z-GGL-pNA	0.09	0.03	0.08	0.03	0.04	0.02
Z-LLE- β NA	0.03	0.03	0.13 $\pm$ 0.01	0.08	1.89 $\pm$ 0.14	0.91 $\pm$ 0.09
Z-GPALA-pAB	0	0	0.48 $\pm$ 0.05	0.23 $\pm$ 0.02	0	0
[¹⁴ C]Methylcasein	5.86 $\pm$ 0.60	6.73 $\pm$ 1.01	39.80 $\pm$ 2.49	29.11 $\pm$ 4.97	6.04 $\pm$ 0.39	5.77 $\pm$ 0.58

Figure 1 Effect of casein substrates on the BrAAP (a), PGPH (b) and trypsin-like (c) activities of lobster proteasome

Reaction mixtures contained 5 μg of MCP, 0.2 mM peptide substrate, and 0–50 μg of casein (■; C), methyleucine (○; M) or FITC-casein (△; F) (see the Experimental section). Heat-activated MCP was used for the BrAAP assays, SDS-activated MCP for the PGPH reactions, and basal MCP for the trypsin-like reactions.

Figure 2 Effect of troponin on the BrAAP (○), PGPH (△), trypsin-like (■) and chymotrypsin-like (●) activities of lobster proteasome

Reaction mixtures contained 5 μg of MCP, 0.2 mM peptide substrate, and 0–50 μg of troponin purified from lobster deep abdominal muscle (see the Experimental section). Heat-activated MCP was used for the BrAAP and chymotrypsin-like assays, SDS-activated MCP for the PGPH assay, and basal MCP for the trypsin-like assay. The hydrolytic rates at 0 μg troponin were 2.76, 1.15, 1.06 and 0.20 μmol of peptide hydrolysed/h per mg of MCP for the BrAAP, PGPH, trypsin-like and chymotrypsin-like activities, respectively.

Table 2 shows the effects of protease inhibitors and cations on the peptidase and [^{14}C]methyleucine activities of the lobster proteasome. The trypsin-like activity was completely inhibited by leupeptin, whereas the PGPH activity was completely inhibited by chymostatin, LiCl or MgCl_2 . NEM strongly inhibited the chymotrypsin-like, BrAAP and [^{14}C]methyleucine activities. In contrast with the four peptidase activities, LiCl and MgCl_2 had a modest stimulatory effect on the [^{14}C]methyleucine activity.

To characterize better the peptidase activity involved in the degradation of myofibrillar proteins by the heat-activated MCP,

Figure 3 Effect of peptide substrates on the degradation of troponin by heat-activated MCP

Lanes: (a), control reaction containing substrate only; (b), control reaction containing substrate and MCP; (c)–(e), reactions containing substrate, MCP, Z-GGR- β NA (0.4 mM), Z-LLE- β NA (0.4 mM), or Z-GPALA-pAB (0.4 mM), respectively. The final concentration of LiCl in the reactions was 50 mM, which completely inhibits the PGPH activity (see Table 2). Reaction mixtures (50 μl , containing 40 μg of troponin and 5 μg of MCP) were incubated for 4 h at room temperature; 2.5 μl samples of each reaction were separated on a SDS/10% polyacrylamide gel and stained with silver (see the Experimental section). Z-LLE- β NA and Z-GPALA-pAB, which are substrates for the PGPH and BrAAP activities respectively, inhibited troponin degradation (lanes d and e), whereas Z-GGR- β NA, the substrate for the trypsin-like activity (lane c), did not. Positions of molecular-mass standards (kDa) are indicated at the left. The proteasome subunits (25–41 kDa) appear as minor bands in lanes (b)–(e). Abbreviations: Tn, troponin; F, dye front.

the effects of inhibitors on the hydrolysis of troponin were determined. Troponin hydrolysis was completely inhibited by SDS (Figure 4, lane h), strongly inhibited by NEM (Figure 4, lane g), and moderately inhibited by DCI or Mg^{2+} (Figure 4, lanes f and i). A surprising result was that chymostatin had differential effects on the degradation of Tn-T₂ and Tn-I isoforms; the compound strongly inhibited the degradation of Tn-I forms, but did not inhibit the degradation of Tn-T₂ (Figure 4, lane d).

Table 2 Effects of inhibitors on the peptidase and proteinase activities of the lobster proteasome

The basal form was used in reactions for trypsin-like and chymotrypsin-like activities; the SDS-activated form was used for PGPH activity; and the heat-activated form was used for BrAAP and methylcaseinolytic activities (see the Experimental section). Data are presented as means $\pm$ 1 S.E.M.; the S.E.M. is not given if less than 0.01. Units are μ mol of peptide hydrolysed/h per mg of MCP and μ g of [14 C]methylcasein hydrolysed/h per mg of MCP for peptidase and proteinase activities, respectively.

Treatment	Activity				
	Trypsin-like	Chymotrypsin-like	PGPH	BrAAP	[14 C]Methylcasein
None	1.46 $\pm$ 0.01 (100%)	0.25 (100%)	1.19 $\pm$ 0.08 (100%)	2.05 $\pm$ 0.02 (100%)	126.1 $\pm$ 1.2 (100%)
Leupeptin (0.05 mM)	0.01 (1%)	0.22 $\pm$ 0.01 (88%)	0.79 $\pm$ 0.06 (66%)	2.13 $\pm$ 0.02 (104%)	105.8 $\pm$ 0.3 (84%)
Chymostatin (0.05 mg/ml)	0.87 $\pm$ 0.03 (60%)	0.16 (64%)	0.06 $\pm$ 0.01 (5%)	0.57 $\pm$ 0.20 (28%)	45.7 $\pm$ 2.3 (36%)
PMSF (1 mM)	1.39 $\pm$ 0.01 (95%)	0.24 $\pm$ 0.01 (96%)	0.79 $\pm$ 0.13 (66%)	2.02 $\pm$ 0.02 (98%)	120.5 $\pm$ 2.9 (96%)
NEM (0.5 mM)	1.41 $\pm$ 0.02 (97%)	0.02 $\pm$ 0.01 (8%)	0.26 $\pm$ 0.02 (22%)	0.27 $\pm$ 0.01 (13%)	15.2 $\pm$ 0.4 (12%)
LiCl (50 mM)	0.77 $\pm$ 0.02 (53%)	0.26 $\pm$ 0.01 (104%)	0.02 $\pm$ 0.01 (2%)	2.03 $\pm$ 0.03 (99%)	160.3 $\pm$ 2.5 (127%)
MgCl ₂ (25 mM)	0.32 (22%)	0.18 (72%)	0.01 (1%)	1.69 $\pm$ 0.16 (82%)	140.3 $\pm$ 2.6 (111%)

Figure 4 Effect of inhibitors on the degradation of troponin by the heat-activated MCP

Lanes: (a), control reaction containing substrate but no MCP; (b), control reaction containing substrate and MCP but no inhibitor; (c)–(i), reactions containing substrate, MCP, and leupeptin (0.05 mM), chymostatin (0.05 mg/ml), PMSF (1 mM), DCI (0.05 mM), NEM (0.5 mM), SDS (0.03%) or MgCl₂ (25 mM), respectively (see the Experimental section). PMSF, DCI and NEM were preincubated for 30 min at room temperature with MCP before addition of substrate; the other compounds were added immediately before the substrate. Reaction conditions and SDS/PAGE were the same as described in the legend to Figure 3. Hydrolysis of the troponin subunits was inhibited to varying extents by NEM, SDS and MgCl₂; hydrolysis was not inhibited by leupeptin or PMSF. Chymostatin inhibited degradation of the Tn-I isoforms, but had no effect on the hydrolysis of Tn-T₂; two novel proteolytic fragments of Tn-T₂ (arrowheads) accumulated in the reaction. Positions of molecular-mass standards (kDa) are indicated at the left. The proteasome subunits (25–41 kDa) appear as minor bands in lanes (b)–(j). Abbreviation: F, dye front.

Hydrolysis of Tn-T₂ (45 kDa) in the presence of chymostatin produced a pair of fragments (41 and 42 kDa) not generated in the other reactions (Figure 4, lane d, arrowheads). This cleavage was not caused by a contaminating protease, since a reaction containing chymostatin and substrate did not generate the fragments (Figure 5a, lane c). Antibody to Tn-T₂ reacted with these fragments, confirming that they were derived from the cleavage of Tn-T₂ (Figure 5b, lane d). The immunoblot also identified a new isoform of troponin-T (Tn-T₄) with a mass of about 35 kDa. The chymostatin-induced cleavage of Tn-T₂ was completely inhibited by leupeptin; DCI, PMSF, and NEM had no effect (Figure 6).

DISCUSSION

The lobster proteasome contains five catalytic activities distinguished by substrate specificity and sensitivity to SDS, heat-treatment, cations and protease inhibitors. Of the five peptidase activities, only the BrAAP component is activated by heat-treatment, suggesting that this activity is involved with the degradation of myofibrillar proteins *in vitro*. The results of the inhibitor and competition experiments support this conclusion.

The BrAAP activity is strongly inhibited by protein substrates (Figures 1 and 2). Conversely, the troponin-degrading activity is inhibited by Z-GPALA-pAB (Figure 3). The sensitivities of the BrAAP and troponin-degrading activities to protease inhibitors are highly correlated (Tables 1, 2; Figure 4). Both are inhibited by DCI, NEM, SDS and chymostatin, but are insensitive to MgCl₂, LiCl, leupeptin and PMSF (the chymostatin-induced cleavage of Tn-T₂ is discussed below). Since the subunit composition of the lobster proteasome is not altered by SDS or heat-treatment (Mykles, 1989b; D. L. Mykles and M. F. Haire, unpublished work), we conclude that these differences in catalytic properties between the three forms result from conformational changes in the complex. SDS has been shown to induce conformational changes in other proteasomes (Saitoh et al., 1989; Djaballah et al., 1993).

The other catalytic components can be eliminated as playing a significant role in the degradation of myofibrillar proteins *in vitro*. The PGPH activity is completely inhibited by 50 mM LiCl (Table 2), conditions in which there is significant hydrolysis of troponin (Figures 3–6). In addition, we have shown that the SDS-activated MCP, which has high PGPH activity, does not degrade troponin or tropomyosin (Mykles and Haire, 1991).

Figure 5 Effect of chymostatin on the degradation of troponin by heat-activated MCP

Lanes: (a), control reaction containing substrate but no MCP or chymostatin; (b), reaction containing substrate and MCP; (c), reaction containing substrate and chymostatin; (d), reaction containing substrate, MCP and chymostatin. Reaction conditions and SDS/PAGE were the same as described in the legend to Figure 3. (a), Gel stained with silver; (b), immunoblot of the same samples shown in panel (a) using an anti-Tn-T₂ antibody. Generation of two 41–42 kDa degradation products (arrowheads), which were recognized by the anti-Tn-T₂ antibody, only occurred in the reaction mixture containing both MCP and chymostatin. Positions of molecular-mass standards (kDa) are indicated at left. The proteasome subunits (25–41 kDa) appear as minor bands in lanes (b)–(d).

Leupeptin completely suppresses the trypsin-like activity (Table 2), but has no effect on troponin degradation (Figure 4). Conversely, DCI does not inhibit the trypsin-like activity (Table

1), but inhibits troponin hydrolysis (Figure 4). Furthermore, the degradation of troponin is not inhibited by the trypsin-like substrate (Figure 3). Although the chymotrypsin-like activity is not activated by heat-treatment, both the chymotrypsin-like and troponin-degrading activities have similar inhibitor sensitivities (Figure 4, Table 2), and thus it could be argued that the conformation induced by heating is more open and allows access of the protein to the chymotrypsin-like catalytic site. However, the competition experiments show that troponin does not inhibit the chymotrypsin-like activity of the heat-activated MCP (Figure 2).

The effects of proteins on the different peptidase activities of the proteasome are complex, and probably result from a combination of competitive inhibition and allosteric modulation. Casein has no effect on the trypsin-like activity, activates the chymotrypsin-like activity, and inhibits the PGPH activity of the rat liver proteasome (Djaballah et al., 1992). In contrast, all three activities of the human brain proteasome are inhibited by casein, with the trypsin-like activity inhibited the least and the PGPH inhibited the most (McDermott et al., 1991). Casein and BSA inhibit the PGPH activity of the bovine pituitary proteasome, but have no effect on the trypsin-like and chymotrypsin-like activities (Wilk and Orlowski, 1983; Orlowski et al., 1991). These differences can be attributed in large measure to differences in the concentrations and kinds of proteins used. Stimulation of the BrAAP, PGPH and chymotrypsin-like activities of the lobster proteasome occurs at low concentrations of protein, whereas inhibition of the BrAAP, PGPH and trypsin-like activities occurs at higher concentrations (Figures 1 and 2); similar effects of protein concentration have been observed with the bovine proteasome (Wilk and Orlowski, 1983). Casein is more effective than BSA, a relatively poor substrate, in suppressing the bovine proteasome (Wilk and Orlowski, 1983; Orlowski et al., 1991), and FITC-casein is a more effective inhibitor than casein or methylcasein in inhibiting the peptidase activities of the lobster enzyme (Figure 1). The selective inhibition of the trypsin-like activity by FITC-casein (Figure 1) may result from negative allosteric effects, since the trypsin-like activity does not appear to have a significant role in the hydrolysis of proteins. The quantification of allosteric and/or competitive effects on the kinetic properties is an important area for future studies.

One surprising result was the activation of a troponin-T₂-cleaving component of the heat-activated MCP by chymostatin. Although we have not characterized this activity completely, it appears that the trypsin-like component is responsible. Leupeptin

Figure 6 Effect of protease inhibitors on the chymostatin-induced degradation of troponin-T₂ by heat-activated MCP

Lanes: (a), reaction containing troponin and MCP only; (b), reaction containing troponin, MCP and chymostatin; (c)–(f), reactions containing troponin, MCP, chymostatin, and leupeptin (c), DCI (d), PMSF (e) or NEM (f). Reaction conditions and SDS/PAGE were the same as described in the legend to Figure 3. Chymostatin-induced cleavage of Tn-T₂ to the two fragments (arrowheads) was completely inhibited by leupeptin. The gel was overstained to accentuate staining of the proteolytic fragments.

is the only compound tested that completely inhibits both the chymostatin-induced cleavage of troponin-T₂ and the trypsin-like activity (Table 2, Figure 6). In addition, the trypsin-like activity is active at the concentrations of chymostatin and LiCl in the reaction mixtures (Table 2). Since the trypsin-like component is unaffected by the heat-treatment, we hypothesize that a conformational change induced by chymostatin allows access of troponin-T₂ to the trypsin-like catalytic site. It is possible, however, that troponin-T₂ cleavage is mediated by an entirely new leupeptin-sensitive component. The production of two fragments in approximately equal amounts suggests that the troponin-T₂ contains two cleavage sites which are susceptible to the chymostatin-induced activity, but only one of the sites in a single polypeptide is cleaved.

It is unlikely that chymostatin is inhibiting the further hydrolysis of troponin-T₂, thus resulting in the accumulation of the 41–42 kDa fragments. These fragments are never observed when proteasome is incubated with troponin in the absence of chymostatin (Figures 5 and 6). More compelling is the fact that leupeptin inhibits the generation of these fragments (Figure 6), even though leupeptin has no effect on the degradation of troponin-T₂ (Figure 4).

The BrAAP component of the lobster proteasome appears to be an important catalytic activity for the initial cleavages of myofibrillar proteins by the heat-activated form *in vitro*. It remains to be determined what role this component plays in the degradation of endogenous proteins in the cell. Increased hydrophobicity appears to be a signal for the selective degradation of oxidatively modified haemoglobin by the proteasome from human erythrocytes (Pacifci et al., 1993). The BrAAP component may also be involved in the processing of proteins for presentation by MHC (major histocompatibility complex) I. Interferon- γ changes the subunit composition and catalytic properties of the proteasome; these proteasomes contain two subunits, LMP2 and LMP7, that are encoded in the MHC II region of the mammalian genome and show enhanced cleavage of substrates at leucine and other hydrophobic residues, as well as at asparagine and basic residues (Driscoll et al., 1993; Gaczynska et al., 1993; Boes et al., 1994; Aki et al., 1994). Since the peptides that bind to MHC I usually end with hydrophobic or basic residues, selective activation of the BrAAP, chymotrypsin-like and trypsin-like components would increase the efficiency at which peptides derived from intracellular proteins are presented by the MHC I pathway (Goldberg and Rock, 1992). Analysis of the effects of subunit composition, allosteric regulators and selective inhibitors on the BrAAP activity will provide important insights into the role of this component in intracellular proteolytic processes.

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