Evidence for the Presence of Five Distinct Proteolytic Components in the Pituitary Multicatalytic Proteinase Complex. Properties of Two Components Cleaving Bonds on the Carboxyl Side of Branched Chain and Small Neutral Amino Acids†

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ABSTRACT: Initial studies on the specificity of the multicatalytic proteinase complex (MPC; EC 3.4.99.46) led to the identification of three distinct proteolytic components designated as trypsin-like, chymotrypsin-like, and peptidylglutamyl-peptide hydrolyzing, all sensitive to inactivation by 3,4-dichloroisocoumarin (DCI), a general serine proteinase inhibitor. The three components cleave the peptidyl–arylamide bonds in the model synthetic substrates, Z-(D)-Ala-Leu-Arg-2-naphthylamide, Z-Gly-Gly-Leu-*p*-nitroanilide, and Z-Leu-Leu-Glu-2-naphthylamide, respectively. We report here evidence for the presence in the MPC of two additional distinct components, neither of them capable of cleaving the three model substrates. One of these components cleaves the Leu–Gly and the Leu–Ala bonds in the substrates Cbz-Gly-Pro-Ala-Leu-Gly-*p*-aminobenzoate and Cbz-Gly-Pro-Ala-Leu-Ala-*p*-aminobenzoate, respectively, and is activated by treatment of the MPC with DCI, *N*-ethylmaleimide, Mg^{2+}, Ca^{2+}, and low concentrations of sodium dodecyl sulfate and fatty acids. This component is apparently identical with the previously identified DCI-resistant component of the MPC that cleaves preferentially bonds on the carboxyl side of branched chain amino acids in natural peptides including neurotensin and proinsulin [Cardozo, C., Vinitsky, A., Hidalgo, M. C., Michaud, C., & Orlowski, M. (1992) *Biochemistry 31*, 7373–7380]. It is probably also identical with the component proposed to be the main factor responsible for the caseinolytic activity [Pereira, M. E., Nguyen, T., Wagner, B. J., Margolis, J. W., Yu, B., & Wilk, S. (1992a) *J. Biol. Chem. 267*, 7949–7955]. The designation "branched chain amino acid preferring" (BrAAP) is proposed for this component. The second component cleaves peptide bonds between the small neutral amino acids Ala-Gly and Gly-Gly in the substrates Cbz-Gly-Pro-Ala-Ala-Gly-*p*-aminobenzoate and Cbz-Gly-Pro-Ala-Gly-Gly-*p*-aminobenzoate, respectively. This component is sensitive to inactivation by DCI, *N*-ethylmaleimide, and organic mercurials, but unlike the BrAAP it is significantly activated neither by Mg^{2+} or Ca^{2+} nor by fatty acids or sodium dodecyl sulfate. The designation "small neutral amino acid preferring" (SNAAP) is proposed for this component. Both components are sensitive to inhibition by the peptidyl-aldehydes *N*-acetyl-Leu-Leu-norleucinal (Ac-LLnL-CHO; calpain inhibitor I) and *N*-acetyl-Leu-Leu-methioninal (Ac-LLM-CHO; calpain inhibitor II) but are resistant to inhibition by Z-LLF-CHO, a potent inhibitor of the chymotrypsin-like activity. The data are consistent with the presence in the MPC of at least five distinct proteolytic components capable of hydrolyzing peptide bonds on the carboxyl side of basic, acidic, aromatic, branched chain, and small neutral amino acids. The synthesis of model substrates that can be used for determination of activity of the latter two components is described.

The multicatalytic proteinase complex (MPC),[1] also referred to as the proteasome, is a high molecular mass particle (~700 kDa; 19S) with a cytoplasmic and nuclear localization, composed of 13–15 low molecular weight (21 000–34 000) nonidentical subunits [for reviews, see Orlowski (1990) and Rivett (1989)]. The subunits are organized in four stacked rings, each composed of 6–8 subunits surrounding a central, water-filled canal. The MPC was found in all eukaryotic cells studied and constitutes up to 0.5–1% of protein in cell homogenates. A structurally simpler form composed of only two subunits was found in archaebacteria (Dahlmann et al., 1989). Preservation of the MPC in evolution is indicated by immunological cross-reactivity between complexes from species as diverse as yeast, *Drosophila*, and mammalian species.

Accumulating evidence indicates that the complex constitutes the catalytic core of a large 26S multisubunit cytoplasmic particle, believed to be necessary for the ubiquitin-dependent pathway of intracellular proteolysis (Rechsteiner, 1987; Hough et al., 1987; Waxman et al., 1987; McGuire et al., 1988; Eytan et al., 1989; Driscoll & Goldberg, 1990). This pathway was also reported to be involved in the degradation of cyclins and could thereby contribute to the regulation of the mitotic cycle of the cell (Glotzer et al., 1991). Other reports have shown the importance of the MPC for cell proliferation and viability.

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[1] Abbreviations: Conventional one- or three-letter abbreviations are used for amino acids; APMSF, (4-amidinophenyl)methanesulfonyl fluoride; Boc, *tert*-butoxycarbonyl; BrAAP, branched chain amino acid preferring; CF_3COOH, trifluoroacetic acid; CH_3CN, acetonitrile; ChT-L, chymotrypsin-like; DCI, 3,4-dichloroisocoumarin; DFP, diisopropylfluorophosphate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; Me_2SO, dimethyl sulfoxide; MPC, multicatalytic proteinase complex; nL, norleucine; 2NA, 2-naphthylamide; pNA, *p*-nitroaniline; pAB, *p*-aminobenzoate; PCMPS, *p*-mercuriphenylsulfonate; PGPH, peptidylglutamyl-peptide hydrolyzing; peptide-CHO, peptidyl-aldehyde; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SNAAP, small neutral amino acid preferring; T-L, trypsin-like; Z or Cbz, benzyloxycarbonyl.

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Table I: Analytical Data for Synthetic Substrates[a]

peptide	retention time (min)	mp (°C)	amino acid composition[b]			
			Ala	Gly	Leu	Phe
(1) Z-Gly-Gly-Gly-pAB	15.40	269–271		2.87		
(2) Z-Gly-Pro-Ala-Leu-Ala-pAB	21.18	262–264	1.7	1.0	.88	
(3) Z-Gly-Pro-Ala-Leu-Gly-pAB	20.24	190–191	1.07	2.0	1.06	
(4) Z-Gly-Pro-Ala-Ala-Gly-pAB	17.24	217–219	2.1	2.0		
(5) Z-Gly-Pro-Ala-Gly-Gly-pAB	16.31	228–230	1.03	3.0		
(6) Z-Gly-Pro-Ala-Ala-Phe-pAB	22.12	186–188	2.15	1.0		0.91

[a] HPLC separation of peptides was carried out as described in Materials and Methods at a solvent flow rate of 1 mL/min. Amino acids composition of the substrates were determined after acid hydrolysis using the *o*-phthalaldehyde method as described in Materials and Methods. The amount of glycine in compound 1 was determined in a weighed sample. The concentration of amino acids in the other substrates was determined in relation to the number of glycine residues in each of the compounds. [b] Proline residues were not determined.

Thus, disruptions in either one of two genes encoding subunits of the MPC arrests proliferation of yeast cells (Fujiwara et al., 1990), and yeast mutants with low chymotrypsin-like activity show accumulation of ubiquitin protein conjugates and a decreased capacity for protein degradation (Heinemeyer et al., 1991; Richter-Ruoff et al., 1992). A structurally intact proteasome seems therefore vital for cell survival.

Several recent reports have implicated the MPC in antigen processing. Low molecular weight peptides (designated as LMPs) structurally similar to, if not identical with, MPC subunits were found to be associated with the major histocompatibility (MHC) class I molecules (Monaco & McDevitt, 1982), and two polymorphic subunits of the LMP complex, homologous to MPC subunits, were found to map in the class II region of the MHC (Martinez & Monaco 1991; Glynne et al., 1991; Kelly et al., 1991; Ortiz-Navarette et al., 1991). Furthermore, the subunit patterns of the LMP complex and the MPC are very similar and the two complexes display serological cross-reactivity (Brown et al., 1991). Collectively these observations indicate that the MPC could be responsible for cytoplasmic processing of MHC class I antigen molecules.

Although research on the biochemistry and function of the MPC has advanced in recent years, basic questions such as how many of the subunits are proteolytically active and what specific activities are expressed by components of the complex remain to be answered. Dissociation of the complex leads to complete loss of proteolytic activity, and attempts to isolate proteolytically active subunits have consistently failed. Accordingly, the integrity of the MPC seems necessary for expression of proteolytic activity, and studies on the specificity of the components must be carried out on the native, intact complex. Initial experiments on the MPC isolated from bovine pituitaries led to the identification of three distinct proteolytic activities, each associated with a different component of the complex. The three activities cleave bonds on the carboxyl side of basic, acidic, and hydrophobic amino acids, (Wilk & Orlowski, 1980, 1983; Orlowski & Wilk, 1981, 1988). On the basis of the structure of the amino acid residue in the P_1 position, the three activities were designated as trypsin-like (cleavage on the carboxyl side of basic residues), chymotrypsin-like (cleavage on the carboxyl side of hydrophobic residues), and peptidylglutamyl-peptide hydrolyzing (cleavage on the carboxyl side of glutamyl residues). We report here evidence for the presence of two additional components, one of them cleaving preferentially peptide bonds on the carboxyl side of branched amino acids and the other cleaving bonds on the carboxyl side of small neutral amino acids alanine and glycine. The synthesis of substrates that can be used for the determination of activities of the two components is also reported.

MATERIALS AND METHODS

Materials

Frozen bovine pituitaries were obtained from Pel Freeze Inc. (Rogers, AR). DCI, *o*-phthalaldehyde, Cbz-Leu-Leu-Glu-2NA, leupeptin, antipain, chymostatin, pepstatin, and Boc- and Z-amino acid derivatives were obtained from Sigma Chemical Co. (St. Louis, MO). Cbz-Gly-Pro was purchased from Bachem (Torrance, CA). *N*-Acetyl-Leu-Leu-nLeu-CHO, *N*-acetyl-Leu-Leu-Met-CHO, and diisopropyl fluorophosphate were obtained from Calbiochem (La Jolla, CA). Cbz-Leu-Leu-Phe-CHO (Vinitsky et al., 1992), Z-(D)-Ala-Leu-Arg-2NA, and Cbz-Gly-Gly-Leu-pNA were synthesized as described previously (Wilk & Orlowski, 1980, 1983). All other reagents of the highest purity were obtained from Fisher Scientific Co. (Fair Lawn, NJ) or from Sigma Chemical Co. (St. Louis, MO).

The MPC was isolated from bovine pituitaries as previously described (Orlowski & Michaud, 1989). Aliquots of the enzyme (1.0 mL; about 0.5 mg of protein) obtained after the last purification step were frozen at −20 °C. The enzyme samples were thawed before use for experiments. Aminopeptidase N (EC 3.4.11.2) was purified from hog kidneys by the method of Pfleiderer (1970) and freed from the contaminating metalloendopeptidase (EC 3.4.24.11) as described previously (Almenoff & Orlowski, 1983).

Methods

Synthetic and Analytical Procedures. Several peptide substrates containing a benzyloxycarbonyl group at the N-terminus and a pAB at the C-terminus were synthesized. The presence of the aromatic amine at the C-terminus facilitated determination of the activity in a coupled enzyme assay in the presence of excess aminopeptidase N. All substrates were synthesized in solution by stepwise elongation from the C-terminus. *N*-Hydroxysuccinimide esters of Boc-amino acids prepared by the method of Anderson et al. (1964) were used for formation of peptide bonds. The synthesis of amino acid arylamides of pAB and elongation of the peptide chain was carried out as described previously (Orlowski et al., 1983). In the final step, the amino termini in peptides 2–6 (Table I) were blocked in a reaction with the *N*-hydroxysuccinimide ester of Z-Gly-Pro and in peptide 1 with the *N*-hydroxysuccinimide ester of Z-Gly. Products were purified by crystallization from ethyl acetate, chloroform, or ethanol–water. Purity of the synthesized peptides was verified by HPLC on a C_{18} reverse-phase μBondapack column 3 × 0.39 cm (Waters Assoc.) and by amino acid analysis. Elution from the HPLC column was carried out with a linear gradient established between an aqueous solution of 0.1% CF_3COOH

Table II: Activity of the MPC toward Synthetic Substrates in the Presence and Absence of Aminopeptidase N (AP-N)[a]

substrate	concn (mM)	activity (μmol/(mg·h)) no additions	activity (μmol/(mg·h)) +AP-N	activity increase (μmol/(mg·h))
(1) Z-Gly-Gly-Leu-↓pNA	0.4	10.8	10.8	0.0
(2) Z-Gly-Gly-Phe-↓pAB	1.0	4.79	4.80	0.0
(3) Z-Leu-Leu-Glu-↓2NA	0.64	25.1	25.1	0.0
(4) Z-(D)Ala-Leu-Arg-↓2NA	0.4	5.96	5.96	0.0
(5) Z-Gly-Gly-Gly-pAB	1.0	0.00	0.00	0.0
(6) Z-Gly-Pro-Ala-Ala-↓Phe-↓pAB	1.0	3.61 (92)	3.93	0.32
(7) Z-Gly-Pro-Ala-Leu-↓Gly-pAB	1.0	0.06 (1)	4.59	4.53
(8) Z-Gly-Pro-Ala-Leu-↓Ala-↓pAB	1.0	0.41 (9)	4.77	4.36
(9) Z-Gly-Pro-Ala-Ala-↓Gly-pAB	0.8	0.077 (4)	2.08	2.00
(10) Z-Gly-Pro-Ala-Gly-↓Gly-pAB	1.0	0.03 (1)	2.55	2.52

[a] Enzyme activities were determined as described in Materials and Methods in 0.1 M Tris-HCl, pH 8.2. Data are mean values of two to four separate determinations. Controls in which either the complex or aminopeptidase N was omitted were also carried through the procedure. Products of enzymatic hydrolysis were isolated by HPLC as described in Materials and Methods, and their amino acid composition was determined after acid hydrolysis. Main cleavage sites are indicated by on-line arrows. Minor cleavage sites are indicated by superscripted arrows. Values in parentheses give the fraction of the substrate cleaved directly between the C-terminal amino acid and the aromatic amine as percent of the total amount of substrate degraded as measured in the presence of aminopeptidase N.

and CH_3CN containing 0.1% CF_3COOH. The initial concentration of CH_3CN was 10%, and its concentration was linearly increased to 60% during 20 min. The latter concentration of CH_3CN was maintained for an additional 5 min before the column was reequilibrated with 10% CH_3CN and 0.1% of TFA. All peptides gave single peaks in HPLC, and amino acid analyses showed the expected amino acid composition. Retention times, melting points, and amino acid composition of the peptides are given in Table I.

Determination of Enzyme Activities. The activities of the trypsin-like (T-L), chymotrypsin-like (ChT-L), and peptidylglutamyl-peptide hydrolyzing (PGPH) were determined as described previously (Wilk & Orlowski, 1980, 1983). Substrate concentrations were 0.4 mM Cbz-D-Ala-Leu-Arg-2NA for determination of the T-L activity, 0.4 mM Cbz-Gly-Gly-Leu-pNA for determination of the ChT-L activity, and 0.64 mM Cbz-Leu-Leu-Glu-2NA for measurements of the PGPH activity. Incubation of the complex with these substrates led to the release of the aromatic amine that could be determined in a diazotization reaction (Bratton & Marshall, 1939; Goldbarg & Rutenburg 1958). Incubation of the MPC with several substrates listed in Table I did not lead to the release of significant amounts of the aromatic amine, but cleavage of these substrates was indicated by the finding that addition to the reaction mixtures of aminopeptidase N led to the appearance of free pAB. Activity toward these substrates was therefore determined in a coupled enzyme reaction in the presence of excess aminopeptidase as shown below using as an example Cbz-Gly-Pro-Ala-Leu-Ala-pAB as the substrate:

$$\text{Cbz-Gly-Pro-Ala-Leu-Ala-pAB} \xrightarrow{\text{MPC}} \text{Cbz-Gly-Pro-Ala-Leu} + \text{Ala-pAB}$$

$$\text{Ala-pAB} \xrightarrow{\text{aminopeptidase N}} \text{Ala} + \text{pAB}$$

In the first reaction the MPC cleaves in a rate-limiting step the Leu–Ala bond of the substrate, and in the second reaction aminopeptidase present in excess releases from Ala-pAB free pAB which is then determined after diazotization. Reaction mixtures contained substrate, Tris-HCl (0.1 M, pH 8.2), MPC, and excess aminopeptidase N (20 μg of protein) in a final volume of 0.25 mL. Reactions were initiated at 37 °C by addition of MPC (2.5–10 μg of protein). After a 15–30-min incubation, reactions were terminated by addition of an equal volume of 10% trichloroacetic acid and diazotization was carried out as previously described (Wilk & Orlowski, 1980, 1983). Controls in which either the MPC or the aminopeptidase was omitted were also carried through the procedure. Activity is expressed in units as the number of micromoles of product generated per hour. Specific activity is expressed in units per milligram of protein.

Identification of Degradation Products. For identification of degradation products derived from cleavage of synthetic substrates, 250 nmol of the peptide was incubated for 3–4 h with the native complex (about 10 μg of protein) in 0.25 mL of 0.01 M Tris-HCl (pH 8.0), and the reaction was terminated by addition of glacial acetic acid (10 μL). Products of degradation were separated on a reverse-phase C_{18} μBondapack column (Waters Assoc.) under conditions described under *Synthetic and Analytical Procedures*. Emerging peaks were collected, and after removal of the solvents under nitrogen, the peptides were hydrolyzed in evacuated tubes in 6 N HCl at 110 °C for 24 h. After removal of HCl the amino acids were dissolved in 0.5 M sodium borate buffer, pH 10.4, and analyzed fluorometrically by reaction with *o*-phthalaldehyde (OPA) (Roth, 1971) as previously described (Cardozo et al., 1992).

Inhibition Kinetics. One microliter of a 1.0 mM solution of 3,4-dichloroisocoumarin in dimethyl sulfoxide (Me_2SO) was added to 99 μL of an enzyme solution containing about 0.5 mg of protein/mL in 0.01 M Tris–EDTA buffer, pH 7.5. The mixtures were incubated at 26 °C, and aliquots containing 1–10 μg of protein were transferred at various time intervals to reaction mixtures at 37 °C (final volume 0.25 mL) for determination of activity as described under *Determination of Enzyme Activities*. The final concentration of Me_2SO did not exceed 4%. The pseudo-first-order inactivation rate constants were obtained from plots of ln (v_t/v_o) versus time.

RESULTS

The results of examination of the activity of the MPC toward a series of synthetic substrates in the presence and absence of aminopeptidase N are summarized in Table II. Cleavage of substrates 1–4 is catalyzed by the previously identified three components of the complex designated as ChT-L (substrates 1 and 2), PGPH (substrate 3), and T-L (substrate 4) (Wilk & Orlowski, 1980, 1983; Orlowski & Wilk 1981). With each of the substrates, hydrolysis occurs between the amino acid in the P_1 position and the aromatic amine. A common property of the three activities is their sensitivity to inactivation by DCI, a mechanism-based general serine proteinase inhibitor

(Harper et al., 1983; Harper & Powers, 1985). Each of the components, however, is inactivated with a distinctly different second-order rate constant (see below). Additional properties that characterize the three activities are inhibition of the ChT-L activity by Z-Gly-Gly-Leu-CHO (Wilk & Orlowski, 1983) and Z-Leu-Leu-Phe-CHO (K_i of 0.46 μM; Vinitsky et al., 1992), inhibition of the T-L activity by leupeptin (K_i = 1.1 μM), and sensitivity to inhibition of the PGPH activity to low concentrations of proteins (Orlowski et al., 1991). Another characteristic of the PGPH component is its activation by low concentrations of SDS and fatty acids and a sigmoidal relationship of plots of velocity versus substrate concentration. Addition of aminopeptidase N to incubation mixtures containing the MPC and either one of these substrates did not increase the amount of reaction products generated, indicating that cleavage occurs exclusively between the C-terminal amino acid and the aromatic amine. It is notable that substrate 5 containing three Gly residues was resistant to hydrolysis, indicating that the Gly–arylamide bond in this substrate is not cleaved by the complex and that in this short peptide the Gly–Gly bond is also resistant to hydrolysis. Similarly, previous experiments (Wilk & Orlowski, 1980) have shown that related substrates containing arylamide bonds between either an Ala or Ser residue and an aromatic amine were very slowly hydrolyzed, indicating that bonds between small neutral amino acids and an aromatic amine in short peptides are poor substrates for the MPC.

A quite different pattern emerged when the length of the peptide chain was increased by introduction of two additional amino acid residues. Thus, analysis of reaction products generated during incubation of the MPC with Z-Gly-Pro-Ala-Ala-Phe-pAB, a substrate containing a Phe residue in the P_1 position (substrate 6, Table II), showed that in addition to cleavage between the Phe residue and the aromatic amine some hydrolysis also occurred at the adjacent Ala–Phe bond. Examination of the effect of DCI on the rate of reaction at the two sites showed that, whereas hydrolysis of the Phe–pAB bond was susceptible to inactivation by this inhibitor, hydrolysis of the adjacent Ala–Phe bond was much more resistant to inactivation. Furthermore, whereas cleavage of the Phe–pAB bond was inhibited by the peptidyl-aldehyde Z-LLF-CHO, the rate of hydrolysis of the Ala–Phe bond was not affected by this inhibitor (data not shown). These results indicated that the Phe–pAB bond is cleaved by the ChT-L activity, and that hydrolysis of the Ala–Phe bond might be catalyzed by a component or components having properties different from the ChT-L activity.

In order to study further this possibility, we examined the reactions of the MPC toward four additional new synthetic substrates (substrates 7–10, Table II). The selection of amino acid sequences in these substrates was guided by the aim to reduce the extent of the reaction between the C-terminal amino acid and the aromatic amine by introducing at this site either an Ala or a Gly residue and minimizing or eliminating thereby cleavage of the amino acid–arylamide bonds by the ChT-L activity. On the basis of the amino acid composition, the four substrates fall into two categories. Substrates 7 and 8 contain the branched chain amino acid leucine in position P_1, whereas substrates 9 and 10 contain the small neutral amino acids glycine or alanine in the same position. The synthesis of the Leu-containing substrates was motivated by previous findings that a component of the complex, resistant to inactivation by DCI, cleaved preferentially bonds on the carboxyl side of branched chain amino acids in natural peptides and proteins (Cardozo et al., 1992). Therefore, the two Leu-containing substrates together with those containing small neutral amino acids at the cleavage sites, could be used to ascertain whether cleavage of bonds on the carboxyl side of branched chain and small neutral amino acids is catalyzed by a single component (for example the BrAAP) or whether these reactions are catalyzed by two distinct components. Each of the extended substrates contained a Pro residue in the P_3 position. The introduction of this residue was based on the finding that cleavage after a branched amino acid residue in the P_1 position seemed to be promoted by the presence of a Pro residue in the P_3 position as shown by the pattern of cleavage in neurotensin and proinsulin (Cardozo et al., 1992).

As shown in Table II, with the exception of substrate 8, these sequence changes virtually eliminated cleavage of bonds between the C-terminal amino acid and the aromatic amine and increased hydrolysis of the bonds between the two adjacent amino acids. Only about 1–4% of the overall hydrolysis of substrates 7, 9, and 10 and 9% of the overall cleavage of substrate 8 could be attributed to direct hydrolysis of the amino acid–arylamide bonds, whereas the bulk of the reaction occurred between the two adjacent amino acids. As a result, the introduction of aminopeptidase N into the incubation mixtures became necessary for detection of bond hydrolysis in these peptides.

The effect of inhibitors on the activity of the MPC toward the two classes of substrates is summarized in Table III. Neither leupeptin, a potent and selective inhibitor of the T-L activity (K_i = 1.1 μM) nor Z-LLF-CHO (K_i = 0.46 μM), chymostatin, or DFP, all inhibitors of the ChT-L-activity, affected degradation of the four substrates even at the high concentrations used. Antipain, an inhibitor of the T-L activity, seemed to exert a weak inhibitory effect on degradation of the Leu-containing substrates but was without effect on degradation of substrates with a Gly or Ala in the P_1 position. Both Z-LLnL-CHO and Z-LLM-CHO markedly inhibited activities toward all four substrates, whereas pepstatin, DFP, PMSF, and APMSF had no effect. The thiol-blocking agents, iodoacetic acid and iodoacetamide, weakly inhibited the activity toward all four substrates, but PCMPS strongly inhibited the activity toward the substrates with Ala or Gly in the P_1 position, but less so the activity toward the Leu-containing substrates. Of interest was the finding that *N*-ethylmaleimide activated cleavage of the Leu-containing substrates but weakly inhibited cleavage of the small neutral amino acid-containing substrates. A markedly different effect on activities toward the two classes of substrates was observed with DCI. This inhibitor greatly activated cleavage of the Leu-containing substrates but inhibited cleavage of the small neutral amino acid-containing substrates. Of the other tested compounds, hemin inhibited the activities toward all four substrates, whereas Mg^{2+}, at the concentrations used, weakly activated cleavage of all four substrates. Both ATP and Mg + ATP inhibited activity with all four substrates.

The inhibitor studies summarized in Table III suggested that the two classes of substrates, one containing Leu residues in the P_1 position, the other containing an Ala or Gly residue in the same position, could be cleaved by two different components of the MPC. This possibility was strongly supported by the finding of contrasting effects of *N*-ethylmaleimide and DCI on the reaction of the MPC with the two types of substrates. We proceeded therefore to examine this possibility in greater detail. Table IV shows the second-order rate constants of inactivation by DCI of the the activity toward the two classes of substrates and a comparison of these data with those obtained for the ChT-L, T-L, and PGPH activities

Table III: Effect of Inhibitors on the Activity of the Pituitary Multicatalytic Proteinase Complex with Four Different Substrates[a]

inhibitor	concn (mM)	substrates			
		Z-GPALA-pAB	Z-GPALG-pAB	Z-GPAAG-pAB	Z-GPAGG-pAB
none		4.87 (100)	3.92 (100)	2.05 (100)]	2.03 (100)
(1) leupeptin	0.02	4.43 (91)	3.37 (86)	2.11 (103)	2.13 (105)
(2) chymostatin	0.05	4.19 (86)	3.92 (100)	2.23 (109)	2.11 (104)
(3) Z-LLF-CHO	0.02	4.53 (93)	3.61 (92)	2.21 (108)	2.21 (109)
(4) antipain	0.05	4.29 (88)	3.21 (82)	2.05 (100)	1.97 (97)
(5) Z-LLnL-CHO	0.02	2.92 (60)	2.43 (62)	0.82 (40)	0.93 (46)
(6) Z-LLM-CHO	0.02	3.21 (66)	2.00 (51)	1.39 (68)	1.46 (72)
(7) pepstatin	0.05	4.38 (90)	3.84 (98)	1.91 (93)	1.91 (94)
(8) DFP	4.0	4.58 (94)	3.96 (101)	1.97 (96)	1.97 (97)
(9) PMSF	1.0	4.82 (99)	3.96 (101)	1.95 (95)	1.91 (94)
(10) APMSF	1.0	4.58 (94)	3.72 (95)	1.97 (96)	2.05 (101)
(11) iodoacetic acid	1.00	4.63 (95)	3.41 (87)	1.70 (83)	1.56 (77)
(12) iodoacetamide	1.00	4.38 (90)	3.76 (96)	1.93 (94)	1.87 (92)
(13) PCMPS	0.10	4.58 (94)	2.63 (67)	0.39 (19)	0.24 (12)
(14) *N*-ethylmaleimide	1.00	8.52 (175)	5.06 (129)	1.80 (88)	1.71 (84)
(15) DCI	0.01	44.5 (914)	24.4 (622)	1.85 (90)	0.95 (47)
(16) hemin	0.05	3.70 (76)	2.35 (60)	0.88 (43)	0.79 (39)
(17) Mg^{2+}	2.0	6.04 (124)	5.02 (128)	2.48 (121)	2.27 (112)
(18) ATP	4.0	3.26 (67)	2.67 (68)	1.31 (64)	1.32 (65)
(19) Mg^{2+}/ATP	2.0/4.0	3.85 (79)	3.10 (79)	1.72 (84)	1.54 (76)

[a] Incubation mixtures for activity determination contained 0.05 M Tris-HCl buffer (pH 8.2), excess aminopeptidase N (20 μg of protein), substrate (1 mM), and the MPC (4.88–9.76 μg of protein). Incubations were at 37 °C for 30 min. One unit represents 1 μmol of substrate degraded/h. Data represent mean specific activities obtained from two to four determinations expressed in units per milligram of protein. All peptidyl-aldehydes (inhibitors 1–6), pepstatin, and hemin were preincubated with the complex for 60 min at 37 °C. Aliquots of the preincubation mixtures were transferred to reaction mixtures at 37 °C containing buffer and substrate for determination of activity as described in Materials and Methods. Inhibitors 8–10 were preincubated with the complex for 60 min at 26 °C before determination of activity. Inhibitors 11–15 were preincubated with the complex for 15 min at 26 °C before determination of activity. The effect of Mg^{2+} and ATP on activity was determined directly without preincubation. EDTA-containing buffers were used during enzyme purification, and all preparations used for assays contained 10 mM Tris-EDTA, pH 7.5. Conventional one-letter abbreviations are used to designate the sequence of amino acids of the substrates.

Table IV: Inhibition of Components of the Complex by 3,4-Dichloroisocoumarin[a]

component	substrate	[S] (mM)	[I] (μM)	$t_{1/2}$ (min)	$k_{obs}/[I]$ (s^{-1} M^{-1})
chymotrypsin-like	Z-GGL-pNA	0.4	10	7.8	151
PGPHA	Z-LLE-2NA	0.64	10	23	51
trypsin-like	Z-(D)ALR-2NA	0.4	10	57	20
SNAAP	Z-GPAGG-pAB	1.0	20	40	16
SNAAP	Z-GPAAG-pAB	1.0	20	77	8.4
BrAAP	Z-GPALG-pAB	1.0	10	activation	
BrAAP	Z-GPALA-pAB	1.0	10	activation	

[a] The complex was incubated with the indicated concentrations of 3,4-dichloroisocoumarin at 26 °C, and aliquots of the incubation mixture were withdrawn at times 0, 10, 20, 30, 45, and 60 min and assayed for activity at 37 °C with the substrates in 0.1 M Tris-HCl at pH 8.2 as described in Materials and Methods. The second-order inactivation rate constants were obtained from plots of ln (v_t/v_o) versus time. Data are mean values obtained from two determinations. SNAAP refers to the small neutral amino acid preferring component and BrAAP refers to the branched chain amino acid preferring component of the complex.

of the MPC. Consistent with previous observations (Orlowski & Michaud, 1989), among the three initially identified components of the complex the ChT-L activity was the most sensitive to inactivation by DCI (k_{obs}/I of 151 s^{-1} M^{-1}) whereas the T-L component was the least sensitive (k_{obs}/I of 20 s^{-1} M^{-1}). The inactivation of the PGPH component proceeded at a rate (k_{obs}/I of 51 s^{-1} M^{-1}) intermediate to those of the other two components. The rate of inactivation of the activity cleaving bonds between small neutral amino acids in the substrates Z-GPAGG-pAB and Z-GPAAG-pAB was even slower than the rate of inactivation of the T-L component. Contrary to the inactivating effect of DCI on all of the substrates listed above, cleavage of the Leu-containing substrates was greatly accelerated by preincubation of the complex with DCI, a finding consistent with data obtained in the inhibitor studies shown in Table III.

Of some concern was the finding that k_{obs}/I values were different for the two small neutral amino acid substrates Z-GPAAG-pAB and Z-GPAGG-pAB (Table IV). This was inconsistent with the assumption that the two substrates are hydrolyzed by the same component since in such a case a single inactivation rate constant would have been anticipated for both substrates. To explain the cause of this discrepancy, we considered the possibility that the slower rate of inactivation of hydrolysis of Z-GPAAG-pAB compared with that of Z-GPAGG-pAB could have resulted from a partial contribution to the hydrolysis of the former substrate by the DCI-activated, BrAAP component. We therefore examined the effect of DCI on the hydrolysis of Z-GPALG-pAB and both Z-GPAAG-pAB and Z-GPAGG-pAB as a function of incubation time. As shown in Figure 1, preincubation of the MPC with DCI caused a rapid and almost 10-fold activation of hydrolysis of Z-GPALG-pAB (panel A). A similar activation was seen when the hydrolysis of Z-GPALA-pAB was examined under the same conditions (data not shown). When the time course of Z-GPAAG-pAB hydrolysis was examined, a small but significant increase in activity was initially observed followed by a steady decrease in the rate of reaction (panel B). By contrast, preincubation of the complex with DCI resulted in a progressive decrease in the rate of hydrolysis of Z-GPAGG-pAB with no indication of an initial activation phase (panel C). These results were consistent with the assumption that the initial activation of Z-GPAAG-pAB hydrolysis by the DCI-treated enzyme could have resulted from a small contribution to this reaction by the BrAAP component and that the subsequent decrease of the rate of hydrolysis of this substrate was the result of a progressive inactivation by DCI of a distinct small neutral amino acids preferring component (SNAAP). The initial activation by DCI of Z-GPAAG-pAB hydrolysis could therefore explain the lower values of k_{obs}/I for this substrate as compared with those for Z-GPAGG-pAB. The progressive inactivation by

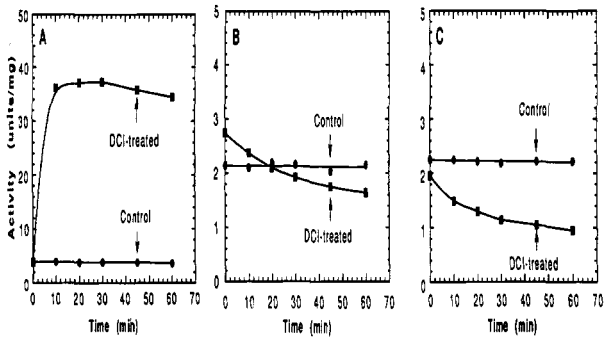

FIGURE 1: Effect of preincubation with DCI on activities of the MPC toward the Leu-containing substrate Z-GPALG-pAB, and the small neutral amino acid containing substrates Z-GPAAG-pAB and Z-GPAGG-pAB. The MPC (0.5 mg of protein/mL) was preincubated at 26 °C with DCI at a final concentration of the inhibitor of 10 μM. Aliquots of the incubation mixture (5–10 μg of protein) were transferred for determination of activity to incubation mixtures at 37 °C containing buffer, substrate, and aminopeptidase N. Activity was determined as described in Materials and Methods. Controls in which the complex was preincubated under the same conditions in the absence of DCI were also carried through the procedure. Panel A shows the effect of preincubation with DCI on hydrolysis of Z-GPALG-pAB, panel B shows the effect on hydrolysis of Z-GPAAG-pAB, and panel C shows the effect on hydrolysis of Z-GPAGG-pAB.

DCI of Z-GPAGG-pAB hydrolysis, with no initial activation phase, suggested that this substrate is hydrolyzed exclusively by a component different from that acting upon the Leu-containing substrates.

The mechanism by which preincubation of the MPC with DCI causes the dramatic increase in hydrolysis of the Leu-containing substrates is not known at this time. Since preincubation with DCI causes inactivation of the ChT-L, T-L, PGPH, and also, albeit slower, the SNAAP component, the possibility was considered that activation of hydrolysis of the Leu-containing substrates could result from the occupation by DCI of the active sites of the other components. However, activation of hydrolysis of the Leu-containing substrates by DCI is already maximal after 10 min (Figure 1), whereas inactivation of the other components of the complex proceeds at a much slower pace. Furthermore, binding of ligands such as leupeptin, chymostatin, antipain, and Z-LLF-CHO to the active sites of the other components of the complex was not observed to result in activation of hydrolysis of the Leu-containing substrates. It is therefore more likely that conformational changes induced by interaction of DCI with the MPC at sites other than the catalytic centers of the other components could be responsible for the activation of the BrAAP component. Such conformational changes could increase the accessibility of the Leu-containing substrates to the active site with a resulting increase in the rate of hydrolysis of these substrates.

The effect of DCI on the kinetic parameters of interaction of the MPC with the two classes of substrates is summarized in Table V. Treatment of the complex with DCI causes a marked decrease in the Michaelis constants for both Leu-containing substrates with a concomitant great increase in V_{max}. This effect is not inconsistent with the interpretation that a conformational change induced by DCI increases the accessibility and thereby the affinity of the Leu-containing substrates to the active site. A rather different result was observed when the effect of DCI on the kinetic parameters of the MPC was examined toward the small neutral amino acid substrates. As expected for an irreversible inhibitor, treatment of the complex with DCI caused a decrease in V_{max} with little change in K_m values. The small decrease in the K_m value for

Table V: Effect of 3,4-Dichloroisocoumarin on the Kinetic Parameters of Reaction with Different Substrates[a]

substrate	range of concn (mM)	enzyme	K_m (mM)	V_{max}
Z-GPALA-pAB	1.6–8.0	native	6.67	26.8
Z-GPALA-pAB	1.6–8.0	DCI-treated	1.3	110
Z-GPALG-pAB	1.6–8.0	native	5.2	19.3
Z-GPALG-pAB	1.6–8.0	DCI-treated	1.9	73.3
Z-GPAAG-pAB	2.4–12.0	native	18.0	27.4
Z-GPAAG-pAB	2.4–12.0	DCI-treated	14.3	22.9
Z-GPAGG-pAB	2.4–12.0	native	6.5	14.2
Z-GPAGG-pAB	2.4–12.0	DCI-treated	7.7	7.4

[a] MPC was preincubated with DCI (10 μM) at 26 °C for 30 min before being used for determination of kinetic constants. In control experiments, the native enzyme was preincubated under the same conditions in the absence of DCI before the measurements. Aliquots of the preincubation mixtures were used for determination of activity by introducing into reaction mixtures containing substrate, buffer, and excess of aminopeptidase N as described in Materials and Methods.

Z-GPAAG-pAB could be interpreted as discussed above by a small contribution of the BrAAP component to the degradation of this substrate.

Several other experiments indicated that cleavage of substrates containing either an Ala or Gly residue in the P_1 position is catalyzed by a component different from that hydrolyzing bonds on the carboxyl side of Leu residues. Figure 2 (panel A) shows the effect of low concentrations of SDS on the activity of the complex toward the two classes of substrates. Thus, SDS greatly activated the hydrolysis of both Leu-containing substrates, although the hydrolysis of Z-GPALA-pAB was markedly more activated (up to 50-fold) than hydrolysis of Z-GPALG-pAB. By contrast, the detergent had no significant effect on cleavage of substrates containing only the small neutral amino acids. A similar activating effect on the hydrolysis of Leu-containing substrates was observed in the presence of lauric acid, (Figure 3, panel B) although the concentrations required for activation were markedly greater than those for SDS.

Additional differences between the activities of the complex toward the two classes of substrates were observed when the effect Mg^{2+} and Ca^{2+} on these activities was examined. Thus, both Mg^{2+} (Figure 3, panel A) and Ca^{2+} (Figure 3, panel B) strongly activated the hydrolysis of the Leu-containing substrates but had only a minor effect on the rate of hydrolysis of the small neutral amino acid-containing substrates. The activating effect was concentration dependent, and rather high concentrations of both ions were needed for full activation.

The pH optima for cleavage of the two classes of substrates were determined in 0.2 M Tris-HCl buffer in the pH range of 7.0–9.0. A rather broad optimum in the pH range of 7.9–8.3 was found for both classes of substrates. All activity determinations described above were therefore carried out at pH 8.2.

DISCUSSION

Early examination of the specificity and properties of the MPC led to the conclusion that this multisubunit intracellular particle contains at least three distinct proteolytic components, cleaving bonds on the carboxyl side of hydrophobic, basic, and acidic amino acid residues (Orlowski & Wilk, 1981; Wilk & Orlowski, 1980, 1983). Evidence has also been presented that the PGPH activity of the complex is expressed by at least two different subunits and that cooperativity between these subunits can be demonstrated (Orlowski et al., 1991; Arribas & Castaño 1990; Djaballah & Rivett, 1992). On the basis of the molecular mass of the MPC of about 700 kDa and the

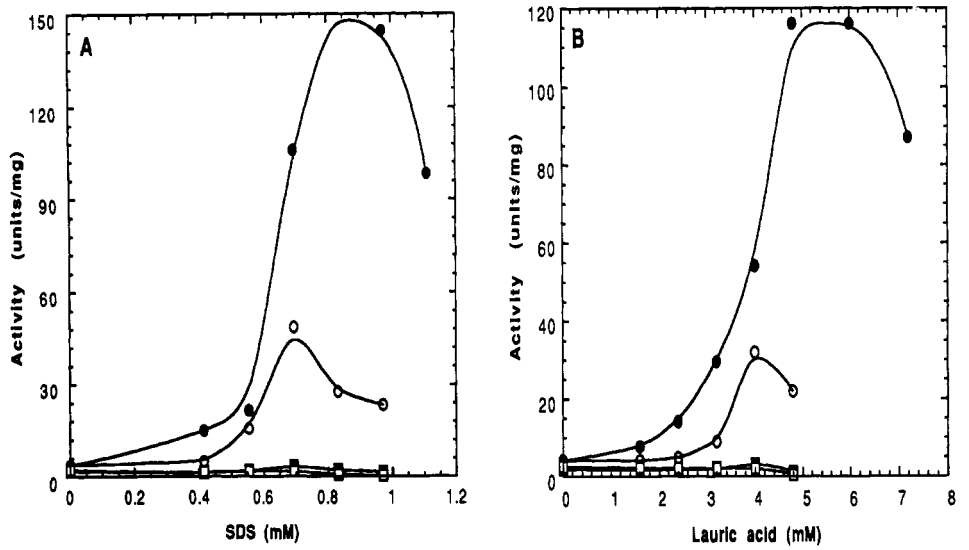

FIGURE 2: Effect of SDS and lauric acid on activities of the complex toward Leu-containing substrates and small neutral amino acid containing substrates. Panel A: Effect of SDS. Incubation mixtures contained the MPC (5–10 μg of protein), 0.1 M Tris-HCl buffer, pH 8.2, aminopeptidase N (20 μg of protein), 1.0 mM substrate, and the indicated concentrations of SDS, in a final volume of 0.25 mL. Incubations were for 30 min at 37 °C. Activities were determined as described in Materials and Methods and are expressed in units per milligram of protein. Controls in which the complex or aminopeptidase N were omitted were also carried through the procedure. Closed circles, open circles, closed squares, and open squares refer to reactions with Z-GPALA-pAB, Z-GPALG-pAB, Z-GPAAG-pAB, and Z-GPAGG-pAB, respectively. Panel B: Conditions of the assay were the same as in panel A.

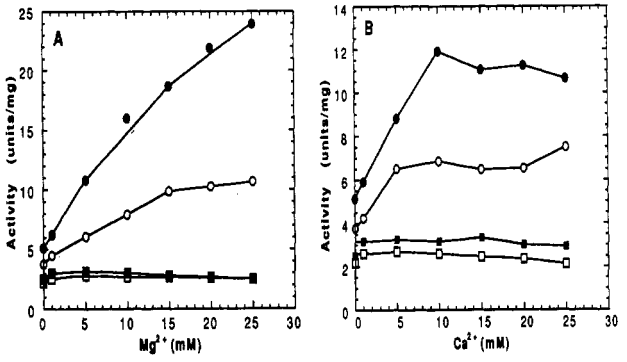

FIGURE 3: Effect of Mg^{2+} and Ca^{2+} on activities of the complex toward two classes of substrates. Conditions were the same as in the legend to Figure 2. Panel A shows the effect of Mg^{2+}, and panel B shows the effect of Ca^{2+}. Closed circles, open circles, closed squares, and open squares refer to reactions with Z-GPALA-pAB, Z-GPALG-pAB, Z-GPAAG-pAB, and Z-GPAGG-pAB, respectively.

molecular masses of the subunits ranging from 21 to 34 kDa, the complex must be composed of about 30 subunits of which at least 13–15 have been shown to be nonidentical. Accordingly, the four identified proteolytic activities could functionally account only for a fraction of the total number of subunits, and therefore the possibility had to be considered either that several other proteolytic activities remained to be identified or alternatively that many of the subunits have other than proteolytic functions. Although molecular cloning has provided the complete amino acid sequence of the majority of the components of the complex (DeMartino et al., 1991; Emori et al., 1991; Fujiwara et al., 1989; Haas et al., 1990; Kumatori et al., 1990; Tamura et al., 1990, 1991; Tanaka et al., 1990), no homology was found between these sequences and the sequences of known proteases. Accordingly, knowledge of the primary sequences has neither been sufficient for identification of the proteolytically active subunits nor led to the identification of the structural elements of the active sites. Preliminary experiments in our laboratory in which the MPC was exposed to [^{14}C]DCI, a general serine proteinase inhibitor (synthesized and kindly provided by Dr. James Powers, Georgia Institute of Technology, Atlanta, GA), have shown that such exposure leads to incorporation of the label into seven nonidentical subunits (unpublished experiments from our laboratory). Assuming that incorporation of the label signifies a reaction with a catalytic center of a proteolytically active subunit, such a result must lead to the conclusion that more than seven nonidentical subunits of the complex express proteolytic activity, since as shown in the experiments with the Leu-containing substrates, at least one of the proteolytically active subunits is resistant to DCI inactivation. We have therefore initiated more detailed studies on the specificity of the complex in order to test this possibility.

Recent work in our laboratory (Cardozo et al., 1992) has shown that an MPC in which the ChT-L, T-L, and PGPH activities have been inactivated by treatment with DCI cleaves a series of natural peptides, including neurotensin, the oxidized B-chain of insulin, and also proinsulin at a greater rate than that of the native uninhibited complex. This indicated the presence in the MPC of a DCI-resistant component with properties different from the three initially identified activities. Analysis of the products derived from degradation of neurotensin and proinsulin by the DCI-treated complex showed that cleavage occurred on the carboxyl side of Ile in neurotensin and on the carboxyl side of Leu and Val residues within the connecting peptide of proinsulin, indicating that the DCI-resistant component cleaves preferentially bonds on the carboxyl side of branched chain amino acids. Several other independent studies suggested the presence in the MPC of an activity distinct from those initially identified. Thus, Mykles and Haire (1991) observed that under certain conditions heating of the MPC from lobster muscles causes the appearance of a casein-degrading activity that is different from the ChT-L, T-L, and PGPH activities measured with model synthetic substrates. However, the relationship of this casein-degrading activity to the BrAAP component described here is uncertain since the effect of DCI on this activity was not tested. Wilk and co-workers (Yu et al., 1991) reported that treatment of the MPC with *N*-acetylimidazole increased its activity toward casein in spite of inactivation of the T-L and PGPH activities and in spite of a progressive inactivation of the ChT-L activity. Pereira et al. (1992a) reported that

Table VI: Summary of Properties of Components of the Multicatalytic Proteinase Complex[a]

	component and model substrate				
effector	trypsin-like Z-ALR-↓2NA	peptidylglutamyl-peptide hydrolyzing Z-LLE-↓2NA	chymotrypsin-like Z-GGF-↓pAB or Z-GGL-↓pNA	branched chain amino acid preferring Z-GPAL↓A-pAB	small neutral amino acid preferring Z-GPAA↓G-pAB
leupeptin	strong inhibition	no effect	no effect	no effect	no effect
chymostatin	no effect	no effect	inhibition	no effect	no effect
Z-LLF-CHO	inhibition	no effect	inhibition	no effect	no effect
DFP	no effect	no effect	inhibition	no effect	no effect
DCI	inhibition	inhibition	inhibition	activation	inhibition
N-ethylmaleimide	strong inhibition	inhibition	inhibition	activation	inhibition
SDS	inhibition	activation	inhibition	activation	no effect
lauric acid	inhibition	activation	inhibition	activation	no effect
protein[b]	no effect	inhibition	no effect	no effect	no effect
Mg^{2+}	inhibition	weak activation	inhibition	activation	weak activation

[a] All properties refer to the native complex as tested with the synthetic substrates listed in the first row of the table. Concentration of inhibitors and activators are the same as shown in Table III. [b] β-Casein and bovine serum albumin, at concentrations of up to 40 μg/mL, were used to test their effect on the activities of the components.

treatment of the MPC with DCI caused an increase of its activity toward casein in spite of inactivation of the other three activities. Similar results have been reported by Strack et al. (1992). All these observations have been interpreted as indicative of the presence in the MPC of a caseinolytic activity that is different from the other three activities. Although none of these studies analyzed the specificity of the caseinolytic activity, its resistance to DCI inactivation, and indeed its activation by DCI, is highly suggestive of identity with the activity shown by us to cleave preferentially bonds on the carboxyl side of branched chain amino acids in natural peptides and proteins (Cardozo et al., 1992).

It is reasonable to assume that the activity of the MPC shown here to cleave the Leu–Ala and the Leu–Gly bonds in Z-GPALA-pAB and Z-GPALG-pAB is identical with that previously reported by us to cleave bonds on the carboxyl side of branched chain amino acid residues in natural peptides and in proinsulin (Cardozo et al., 1992). We have therefore proposed the designation "branched chain amino acid preferring" (BrAAP) for this component. With respect to specificity, this activity resembles the ChT-L activity of the MPC. However, unlike the ChT-L activity the BrAAP component of the MPC is neither capable of cleaving the Leu-pNA or Phe-pAB bonds in Cbz-Gly-Gly-Leu-pNA and in Cbz-Gly-Gly-Phe-pAB, respectively, nor inhibited by the aldehyde inhibitors chymostatin and Z-LLF-CHO. Furthermore, unlike the ChT-L activity, which is the only component of the complex sensitive to inhibition by DFP (Wagner et al., 1986; Ishiura et al., 1986; Orlowski & Michaud, 1989), this component is resistant to inactivation by this inhibitor. Other properties distinguish this activity from the T-L and PGPH activities of the MPC. Thus, unlike the T-L and PGPH activities of the MPC, the BrAAP component is not capable of cleaving Cbz-(D)Ala-Leu-Arg-2NA and Cbz-Leu-Leu-Glu-2NA, the two substrates used for measuring these activities, and is not inhibited by leupeptin and antipain, inhibitors of the T-L activity.

Early studies have shown that exposure of the MPC to low concentrations of SDS and fatty acids greatly activates the PGPH activity (Wilk & Orlowski 1980, 1983). This activation has been later found to be a property of all preparations of the MPC isolated from diverse sources (Dahlmann et al., 1985; Ray & Harris 1986; Tanaka et al., 1988). It was therefore of interest that the BrAAP component is also activated by the same agents. That these activities, however, are expressed by different components is indicated by several lines of evidence. Thus, the BrAAP component is activated by DCI and *N*-ethylmaleimide (a thiol-blocking agent that inhibits all other components of the MPC) whereas the PGPH activity is inhibited by the same agents. Also, unlike the PGPH activity which is strongly inhibited by the presence of low concentrations of such proteins such as casein, albumin, and lysozyme (Orlowski et al., 1991), the activity of the BrAAP component is not inhibited by the presence of these proteins at concentrations up to 40 μg/mL (unpublished experiments).

Previous work in our laboratory has shown that in addition to cleaving the Leu–pNA bond in Cbz-Gly-Gly-Leu-pNA and the Phe–pAB bond in Cbz-Gly-Gly-Phe-pAB the ChT-L activity of the MPC cleaves bonds on the carboxyl side of aromatic amino acid residues in natural peptides such as luteinizing hormone-releasing hormone and bradykinin (Vinitsky et al., 1992) and that these bonds are not cleaved by the BrAAP component. Thus, two distinct activities capable of cleaving bonds on the carboxyl side of hydrophobic amino acid residues are an integral part of the MPC; one is DCI-resistant and prefers bonds with branched amino acids in the P_1 position, the other is DCI-sensitive and prefers bonds on the carboxyl side of aromatic residues in natural peptides. That the specificities of the two components are not absolute is indicated by the ability of the ChT-L component to cleave the Leu–pNA bond in Cbz-Gly-Gly-Leu-pNA. The finding that the DCI-treated MPC cleaves natural peptides and proteins such as β-casein and proinsulin at a rate faster than the native complex indicates that the BrAAP component is probably the major factor responsible for the protein degrading activity of the MPC. Although Pereira et al. (1992b) and Mykles and Haire (1991) reported that SDS inhibits the caseinolytic activity of the MPC, it is still likely that the BrAAP component, although activated by SDS, is identical with the caseinolytic activity since the effect of SDS might be dependent on the source and nature of the enzyme preparation (latent or activated). Thus, unlike these authors, we and others have observed activation of casein degradation by low concentrations of SDS (Orlowski & Michaud, 1989; McGuire et al., 1989; Tanaka et al., 1986). Since expression of the full activity of the BrAAP component requires the presence of various activators such as SDS, fatty acids, DCI, and also divalent cations such as Mg^{2+} or Ca^{2+}, this component joins the PGPH activity of the MPC as a contributing factor to the latency of the complex described by many investigators (Orlowski & Wilk 1980; Wilk & Orlowski 1983; Dahlmann et al., 1985; Ray & Harris 1986; Tanaka et al., 1988; Mykles, 1989; McGuire et al., 1989).

The results presented here indicate that in addition to the BrAAP component the MPC contains another distinct activity capable of cleaving bonds between small neutral amino acids,

designated here as small neutral amino acid preferring (SNAAP). Although the exact specificity of this component needs to be further examined, several properties distinguish it from the other activities of the MPC. Thus, unlike the ChT-L activity this component is not inhibited by the aldehyde inhibitors chymostatin, and Z-LLF-CHO, and unlike the T-L activity it is not inhibited by leupeptin. Sensitivity to inhibition by DCI and *N*-ethylmaleimide, a thiol-blocking agent that activates the BrAAP component but inhibits the ChT-L, T-L, and PGPH activities of the pituitary MPC (Wilk & Orlowski 1980; Cardozo et al., 1992), distinguishes this component from the BrAAP component. Also, concentrations of SDS and lauric acid that activate the BrAAP and PGPH activities and inhibit the ChT-L and T-L activities have little effect on the activity of this component.

Identification of the BrAAP and SNAAP components increases the number of distinct proteolytic activities of the MPC to five. A summary of the properties of the five components is given in Table VI. The presence, however, in the MPC of 13–15 nonidentical subunits raises the possibility that other, still not identified activities, could be associated with the complex. It is therefore possible that the number of proteolytically active subunits will further increase as the activity of the complex toward a wider spectrum of synthetic and natural substrates is examined. Attempts to isolate proteolytically active subunits have invariably failed, and dissociation of the complex leads to a complete loss of all proteolytic activity. This indicates that the integrity of the complex is necessary for expression of activity and that interaction between subunits of the complex might be necessary for its function. The presence in a single particle of activities capable of cleaving bonds after basic, acidic, aromatic, branched chain amino acids, and small neutral amino acids has to be considered as an advantage in the process of degradation of large protein molecules with their inherent diversity of peptide bonds. Coordinated interaction of these activities could lead to the disintegration of a protein molecule without accumulation of intermediate large peptide fragments, and possibly without the release of such fragments into cytoplasmic spaces. That all or none degradation of proteins seems to be typical of cytoplasmic proteolysis (Rechsteiner, 1987) may therefore argue for the role of the MPC in this process.

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