Evidence That the Nature of Amino Acid Residues in the P_3 Position Directs Substrates to Distinct Catalytic Sites of the Pituitary Multicatalytic Proteinase Complex (Proteasome)†

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Received January 13, 1994; Revised Manuscript Received March 22, 1994®

ABSTRACT: Cleavage of bonds after neutral amino acids by the multicatalytic proteinase complex (MPC) has been recently shown to be catalyzed by at least three distinct components [Orlowski, M., Cardozo, C., & Michaud, C. (1993) *Biochemistry 32,* 1563–1572]. One component, designated as chymotrypsin-like (ChT-L), cleaves peptide bonds on the carboxyl side of hydrophobic residues and is also active toward peptidyl–arylamide bonds. A second component, designated as branched-chain amino acid preferring (BrAAP), and a third component, designated as small neutral amino acid preferring (SNAAP), cleave preferentially bonds on the carboxyl side of branched-chain amino acids and between small neutral amino acids, respectively. Evidence indicates that the BrAAP component is a major factor responsible for degradation of protein by the MPC. The purpose of the present study was to identify the structural requirements that determine the involvement of these components in cleavage of peptides after different neutral amino acids. A series of substrates was synthesized with the aim of probing the role of residues beyond those flanking the scissile bond in directing substrates to defined catalytic sites. The data indicate that a proline or glycine residue in the P_3 position directs the substrate to the catalytic site of the BrAAP component provided that a branched-chain amino acid is present in the P_1 position. A proline residue in P_3 is also important for involvement of the SNAAP component in substrate degradation. The presence of this residue interferes with substrate binding to the catalytic site of the ChT-L activity, even in the presence of a phenylalanine residue in the P_1 position. Substrates with a proline in P_3 and a phenylalanine in P_1 positions are poorly cleaved by both the BrAAP and ChT-L components, further supporting the preference of the former for branched-chain amino acid residues in the P_1 position. Replacement of proline by hydrophobic residues shifts the activity to a catalytic site with properties of the ChT-L component. Substrates with a glycine in the P_3 position can be cleaved by either the BrAAP or the ChT-L component, depending on the nature of the residue in the P_1 position. A substrate with glycine residues in both the P_3 and P_4 position, and a leucine residue in P_1 (Cbz-Gly-Gly-Ala-Leu↓-Ala-pAB), was cleaved by the BrAAP component with sigmoidal kinetics and an unusually high V_{max}, suggesting positive cooperativity between two or more active sites of the MPC, and possible involvement of both overt and latent activities of the MPC in substrate hydrolysis.

The multicatalytic proteinase complex (MPC[1]; multicatalytic endopeptidase complex; EC 3.4.99.46), also referred to as the proteasome, is a high molecular mass (~700 kDa; 19S) intracellular particle [for reviews see Orlowski, (1990, 1993), Rechsteiner et al. (1993) and Rivett (1993)] composed of 13–15 low molecular weight nonidentical subunits (21 000–34 000). It is organized into four stacked rings, each containing 6–8 subunits surrounding a water-filled tunnel (Kopp et al., 1986; Tanaka et al., 1986; Puhler et al., 1992; Dahlmann et al., 1989). The importance of the complex is indicated by the finding that it is present in all eukaryotic cells, that it is highly conserved in evolution, and that it is essential for cell growth. Its localization in both the cytoplasm and nucleus (Akhayat et al., 1987; Arrigo et al., 1988) suggests a functional role in both of these compartments.

The MPC is credited with diverse cellular functions. As the "catalytic core" of a larger (26S) complex, it is thought to be necessary for the ubiquitin-dependent and ubiquitin-independent pathways of intracellular proteolysis (Hough et al., 1987; Waxman et al., 1987; Driscoll & Goldberg, 1989; Eytan et al., 1989). Degradation of cyclins (Glotzer et al., 1991) and short-lived proteins such as nuclear oncogene products (Ciechanover et al., 1991) and ornithine decarboxylase (Murakami et al., 1992) is dependent on these pathways, indicating a possible role in the regulation of cell growth and division. Findings that distribution of the MPC changes with stages of the cell cycle (Kawahara & Yokosawa, 1992; Amsterdam et al., 1993), and that disruption of one of several genes encoding subunits of the MPC causes arrest of cell division (Fujiwara et al., 1990; Emori et al., 1991; Heinemeyer et al., 1991), also suggest a role for the MPC in cell growth. Other reports suggest that the complex participates in processing of antigens for presentation on major histocom-

† This work was supported by Grants DK 25377 (to M.O.) and KO8 HL 08254 (to C.C.) from the National Institutes of Health, and by a postdoctoral fellowship (to A.V.) from the National Institute of Drug Abuse (DA 07135).

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® Abstract published in *Advance ACS Abstracts,* May 1, 1994.

[1] Abbreviations: Conventional one and three-letter abbreviations are used for amino acids. AP-N, aminopeptidase N; BrAAP, branched-chain amino acid preferring; ChT-L, chymotrypsin-like; DCI, 3,4-dichloroisocoumarin; HPLC, high-pressure liquid chromatography; MPC, multicatalytic proteinase complex; pAB, *p*-aminobenzoate; PGPH, peptidylglutamyl peptide hydrolyzing; SDS, sodium dodecyl sulfate; SNAAP, small neutral amino acid preferring; TFA, trifluoroacetic acid; T-L, trypsin-like; Z or Cbz, benzyloxycarbonyl; peptide-CHO, peptidylaldehyde.

0006-2960/94/0433-6483$04.50/0

patability class I molecules (Brown et al., 1991; Kelly et al., 1991; Martinez & Monaco, 1991; Ortiz-Navarrete et al., 1991).

Biochemical studies have begun to elucidate the number and specificity of proteolytic activities of the complex. Early studies provided evidence for the presence of three distinct activities cleaving bonds on the carboxyl side of acidic, basic, and hydrophobic amino acids (Orlowski & Wilk, 1981; Wilk & Orlowski, 1980, 1983). The activities were designated as peptidylglutamyl peptide hydrolyzing (PGPH), trypsin-like (T-L), and chymotrypsin-like (ChT-L), respectively, on the basis of the nature of the amino acid residue in the P_1 position of the scissile bond.[2] The three activities are inactivated by 3,4-dichloroisocoumarin (DCI) (Orlowski & Michaud, 1989), a compound that was shown to react with serine residues in the active center of proteases (Harper & Powers, 1985; Harper et al., 1985). More recent studies in this laboratory led to the identification of two additional distinct components of the MPC (Cardozo et al., 1992; Orlowski et al., 1993). One of these hydrolyzed preferentially bonds between small neutral amino acids and was designated as small neutral amino acid preferring (SNAAP). The other hydrolyzed preferentially bonds on the carboxyl side of branched-chain amino acids and was designated as branched-chain amino acid preferring (BrAAP). The latter component was resistant to inactivation by DCI, and indeed exposure of the MPC to DCI greatly activated the BrAAP component. Evidence was presented that the BrAAP component represents a major factor in degradation of proteins and that it may be responsible for most of the protein-degrading activity of the MPC.

The presence of three distinct components cleaving bonds on the carboxyl side of neutral amino acids (ChT-L, SNAAP, and BrAAP) pointed to a need for identifying those structural elements of the substrate that determine or influence the involvement of one of the three catalytic centers in peptide bond hydrolysis. Identification of such structural elements could lead to the rational design and synthesis of specific inhibitors of components of the complex that could be used in studies on their role in protein degradation and in studies on the intracellular function of the complex. We report here substrate and inhibitor studies of the three components cleaving bonds after neutral amino acids, with emphasis on contribution of residues in the P_3, P_4, and P_1 positions to substrate binding and catalysis.

MATERIALS AND METHODS

Materials

Frozen bovine pituitaries were obtained from Pel Freeze (Rogers, AR). Leupeptin, DCI, *o*-phthaldialdehyde, and Cbz-Gly-Pro, Cbz-Gly-Gly, Cbz-Ala-Phe, Cbz-Gly-Phe, and other Boc- and Cbz-amino acid derivatives were obtained from Sigma Chemical Co. (St. Louis, MO). Cbz-Pro-Gly was obtained from Bachem Bioscience Inc. (Philadelphia, PA). Cbz-Leu-Leu-Phe-CHO was synthesized as described previously (Vinitsky et al., 1992).

The MPC was isolated from bovine pituitaries as previously described (Orlowski & Michaud, 1989). Aliquots of the enzyme from the last purification step were kept frozen at −20 °C. The enzyme was then thawed for use in the experiments. Aminopeptidase N (EC 3.4.11.2) was purified from hog kidneys using the method of Pfleiderer (1970) and freed from contaminating metalloendopeptidase (EC 3.4.24.11; neprilysin) as described previously (Almenoff & Orlowski, 1983).

[2] The nomenclature of Schechter and Berger (1967) is used to describe the position (P) of the residues in the substrate and the corresponding subsites (S) in the active site of the enzyme.

Table 1: Analytical Data for Synthetic Substrates[a]

	peptide	mp (°C)	amino acid composition				retention time (min)
			Ala	Gly	Leu	Phe	
1.	Z-GGALA-pAB	245–246	1	2.09	0.88		20.67
2.	Z-GGAGG-pAB	244–246	1	3.99			16.32
3.	Z-GLAGG-pAB	180–182	1	3.01	1.02		19.82
4.	Z-GLALA-pAB	215–217	2	1.04	2.08		24.14
5.	Z-AFAGG-pAB	263–264	2	2.11		0.94	21.00
6.	Z-AFALA-pAB	266–267	3		0.95	1.00	25.23
7.	Z-LGAGG-pAB	225–227	1	3.07	0.97		20.20
8.	Z-LGALA-pAB	155–156	2	1.1	1.58		24.67
9.	Z-PGALA-pAB	254–256	2	1.10	1.05		22.71
10.	Z-GPAFA-pAB	160–162	2	1.09		0.78	22.74
11.	Z-GPAFG-pAB	239–240	1	1.92		0.86	22.13

[a] Substrates were subjected to acid hydrolysis and amino acid analysis as described in Materials and Methods. The amounts of each amino acid are expressed in relation to the number of alanine residues present in each of the compounds. The number of proline residues was not determined.

Methods

Synthesis of Substrates. Several peptide substrates containing a benzyloxycarbonyl group at the N-terminus and a pAB at the C-terminus were synthesized. Cbz-Gly-Pro-Ala-Leu-Ala-pAB, Cbz-Gly-Pro-Ala-Gly-Gly-pAB, and Cbz-Gly-Pro-Ala-Ala-Phe-pAB were synthesized as described previously (Orlowski et al., 1993). The tripeptides Ala-Leu-Ala-pAB, Ala-Gly-Gly-pAB, Ala-Phe-Ala-pAB, and Ala-Phe-Gly-pAB 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. Formation of the amino acid–arylamide bond with pAB was carried out in the presence of 1-hydroxybenzotriazole as described previously (Orlowski et al., 1983). The tripeptide was then coupled to the *N*-hydroxysuccinimide ester of the appropriate Cbz-dipeptide. Products were purified by crystallization from ethyl acetate, chloroform, or ethanol–water. The purity of peptides was determined by reverse-phase HPLC on a C_{18} μBondapak column, (3.9 × 30 cm, Waters Associates) and by amino acid analysis (Table I). Elution from the reverse-phase column was carried out with a linear gradient between an aqueous solution of 0.1% TFA and acetonitrile containing 0.1% TFA. The initial concentration of acetonitrile was 10%, and its concentration was linearly increased to 60% over 20 min and then held at 60% for an additional 5 min. All peptides gave single peaks by HPLC. For amino acid analysis, peptides were hydrolyzed in evacuated tubes in HCl–propionic acid at 150 °C for 15 min (Westall & Hesser, 1974). The acid was evaporated under nitrogen, and amino acids were analyzed fluorometrically after reaction with *o*-phthaldialdehyde (Roth, 1971) as described previously (Cardozo et al., 1992). Amino acid analyses yielded the expected amino acid composition. Melting points, retention times by HPLC, and amino acid composition of the peptides are summarized in Table 1.

Determination of Enzyme Activities. Reactions are based upon liberation of an aromatic amine which is then detected by diazotization as described previously (Bratton & Marshall, 1939; Goldbarg & Rutenburg, 1958; Orlowski et al., 1993). The assay for cleavage of internal peptide bonds is based on a coupled enzyme assay in the presence of excess aminopeptidase N (Orlowski et al., 1993). Reaction mixtures contained substrate, Tris-HCl buffer (0.05 M, pH 8.0), hog aminopep-

tidase N (20 μg), and the MPC in a total volume of 0.25 mL. Reactions were initiated at 37 °C and were continued for 15–30 min. Reactions were stopped by the addition of an equal volume of 10% trichloroacetic acid. Activity is expressed in units as the number of micromoles of product generated per hour. Specific activity is expressed as units/mg of protein, where the protein concentration is determined by absorbance at 280 nm.

Identification of Degradation Products. The peptide (500 nmol) was incubated with the MPC (10–50 μg of enzyme) at 37 °C in 0.05 M Tris-HCl, pH 8.0 (total volume 0.25 mL), for periods from 1 to 8 h. Reaction was terminated by freezing. Products of degradation were separated by HPLC as described above. Emerging peaks were collected manually and dried under nitrogen. The amino acid composition of the peaks was determined after acid hydrolysis. Free pAB was detected in the hydrolysates by a diazotization procedure.

Kinetics of Inhibition by DCI. 3,4-Dichloroisocoumarin (10 mM) in Me_2SO was added to enzyme solutions containing 0.5 mg/mL of the MPC in 0.01 M Tris–EDTA buffer, pH 7.5, to achieve a final concentration of the inhibitor of 10 μM. The mixtures were incubated at 26 °C, and at various times, aliquots were transferred to reaction mixtures (total volume 0.25 mL) that had been warmed to 37 °C for determination of activity as described under Determination of Enzyme Activities. The final concentration of Me_2SO in the assay mixtures did not exceed 4%. Second-order inactivation rate constants were obtained from plots of ln (v_t/v_0) versus time, where v_t is the rate of the reaction at time t, and v_0 is the rate of the reaction at time zero of the incubation of the enzyme with DCI.

RESULTS

Previous work in this laboratory has shown that two distinct components of the MPC, one preferring substrates with branched-chain amino acids in the P_1 position (BrAAP), the other preferring small neutral amino acids in the same position (SNAAP), show an apparent preference for substrates with a proline residue in the P_3 position. This raised the question of the importance of this residue to binding and alignment of substrates to the different active centers of the MPC. Also, findings that the two components showed preference for extended peptide substrates, and an inability to cleave peptidyl–arylamide bonds, prompted examination of the importance of residues beyond those forming the scissile bond for substrate binding and catalysis. A series of new substrates, analogs of Z-GPALA-pAB and Z-GPAGG-pAB, model substrates of the BrAAP and SNAAP components, respectively, were therefore synthesized having amino acid replacements in the P_3, P_4, and P_1 position (substrates 2–6 and 9–14, Table 2). With the exception of substrates 5, 7, 11, and 13 (Table 2), activity toward these substrates was low unless aminopeptidase N was included in the incubation mixtures, indicating that MPC poorly hydrolyzes the Ala-pAB and Gly-pAB arylamide bonds when a Pro is present in the P_3 position, and the P_1 position is occupied by either a Leu or Gly residue. Activities, however, were increased for all substrates, albeit to a different extent, when aminopeptidase N was present in the incubation mixtures.

Table 2 summarizes activity data obtained with a series of substrates having different amino acid replacements in the P_3, P_4, and P_1 position, together with ratios of activities measured in the presence and absence of aminopeptidase N (AP-N) and the sites of cleavage of peptide bonds deduced from amino acid analyses of degradation products. The magnitude of the ratios of activities measured in the presence and absence of AP-N expresses the ratio of the rates of cleavage occurring inside the peptide chain to that occurring at the amino acid–arylamide bond. The finding that this ratio was high for the model substrate of the BrAAP component (substrate 1, Table 2) is consistent with previous observations (Cardozo et al., 1992; Orlowski et al., 1993). The minor cleavage occurring at the Ala-pAB bond was inhibited by Z-LLF-CHO, an inhibitor of the ChT-L component, and was thus attributed to the action of this component.

Table 2: 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 (I)	+ AP-N (II)	increase	ratio (II/I)
1. Z-GPAL↓A↓-pAB	1.0	0.61 (13)	4.64	4.03	7.6
2. Z-PGAL↓A↓-pAB	1.0	0.44 (10)	4.42	3.98	10.0
3. Z-GGAL↓A↓-pAB	1.0	0.34 (8.6)	3.97	3.63	11.7
4. Z-LGA↓L↓A↓-pAB	1.0	0.76 (17)	4.46	3.7	5.9
5. Z-GPAF↓A↓-pAB	1.0	2.17 (75)	2.86	0.69	1.3
6. Z-GPAF↓G↓-pAB	1.0	0.76 (30)	2.51	1.75	3.3
7. Z-GPAA↓F↓-pAB*	1.0	3.61 (92)	3.93	0.32	1.1
8. Z-GPAG↓G-pAB	1.0	0.15 (4.3)	3.52	3.37	23.5
9. Z-GGAG↓G-pAB	1.0	0 (0)	0.37	0.37	-
10. Z-LGAG↓G-↓pAB	1.0	0.048 (13)	0.37	0.32	7.7
11. Z-GL↓A↓L↓A↓-pAB	1.0	2.66 (7.2)	37.1	34.44	13.9
12. Z-GL↓AG↓G↓-pAB	1.0	0.14 (7.1)	1.98	1.84	14.1
13. Z-AF↓A↓L↓A↓-pAB	0.2	15.2 (15)	60.6	45.2	4.0
14. Z-AF↓A↓G↓G↓-pAB	1.0	0.35 (45)	0.78	0.38	2.2

[a] Enzyme activities were determined as described in Materials and Methods. Incubation mixtures contained 0.5–10 μg of MPC. Data are mean values of 2 separate determinations. Controls in which either the MPC or aminopeptidase N was omitted were also carried through the procedure. Values in parentheses represent percent of total activity, with the activity in the presence of AP-N arbitrarily set at 100. Large and small arrows indicate predominant and minor cleavage sites, respectively. Products of enzymatic hydrolysis were isolated by HPLC as described in Materials and Methods, and their amino acid composition was determined after acid hydrolysis. *Data obtained from Orlowski et al. (1993).

A similar pattern of cleavage (and a high ratio of activity in the presence of AP-N to that in its absence) was also observed with substrates in which the Pro residue in the P_3 position was replaced by a Gly (substrates 2–4, Table 2). These substrates were also predominantly cleaved on the carboxyl side of the Leu residue in the P_1 position, with little cleavage at the amino acid–arylamide bond. The effect of several inhibitors and activators on the rate of reaction was examined in order to identify the component responsible for degradation of these substrates. The results in Tables 3 and 4 show that treatment of the MPC with DCI, *N*-ethylmaleimide (NEM), Mg^{2+}, Ca^{2+}, or SDS increased the rate of substrate cleavage, indicating involvement of the BrAAP component of the MPC, since such activation was previously shown to be a distinguishing property of this component (Cardozo et al., 1992; Orlowski et al., 1993). This contrasts with the inhibitory effects of these agents on the ChT-L and T-L components (Wilk & Orlowski, 1983; Orlowski et al., 1993). The finding that leupeptin (Table 3) and Z-LLF-CHO (Table 4), potent inhibitors of the T-L (Wilk & Orlowski, 1983) and ChT-L components (Vinitsky et al., 1992), respectively, had little effect on the rates of reaction is also consistent with this conclusion. That the PGPH component is not involved is indicated by the finding that lysozyme, a protein that greatly inhibits the PGPH activity of the MPC (Orlowski & Michaud, 1989; Orlowski et al.,

Table 3: Effects of Inhibitors on Degradation of Selected Synthetic Substrates[a]

	substrate	act. remaining (%) DCI (10 μM)	NEM (1 mM)	leupeptin (50 μM)
1.	Z-GPALA-pAB	914	175	91
2.	Z-PGALA-pAB	548	130	93
3.	Z-GGALA-pAB	513	206	85
4.	Z-LGALA-pAB	444	147	89
5.	Z-GPAFA-pAB	133	105	89
6.	Z-GPAFG-pAB	84	102	91
7.	Z-GPAGG-pAB	47	84	105
8.	Z-GGAGG-pAB	20	67	87
9.	Z-LGAGG-pAB	5.7	80	100
10.	Z-GLALA-pAB	12	91	84
11.	Z-GLAGG-pAB	15	88	93
12.	Z-AFALA-pAB	1	54	122
13.	Z-AFAGG-pAB	5	43	98

[a] Activities were determined in the presence of aminopeptidase N as described in Materials and Methods and are expressed as percent relative to those determined in the absence of inhibitors. Data represent mean values of 2 separate determinations. For experiments with DCI, activity was determined after preincubation of the MPC with 10 μM DCI for 60 min at 25 °C, those with NEM after preincubation of the enzyme with 1 mM NEM for 15 min at 25 °C, and those with leupeptin after 60-min preincubation of the enzyme with the inhibitor (50 μM) at 37 °C.

Table 4: Effect of Activators and Inhibitors on Degradation of Substrates of the BrAAP Component[a]

substrate	activity (% of control) $MgCl_2$ (10 mM)	$CaCl_2$ (1 mM)	SDS (0.04%)	lysozyme (2 μg)	Z-LLF-CHO (50 μM)
Z-GPALA-pAB	254	157	1400	144	78
Z-LGALA-pAB	128	117	410	89	86
Z-GGALA-pAB	222	224	446	118	78
Z-PGALA-pAB	156	180	755	112	100

[a] Data represent mean values for 2 separate determinations. For determination of activity, assays contained about 2.5 μg of the MPC and substrate (1 mM) and were performed in 0.05 M Tris-HCl buffer, pH 8.0, in the presence of aminopeptidase N as described in Materials and Methods. For experiments with Z-LLF-CHO, the MPC was preincubated with the inhibitor for 60 min at room temperature.

1991, 1993), had essentially no effect on the rate of degradation of these substrates (Table 4). Thus, the findings suggest that the presence of either a Pro or Gly in the P_3 position, and a branched-chain amino acid (such as Leu) in the P_1 position, yielded substrates cleaved predominantly by the BrAAP component. The nature of the residue in the P_4 position did not seem to affect markedly the reaction since in this series of substrates this residue could be replaced by either a glycine, proline, or leucine residue (Table 3 and 4).

The kinetic parameters of degradation of substrates cleaved by the BrAAP component are summarized in Table 5. Substrates with a leucine or proline in P_4 and a Gly in P_3 (substrates 3 and 4 in Table 5) displayed Michaelis–Menten kinetics and specificity constants similar to those of the prototypic substrates (substrates 1 and 2, Table 5). K_m and k_{cat} values for the substrate containing proline in P_4 (Z-PGALA-pAB) were similar to those for the prototypic substrate (Z-GPALA-pAB). Introduction of a leucine residue in P_4 resulted in a substrate (Z-LGALA-pAB) with a higher K_m than the other substrates. However, the estimated k_{cat} for this substrate was commensurately increased, with the result that its specificity constant was similar to that of the other BrAAP substrates.

Of interest are the values obtained for the substrate in which the Pro residue in Z-GPALA-pAB was replaced by a glycine (Z-GGALA-pAB). The relationship between substrate concentration and reaction velocity was sigmoidal, and the V_{max} reached values of 300 units/mg of protein. A Hill plot of the data yielded a value of 6.0 ($r = 0.973$), suggesting positive cooperative interactions between several substrate binding sites. Previous work has indicated that the BrAAP component has an overt component and also a latent component that is exposed after treatment of the MPC with such activators as SDS and DCI (Orlowski et al., 1993). One explanation for the unusually high V_{max} observed for hydrolysis of Z-GGALA-pAB could be the involvement of both the overt and latent components in the degradation of this substrate.

Table 5: Kinetic Parameters for Degradation of Synthetic Substrates by the BrAAP Component[a]

	substrate	[S]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
1.	Z-GPALG-pAB*	1.6–8.0	5.2	3.75	720
2.	Z-GPALA-pAB*	1.6–8.0	6.7	5.2	780
3.	Z-LGALA-pAB	1.6–8.0	30	24	820
4.	Z-PGALA-pAB	1.6–8.0	8.7	8.4	960
5.	Z-GGALA-pAB	0.8–4.8	2.5	58	23300

[a] Data represent mean values for 2 separate determinations. For substrates with Michaelis–Menten kinetics, K_m and k_{cat} were determined from the regression line fitted to plots of $1/v$ vs $1/s$. Values for k_{cat} were calculated assuming a molecular mass of 700 kDa and assuming one active site per molecule of MPC. Substrate 5 displayed sigmoidal kinetics. The apparent K_m for this substrate is taken as the substrate concentration at half of the V_{max} observed. k_{cat} for this substrate was calculated assuming a V_{max} of 300 units/mg and assuming one active site per molecule of MPC. *Data from Orlowski et al. (1993).

In order to further examine the importance of the leucine residue in the P_1 position for activity of the BrAAP component, we replaced this residue with a Phe residue. This caused a dramatic decrease in the ratio of activity measured in the presence of AP-N to that measured in the absence of this enzyme (substrates 5 and 6 in Table 2), indicating a shift of the predominant site of cleavage to the amino acid–arylamide bond, a bond previously suggested to be resistant to hydrolysis by the BrAAP component (Orlowski et al., 1993). A similar effect was observed when the Leu residue was replaced by an Ala and a Phe residue was introduced in the P_1' position (substrate 7, Table 2). These results indicated involvement of a catalytic site different from that of the BrAAP component. Evidence supporting this conclusion was obtained from experiments showing that treatment of the MPC with DCI or NEM had little effect on activity with these substrates (substrates 5 and 6 in Table 3), but examination of the degradation products indicated that treatment with DCI completely inhibited cleavage of the amino acid–arylamide bond, suggesting the involvement of the ChT-L component, which is known to be sensitive to inactivation by this inhibitor. While inhibiting cleavage of the amino acid–arylamide bond, treatment with DCI stimulated hydrolysis of the phenylalanyl–amino acid bond in Z-GPAFA-pAB and Z-GPAFG-pAB by 652% and 167%, respectively, indicating that the BrAAP component could cleave, albeit poorly, the bond on the carboxyl side of phenylalanine (data not shown). Taken together, these results indicate that cleavage of a phenylalanyl–amino acid bond by either the BrAAP or the ChT-L component is unfavorable when a Pro residue is positioned at the P_3 site and that the latter residue contributes to directing the substrate to the catalytic site of the BrAAP component.

The pattern of substrate cleavage was quite different when the Pro residue in the P_3 position was replaced by either a Phe or Leu (substrates 11–14, Table 2, and 10–13, Table 3). Such substrates were cleaved at several sites, suggesting possible involvement of more than one catalytic site. It is noteworthy

Table 6: Kinetic Constants for Inactivation by DCI for Several Substrates[a]

substrate	[S] (mM)	[I] (μM)	$t_{1/2}$ (min)	$k_{obs}/[I]$ (s^{-1} M^{-1})
1. Z-GLAGG-pAB	1.0	10	6.6	178
2. Z-GLALA-pAB	1.0	10	5.0	238
3. Z-AFAGG-pAB	1.0	10	5.0	231
4. Z-AFALA-pAB	1.0	10	5.4	220
5. Z-GGAGG-pAB	2.0	10	6.4	180
6. Z-LGAGG-pAB	2.0	10	5.4	213

[a] Data represent the mean of 2 determinations. The MPC was incubated with 10 μM DCI at 25 °C, and aliquots of the incubation mixture were removed at 0, 5, 10, 15, and 20 min and assayed for activity as described in Materials and Methods. The second-order rate constants were obtained from plots of $\ln(v_t/v_0)$ vs time.

that the most rapidly cleaved substrates were those that contained a Leu residue in the P_1 position and either a Phe or Leu residue in the P_3 position. Activity toward each of the four substrates was highly sensitive to inactivation by DCI, indicating that they were cleaved by an activity other than the BrAAP component. It was previously shown that the ChT-L, PGPH, T-L, and SNAAP components are inactivated by DCI with distinctly different second-order rate constants ($k_{obs}/[I]$). Thus, information regarding the identity of the component cleaving a particular bond can be obtained by determining values for $k_{obs}/[I]$. As shown in Table 6, values for $k_{obs}/[I]$ were close to 200 s^{-1} M^{-1} for all four substrates, rates that are markedly greater than those observed for the SNAAP, PGPH, and T-L components (Orlowski et al., 1993), while being similar to those previously observed for the chymotrypsin-like component, suggesting that these substrates are cleaved by a chymotrypsin-like component.

Previous studies probing the primary specificity of the BrAAP component by use of synthetic substrates led to identification of a fifth distinct proteolytic component of the MPC that cleaves preferentially bonds between small neutral amino acids and is characterized by a slow rate of inactivation by DCI (Orlowski et al., 1993). On the basis of the nature of residues flanking the scissile bond, the component was designated as small neutral amino acid preferring (SNAAP). The prototypic substrate for the SNAAP component, Z-GPAGG-pAB (substrate 8, Table 2), is cleaved exclusively at the Gly–Gly bond (Orlowski et al., 1993) and, like the prototypic substrate for the BrAAP component, contains a proline residue in the P_3 position. To probe the importance of this proline residue, substrates containing replacements in the P_3 and P_4 positions were synthesized (substrates 9 and 10, Table 2). As shown in Table 2, the predominant site of cleavage of these substrates was, like for Z-GPAGG-pAB, at the Gly–Gly bond. Exposure of the MPC to DCI led to inactivation of the component that cleaved the new substrates with a second-order rate constant close to 200 s^{-1} M^{-1}, a value much higher than that obtained for the SNAAP component. These results indicate involvement of the ChT-L activity and show that replacement of the Pro residue in P_3 by a Gly does not favor binding to the catalytic site of the SNAAP component.

DISCUSSION

On the basis of the cleavage sites in natural peptides such as neurotensin, proteins such as proinsulin, and synthetic substrates, the primary specificity of the BrAAP component was shown to be for cleavage of peptide bonds on the carboxyl side of the branched-chain amino acids Leu, Ile, and Val when a Pro residue was present in the P_3 position (Cardozo et al., 1992; Orlowski et al., 1993). That the Pro is important for directing the substrate to the active site of the BrAAP component is indicated in this study by the data obtained with substrates in which the Leu residue in the P_1 position was replaced by a Phe (substrates 5 and 6 in Table 2). Such substrates were cleaved, albeit slowly, by the BrAAP component on the carboxyl side of phenylalanine, although cleavage after this residue is a preferred site of attack by the ChT-L component in substrates not containing a Pro in P_3. It is also notable that the presence of the Pro residue in the P_3 position shifted the main cleavage site in Z-GPAFA-pAB to the amino acid–arylamide bond between Ala-pAB even in the presence of a neighboring Phe residue (substrate 5 in Table 2). This indicates that the presence of the proline residue interferes with the binding of the Phe residue to the catalytic center of the ChT-L component. This interpretation was also confirmed in recent studies in our laboratory by Vinitsky et al. (results to be published in a separate report) showing that an aldehyde inhibitor containing a Pro residue in P_3 and a phenylalaninal residue instead of a leucinal residue in P_1 inhibits poorly both the BrAAP and ChT-L components.

The importance of the Pro residue in the P_3 position for substrate binding to the active site of the BrAAP component is also emphasized by the results of studies with substrates in which this residue was replaced by either a Phe or Leu. Activity toward such substrates became highly sensitive to inactivation by DCI, suggesting that they are cleaved by the ChT-L component. Apparently, the S_3 subsite of the BrAAP component poorly accommodates bulky hydrophobic groups.

The findings presented here also provide a basis for further definition of the secondary specificity of the BrAAP component. Several substrates with a Gly residue in the P_3 position have been shown to be readily degraded by the BrAAP component, indicating that this small neutral amino acid can successfully replace the Pro residue in this position. Indeed, one of these substrates, Z-GGALA-pAB, showed a sigmoidal relationship between substrate concentration and reaction velocity, and the highest specificity and turnover rate constants of all currently available substrates. Since the rate of reaction with all substrates cleaved by the BrAAP component was greatly increased in the presence of several activators such as SDS, DCI, and Mg^{2+}, possibly indicating activation of a latent form of this component, it is reasonable to assume that the high activity obtained with Z-GGALA-pAB is an expression of participation of both the overt and latent components of the MPC in the hydrolysis of this substrate.

Findings that cleavage of some synthetic substrates by the MPC proceeds at a maximal rate only in the presence of activators have led some investigators to propose that the complex exists in a latent state and that in vivo activation by as yet undefined agents is needed for expression of full activity. Indeed, forms of the complex have been described that are unable to degrade protein substrates such as casein unless they are activated by treatments such as dialysis or heating (McGuire et al., 1989; Mykles, 1989). Findings that agents such as detergents (Wilk & Orlowski, 1983; Dahlmann et al., 1985b; Ray & Harris, 1986; McGuire et al., 1989; Orlowski & Michaud, 1989; Orlowski et al., 1993), fatty acids, divalent cations (Dahlmann et al., 1985a; Ishiura et al., 1985; Ray & Harris, 1986; Pereira et al., 1992; Orlowski, 1993), and polyamines (Orlowski & Michaud, 1989; Mellgren, 1990) activate degradation of chromogenic substrates, in conjunction with the discovery of specific protein activators of the MPC (Yukawa et al., 1991; Chu-Ping et al., 1992a; Dubiel, 1992), could also be interpreted as evidence of the existence of a

latent form of the MPC. The finding that cleavage of Z-GGALA-pAB by the BrAAP component proceeded at an unusually rapid rate raises the question whether the latent and overt activities of the MPC described by many authors should be attributed to different forms of the complex or rather to differences in the structure of the substrate or in its interaction with the enzyme.

The presence in the MPC of 13–15 nonidentical subunits (Wilk & Orlowski, 1983; Tanaka et al., 1986; Lee et al., 1990) assembled into a cylindrical particle composed of a total of 28–32 subunits (Kopp et al., 1986; Tanaka et al., 1986; Puhler et al., 1992) implies the presence in the complex of multiple copies of some of the subunits. This provides a structural basis for postulating the existence of multiple substrate binding sites, and also of allosteric interactions between substrate binding sites. Such positive cooperative interactions typically yield sigmoidal substrate–velocity curves. Sigmoidal kinetics have been reported for the PGPH component (Arribas & Castaño, 1990; Orlowski et al., 1991; Djaballah & Rivett, 1992), providing evidence for multiple binding sites for substrates cleaved by this component. Findings in the current study that the BrAAP substrate Z-GGALA-pAB was cleaved in a manner yielding sigmoidal substrate–velocity curves provides evidence that this component also is subject to cooperative interactions between multiple substrate bindings sites. By contrast, substrates tested in this study with a Pro in P_3 or P_4 or with a Leu in P_4 (Table 5) yielded typical Michaelis–Menten kinetics. This indicates that the nature of residues in these positions is also important for inducing cooperative interactions between different substrate binding sites.

The SNAAP component is characterized by a preference for cleavage of peptide bonds between small neutral amino acids and a sensitivity to inactivation by DCI, albeit with a rather low second-order rate constant. Substrates for the SNAAP component contain, like those for the BrAAP component, a Pro residue in the P_3 position. Replacement of this residue by a Gly, Leu, or Phe (substrates 9, 10, 12, and 14, Table 2) prevents degradation by the SNAAP component, suggesting a rather strict requirement for the presence of a Pro residue in the P_3 position.

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