A 3,4-Dichloroisocoumarin-Resistant Component of the Multicatalytic Proteinase Complex†

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ABSTRACT: The multicatalytic proteinase complex (MPC) exhibits three proteolytic activities designated as trypsin-like, chymotrypsin-like, and peptidylglutamyl-peptide hydrolyzing (PGPHA). Evidence based on inhibitor and specificity studies indicates that each of the three activities is associated with a different component of the complex. Inactivation of the three activities by the serine proteinase inhibitor, 3,4-dichloroisocoumarin (DCI), reveals the presence of an additional DCI-resistant component that cleaves natural peptides including neurotensin, dynorphin, angiotensin II, the oxidized B-chain of insulin, and also proinsulin at a rate greater than that of the native uninhibited complex. Examination of the reaction products of neurotensin (NT) and proinsulin degradation showed cleavage of the Ile^{12}–Leu^{13} bond in NT and cleavage of the Leu^{44}–Ala^{45} and Val^{39}–Gly^{40} bonds within the connecting peptide (C-chain) of bovine proinsulin, suggesting preferential cleavage of bonds on the carboxyl side of branched chain amino acids. Although resistant to inhibition by DCI, the component was sensitive to inhibition by the isocoumarin derivatives, 7-amino-4-chloro-3-[3-(isothioureido)propoxy]isocoumarin and 4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin. Degradation of NT was activated by leupeptin, chymostatin, and antipain indicating that binding of these aldehyde inhibitors at one site can stimulate proteolytic activity at a different site of the complex. The DCI-resistant component seems to constitute a major component of the complex active in degradation of natural peptides and proteins.

The multicatalytic proteinase complex (MPC),[1] also referred to as proteasome, is an unusually high molecular mass (~700 kDa; 19S) proteinase composed of multiple low molecular weight (21 000–32 000) nonidentical subunits that represents a major extralysosomal proteolytic system [for reviews, see Orlowski (1990) and Rivett (1989)]. The growing interest in the MPC is stimulated by the finding that it is present in all eukaryotic cells, that it constitutes up to 0.5–1.0% of protein in tissue homogenates, that it is highly conserved in evolution, and that it is essential for cell proliferation (Fujiwara et al., 1991). Evidence was also presented that the MPC constitutes the proteolytic core (Eytan et al., 1989; Driscoll & Goldberg, 1990) of the ubiquitin-dependent pathway of intracellular proteolysis (Hershko, 1988; Hough et al., 1986, 1987, 1988; Rechsteiner, 1987). The conclusion that the MPC might be involved in the ubiquitin-dependent proteolytic system was foreshadowed by earlier work showing that a high molecular weight proteinase is involved in the ATP-dependent proteolytic system and that immunoprecipitation of the MPC by specific antibodies seemed to completely remove the ATP-dependent activity toward lysozyme and lysozyme–ubiquitin conjugates (Waxman et al., 1987; Ganoth et al., 1988; Matthews et al., 1989; McGuire et al., 1988, 1989; McGuire & DeMartino, 1989). This pathway could be involved in the degradation of cytoplasmic and probably nuclear proteins and also, as recently reported in the degradation of cyclins, could thereby influence the mitotic cycle of the cell (Glotzer et al., 1991).

Early studies (Orlowski & Wilk, 1981; Wilk & Orlowski, 1980, 1983) provided evidence that the complex exhibits three endopeptidase activities, cleaving peptide bonds after basic, acidic, and hydrophobic amino acids in synthetic and natural peptides. The three activities were designated as trypsin-like (cleavage on the carboxyl side of basic amino acid residues), chymotrypsin-like (cleavage on the carboxyl side of hydrophobic residues), and peptidylglutamyl-peptide hydrolyzing (PGPHA) (cleavage on the carboxyl side of acidic residues). These activities can be determined with model synthetic substrates such as Cbz-D-Ala-Leu-Arg-2NA, Cbz-Gly-Gly-Leu-pNA, and Cbz-Leu-Leu-Glu-2NA, respectively. Inhibitor and activator studies provided evidence that each of the three activities is associated with a different component of the complex. The complex contains a total of 28–32 subunits organized in a particle (19S–20S) consisting of four stacked rings, each containing 6–8 subunits (Kopp et al., 1986). Thirteen to fourteen nonidentical subunits can be separated by HPLC or by PAGE. In spite of great progress achieved in studies of the complex, fundamental questions remain unanswered. How many distinct proteolytic activities are expressed by the complex, and which and how many of the subunits are proteolytically active?

In a previous report from this laboratory, we have shown that the initially identified three activities of the MPC can be inactivated by incubation with DCI (Orlowski & Michaud, 1989), a general serine proteinase inhibitor (Harper et al., 1985). Here we present evidence that after inactivation of

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[1] Abbreviations: AMp, 2-amino-2-methyl-1-propanol; APMSF, (4-amidinophenyl)methanesulfonyl fluoride; Bz, benzoyl; Cbz or Z, benzyloxycarbonyl; DCI, 3,4-dichloroisocoumarin; DFP, diisopropyl fluorophosphate; 2NA, 2-naphthylamide; HPLC, high-pressure liquid chromatography; LHRH, luteinizing hormone-releasing hormone; MPC, multicatalytic proteinase complex; NT, neurotensin; OPA, *o*-phthalaldehyde; PGPHA, peptidylglutamyl-peptide hydrolyzing activity; PMSF, phenylmethanesulfonyl fluoride; pNA, *p*-nitroanilide; TFA, trifluoroacetic acid.

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the three activities by incubation with DCI a remaining, DCI-resistant component of the MPC cleaves peptide bonds in natural peptides such as neurotensin (NT), substance P, dynorphin, α-neoendorphin, angiotensin II, and the oxidized B-chain of insulin and also in proteins such as proinsulin. Some initial results of studies on the specificity of this component and its profile of inhibition by various protease inhibitors are also reported.

MATERIALS AND METHODS

Materials

Frozen bovine pituitaries were obtained from Pel Freeze Inc. (Rogers, AR). Peptide substrates, 2-amino-2-methyl-1-propanol (AMp), DCI, 9-fluorenylmethyloxycarbonyl-(Fmoc-) amino acids, *o*-phthalaldehyde, and Cbz-Leu-Leu-Glu-2NA were obtained from Sigma Chemical Co. (St. Louis, MO). Z-(D)-Ala-Leu-Arg-2NA and Cbz-Gly-Gly-Leu-pNA were synthesized as described previously (Wilk & Orlowski, 1980, 1983). Bovine proinsulin was generously provided by the Eli Lilly Co. (Indianapolis, IN). 7-Amino-4-chloro-3-[3-(isothioureido)propoxy]isocoumarin and 4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin and the chloromethyl ketone inhibitors were generously provided by Dr. James Powers, Georgia Institute of Technology, Atlanta, GA.

Neurotensin$^{1-4}$ (pGlu-Leu-Tyr-Glu) was synthesized using the Fmoc procedure and an Applied Biosystems 430A peptide synthesizer according to the manufacturers' recommendation. Fmoc-glutamine rather than pyroglutamate was used in the last cycle. The N-terminal glutamine was quantitatively cyclized to pyroglutamate by heating the peptide in 10 mM sodium phosphate buffer, pH 6.8, for 24 h at 70 °C. The final product was more than 90% pure by HPLC. Acid hydrolysis gave the expected amino acid composition.

The MPC was isolated from bovine pituitaries as previously described (Orlowski & Michaud, 1989).

Methods

Determination of Enzyme Activity with Synthetic Substrates. Enzyme activities 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 trypsin-like activity, 0.4 mM Cbz-Gly-Gly-Leu-pNA for determination of the chymotrypsin-like activity, and 0.64 mM Cbz-Leu-Leu-Glu-2NA for measurements of the PGPHA. Activity is expressed in units as the number of nanomoles of product generated per hour. Specific activity is expressed in units per milligram of protein.

Treatment of the Enzyme with DCI. The isolated enzyme (0.99 mL; about 0.5 mg of protein) was incubated at 26 °C for 90 min with 10 μL of a 1 mM solution of DCI (Harper et al., 1985) in dimethyl sulfoxide (final DCI concentration 10 μM). An equal amount of enzyme was incubated under the same conditions with 10 μL of dimethyl sulfoxide as a control. Both enzymes were then dialyzed for 24 h against a Tris-HCl buffer (1 mM, pH 7.5). Determination of activity showed inactivation of about 90% of the activity against all three synthetic substrates when compared with the control. The inactivated enzyme did not regain any of the activity toward synthetic substrates for a period of at least 20 days.

Determination of Activity with Natural Peptides and Proteins. Unless otherwise stated, incubation mixtures contained Tris-HCl buffer (0.05 M, pH 8.0), 10 nmol of peptide (final concentration 125 μM) and the MPC (5–10 μg of protein) in a final volume of 80 μL. Incubations were at 37 °C for 30–120 min. Reactions were terminated by the addition of 20 μL of glacial acetic acid, and 80–90 μL of the mixture was subjected to high-pressure liquid chromatography (HPLC) on a reverse-phase 5-μm Resolve C_{18}, 90-Å column (3.9 × 150 mm; Waters). Peptides were eluted with a linear gradient established between 0.1% trifluoroacetic acid (TFA) in acetonitrile and an 0.1% aqueous solution of TFA. The initial concentration of acetonitrile was 10%, and its concentration was increased to 35–55% during 20–40 min depending on the peptide. The rate of peptide degradation was determined by measuring the decrease of the peak height of the peptide compared with controls in which the enzyme was omitted. The amount of peptide degraded was determined from a standard curve prepared by measuring the peak heights versus amount of peptide. Alternatively degradation of the peptide was determined by integrating the area under the peak of the peptide at time zero and determining the decrease in the area after incubation with the enzyme. Both methods gave comparable results. Activity is expressed in nanomoles of peptide degraded per hour. Specific activity is expressed in nanomoles of peptide degraded per milligram of enzyme protein per hour.

For measuring the rate of degradation of the B-chain of insulin, incubation mixtures contained 10 μL of a 0.5 mM solution of the peptide, 20 μL of a Tris-HCl buffer (0.05 M, pH 8.0) and 20 μL of the enzyme (10 μg of protein for the native enzyme and about 4 μg of the DCI-inhibited enzyme). Incubations were carried out at 37 °C for various times, and the reaction was stopped by the addition of 10 μL of glacial acetic acid. A total of 50 μL of the mixture was subjected to HPLC as described above, and the amount of oxidized B-chain degraded was determined from the decrease in the peak height of the emerging peak as described above.

For measuring the rates of degradation of proinsulin, incubation mixtures contained 20 μL of a proinsulin solution (1 mg/mL in 2 mM HCl; final concentration 22 μM), 70 μL of 0.05 M Tris-HCl buffer, pH 8.0, and 10–20 μL of the MPC (5 μg of native or DCI-inhibited enzyme). Incubations were at 37 °C and aliquots of the incubation mixture (50 μL) were removed at time 0 and 180 min and treated with 10 μL of glacial acetic acid. The mixtures were subjected to HPLC on a Deltapak column (C4; 5 μm; 300 Å; 3.9 × 150 mm; Waters) and eluted with a linear gradient from 20 to 30% acetonitrile containing 0.1% trifluoroacetic acid over 50 min. The degradation rate was determined from the decrease in the peak height of the substrate as described above.

Identification of Degradation Products. For identification of degradation products of NT, 100 nmol of the peptide was incubated with either the native (about 200 μg of protein) or DCI-inhibited enzyme (about 100 μg of protein) for 5 h in a final volume of 0.5–0.6 mL at pH 8.0 (Tris-HCl, 0.01 M), and the reaction was terminated by the addition of glacial acetic acid (70 μL). The amino acid composition of the degradation products was determined as described below.

Products of degradation were separated on a reverse-phase 5 μm Resolve C_{18} column as described above, and the emerging peaks were collected. Solvents were then removed under nitrogen, and 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). The OPA-derivatizing reagent (5 mg of OPA in 0.4 mL of methanol, 0.025 mL of 2-mercaptoethanol, and 0.575 mL of 0.5 M sodium borate buffer, pH 10.4) was added in a 2-fold excess to amino acid hydrolysates and allowed to react for 1 min. The reaction

Table I: Rate of Degradation of Synthetic and Natural Substrates, by the Native and 3,4-Dichloroisocoumarin-Treated Multicatalytic Proteinase Complex[a]

substrate	activity (nmol/(mg of enzyme·h)) native enzyme	DCI-treated	treated/native
1. Z-D-Ala-Leu-Arg-2NA	8870 (3)	630 (3)	0.07
2. Z-Gly-Gly-Leu-pNA	8300 (3)	115 (3)	0.01
3. Z-Leu-Leu-Glu-2NA	12600 (3)	1100 (3)	0.09
4. LHRH	10600 (6)	590 (5)	0.06
5. neurotensin$^{1-13}$	74 (11)	159 (14)	2.1
6. neurotensin$^{1-11}$	9.9 (2)	21 (2)	2.1
7. α-neoendorphin	89 (2)	275 (2)	3.1
8. angiotensin II	67 (2)	104 (2)	1.6
9. ox. B-chain of insulin	103 (3)	1460 (3)	14
10. proinsulin	25.4 (3)	54.6 (3)	2.1

[a] Treatment of the MPC with DCI and determination of activities are described in Materials and Methods. The activity toward peptides was determined at a substrate concentration of 0.125 mM and pH 8.0 (0.05 M Tris-HCl buffer). The concentration of LHRH in the incubation mixtures was 0.25 mM. Products were separated and quantitated by HPLC. Data are mean values of the number of determinations shown in parentheses.

mixtures were then subjected to HPLC on a 5-μm Resolve C_{18}, 90-Å column (3.9 × 150 mm). Elution of OPA amino acid derivatives was carried out with a linear gradient established between 0.05 M sodium acetate–0.05 M Na_2HPO_4, pH 7.0, containing 2% methanol and 2% tetrahydrofuran (solvent A) and a 65:35 mixture of methanol and water (solvent B). The initial concentration of B was increased linearly from 0 to 100% over a 40-min period at a flow rate of 1.5 mL/min. The fluorescence was monitored with a Waters fluorescence detector equipped with a 338-nm excitation filter and a 425-nm pass emission filter. Alternatively, the composition of some degradation products was determined by amino acid analysis using the Waters Pico-Tag system.

For identification of degradation products of proinsulin, incubation mixtures contained 50 μg of proinsulin, 25 μg of enzyme, 25 mM magnesium chloride, and Tris-HCl buffer (0.05 M, pH 8.0) in a final volume of 270 μL. Magnesium chloride was used to accelerate the rate of degradation of proinsulin. The same products were obtained in the presence and absence of this metal ion. Products were eluted with a linear gradient from 25 to 32% of acetonitrile and 0.1% TFA over 80 min, at a flow rate of 1 mL/min. Emerging peaks were collected manually and subjected to five cycles of amino acid sequencing in an Applied Biosystems 470 gas-phase protein sequencer.

RESULTS

Previous work (Orlowski & Michaud, 1989) has shown that all three initially identified components of the MPC are susceptible to inactivation by DCI, a general serine proteinase inhibitor, although at different second-order rate inhibition constants. Data in Table I show that incubation of the complex for 90 min with 10 μM DCI followed by overnight dialysis against a 1 mM Tris-HCl buffer (pH 7.5) leads to a 90% or more inactivation of all activities toward the three synthetic substrates (substrates 1, 2, and 3; Table I). It was, however, important to determine whether inactivation of these three activities eliminates all activities of the MPC toward structurally more diversified peptides and proteins. We have therefore studied the rate of degradation of NT, luteinizing hormone-releasing hormone (LHRH), and several other natural peptides and proteins by both the native and the DCI-inactivated enzyme. Data summarized in Table I show that whereas the DCI-treated complex showed less than 10% of the activity of the native enzyme toward LHRH, it degraded all other peptides as well as proinsulin at a faster rate than the native enzyme. Indeed, the rate of degradation of most peptides and proinsulin was 2–3 times faster by the DCI-treated than by the native enzyme and the rate of degradation of the oxidized B-chain of insulin was as much as 14 times faster. Similar results were also obtained with dynorphin and substance P (data not shown). This suggested the presence in the complex of a DCI-resistant component, apparently distinct from the previously identified three activities. The possibility that this component represents a contaminant is eliminated by the observation that it cannot be removed by any of the steps used in conventional protein purification and by the finding that it represents the major activity of the complex capable of degrading natural peptides and proteins.

Table II: Products of Degradation of Neurotensin Generated by the Native and Dichloroisocoumarin-Inhibited Enzyme[a]

enzyme	identified products	classification
dichloroisocoumarin-inhibited	pE-L-Y-E	minor
	pE-L-Y-E-N	minor
	pE-L-Y-E-N-K-P-R-R-P-Y-I	major
	N-K-P-R-R-P-Y-I	minor
	N-K-P-R-R-P-Y-I-L	minor
native	Y-I	minor
	E-N	minor
	pE-L-Y	minor
	K-P-R	minor
	pE-L-Y-E	major
	pE-L-Y-E-N	minor
	K-P-R-R-P-Y-I	minor
	N-K-P-R-R-P-Y-I-L	minor
	E-N-K-P-R-R-P-Y-I	minor
	pE-L-Y-E-N-K-P-R-R-P-Y-I	minor

[a] Conventional one-letter abbreviations are used to show the amino acid sequences. Degradation products were isolated by HPLC and identified by amino acid analysis after acid hydrolysis as described in Material and Methods. A major cleavage product is defined as that which quantitatively dominates other reaction products.

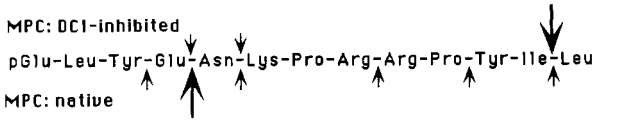

FIGURE 1: Amino acid sequence of neurotensin and cleavage sites in reactions with the native and DCI-inhibited MPC.

In order to more clearly characterize this activity, we examined the cleavage products generated during incubation of NT and proinsulin with either the DCI-inactivated or native complex. Table II contains a listing of the products identified in reaction mixtures of the two enzyme preparations with NT, and Figure 1 indicates the amino acid sequence of NT and the cleavage sites catalyzed by the two enzyme forms. When NT was incubated with the DCI-inhibited enzyme, the primary cleavage site was at the Ile^{12}–Leu^{13} bond giving rise to NT$^{1-12}$ as the main reaction product (Figure 2; panel A). This product accumulated during the course of the reaction and persisted even during prolonged incubation periods, suggesting that it is rather resistant to further degradation. Minor amounts of NT$^{1-4}$, and even smaller amounts of NT$^{1-5}$ also appeared in the reaction mixtures, suggesting some cleavage of the Glu^4–Asn^5 and the Asn^5–Lys^6 bonds. The appearance of these products posed the question of whether their formation is catalyzed by the DCI-resistant component of the complex or by the residual activities remaining after treatment of the enzyme with DCI. Several considerations argue for the second

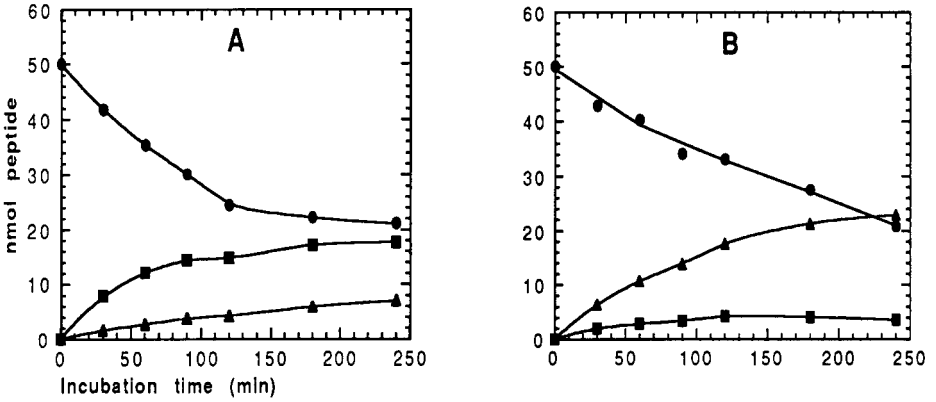

FIGURE 2: Degradation of neurotensin and formation of neurotensin$^{1-12}$ and neurotensin$^{1-4}$ in reactions with the native and 3,4-dichloroisocoumarin-treated enzyme. Incubation mixtures (at 37 °C; final volume 0.2 mL) contained 50 nmol of neurotensin, Tris-HCl buffer (0.0125 M, pH 8.0), and either 34 μg of the DCI-inhibited (panel A) or 28 μg of the native (panel B) complex. Aliquots of the incubation mixtures (25 μL) were withdrawn at the indicated time intervals and treated with 5 μL of glacial acetic acid. A total of 20 μL of these mixtures was subjected to HPLC, and the separated peptides were quantitated as described in Material and Methods. Neurotensin$^{1-13}$ is indicated by circles, neurotensin$^{1-12}$ by squares, and neurotensin$^{1-4}$ by triangles. Data are mean values obtained from two separate experiments.

Table III: Effect of Some Inhibitors on Neurotensin Degradation and Formation of Products by the Native Complex[a]

inhibitor	concn (mM)	relative activity: degradation of NT	relative activity: formation of NT$^{1-12}$	relative activity: formation of NT$^{1-4}$
none		100	100	100
1. *N*-ethylmaleimide	1.0	72	168	40
2. leupeptin	0.02	82	118	61
3. chymostatin	0.05	85	173	50
4. antipain	0.05	81	110	66
5. hemin	0.05	99	152	50

[a] Activity was determined as described under Materials and Methods at a neurotensin concentration of 0.125 mM. Inhibitors 1–5 were pre-incubated with the enzyme for 15 min at 26 °C before initiation of the reaction by the addition of substrate. Activities are relative to those obtained with the enzyme not exposed to the inhibitors and arbitrarily set at 100.

possibility. Thus, the rate of formation of these fragments was very low compared with the rate of formation of NT$^{1-12}$. Furthermore, the appearance of the NT$^{1-4}$ fragment required cleavage of the Glu^4–Asn^5 bond, the main site presumably attacked by the PGPHA of the native enzyme, an activity not fully eliminated by treatment with DCI (Table I). Also, experiments with leupeptin, chymostatin, and antipain showed that these aldehydes inhibit the overall rate of degradation of NT by the native enzyme but increase the generation of NT$^{1-12}$ with a concomitant inhibition of NT$^{1-4}$ formation (Table III). Although the same aldehydes caused an increase in degradation of NT by the DCI-treated enzyme (Table IV), this was again associated with an increase in NT$^{1-12}$ generation and a simultaneous inhibition of NT$^{1-4}$ formation (data not shown). These results are consistent with the conclusion that formation of NT$^{1-12}$ is primarily catalyzed by the DCI-resistant component of the complex and that the aldehyde inhibitors act by interfering with the degradation of this peptide by the other components of the complex.

Quite different results from those described above were obtained when NT was incubated under the same conditions with the native enzyme (Figure 2; panel B). The amount of NT$^{1-12}$ formed was less than that formed by the DCI-inhibited enzyme. Also, there was a great increase in generation of NT$^{1-4}$, indicating that the main cleavage site was the Glu^4–Asn^5 bond. Indeed, the NT$^{1-4}$ fragment became the main degradation product, increasing by about 5-fold relative to NT$^{1-4}$ formed by the DCI-treated enzyme. In addition to NT$^{1-4}$ and NT$^{1-12}$, the appearance of several other degradation products indicated additional cleavage sites at the Tyr^3–Glu^4, Asn^5–Lys^6, Arg^8–Arg^9, and Pro^{10}–Tyr^{11} bonds. These latter cleavage sites are apparently an expression of the chymotrypsin-like, trypsin-like, and PGPHA activities of the complex, all being inhibited by treatment with DCI, and therefore not seen in reactions of the DCI-inhibited enzyme with NT.

The degradation products of NT (with the exception of NT$^{1-12}$) observed in reaction mixtures with the native enzyme could have been formed directly from cleavage of the full-length peptide by the three DCI-sensitive components or also in a sequential reaction from NT$^{1-12}$ formed initially in a reaction catalyzed by the DCI-resistant component. That the latter reaction sequence is indeed a factor in the overall reaction is indicated by the finding that the amount of the NT$^{1-12}$ fragment tended to decrease during prolonged incubation periods with the native enzyme (Figure 2; panel B) and that this decrease was associated with a concomitant increase of the amount of NT$^{1-4}$ and other degradation fragments of NT. Other observations also suggest that NT$^{1-4}$ and other degradation fragments are at least partially derived from cleavage of the NT$^{1-12}$. For example, the aldehyde inhibitors, leupeptin, chymostatin, and antipain, increased the amount of NT$^{1-12}$ when added to reaction mixtures containing the native enzyme while at the same time decreasing the formation of NT$^{1-4}$ and other degradation products (Table III). Similar effects were seen when the native enzyme was incubated with NT in the presence of *N*-ethylmaleimide and hemin.

For further examination of the specificity of the DCI-resistant component of the complex, we have studied the degradation of bovine proinsulin, a precursor that, unlike insulin, is readily attacked by the MPC. The reactions were carried out in the presence of magnesium chloride (final concentration 25 mM) which increased the rate of degradation of proinsulin without changing the composition of the degradation products. The retention times and the ratios of peak heights of the products were the same for the native and DCI-inhibited enzyme, suggesting that the same products are generated by both forms of the MPC. This observation together with the finding that treatment of the native complex with DCI leads to a 2-fold increase in proinsulin degradation (Table I) suggested that proinsulin is primarily degraded by the DCI-resistant component of the complex. Amino acid sequencing showed that cleavages occurred within the C-chain of proinsulin. Each of the isolated products contained the N-terminal sequence of proinsulin in addition to a sequence resulting from cleavage by the enzyme. The amino acid

Table IV: Effect of Inhibitors on the Enzymatic Activities of the Multicatalytic Proteinase Complex[a]

inhibitor	concn (mM)	relative activity of components			
		DCI-resistant	chymotrypsin-like	trypsin-like	PGPHA
none		100	100	100	100
1. iodoacetamide	1.0	93	96	108	100
2. *N*-ethylmaleimide	1.0	31	71	18	95
3. iodoacetic acid	1.0	86	102	90	83
4. *p*-Mercuriphenylsulfonate	0.1	7	2	0	0
5. leupeptin	0.02	156	101	9	92
6. chymostatin	0.05	121	45	51	97
7. antipain	0.05	125	101	40	78
8. pepstatin	0.05	91	106	96	105
9. Boc-FLF-CH_2Cl	0.1	98	95	99	116
10. FLF-CH_2Cl·HCl	0.1	71	83	104	99
11. Boc-FFR(Tos)-CH_2Cl	0.1	52	93	103	111
12. Suc-FLF-CH_2Cl	0.1	145	92	100	130
13. DFP	4.0	113	43	106	112
14. APMSF	1.0	68	105	96	103
15. PMSF	1.0	78	99	97	103
16. DCI	0.04	69	2	20	17
17. 7-amino-4-chloro-3-[3-(isothioureido)propoxy]isocoumarin	0.1	13	nd	nd	nd
18. 4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin	0.1	38	162	95	49
19. ATP/Mg^{2+}	2.0/4.0	71	92	71	80
20. hemin	0.05	59	157	81	69

[a] Inactivation of the enzyme by DCI (10 μM) was carried out as described in Material and Methods. The activity of the DCI-treated enzyme was determined with NT as the substrate. The other activities were determined with the synthetic substrates as described in Materials and Methods. Inhibitors 1–8 were preincubated with the enzyme for 15 min at 26 °C and inhibitors 9–18 were preincubated with the enzyme for 60 min at 26 °C before the reaction was initiated at 37 °C by the addition of substrate. The effect of inhibitors 19 and 20 was determined without preincubation with the enzyme. Controls with the appropriate solvents were also carried through the procedures. Incubations were for 120 min. Activities are relative to those obtained with the enzyme not exposed to the inhibitor arbitrarily set at 100. Data are mean values obtained from 2 to 3 separate determinations. The conventional one-letter abbreviations are used for the amino acid residues in the structure of the peptidylchloromethyl ketones. The effect of 7-amino-4-chloro-3-[3-(isothioureido)propoxy]isocoumarin on the three activites was not determined (nd) because of interference by this compound with the diazotization reaction.

sequences of the degradation products indicated cleavage at the Val^{39}–Gly^{40} and the Leu^{44}–Ala^{45} bonds in the connecting peptide of proinsulin.

The pH optimum for degradation of NT by the DCI-treated complex was determined in Tris-HCl buffers and AMp buffers (0.2 M) in the pH range of 7.06–9.38. Optimal activity was found at about pH 9.0. At pH 8.0, the rate of degradation of NT was about 50% of that at pH 9.03. The K_m for NT at pH 8.0 was determined to be 0.42 mM.

A summary of the effect of a series of inhibitors on degradation of NT by the DCI-treated enzyme and comparative data showing the effect of the same inhibitors on the cleavage of the three model synthetic substrates by the native enzyme are given in Table IV. All four activities of the complex were susceptible to inactivation by the organic mercurial *p*-mercuriphenylsulfonate and were quite unaffected by exposure to iodoacetamide and iodoacetic acid. The DCI-resistant component was less inhibited by *N*-ethylmaleimide than the trypsin-like activity but distinctly more inhibited than either the PGPHA or the chymotrypsin-like activity. Of interest was the effect of the inhibitors of bacterial origin. All three aldehyde inhibitors, leupeptin, chymostatin, and antipain, promoted the degradation of NT by the DCI-resistant component of the complex, but they had variable effects on the other three activities. Leupeptin at 0.02 mM strongly inhibited the trypsin-like activity and had virtually no effect on the PGPHA and chymotrypsin-like activities, a finding consistent with previous observations (Orlowski & Wilk, 1981; Wilk & Orlowski, 1983). Chymostatin inhibited primarily the chymotrypsin-like and trypsin-like activities of the complex, while antipain, like leupeptin, primarily inhibited the trypsin-like activity. Pepstatin, an inhibitor of the class of aspartate proteases, had no effect on any of the four activities. The mechanism of activation by aldehyde inhibitors of NT degradation by the DCI-resistant component of the complex is not clear. It is possible that binding of the aldehydes at some site(s) of the complex induces conformational changes in the complex that favor catalysis at the active site of the DCI-resistant component.

None of the four chloromethyl ketones studied (Table IV; inhibitors 9–12) had any effect on the three components of the complex as measured with the synthetic substrates. However, two of the inhibitors, Phe-Leu-Phe-CH_2Cl and Boc-Phe-Phe-Arg(Tos)-CH_2Cl markedly inhibited the DCI-resistant component, and succinyl-Phe-Leu-Phe-CH_2Cl weakly inhibited the chymotrypsin-like activity but activated both the DCI-resistant component and the PGPHA. This latter effect adds succinyl-Phe-Leu-Phe-CH_2Cl to the list of compounds which by binding to one site of the complex either inhibit or stimulate activity at different sites, suggesting interactions between components of the MPC.

It was of interest to examine the effect of serine protease inhibitors on the activities of the MPC in view of the uncertainties about the catalytic mechanisms involved in the function of the complex. As shown in Table IV, DFP, a serine protease inhibitor, had no effect on the DCI-resistant component at concentrations as high as 4 mM. Of the other three activities, only the chymotrypsin-like activity was inhibited, a finding consistent with previous reports (Wagner et al., 1986; Orlowski & Michaud, 1989). APMSF and PMSF weakly inhibited the DCI-resistant component, but had no effect on the other three activities. DCI, a general serine proteinase inhibitor, had a strong inhibitory effect on the chymotrypsin-like, trypsin-like, and PGPHA activities of the complex at a concentration of 40 μM. As shown in Table I, even at a concentration 10 μM, 90% or more of the activity of these components was inactivated, leading to the manifestation of the DCI-resistant component. That the DCI-resistant component of the complex is somewhat susceptible to inhibition by isocoumarin derivatives is shown by the finding that after

inactivation of the three activities by treatment with 10 μM DCI additional treatment with 40 μM DCI caused about a 30% inhibition of this component and that two other isocoumarin derivatives, 7-amino-4-chloro-3-[3-(isothioureido)-propoxy]isocoumarin and 4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin, caused respectively a 90% and 60% inhibition at a concentration of 100 μM.

In view of reports that the 19S MPC constitutes the proteolytic core of the 26S complex involved in the ubiquitin-dependent pathway of protein degradation (Eytan et al., 1989; Driscoll & Goldberg, 1990), it was of interest to examine the effect of ATP and hemin on the activities of the MPC. ATP was reported to stimulate and hemin was reported to inhibit the proteolytic activity of the 26S complex, as well as certain preparations of the MPC from muscle (Driscoll & Goldberg, 1989). However, as shown in Table IV, ATP in the presence of Mg^{2+} had an inhibitory effect on both the DCI-resistant component and also as previously reported (Orlowski & Michaud, 1989) on the other three components. Hemin inhibited the DCI-resistant component of the complex but had a mixed effect on the other three activities. Both the PGPHA and trypsin-like activities were inhibited, whereas the chymotrypsin-like activity was markedly stimulated. Thus, hemin should be added to the group of inhibitors that show composite effects on different components.

DISCUSSION

Early evidence based on specificity studies and on the effect of inhibitors and activators indicated that the MPC exhibits at least three proteolytic activities cleaving bonds on the carboxyl side of hydrophobic, basic, and acidic amino acid residues and that each of these activities is associated with a different component of this multimeric protein (Orlowski & Wilk, 1981; Wilk & Orlowski, 1980, 1983). This induced us to propose the name "multicatalytic proteinase complex" in recognition of the possibility that at least several of the nonidentical subunits of the complex could express proteolytic activity. It is now recognized that the complex is composed of some 30 low molecular mass subunits (21–32 kDa) of which 13–15 are nonidentical. It is, however, still not clear how many of the subunits are proteolytically active and what activities are expressed by particular subunits. More recently, examination of the PGPHA of the complex led us to conclude that this activity is apparently expressed by at least two subunits, increasing thereby the number of proteolytically active components to at least four (Orlowski et al., 1991). The finding that DCI can be used for the inactivation of all three initially identified activities of the complex (Orlowski & Michaud, 1989) made it possible to explore for the presence of an activity or activities that are resistant to inactivation by this inhibitor. Data presented here indeed provide evidence for the presence of such a DCI-resistant component. In addition to resistance to DCI inactivation, properties that distinguish this activity from those previously identified include failure to cleave Cbz-Gly-Gly-Leu-pNA, Cbz-D-Ala-Leu-Arg-2NA, and Cbz-Leu-Leu-Glu-2NA, the three synthetic substrates cleaved by the other components, activation by leupeptin, chymostatin, and antipain of NT degradation, inhibition by 7-amino-4-chloro-3-[3-(isothioureido)propoxy]isocoumarin and 4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin, increased activity toward most natural peptides and proinsulin, and apparent preference toward bonds on the carboxyl side of branched chain amino acids.

The possibility of the existence in the MPC of an activity other than the three initially identified has been raised on the basis of studies of the multicatalytic proteinase from lobster muscles (Mykles & Haire, 1991). Heating of a basal form of the enzyme induced the appearance of a caseinolytic activity that was apparently distinct from the three activities measured with synthetic substrates. Wilk and co-workers (Yu et al., 1991) observed that chemical modification of the complex by reaction with *N*-acetylimidazole causes a rapid inactivation of the PGPHA and the trypsin-like activity, a slow inactivation of the chymotrypsin-like activity but an acceleration of casein degradation. These observations were interpreted as indicating that the caseinolytic activity of the complex is catalyzed by a component that is distinct from the other three activities, although the participation of the chymotrypsin-like activity in degradation of casein could not be ruled out. Pereira et al. (1992) have observed what may be a related activity of the MPC; inhibition of the PGPHA, trypsin-like, and chymotrypsin-like activities of the complex by DCI led to a stimulation of the caseinolytic activity of the complex by 5–15-fold. It is possible that the DCI-resistant component described here is identical with the activity described by those authors, although none of those studies has analyzed the nature, properties, or specificity of this activity. The data presented here add credence to the conclusion that the complex contains a distinct activity that differs with respect to specificity and sensitivity to inhibitors from those of the three previously identified activities and that this activity is capable of degrading both oligopeptides and proteins. Thus, the proteolytically active components must now be considered to number at least 5 since the PGPHA activity is apparently expressed by at least two components (Orlowski et al., 1991). It is conceivable that the count of catalytically active components of the complex will keep increasing as the catalytic properties of the complex are further scrutinized and as its activity toward different types of peptide bonds is further examined. It is notable in this regard that the reaction of the complex with [^{14}C]DCI (prepared by Dr. J. Powers, Georgia Institute of Technology, Atlanta, GA) leads to labeling of at least 7 different subunits (unpublished experiments from this laboratory).

Cleavage sites catalyzed by the DCI-resistant component in NT and proinsulin indicate a preference toward bonds on the carboxyl side of branched chain amino acids. It is therefore of interest that Cbz-Gly-Gly-Leu-pNA, a substrate for the previously identified chymotrypsin-like activity, is resistant to hydrolysis by the DCI-resistant component. This resistance might be the result of steric hindrance by the aromatic amine causing interference with substrate binding or could also indicate the need for a longer peptide structure for binding. The chymotrypsin-like activity of the complex cleaves not only the Leu-pNA bond in Cbz-Gly-Gly-Leu-pNA but also bonds in which the Leu residue in this substrate was replaced by either a Phe or Tyr residue. That the native complex is capable of cleaving bonds after aromatic residues in natural peptides has been previously reported (Wilk & Orlowski, 1980). Thus, for example, the main cleavage sites in LHRH were at the Tyr^5–Gly^6 and the Trp^3–Ser^4 bonds and the main cleavage site in bradykinin was at the Phe^5–Ser^6 bond (Wilk & Orlowski, 1980). Cleavage, however, of LHRH was inhibited by as much as 95% (Table I) after exposure to DCI, and bradykinin was not significantly cleaved by the DCI-inhibited enzyme (data not shown), indicating that the DCI-resistant component does not catalyze these reactions. This conclusion is also supported by the failure of this component to cleave the Tyr^3–Glu^4 and the Tyr^{11}–Ile^{12} bonds in NT, the latter of the two bonds being adjacent to the cleaved Ile^{12}–Leu^{13} bond. These results indicate the presence in the complex

of at least two activities with primary specificities directed toward bonds with hydrophobic residues in the P_1 position, although the DCI-resistant component seems to discriminate between the nature of the hydrophobic side chains of these residues. That the selection of bonds undergoing cleavage might also depend on the conformation of the peptide chain and the amino acid residues not directly involved in the formation of the scissile bond is indicated by the fact that only those bonds located in the connecting peptide of the proinsulin molecule are attacked, while similar bonds located within the mature insulin molecule seem to be resistant to cleavage. It is of interest in this respect that the connecting peptide (C-chain) in proinsulin was reported to be largely unstructured (Weiss et al., 1990). Further studies are needed, however, to more exactly identify the specificity of these components and the contribution to specificity of sequences positioned beyond the immediate vicinity of the scissile bond.

The mechanism of activation of the DCI-resistant component of the complex by the aldehyde inhibitors, such as leupeptin, chymostatin, and antipain, is unclear. Dick et al. (1991) observed in an extensive study on the degradation of the oxidized B-chain of insulin that leupeptin, which inhibits rather selectively the trypsin-like activity toward synthetic substrates, also inhibited cleavage of the Gln^4–His^5 bond in the oxidized B-chain of insulin, suggesting that the trypsin-like activity of the complex also cleaves bonds other than those involving basic residues. These investigators, however, also observed that these aldehyde inhibitors stimulated the appearance of other degradation products of the oxidized B-chain of insulin. Since many of the cleavage sites in this substrate involved bonds after branched chain amino acids, it is reasonable to assume that they could have been derived from reactions catalyzed by the DCI-resistant component described here. This is also consistent with our finding that treatment of the complex with DCI caused a 14-fold increase in the rate of degradation of the oxidized B-chain of insulin and also with our results showing stimulation by the aldehydes of the cleavage of the Ile^{12}–Leu^{13} bond in NT by the DCI-resistant component. Binding of the aldehyde inhibitors to the active site of the DCI-resistant component would be expected to have an inhibitory rather than a stimulatory effect. It is unlikely that the aldehydes bind to the active sites of the three components inactivated by DCI treatment. Therefore, the possibility needs to be considered that the aldehydes exert their effect by binding to allosteric sites irrespective of whether these are, or are not, involved in catalysis.

The mechanistic classification of the activities of the complex has been the subject of controversy with arguments being cited both for classification as thiol proteinases (Dahlmann et al., 1985; Rivett, 1985; Wagner et al., 1986; McGuire & DeMartino, 1986) and serine proteinases (Dahlmann et al., 1989; Orlowski & Michaud, 1989; Mykles, 1989). This controversy has been further increased by the finding that the primary structure of some 14 subunits of the MPC from different species, deduced from the nucleotide sequences of recombinant cDNA clones, shows no homology to any of the known amino acid sequences of proteases (Haass et al., 1989, 1990a,b; Tamura et al., 1991; Fujiwara et al., 1991; DeMartino et al., 1991). This, however, does not exclude the possibility that an active serine residue participates in catalysis, although perhaps not necessarily as part of a typical catalytic triad. Indeed, the presence of an active serine residue in the catalytic center of all three initially identified components of the MPC is indicated by their sensitivity to inactivation by DCI (Orlowski & Michaud, 1989), an inhibitor shown not to inhibit cysteine proteinases at the concentrations used (Harper et al., 1985). Like the other three activities of the MPC, the DCI-resistant component was also resistant to inactivation by DFP, PMSF, and APMSF. However, the possible presence of an active serine residue is also here indicated by the sensitivity to inactivation by isocoumarin derivatives other than DCI, such as 7-amino-4-chloro-3-[3-(isothioureido)propoxy]isocoumarin and 4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin. The resistance of the MPC to inactivation by DFP, PMSF, and APMSF could result from inaccessibility of its active site(s) to these inhibitors. In turn, the increased activity of the complex toward a wide spectrum of natural peptides observed after treatment with DCI could result from increased substrate accessibility to the active site of the DCI-resistant component possibly induced by structural changes after reaction with DCI.

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